ACTA BIOLOGICA CRACOVIENSIA Series Botanica 50/2: 49–54, 2008

MICROSTRUCTURE AND CHEMICAL COMPOSITION OF LEAF CUTICULAR WAXES IN TWO *SALIX* SPECIES AND THEIR HYBRID

BEATA SZAFRANEK[1*], DOMINIK TOMASZEWSKI[2], KATARZYNA POKRZYWIŃSKA[1] AND MAREK GOŁĘBIOWSKI[1]

[1]*Faculty of Chemistry, University of Gdańsk, ul. Sobieskiego 18/19, 80–952 Gdańsk, Poland,*
[2]*Institute of Dendrology, Polish Academy of Sciences, ul. Parkowa 5, 62–035 Kórnik, Poland*

Received November 27, 2007; revision accepted October 6, 2008

Leaf epicuticular wax morphology and chemical composition of total cuticular waxes were studied in two *Salix* species (*Salix alba* and *S. fragilis*) and their hybrid (*S.* ×*rubens*). A smooth wax layer with small, scattered wax structures covered the adaxial leaf surface in all three taxa, and a crustlike wax layer composed of terminally fused wax filaments was present on the abaxial surface. The leaf cuticular waxes, both epicuticular and intracuticular, were obtained by hot extraction in chloroform and then analyzed by gas chromatography and mass spectrometry. The principal components of the waxes were primary alcohols, fatty acids, aldehydes, *n*-alkanes and wax esters. The qualitative composition of the waxes was quite similar but there were quantitative differences between the taxa. The epicuticular crystalline waxes are composed of very-long-chain aldehyde polymers.

Key words: *Salix*, willow, epicuticular and intracuticular waxes, SEM analysis, chemical analysis, wax extraction.

INTRODUCTION

Epicuticular waxes form the outer layer of plant cuticles (Baker, 1982; Bianchi, 1995; Wettstein-Knowles, 1995; Barthlott et al., 1998). They are composed mainly of very-long-chain aliphatic components and exist as an amorphous film or as crystalline structures. Additional intracuticular waxes are embedded in cutin. The classical method for obtaining epicuticular waxes is one-minute extraction in cold chloroform or other solvents. Epicuticular wax can now also be isolated by mechanical methods (Ensikat et al., 2000; Riedel et al., 2007) which distinguish the epicuticular wax outside the plant cuticle from the cuticular wax embedded in the cuticle matrix.

The chemical composition of cuticular waxes has been studied in some *Salix* species, using the classical extraction method with $CHCl_3$ or CH_2Cl_2 (Hietala et al., 1995; Hietala et al., 1997; Cameron et al., 2002). Those analyses found *n*-alkanes, *n*-alcohols, *n*-aldehydes, wax esters and free fatty acids in willow waxes.

The epicuticular wax layer on the leaves of four European *Salix* species occurring in Poland (*S. alba*, *S. fragilis*, *S. trianda*, *S. pentandra*) has been examined by scanning electron microscopy (SEM) (Tomaszewski, 2004). That study showed the presence of characteristic wax structures in the first three species. In preliminary experiments with standard cold $CHCl_3$ and CH_2Cl_2 extraction, the solubility of the epicuticular waxes was found to be very low. To our knowledge there are no previous reports on the cuticular wax composition of these *Salix* species.

Most of the epicuticular waxes in rice and sugar cane were also found to be insoluble in organic solvents at room temperature (Haas et al., 2001). Complete removal of all those waxes was achieved by extraction with $CHCl_3$ at 60°C. Aldehydes were responsible for the incomplete extraction with $CHCl_3$ at room temperature because of their presence in an insoluble polymeric form. They did dissolve at elevated temperature, however, following cleavage of polymeric bonds. The wax crystals in *Nepenthes alata* also contained polymeric forms of very-long-chain aldehydes (Riedel et al., 2003).

The present work studies the microstructure and chemical composition of leaf cuticular waxes in two *Salix* species and their natural hybrid. Owing to

*e-mail: Beata.Szafranek@univ.gda.pl

PL ISSN 0001-5296

the low solubility of the waxes, hot chloroform extraction was applied in order to remove the crystalline wax from the willow leaves.

MATERIALS AND METHODS

PLANT MATERIAL

The material for analysis consisted of fresh leaves of two *Salix* species (*S. alba* L., *S. fragilis* L.) and their hybrid (*S. ×rubens* Schrank). The trees were adults growing near Kórnik in Wielkopolska Province, west-central Poland (~52°14'N, 17°05'E). The leaves were taken in September 2003. Only mature and healthy leaves from the middle part of the shoot were collected; these were immediately weighed (~200 g per taxon). Specimens have been deposited in the Herbarium of the Institute of Dendrology in Kórnik (KOR).

SCANNING ELECTRON MICROSCOPY (SEM)

Samples (~1 × 1 cm squares) were taken from the central part of the leaf blade (two leaves per plant) and air-dried. The surface was coated with gold and viewed at 15 kV with a Hitachi S3000N SEM from the Institute of Plant Protection in Poznań. Micrographs produced from plants sourced from different locations in Poland and elsewhere were used as well.

WAX EXTRACTION

The leaves were harvested, transferred to the laboratory, weighed and extracted. The leaf cuticular waxes were extracted by dipping and shaking the leaves, enclosed in a tea ball, in hot $CHCl_3$ (60°C) for 90 sec. The procedure was repeated for ten portions of leaves and the resulting extracts were pooled. For quantitative analysis the solvent was spiked with internal standards (*n*-docosane, *n*-hexatriacontane). The wax extracts, composed of both epi- and intracuticular waxes, were then filtered, dried with Na_2SO_4, and concentrated. The total surface area of the leaves was estimated by photocopying the leaves, cutting out the paper copies and weighing them.

CHEMICAL ANALYSES

The GC-FID analyses were carried out with a GC 8000 TOP (CE Instruments) gas chromatograph equipped with an FID detector (argon carrier gas). Mass spectra (70 eV) were recorded with a TRIO-2000 quadrupole mass spectrometer. The samples were introduced through a Hewlett-Packard 5890 gas chromatograph (helium carrier gas).

The alkanes, wax esters, alcohols and aldehydes were analyzed directly in native samples; the fatty acids were analyzed as TMSi derivatives. A silylation procedure was employed using a mixture of N,O-bis(trimethylsilyl)acetamide (BSA) and trimethylchlorosilane (TMCS) (85:15, v/v) at 70°C for 30 min. Aldehydes and free aliphatic alcohols can be analyzed without derivatization on a capillary column (Evershed, 1992a). Aldehydes were quantified using GC analyses of native samples because these compounds could not be analyzed quantitatively after wax treatment with BSA. Aldehydes form artefacts with a variety of silylation reagents (Little, 1999) and should be analyzed without derivatization (Nass et al., 1998).

The GC-MS and GC-FID analyses of alkanes, alcohols, aldehydes and fatty acids were performed using an RTX-1 WCOT capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Restek). The oven temperature increase was programmed from 200°C to 320°C at 4°C/min and then held for 15 min, with 80 kPa carrier gas pressure and 330°C injector and detector temperature, 1:30 split ratio and ~1 μl sample injection volume.

Compounds were GC-MS-identified by comparing their mass spectra with published data (Christiansen et al., 1968; MSDC, 1991; Evershed, 1992b; Christie, 1994; Kitson et al., 1996; Szafranek and Synak, 2006) and also analyzed by GC-FID. GC-FID quantitative analysis was performed in three replicates (RSD 5–10%).

Wax esters were GC-FID analyzed only (oven at 200°C, 4 C/min to 380°C, 20 min at 380°C; injector 360°C; FID detector with ceramic flame jet, temp. 400°C; carrier gas at 100 kPa; 30 m × 0.25 mm i.d., film thickness 0.1 μm DB-1HT WCOT capillary column; J&W Scientific). Compounds were identified by comparing their retention times with commercial standards (behenic acid behenyl ester and behenic acid arachidyl ester; Sigma, Poland).

RESULTS

SEM ANALYSIS OF LEAF EPICUTICULAR WAXES

Scanning electron microscopy showed a homogenous, smooth epicuticular wax layer (although sparse fine structures may be present) covering the adaxial (upper) surface of the leaf blade in all three taxa, and specific wax structures ("conicoids") on the abaxial (lower) surface (Fig. 1a,c,e), consisting of terminally fused wax filaments. *Salix alba* L. had very large, loosely structured conicoids (Fig. 1a). In *S. fragilis* L. they were smaller and more compact. *S. ×rubens* Schrank (= *S. alba* × *S. fragilis*) resembles *S. fragilis* in this regard (Fig. 1c,e). Simple unbranched trichomes with smooth cuticular ornamentation appeared on both leaf surfaces in both *S. alba* and *S. ×rubens* (more densely in the former);

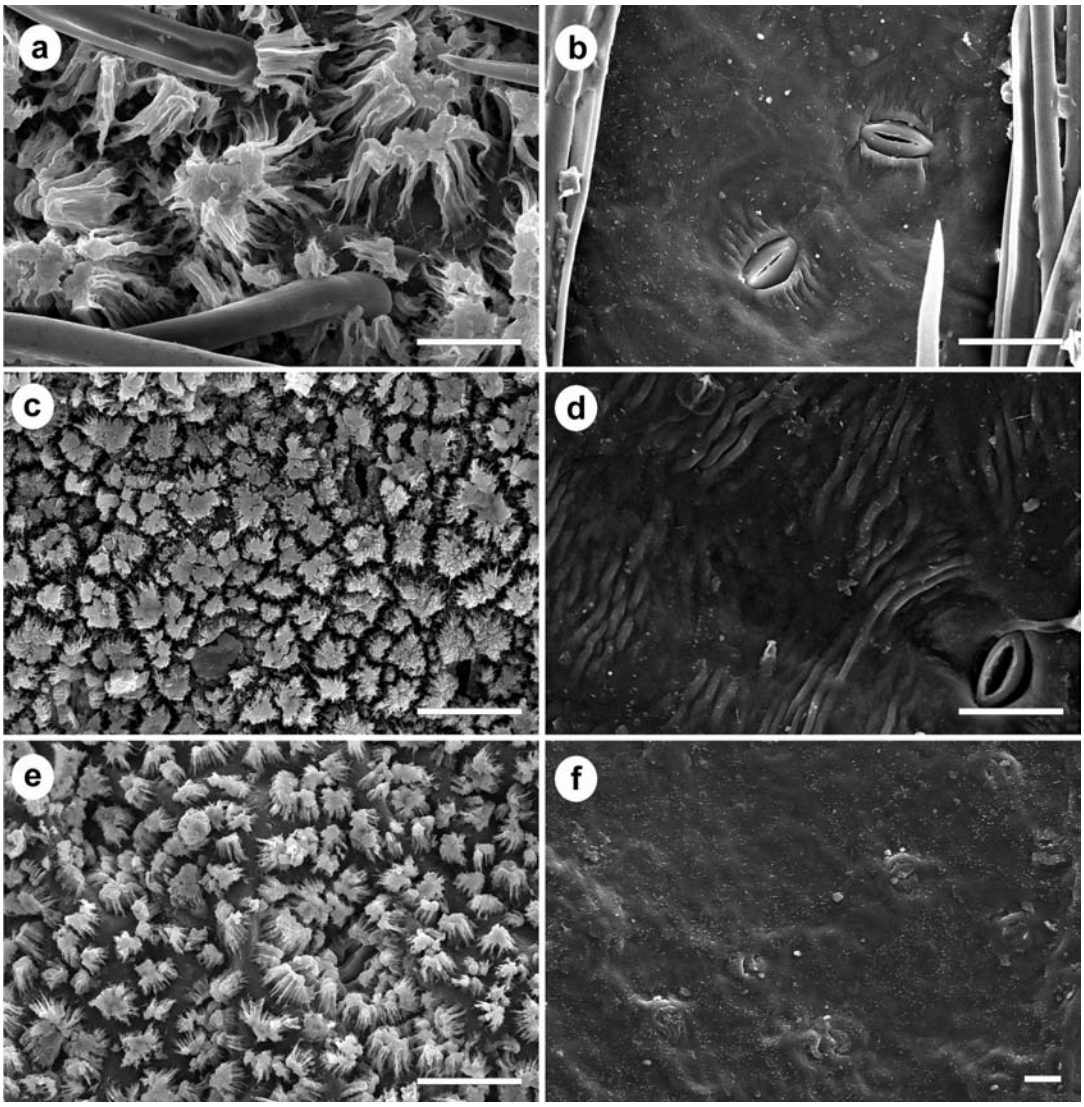

Fig. 1. *Salix* leaf surfaces (SEM). *S. alba* – abaxial (**a**) and adaxial (**b**), *S. fragilis* – abaxial (**c**) and adaxial (**d**), *S.* ×*rubens* – abaxial (**e**) and adaxial (**f**). Bar = 25 μm.

in *S. fragilis* the leaf blade is glabrous. Similar conical wax structures are probably present on the abaxial leaf surface in the majority of *Salix* species (Fig. 2 a–f), differing only in size and the compactness of their filaments.

CHEMICAL COMPOSITION

The gravimetrically estimated wax yield of the cuticular wax layers on the leaves of the willow taxa were as follows: *S. alba* 98 μg/cm^2, *S. fragilis* 75 μg/cm^2 and *S.* ×*rubens* 100 μg/cm^2.

The principal components of the *Salix* waxes were very-long-chain primary alcohols (8–18 μg/cm^2) (Tab. 1). They ranged in length from C_{22} to C_{30}, with even-numbered compounds predominating, but traces of C_{25} and C_{27} alcohols were detected as well (Fig. 3). The most abundant alcohol was *n*-hexacosanol (C_{26}). The distribution pattern was similar in all three taxa.

The second major class of wax compounds consisted of fatty acids (Tab. 1), their yields varying from 4 μg/cm^2 (*S. fragilis*) to 15 μg/cm^2 (*S. alba*). This fraction consisted of a homologous series of saturated, straight-chain fatty acids with even and odd numbers of carbon atoms from C_{20} to C_{30} (Fig. 3). The most abundant acids were hexacosanoic (C_{26}) (18–43% of total fatty acids), octacosanoic (C_{28}) (21–40%) and tetracosanoic acid (C_{24}) (13–18%). *S. fragilis* and *S.* ×*rubens* displayed similar distributions of fatty acid homologues; *S. alba* contained a relatively higher percentage of octacosanoic acid (40%).

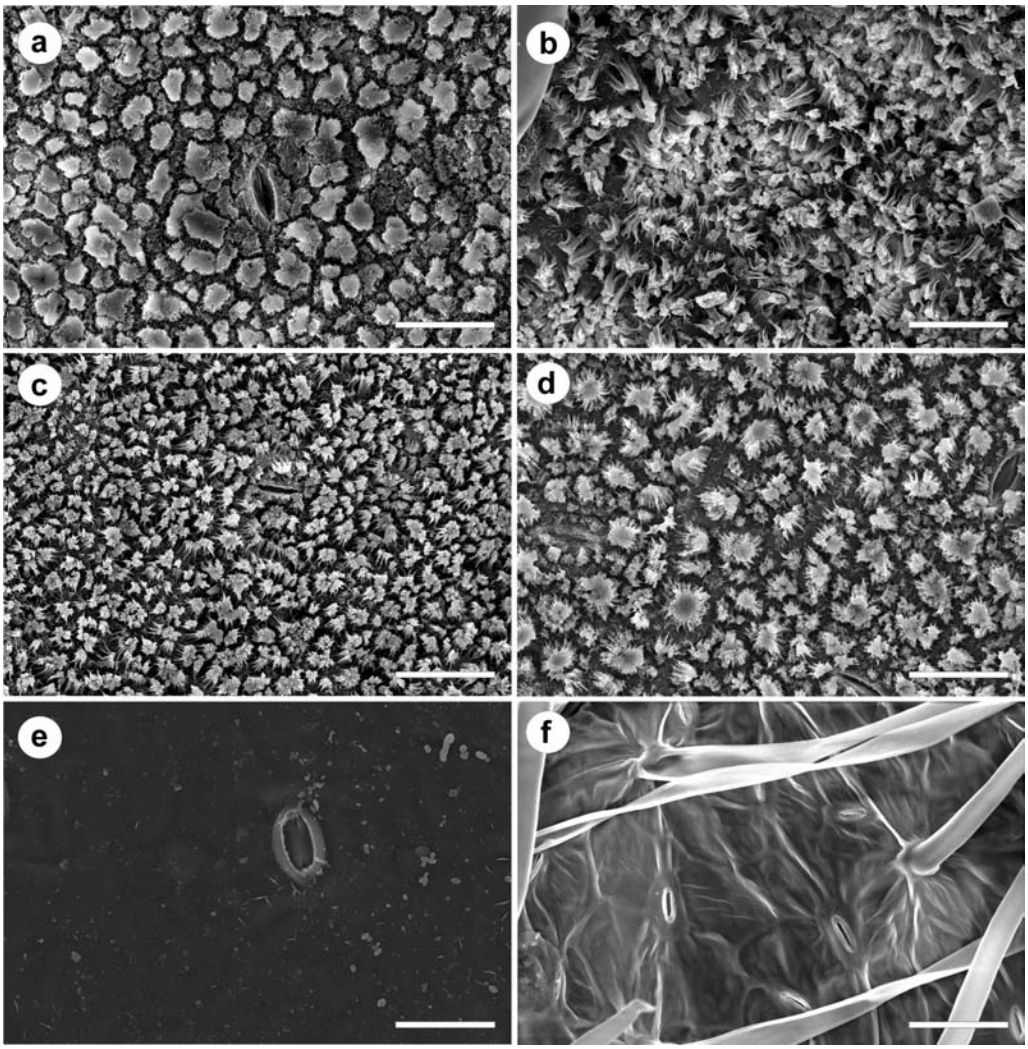

Fig. 2. Abaxial leaf surface of several *Salix* species (SEM). (**a**) *S. purpurea* – Poland, (**b**) *S. atrocinerea* – Spain, (**c**) *S. phylicifolia* – Russia, (**d**) *S. triandra* – Poland, (**e**) *S. alpina* – Poland, (**f**) *S. viminalis* – Poland. Bar = 25 μm.

Aldehydes were present in the cuticular waxes in quantities from 3.8 to 11.6 μg/cm^2 (Tab. 1). They consisted of homologous series of straight-chained compounds with odd and even numbers of carbon atoms from C_{22} to C_{30}, the main component being *n*-hexacosanal (43–79% of total aldehydes) (Fig. 3). Their percentage distribution was quite similar in all the taxa studied.

Salix waxes also contained detectable levels of *n*-alkanes (1.4–5 μg/cm^2) with carbon atom numbers from C_{23} to C_{30} (Tab. 1). The most abundant of these were heptacosane (C_{27}), nonacosane (C_{29}) and pentacosane (C_{25}) (Fig. 3).

Homologous esters of very-long-chain fatty acids with primary alcohols (wax esters) were present at similar levels, between 0.8 and 1.4 μg/cm$_2$ (Tab. 1). They were GC-separated according to the number of carbon atoms. *Salix* cuticular waxes contained a wide range of different wax ester isomers with chain lengths from C_{38} to C_{56} (Fig. 3). The principal wax esters were those with carbon numbers C_{48}, C_{50} and C_{52}. *Salix* waxes also contained ketones, secondary alcohols and some unidentified components, but they were present in very small concentrations and were not analyzed thoroughly. Interestingly, we found traces of free alkan-2-ols (C_{25}, C_{27}, and C_{29}) in the willow waxes. They had previously been found only in potato cuticular waxes (Szafranek and Synak, 2006).

DISCUSSION

In this study, *Salix* alba, *S. fragilis* and *S.* ×*rubens* were selected for comparison of leaf wax microstructure and chemical composition in two

TABLE 1. Cuticular wax composition in three *Salix* taxa (μg/cm^2). Data are means ± SD, N = 3.

Wax class	*S. alba*	*S. fragilis*	*S. ×rubens*
Alcohols	13.0±0.2	7.8±0.1	18.1±1.0
Fatty acids	14.7±0.8	3.6±0.4	12.3±1.2
Aldehydes	5.2±0.1	3.8±0.3	11.6±0.2
Alkanes	5.0±0.1	1.4±0.1	3.9±0.1
Wax esters	1.2±0.1	0.8±0.1	1.4±0.1
Ketones*	0.6±0.1	n.d.	0.4±0.1
Alkan-2-ols*	0.2±0.1	0.1±0.1	0.1±0.1
Unidentified	2.9±0.1	1.9±0.1	2.6±0.1
Total	42.8	19.4	50.4

*tentatively identified

willow species and their hybrid. To our knowledge this is the first report on the cuticular wax composition of these *Salix* taxa.

Scanning electron microscopy showed epicuticular waxes in the form of conicoids on the abaxial surface of the willow leaves. To the best of our knowledge, their presence in some taxa and absence in others is independent of the particular combination of environmental/geographical factors (Tomaszewski unpubl.). The abaxial surface conicoids found in this study cannot be compared with waxes Cameron et al. (2002) found; in that study, SEM images were made only from the adaxial leaf surfaces, where conicoids are normally absent.

Salix cuticular waxes were obtained by hot extraction in $CHCl_3$ according to the method described by Haas et al. (2001), and then GC-MS and GC-FID analyzed. At room temperature the waxes were practically insoluble in chloroform. Since the solvent method of wax extraction releases not only epi- but also intracuticular waxes (Jetter et al., 2000; Riedel et al., 2003; Riedel et al., 2007), the compounds extracted from the surface of *Salix* leaves and described in this study represent total cuticular waxes.

The total amounts of polar components of the cuticular waxes (*n*-alcohols, free fatty acids and *n*-aldehydes) were higher than reported in previously examined *Salix* taxa (*S. purpurea*, *S. dasyclados*, *S. eriocephala, S. myrsinifolia, S. viminalis, S. dasyclados* × *S. triandra*) (Hietala et al., 1995; Hietala et al., 1997; Cameron et al., 2002). For example, aldehydes identified in the *Salix* clones described by Hietala et al. (1995, 1997) varied from 0.4 to 4 μg/cm^2, but were present as minor components in waxes analyzed by Cameron et al. (2002). The differences may be attributable to genetic and environmental factors. The amounts and composition of these compounds may be affected by chemicals, light, temperature and mutations (Bianchi, 1995). Cameron et al. (2002) reported significant differences in total wax load and

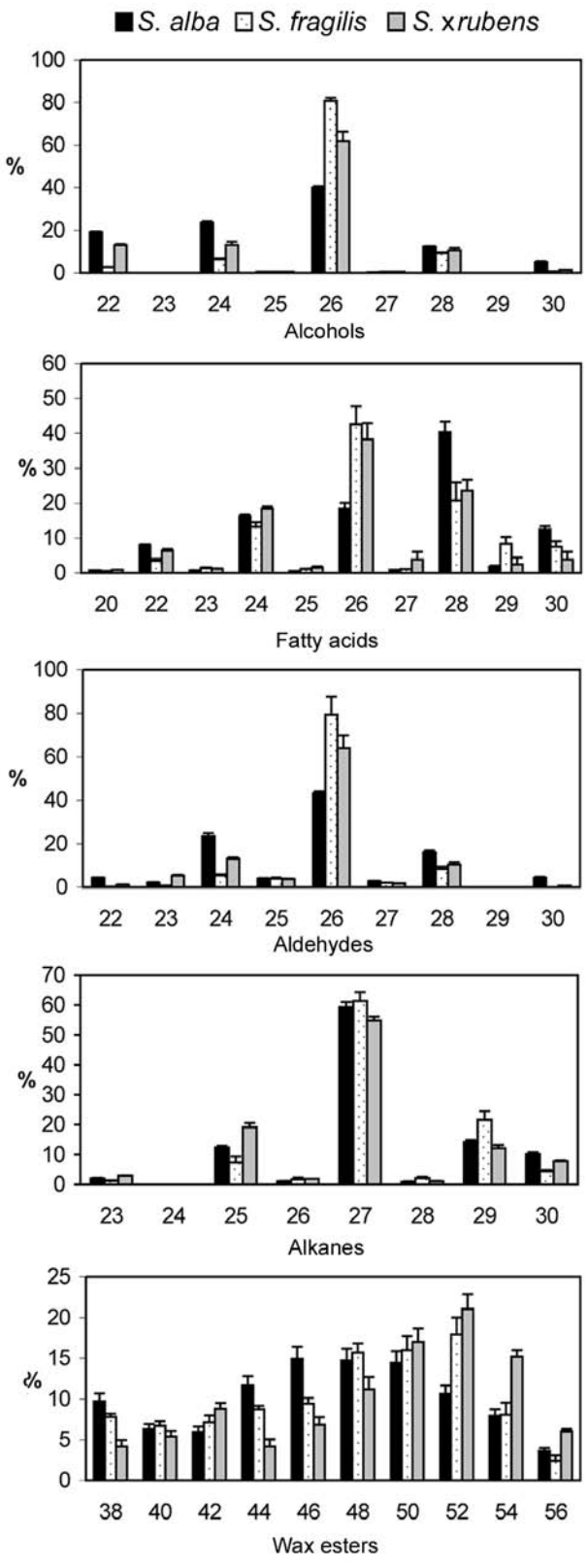

Fig. 3. Relative abundance (means ± SD, N = 3) of primary alcohols, fatty acids, aldehydes, *n*-alkanes and wax esters identified in cuticular waxes of *Salix* taxa.

wax composition in species over the growing season, but the highest yields of *n*-alcohols and *n*-aldehydes were still smaller than in the present study. Another explanation could involve differences in extraction methods. In sugar cane and rice, the yield of wax was lower with $CHCl_3$ extraction at room temperature than with hot $CHCl_3$ extraction (Haas et al., 2001); the yield of aldehydes and other polar compounds (primary alcohols and fatty acids) increased when hot extraction was applied. The most abundant compound classes in the sugar cane and rice waxes were aldehydes, primary alcohols and free fatty acids, as in *Salix* waxes. Haas et al. (2001) concluded that the aldehydes were present in polymeric form because they were insoluble at room temperature. We suggest that the aldehydes in the *Salix* waxes are also present in polymeric form.

The SEM images collected from the adaxial and abaxial surfaces of *Salix* leaves differed significantly (Fig. 1 a–f). The wax samples were obtained by solvent extraction from the entire leaf area. In consequence, the extract was a mixture of cuticular wax components from both surfaces. The epicuticular crystalline waxes were dissolved only by hot extraction. The solubility data indicate that the wax structures on the leaf surface of *Salix* consist of very-long-chain aldehydes present in polymeric form, as in earlier studies of rice and sugar cane (Haas et al., 2001), and *Nepenthes* epicuticular waxes (Riedel et al., 2003, 2007).

ACKNOWLEDGEMENTS

This work was financed in part by the Polish Ministry of Science and Higher Education (DS/8200–4–0085–8) and by the Institute of Dendrology in Kórnik. The authors are grateful to Magdalena Gawlak and Dr. Marzenna Guzicka for their help in preparing the SEM images, and to the anonymous referees for their helpful comments.

REFERENCES

BAKER EA. 1982. Chemistry and morphology of plant epicuticular waxes. In: Cutler DF, Alvin KL, and Price CE [eds.], *The plant cuticle*, 139–165. Academic Press, London.

BARTHLOTT W, NEINHUIS C, CUTLER D, DITSCH F, MEUSEL I, THEISEN I, and WILHELMI H. 1998. *Botanical Journal of Linnean Society* 126: 237–260.

BIANCHI G. 1995. Plant waxes. In: Hamilton RJ [ed.], *Waxes: Chemistry, molecular biology and functions*, 175–222. The Oily Press, Dundee, Scotland.

CAMERON KD, TEECE MA, BEVILACQUA E, and SMART LB. 2002. Diversity of cuticular wax among *Salix* species and *Populus* species hybrids. *Phytochemistry* 60: 715–725.

CHRISTIANSEN K, MAHADEVAN V, VISWANATHAN CV, and HOLMAN RT. 1968. Mass spectrometry of long-chain aliphatic aldehydes, dimethyl acetals and alk-1-enyl ethers. *Lipids* 4: 421–427.

CHRISTIE WW. 1994. *Gas chromatography and lipids*. The Oily Press, Ayr, Scotland.

ENSIKAT HJ, NEINHUIS C, and BARTHLOTT W. 2000. Direct access to plant epicuticular wax crystals by a new mechanical isolation method. *International Journal of Plant Sciences* 161: 143–148.

EVERSHED RP. 1992a. Gas chromatography of lipids. In: Hamilton RJ, and Hamilton S [eds.], *Lipid analysis. A practical approach*, 113–151. Oxford University Press, Oxford.

EVERSHED RP. 1992b. Mass spectrometry of lipids. In: Hamilton RJ, and Hamilton S [eds.], *Lipid analysis. A practical approach*, 263–308. Oxford University Press, Oxford.

HAAS K, BRUNE T, and RÜCKER E. 2001. Epicuticular wax crystalloids in rice and sugar cane leaves are reinforced by polymeric aldehydes. *Journal of Applied Botany* 75: 178–187.

HIETALA T, LAAKSO S, and ROSENQVIST H. 1995. Epicuticular waxes of *Salix* species in relation to their overwintering survival and biomass productivity. *Phytochemistry* 40: 23–27.

HIETALA T, MOZES N, GENET MJ, ROSENQVIST H, and LAAKSO S. 1997. Surface lipids and their distribution on willow (*Salix*) leaves: a combined chemical, morphological and physicochemical study. *Colloids and Surfaces* B: *Biointerfaces* 8: 205–215.

JETTER R, SCHÄFFER S, and RIEDERER M. 2000. Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. *Plant, Cell and Environment* 23: 619–628.

KITSON FG, LARSEN BS, and MCEWEN CN. 1996. *Gas chromatography and mass spectrometry*, 162–163. Academic Press, London.

LITTLE JL. 1999. Artifacts in trimethylsilyl derivatisation reactions and ways to avoid them. *Journal of Chromatography* A 844: 1–22.

MSDC 1991. *Eight peak index of mass spectra*. The Royal Society of Chemistry, Cambridge.

NASS R, MARKSTÄDTER C, HAUKE V, and RIEDERER M. 1998. Quantitative gas chromatographic analysis of plant cuticular waxes containing long-chain aldehydes. *Phyto-chemical Analysis* 9: 112–118.

RIEDEL M, EICHNER A, and JETTER R. 2003. Slippery surfaces of carnivorous plants: composition of epicuticular wax crystals in *Nepenthes alata* Blanco pitchers. *Planta* 218: 87–97.

RIEDEL M, EICHNER A, MEIMBERG H, and JETTER R. 2007. Chemical composition of epicuticular wax crystals on the slippery zone in pitchers of five *Nepenthes* species and hybrids. *Planta* 225: 1517–1534.

SZAFRANEK B, and SYNAK E. 2006. Cuticular waxes from potato (*Solanum tuberosum*) leaves. *Phytochemistry* 67: 80–90.

TOMASZEWSKI D. 2004. The wax layer and its morphological variability in four European *Salix* species. *Flora* 199: 320–326.

WETTSTEIN-KNOWLES P. 1995. Biosynthesis and genetics of waxes. In: Hamilton RJ [ed.], Waxes: *Chemistry, molecular biology and functions*, 91–130. The Oily Press, Dundee, Scotland.