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Nanotubules on plant surfaces: Chemical composition of epicuticular wax crystals on needles of *Taxus baccata* L.

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This paper is dedicated to Professor Rodney Croteau on the occasion of his 60th birthday.

Abstract

Needles of *Taxus baccata* L. were covered with tubular epicuticular wax crystals varying in diameters (100 and 250 nm) and lengths (300–500 and 500–1000 nm) on the abaxial and adaxial surfaces, respectively. Various sampling protocols were employed to study the chemical composition of the needle waxes on three different levels of spatial resolution. First, a dipping extraction of whole needles yielded the total cuticular wax mixture consisting of very long chain fatty acids (21%), alkanediols (19%), phenyl esters (15%), and secondary alcohols (9%) together with small amounts of aldehydes, primary alcohols, alkanes, alkyl esters, and tocopherols. Second, waxes from both sides of the needle were sampled separately by brushing with $CHCl_3$-soaked fabric glass. Both sides showed very similar qualitative composition, but differed drastically in quantitative aspects, with nonacosan-10-ol (18%) and alkanediols (33%) dominating the abaxial and adaxial waxes, respectively. Third, the epi- and intracuticular wax layers were selectively sampled by a combination of mechanical wax removal and brushing extraction. This provided direct evidence that the tubular wax crystals contained high percentages of nonacosane-4,10-diol and nonacosane-5,10-diol on the abaxial surface, and nonacosan-10-ol on the adaxial surface of the needles. Together with these compounds, relatively large amounts of fatty acids and smaller percentages of aldehydes, primary alcohols, alkyl esters, and alkanes co-crystallized in the epicuticular layer. In comparison, the intracuticular wax consisted of higher portions of cyclic constituents and aliphatics with relatively high polarity. The formation of the tubular crystals is discussed as a spontaneous physicochemical process, involving the establishment of gradients between the epi- and intracuticular wax layers and local phase separation.

Keywords: *Taxus baccata*; Taxaceae; Cuticular wax; Yew needles; Surface composition; Secondary alcohols

1. Introduction

The surfaces of leaves, flowers and fruits, and non-woody stems are covered with a cuticle that consists of cutin and waxes (Walton, 1990). 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). The primary physiological function of the cuticle, to limit non-stomatal water loss, is likely associated with the intracuticular waxes (Baur, 1998), but the epicuticular waxes might also contribute to the transpiration barrier. For gymnosperm needles, epicuticular waxes also serve two other physiological functions: protecting the tissue against UV light (Reicosky and Hanover, 1978) and reducing stomatal water loss by moderating the gas exchange through stomatal antechambers (Jeffree et al., 1971). On the abaxial side of gymnosperm needles, these functions have also been discussed in the context of Florin rings, special surface protrusions on pavement cells around stomatal pores (Kim et al., 1999). Besides, the epicuticular waxes form the true surface of the plant organs and therefore must serve important ecological functions in the interaction with insects and pathogens (Eigenbrode and Espelie, 1995).

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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 certain other species. These epicuticular crystals exhibit a wide range of forms, e.g., platelets, ribbons or tubules (Barthlott et al., 1998), and specific shapes were found to correlate with various prominent wax constituents (Baker, 1982). This indirect evidence suggested that these compounds both initiate crystallization and accumulate in the crystals. For instance, it was assumed that tubule-shaped crystals on the needle surface of diverse gymnosperms contained the secondary alcohol nonacosan-10-ol (Jeffree et al., 1975; Holloway et al., 1976; Jetter and Riederer, 1994). Hence, the presence of individual compounds at the very surface could qualitatively be inferred. But as neither the crystal-forming compounds nor possible admixtures could be quantified, the exact surface composition remained unknown.

The composition of plant cuticular waxes has long been studied using superficial extraction of intact plant material with organic solvents (Walton, 1990). It was shown that solvent molecules rapidly enter into the deeper layers of the cuticle and therefore typically release a mixture of both epi- and intracuticular waxes (Jetter et al., 2000). The resulting extracts must reflect the total wax composition, averaging over the entire depth of the cuticular wax layers. Wax mixtures of diverse plant species were reported to consist of homologous series of very long chain fatty acid derivatives, i.e., fatty acids, aldehydes, primary and secondary alcohols, ketones, and alkanes of chain lengths C_{20}–C_{36}, as well as alkyl esters with C_{38}–C_{70} (Walton, 1990). In addition, characteristic compounds such as triterpenoids, tocopherols, or aromatic compounds can be present, in some species only in trace amounts and in others dominating the mixture (Baker, 1982; Wollenweber et al., 1998). For the total wax mixture of *Taxus baccata* needles, four novel homologous series of esters have recently been described as characteristic compounds. These series contain alcohol moieties with phenyl propanoid and phenyl butanoid structures (Jetter et al., 2002).

Only in recent years methods have been developed that allow independent and selective sampling of both the epi- and intracuticular wax layers (Jetter et al., 2000; Jetter and Schäffer, 2001). They have successfully been employed to analyze the composition of epicuticular wax crystals on leaves of *Pisum sativum* (Gniwotta et al., 2005) and in the pitcher traps of the carnivorous plant *Nepenthes alata* (Riedel et al., 2003). Hence, it is now possible to describe the composition of plant surfaces more accurately. For example, it can be tested whether species-characteristic compounds are present at or near the plant surface and are hence available for direct contact with insect herbivores or with bacterial and fungal pathogens landing on the plant cuticle.

In the current study, we have performed chemical analyses on *T. baccata* needles to address the questions: (1) whether the cuticular waxes on adaxial and abaxial needle surfaces differ, (2) which compounds constitute the epicuticular wax crystals on the surface of both needle sides, (3) whether gradients between the intra- and epicuticular wax layers on both sides of the needles exist, and (4) how much of the total cuticular wax is contributed by the intra- and epicuticular waxes.

2. Results and discussion

Scanning electron microscopy revealed that the epidermal pavement cells on both sides of mature *T. baccata* needles had a rectangular shape (Fig. 1A, B, and D). The adaxial needle surfaces were devoid of stomata and had an overall flat appearance. In contrast, the abaxial sides showed cuticular surface sculptures, forming a few broad protrusions on each pavement cell and Florin rings around stomatal pores. All of these larger structures were covered with epicuticular wax crystals (Fig. 1B, C, E, and F) that had the characteristic tubule shape described previously for gymnosperm surfaces (Riederer, 1989). The tubules on abaxial and adaxial surfaces had clearly differing sizes, with diameters of approximately 100 and 250 nm, while lengths varied between 300–500 and 500–1000 nm, respectively. Both needle surfaces further differed in numbers and local distribution of crystals, covering the abaxial side in a dense continuous network and the adaxial side in small clusters of tubules between zones where a smooth epicuticular wax film was visible.

The chemical composition of the yew needle wax was investigated in three separate experiments designed to give varying levels of spatial resolution and to allow comparisons with the literature data. In a first experiment, the combined wax from both needle sides was extracted by brief immersion of intact *T. baccata* needles in $CHCl_3$. The overall wax load was 34.1 ± 1.3 μg/cm^2, containing fatty acids (21%), alkanediols (19%), phenyl esters (15%), and secondary alcohols (9%) together with smaller amounts of aldehydes, primary alcohols, alkanes, alkyl esters, and tocopherols (Table 1). Overall, 96 compounds were identified that accounted for 85% of the wax load, while only 15% of the wax remained unidentified. The most prominent constituents were nonacosan-10-ol, nonacosane-4,10-diol and nonacosane-5,10-diol. No other (homologous or isomeric) secondary alcohols were present, and only trace amounts of other isomeric alkanediols were detected (nonacosane-3,10-diol, nonacosane-6,10-diol, nonacosane-7,10-diol and nonacosane-10,13-diol). Complete homologous series of fatty acids (C_{20}–C_{32}), primary alcohols (C_{21}–C_{32}) and aldehydes (C_{26}–C_{32}) were present. They all had similar chain length distributions, dominated by even-numbered homologs, whereas the series of *n*-alkanes (C_{25}–C_{29}) was dominated by odd numbered chain lengths. The chain length distribution of alkyl esters (C_{42}–C_{46}) showed a maximum at C_{44}. According to their mass spectral fragmentation patterns, these alkyl esters were formed by C_{18}–C_{24} alcohols and C_{20}–C_{26} fatty acids. The phenyl esters

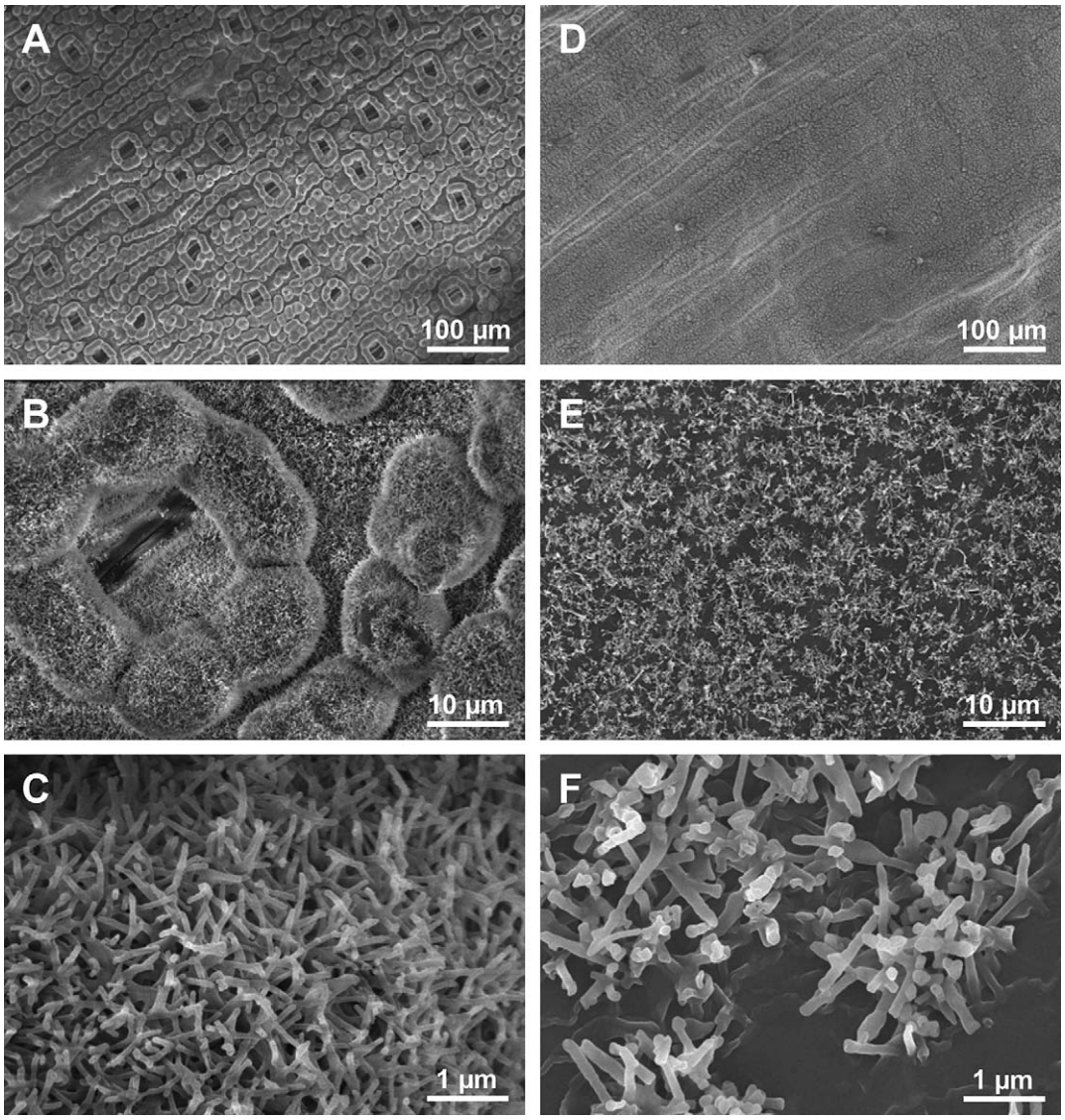

Fig. 1. Scanning electron micrographs of *T. baccata* needle surfaces. (A–C) Abaxial surface. (D–F) Adaxial surface.

consisted of 4-(3′,4′-dihydroxyphenyl)-2-butyl esters of C_{20}–C_{26} fatty acids, 3-(4′-hydroxyphenyl)-propyl esters of C_{22}–C_{28} fatty acids, and 3-(3′,4′-dihydroxyphenyl)-propyl esters of C_{22}–C_{26} fatty acids. The only other cyclic compounds identified were α-, β-, δ-, and γ-tocopherol. *T. baccata* thus had a needle wax composition relatively similar to that of diverse other gymnosperm species (Franich et al., 1978; Riederer, 1989).

A second chemical analysis differentiated between the waxes on the abaxial and adaxial surfaces of *T. baccata* needles. Previously, protocols for the selective extraction of wax from one leaf side had been described only for broadleaf plant species (Jetter et al., 2000). As they could not be applied to the small yew needles, new methods had to be devised. To this end, the needles were gently brushed with fabric glass that had been pre-extracted and soaked with $CHCl_3$. SEM inspection confirmed that this was selectively extracting the wax from only one side, as the epicuticular wax crystals were completely removed from the treated surface, while they remained intact on the opposite side of the needle (data not shown). In order to remove the wax exhaustively, individual needles were extracted by brushing more than 50 times, with the extraction repeated three times. The combined extracts contained 31.1 ± 2.2 μg/cm^2 for the abaxial surface and 17.7 ± 0.8 μg/cm^2 on the adaxial side (Table 2). Taken together, these results correspond to an average wax load of 24.4 μg/cm^2 for the entire needle surface, and are significantly lower than the total coverage found in the previous experiment using whole needle extraction (t test, $t(8) = 5.383$, $P = 0.001$).

It seemed likely that the discrepancy between the first and second experiments was caused by the different extraction protocols used. To test whether the brushing extraction might not be exhaustive, possibly due to the relatively short contact between fabric glass and needle surface, further control experiments were performed. After brushing both needle surfaces with $CHCl_3$-soaked fabric glass, the needles were dipped into $CHCl_3$. This additional extraction step yielded

Table 1
Composition of total cuticular waxes of *T. baccata* needles

Chain length	Aldehydes	Alkanes	Fatty acids	Alkyl esters	Primary alcohols	Dihydroxy-phenyl-butanoids[b]	Hydroxy-phenyl-propanoids[b]	Dihydroxy-phenyl-propanoids[b]	Tocopherols	Secondary alcohols	*sec.sec.* Diols	Unidentified
20	–	–	0.1 ± 0.01	–	–	0.6 ± 0.2	–	–	–	–	–	–
21	–	–	tr[a]	–	tr	0.2 ± 0.1	–	–	–	–	–	–
22	–	–	0.5 ± 0.02	–	0.3 ± 0.04	0.8 ± 0.1	0.3 ± 0.04	–	–	–	–	–
23	–	–	0.1 ± 0.01	–	tr	tr	0.2 ± 0.04	0.1 ± 0.04	–	–	–	–
24	–	–	1.3 ± 0.1	–	0.2 ± 0.04	0.5 ± 0.1	0.8 ± 0.1	tr	–	–	–	–
25	–	0.1 ± 0.04	0.2 ± 0.02	–	tr	–	0.1 ± 0.04	0.7 ± 0.1	–	–	–	–
26	0.1 ± 0.04	0.1 ± 0.01	0.7 ± 0.1	–	0.1 ± 0.04	tr	0.4 ± 0.04	0.1 ± 0.04	–	–	–	–
27	0.2 ± 0.01	0.3 ± 0.04	0.4 ± 0.04	–	0.1 ± 0.02	–	tr	0.3 ± 0.1	–	–	–	–
28	0.9 ± 0.04	tr	2.1 ± 0.2	–	0.2 ± 0.04	–	tr	–	–	–	–	–
29	0.1 ± 0.04	0.2 ± 0.02	0.4 ± 0.04	–	–	–	–	–	–	3.0 ± 0.4	6.4 ± 0.2	–
30	0.3 ± 0.1	–	1.0 ± 0.1	–	0.1 ± 0.04	–	–	–	–	–	–	–
31	–	–	tr	–	tr	–	–	–	–	–	–	–
32	0.3 ± 0.02	–	0.3 ± 0.2	–	tr	–	–	–	–	–	–	–
Total	1.9 ± 0.1	0.7 ± 0.1	7.2 ± 0.8	2.4 ± 0.4	1.2 ± 0.3	2.2 ± 0.1	0.9 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	3.0 ± 0.4	6.4 ± 0.2	5.0 ± 0.5

Mean values ($n = 5$) and SE are given for the coverage (μg/cm^2) of individual homologs of the major compound classes.
[a] tr, traces, i.e., less than 0.05 μg/cm^2.
[b] Chain length refers to the carbon number of the fatty acid moiety of the aromatic esters.

Table 2
Composition of waxes sampled by diverse methods from abaxial and adaxial sides of *T. baccata* needles

Compound classes	Abaxial			Adaxial			Both sides
	Brushing extraction	Total of three gum arabic treatments	Brushing extraction after gum arabic treatments	Brushing extraction	Total of three gum arabic treatments	Brushing extraction after gum arabic treatments	Residues left after brushing extraction
Aldehydes	1.0 ± 0.3	2.0 ± 0.3	0.7 ± 0.3	2.0 ± 0.4	3.8 ± 0.6	–	0.2 ± 0.3
Alkanes	0.7 ± 0.1	1.3 ± 0.2	0.4 ± 0.04	3.0 ± 0.2	5.7 ± 0.8	tr[a]	0.6 ± 0.5
Fatty acids	9.4 ± 2.0	17.3 ± 4.4	5.4 ± 1.1	18.1 ± 2.8	21.9 ± 2.8	2.2 ± 0.4	12.6 ± 6.5
Alkyl esters	2.0 ± 0.5	3.7 ± 0.4	2.5 ± 0.6	4.7 ± 0.9	6.5 ± 0.5	3.0 ± 0.8	3.9 ± 1.8
Primary alcohols	1.2 ± 0.1	3.6 ± 1.9	1.1 ± 0.3	3.5 ± 0.6	4.7 ± 0.4	0.9 ± 0.3	1.6 ± 0.6
Dihydroxyphenyl-butanoids	4.2 ± 1.5	1.0 ± 0.4	2.2 ± 0.5	12.7 ± 2.7	3.1 ± 1.0	24.7 ± 2.6	21.6 ± 7.4
Hydroxphenyl-propanoids	4.4 ± 0.4	1.8 ± 0.5	3.1 ± 0.3	4.1 ± 0.5	1.1 ± 0.2	8.6 ± 1.7	6.5 ± 1.8
Dihydroxyphenyl-propanoids	2.7 ± 0.4	1.3 ± 0.3	2.0 ± 0.5	4.9 ± 0.9	2.0 ± 0.4	15.5 ± 2.5	9.0 ± 1.3
Tocopherols	2.9 ± 1.3	–	4.8 ± 0.5	3.7 ± 0.5	–	12.6 ± 1.3	10.7 ± 1.3
Secondary alcohols	9.1 ± 0.8	13.9 ± 1.3	7.8 ± 0.4	18.4 ± 1.9	27.3 ± 2.1	3.8 ± 0.5	2.7 ± 0.5
sec.sec. Diols	33.1 ± 1.4	41.4 ± 4.9	37.3 ± 4.1	6.5 ± 0.7	5.3 ± 0.4	11.0 ± 2.1	6.0 ± 4.1
Unidentified	27.0 ± 1.5	20.4 ± 2.0	32.1 ± 3.0	15.4 ± 1.1	16.4 ± 0.3	14.5 ± 2.4	24.5 ± 5.0
Total wax yields	31.1 ± 2.2	17.5 ± 1.7	13.9 ± 0.9	17.7 ± 0.8	15.2 ± 0.4	3.3 ± 0.3	5.8 ± 0.1

Percentages of compound classes in respective samples and total wax yields in μg/cm^2 are given as mean values ($n = 5$) and SE.
[a] tr, traces, i.e., less than 0.1%.

5.8 ± 0.1 μg/cm^2 of wax (Table 2), thus increasing the overall wax load found by consecutive treatments to more than 30 μg/cm^2. This result confirmed the wax load found for whole needle extraction (see above, t test, $t(8) = 2.116$, $P = 0.067$), at the same time showing that the brushing protocol had yielded approximately 80% of the wax. The latter thus proved to be a reliable method for differentiating waxes on the abaxial surface from adaxial surface of needles on both quantitative and qualitative levels. To our knowledge, this is the first study to systematically explore and quantitatively evaluate the use of solvent-soaked spongy materials for the extraction of plant cuticular wax.

In the waxes from both sides of the needles the same compound classes and homologs were identified, including all the compounds previously detected in the whole needle extract. The relative amounts of all compounds differed significantly on both sides of the needles (t test, $P \leqslant 0.01$, except: hydroxphenylpropanoids $P = 0.364$, tocopherols $P = 0.205$). Alkanes, aldehydes, fatty acids, alkyl esters and phenyl esters were approximately twofold higher on the adaxial surface than on the abaxial surface (Table 2). Interestingly, both sides of the needle differed also in the percentage of the two compound classes that had previously been implicated in formation of epicuticular wax tubules. While nonacosan-10-ol amounted to $18.4 \pm 1.9\%$ in the wax of the adaxial surface, it was present in much lower percentage on the abaxial side ($9.1 \pm 0.8\%$). Conversely, the closely related *sec.sec.* alkanediols dominated the composition of wax from the abaxial surface ($33.1 \pm 1.4\%$), but were found at only $6.5 \pm 0.7\%$ in the wax of the adaxial surface.

A third experiment was designed to assess the composition of the tubular wax crystals on the adaxial and abaxial surfaces of *T. baccata* needles. To this end, both the epicuticular and intracuticular wax layers had to be sampled separately from the two sides of the needles, thus distinguishing four different wax compartments. 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 (Riedel et al., 2003; Gniwotta et al., 2005). As organic solvents like $CHCl_3$ 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 employing adhesives that are free of organic solvents. After exhaustive removal of epicuticular wax, the remaining intracuticular wax can be extracted. Unfortunately, none of these protocols had previously been employed for the analysis of surface waxes of gymnosperms, due to the small size and extreme geometry of needles.

Preliminary experiments showed that gum arabic, applied in aqueous solution, was the adhesive that allowed most reproducible removal of surface wax from *T. baccata* needles (data not shown). Three consecutive adhesive treatments of the adaxial surface yielded wax amounts drastically decreasing from 14.2 ± 0.5 to 0.2 ± 0.2 μg/cm^2 (Fig. 2), the latter value being not significantly different from zero (t test, $t(4) = 1.476$, $P = 0.214$). In sharp contrast, subsequent extraction of the adaxial surface using $CHCl_3$-soaked fabric glass yielded 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 gum arabic application ensured the exhaustive removal of epicuticular material, and, consequently, the remaining material released in the final extraction step can be interpreted as intracuticular wax.

Consecutive gum arabic treatments of the abaxial surface also yielded steadily decreasing wax amounts (Fig. 2). The third adhesive removal step still released 1.7 ± 0.3 μg/cm^2 of wax, i.e., amounts significantly different from zero (t test, $t(4) = 5.858$, $P = 0.004$). As the treatment could not be further repeated without damaging the needles, it was impossible to continue the adhesive sampling until no further material was released. Extraction following the third gum arabic treatment yielded relatively high amounts of wax. Overall, it can be concluded that the initial gum arabic sampling of this side of the needle was selective but not exhaustive for the epicuticular material, and the final extraction gave intracuticular wax together with small amounts of the remaining epicuticular material.

Using SEM, the effect of gum arabic on either the abaxial or adaxial side of the needles could be further confirmed (Fig. 3). A single adhesive treatment removed the wax crystals only from the top portions of the cuticular ridges on the abaxial surface, but did not affect the tubules in the troughs between (Fig. 3A). After three consecutive gum arabic treatments of the abaxial surface, only small portions of the crystals in deep depressions remained (Fig. 3B). From the adaxial surface, the majority of tubular wax crystals were removed already after the first adhesive

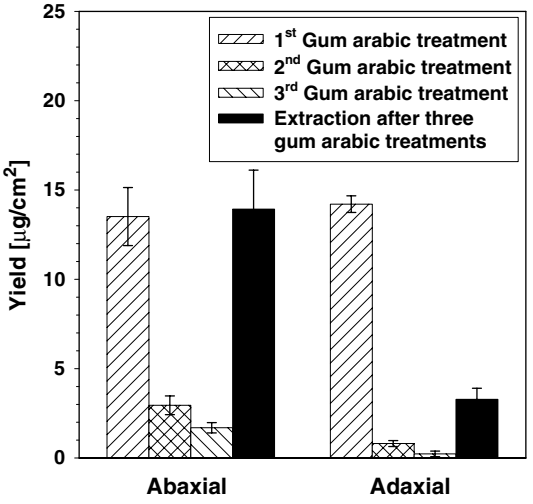

Fig. 2. Wax yields from *T. baccata* needles sampled by a combination of mechanical and extractive methods. Three consecutive treatments with the adhesive gum arabic were employed to remove the epicuticular wax layer, and the following extraction by brushing with $CHCl_3$-soaked fabric glass was employed to sample the intracuticular wax layer.

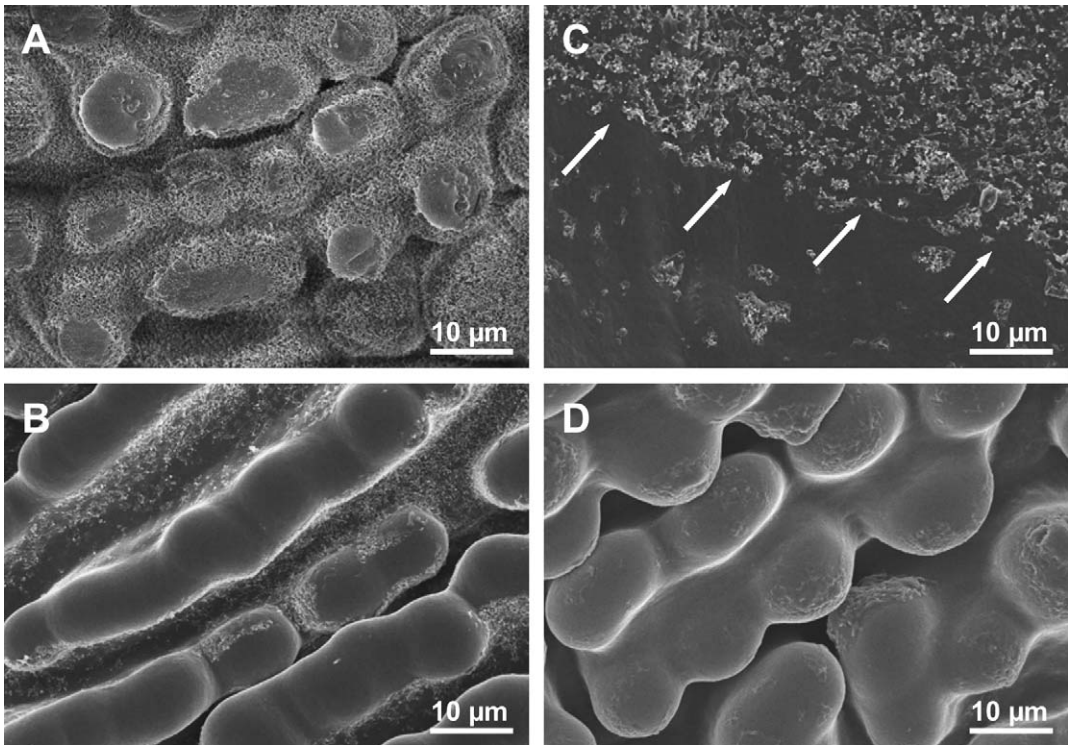

Fig. 3. Scanning electron micrographs of *T. baccata* needle surfaces after the application of various wax sampling protocols. (A) Abaxial surface after single treatment with gum arabic. (B) Abaxial surface after three consecutive treatments with gum arabic. (C) Adaxial surface after single treatment with gum arabic. The line between the treated area (bottom half of the picture) from the neighbouring untreated zone (top half) is highlighted by arrows. (D) Abaxial surface after dipping extraction in $CHCl_3$.

treatment, leaving some smaller structures and only small patches of tubules behind (Fig. 3C). An irregularly curved line sharply delineated the treated area from the neighbouring untreated zone, where the tubules remained unchanged. After superficial extraction of needles using $CHCl_3$, all tubular wax crystals had been completely removed from both surfaces, including the areas between the cuticular ridges (Fig. 3D). In contrast, the Florin rings and the cuticular ridges on pavement cell ridges on the abaxial side remained intact after extraction, demonstrating that these larger structures are not formed by cuticular wax.

After treating the yew needle surfaces with gum arabic, the removed waxes were visible on the lower side of the dry adhesive film, from which they protruded as tubules with diameters identical to the native crystals (Fig. 4). Waxes from the abaxial surface were visible as discrete spots matching the geometry of the cuticular ridges on this needle side (Fig. 4A and B). Thus, the gum arabic imprints confirmed the interpretation that during the first adhesive treatment only the epicuticular wax from the tops of the ridges had been sampled. On the contrary, the gum arabic imprint from the adaxial surface was entirely covered with wax (Fig. 4D and E), reflecting the nearly quantitative removal of crystals from this side. Small areas of the adhesive preparations from both needle surfaces carried a smooth film of wax on top of the embedded tubular crystals (Fig. 4C and F). It is plausible that in these instances the crystals had been torn off the surface together with the surrounding epicuticular wax film. All these results are in good accordance with the chemical data (cf. Fig. 2), thus illustrating the selective mechanical sampling of epicuticular wax (crystals).

Adding the yields of consecutive gum arabic treatments, the abaxial needle surface was found covered with 17.5 ± 1.7 µg/cm^2 of epicuticular wax, while intracuticular wax amounted to 13.9 ± 0.9 µg/cm^2 (Table 2). Correspondingly, the adaxial side had an epicuticular wax load of 15.2 ± 0.4 µg/cm^2 and an intracuticular wax layer of 3.3 ± 0.3 µg/cm^2. The total wax yields of consecutive gum arabic treatments and brushing extraction were 31.4 µg/cm^2 for the abaxial and 18.5 µg/cm^2 for the adaxial surface, in very good agreement with the results for direct extraction (t test, abaxial, $t(8) = 0.126$, $P = 0.903$; adaxial, $t(8) = 0.817$, $P = 0.438$). The second and third experiments thus mutually confirm each other.

On the abaxial surface, the relative amounts of most compound classes differed significantly between the epi- and intracuticular wax layers ($P < 0.05$ except: *sec.sec.* diols $P = 0.182$). Percentages of aldehydes, alkanes, fatty acids, primary alcohols and alkyl esters in the epicuticular wax were between one- and twofold higher than in the intracuticular layer (Table 2). Tocopherols were absent in the epicuticular wax, but were detected in the intracuticular material ($4.8 \pm 0.5\%$). Similarly, the aromatic esters, comprising phenyl propanoids and phenyl butanoids, were located mainly in the intracuticular layer. Diols

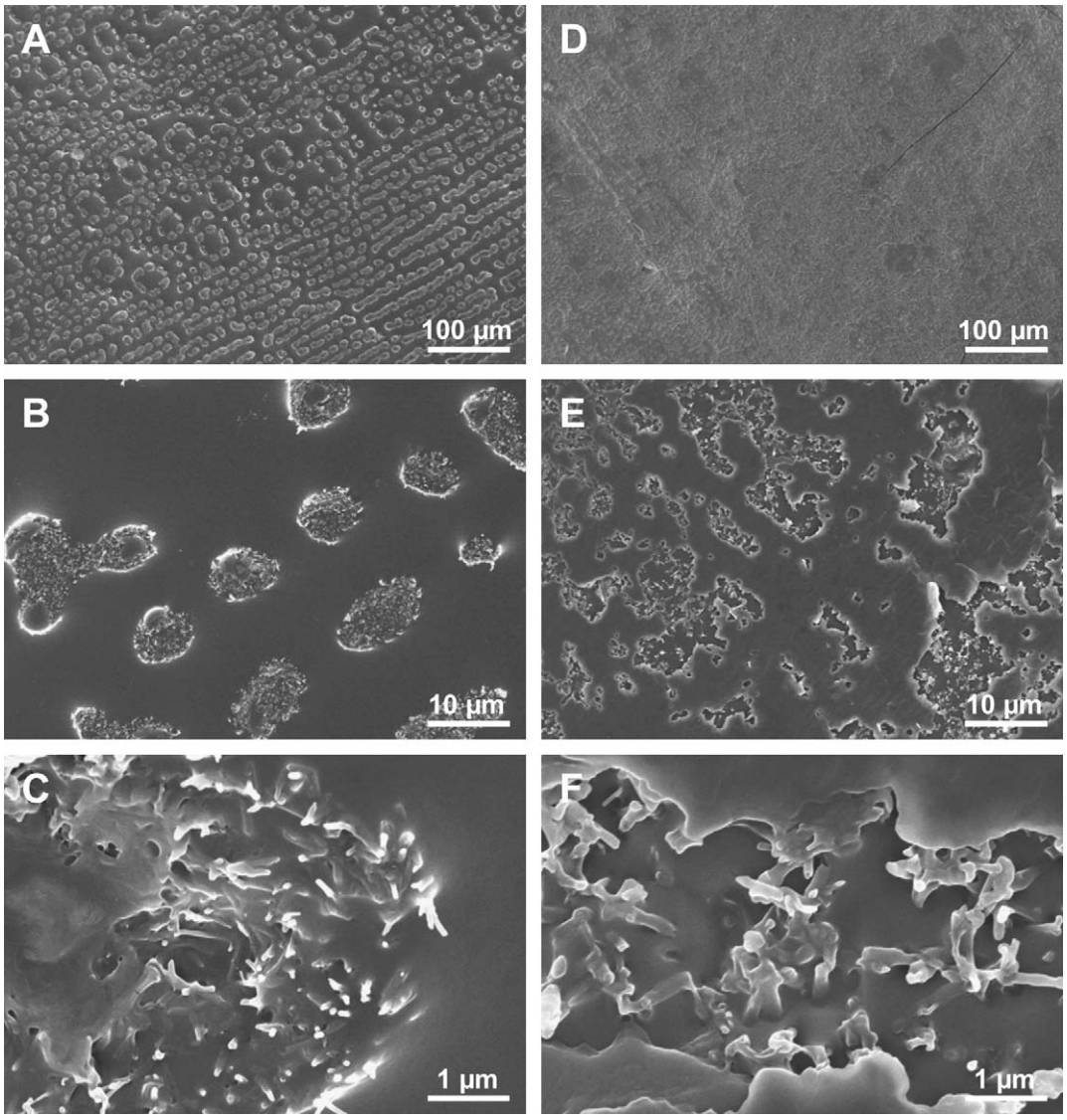

Fig. 4. Scanning electron micrographs of the lower surface of gum arabic films after they had been applied to *T. baccata* needle surfaces and peeled off. (A–C) Abaxial surface. (D–F) Adaxial surface.

predominated in both the epicuticular ($41.4 \pm 4.9\%$) and the intracuticular layers ($37.3 \pm 4.1\%$).

The chain length distribution of the fatty acids and primary alcohols differed substantially between the epi- and intracuticular wax layers on the abaxial side of the needle (Fig. 5A and B). In the epicuticular layer C_{20}–C_{32} fatty acids were present, the prevalent homolog being octacosanoic acid (C_{28}, 36%), while the series of primary alcohols showed a broad homolog distribution between C_{21} and C_{28}. In contrast, the intracuticular wax was found to contain fatty acids dominated by tetracosanoic acid (C_{24}, 34%), and primary alcohols with a very high percentage of docosanol (C_{22}, 48%).

On the adaxial surface, the compound class distribution pattern between the epi- and intracuticular layers was overall very similar to the abaxial surface. Gradients between both layers on the adaxial side existed for all compound classes ($P < 0.001$), most drastically for the secondary alcohols and diols. In contrast to the abaxial surface, here the secondary alcohol nonacosan-10-ol was found to dominate in the epicuticular wax ($27.3 \pm 2.1\%$), while the corresponding nonacosanediols played a minor role ($5.3 \pm 0.4\%$). Tocopherols were not detectable in the epicuticular wax, but accumulated in the intracuticular wax instead. Aromatic esters accounted for 48.8% of the intracuticular wax, while they accumulated to only 6% in the neighbouring epicuticular compartment.

Fatty acids with a broad chain length distribution (C_{20}–C_{32}), dominated by octacosanoic acid (C_{28}, 33%), accounted for 22% of the epicuticular wax of the adaxial surface, while only C_{20}, C_{22} and C_{24} fatty acids were detected in the intracuticular wax (Fig. 5C). The primary alcohol fraction of the epicuticular wax contained all chain lengths from C_{21} to C_{28}, dominated by docosanol (C_{22}, 25%), while only relatively short alcohol homologs were detectable in the intracuticular wax with an extremely high percentage of C_{22} (63%) (Fig. 5D).

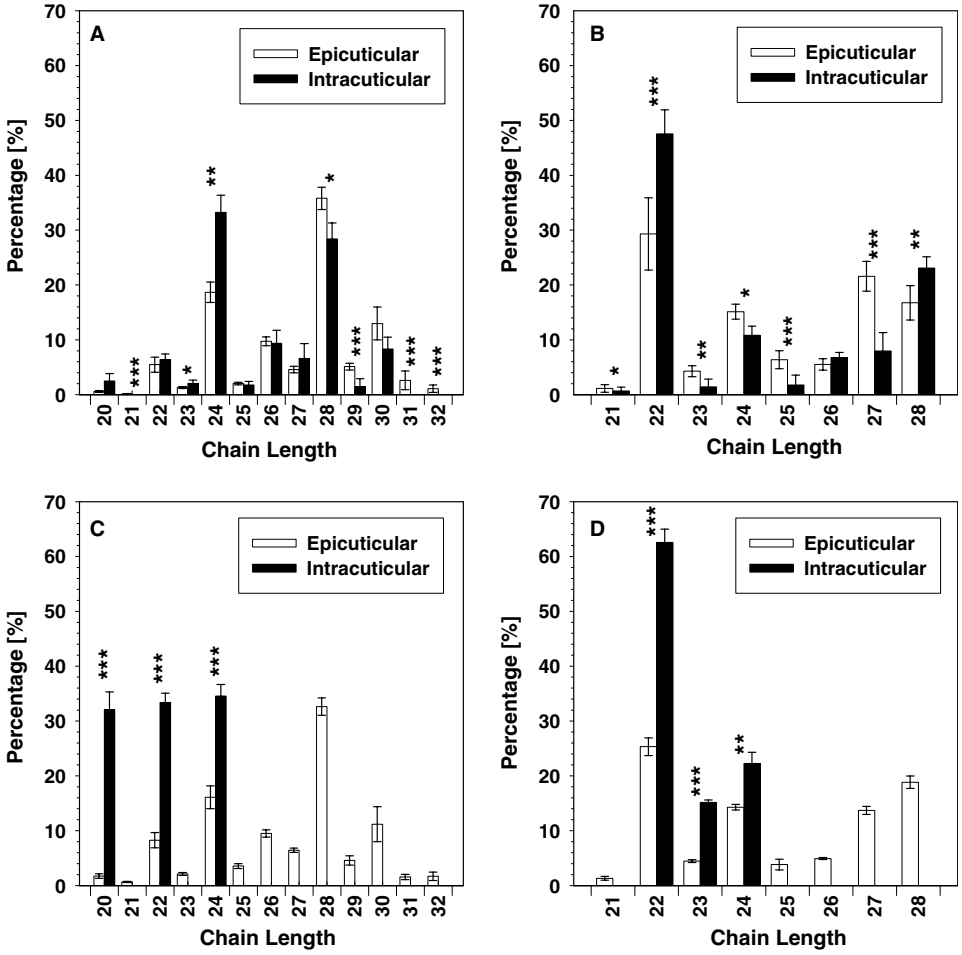

Fig. 5. Chain length distribution of individual wax components in the epicuticular and intracuticular waxes of *T. baccata* needles. Mean percentages of individual homologs ($n = 5$, ±SE) are shown for the series of: (A) abaxial fatty acids, (B) abaxial primary alcohols, (C) adaxial fatty acids, and (D) adaxial primary alcohols ($^{***}P \leqslant 0.001$, $^{**}P \leqslant 0.01$, $^{*}P \leqslant 0.05$).

Based on the current results, the chemical composition of the tubular wax crystals on both sides of *T. baccata* needles can now be judged. To this end, it is important to note that the sampling procedure proved to be selective for the epicuticular material, i.e., it successfully excluded the intracuticular wax. The epicuticular samples consisted of crystals, possibly together with (pieces of) the epicuticular wax film surrounding them on the plant surface. However, since the epicuticular crystals form a much thicker layer than the wax film, the tubules must dominate the material sampled by adhesive removal. Consequently, the composition of the gum arabic samples should accurately reflect the composition of the tubular epicuticular wax crystals. Our results for the first time give direct evidence that the major crystal components are a mixture of nonacosane-4,10-diol and nonacosane-5,10-diol on the abaxial surface, and nonacosan-10-ol on the adaxial surface of the needles. This finding is consistent with previous studies which, based on indirect evidence, concluded that the tubular crystals on other gymnosperm needles were dominated by secondary alcohols and/or their derivatives (Jeffree et al., 1975; Holloway et al., 1976; Jetter and Riederer, 1994; Jetter and Riederer, 1995; Jetter and Riederer, 1996). Together with the diols and secondary alcohol, relatively large amounts of fatty acids and smaller percentages of aldehydes, primary alcohols, alkyl esters, and alkanes co-crystallized. Our results show that the presence of wax components other than diols and secondary alcohols does not affect the formation of tubular crystals significantly.

In addition to the qualitative aspects discussed above, our results revealed quantitative differences between the compositions of epicuticular wax crystals on both needle surfaces. Most importantly, the nanotubules on the adaxial surface of yew needles were dominated by nonacosan-10-ol, while the corresponding crystals on the abaxial surface contained particularly high concentrations of *sec.sec.* diols. These differences in the chemical compositions of tubular crystals on adaxial and abaxial surfaces of *T. baccata* needles were reflected by differences in their sizes.

It is noteworthy that both the relative concentrations (%) and the absolute amounts (μg/cm^2) of nonacosan-10-ol were significantly higher in the epicuticular wax of both needle sides than in the corresponding intracuticular compartments. This compound, albeit present in the intracuticular layer, is clearly enriched in the surface wax. Coincident with crystal formation, the secondary alcohol establishes a

gradient from the inner parts of the cuticle to the outer layers. This confirms previous hypotheses, describing the formation of tubular wax crystals as a spontaneous phase separation, driven by the accumulation of secondary alcohol molecules near the outermost plant surface (Jetter and Riederer, 1994). A moderate capacity for the biosynthesis of this compound in the adaxial epidermis, aided by its spontaneous enrichment near the surface, thus suffices to accumulate the amounts necessary for crystal formation.

Surprisingly, we found that alkanediols were present at similar levels in the epi- and intracuticular layers of the abaxial wax. Thus, gradients between compartments in this case do not contribute to the accumulation near the surface, and to the crystal formation there. Instead, relatively high overall amounts of diols are biosynthesized and exported to the abaxial cuticle. The high capacity for alkanediol biosynthesis in the abaxial epidermis, without the aid of spontaneously formed gradients within the cuticle, is thus responsible for the accumulation of these compounds near the surface, and consequently for the formation of tubular crystals.

Earlier work had 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). The chain length distributions of fatty acids and primary alcohols especially in the waxes on the adaxial side of *T. baccata* needles show a similar trend. Although the epicuticular wax contained more fatty acids and primary alcohols, the intracuticular wax was found to have higher percentages of shorter chain-length homologs in both compound classes. Within a given compound class, representatives with shorter chain length are slightly more polar than the higher homologs. Hence, our observations on epi- and intracuticular chain length distributions support the hypothesis that polar components tend to accumulate in the intracuticular compartment.

Our results on yew needle waxes further revealed that tocopherols and phenyl esters accumulated to higher concentrations in the intracuticular wax layers. A similar gradient had been reported for triterpenoids in the waxes of *Prunus laurocerasus* leaves (Jetter et al., 2000) and of *Lycopersicon esculentum* fruits (Vogg et al., 2004). Triterpenoids, tocopherols and phenyl esters, all characterized by carbon ring systems, have molecular geometries largely differing from very-long-chain fatty acid derivatives. The cyclic compounds should therefore form solid phases separate from the predominant aliphatic wax constituents. As the cyclic compounds have slightly higher polarity than the open-chain hydrocarbons, it seems plausible that the separate alicyclic phases accumulate in the intracuticular compartment.

3. Conclusion

Our analyses of the surface waxes on needles of *T. baccata* for the first time provided direct evidence for the composition of tubular epicuticular crystals. The formation of these natural nanostructures was shown to be dependent on the accumulation of high amounts of the secondary alcohol nonacosan-10-ol and two alkanediol isomers on the adaxial and abaxial sides of the needles, respectively. These compounds crystallize together with diverse other wax constituents. The underlying intracuticular wax layers have relatively low concentrations of the compounds co-crystallizing at the surface. Crystal formation can be described as a spontaneous process driven by molecular characteristics of predominant compounds in the mixture, mediated through the formation of chemical gradients within the cuticle and local phase separation.

4. Experimental

4.1. Plant material

Twigs were harvested in spring from plants of *T. baccata* L. growing continuously on the campus of the University of British Columbia. Mature needles were cut from the twigs using razor blades. Batches of 30–40 needles were used for total wax analysis, while 5–8 needles were used for brushing extractions and gum arabic treatments. Five independent samples were taken with each method or combination of methods.

4.2. Mechanical wax removal

Gum arabic was employed as an 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 $CHCl_3$ to remove any soluble lipids and residues. An aqueous solution of the adhesive (1 g/ml) was applied onto the entire abaxial or adaxial surface of the needles using a small paintbrush. After 30 min, the solution was dry and a thin polymer film could be peeled off in pieces that were collected and extracted with $CHCl_3$ at room temperature. A defined amount of *n*-tetracosane was added to the extracts as an internal standard. Needle widths and lengths were measured using a sliding caliper, and the surface area was calculated assuming rectangular needle shape and flat surfaces.

4.3. Wax extraction

For total wax extraction, cut needles were immediately immersed twice for 30 s in $CHCl_3$ at room temperature. To selectively extract waxes from the abaxial and adaxial surfaces of needles, either of the two surfaces was brushed gently with fabric glass that had been pre-extracted (in a Soxhlet apparatus) and soaked with $CHCl_3$. A similar procedure was used to extract intracuticular waxes after mechanical removal of epicuticular waxes from the abaxial and adaxial surfaces. The needles were brushed more than 50 times per extraction, and three consecutive extractions were carried out to exhaustively sample the waxes. The

three extracts were separately collected and a defined amount of *n*-tetracosane was added as an internal standard. The resulting solutions were filtered, dried, and stored at 4 °C until they were analyzed.

4.4. Chemical analysis

Prior to GC analysis, $CHCl_3$ was evaporated from the samples under a gentle stream of N_2 while heating to 50 °C. Then the wax mixtures were treated with bis-*N*,*N*-(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma–Aldrich) in pyridine (30 min at 70 °C) to transform all hydroxyl-containing compounds into the corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was studied with capillary GC (5890N, Agilent, Avondale, PA; 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 mass spectrometric detector (5973N, Agilent). GC was carried out with temperature-programmed 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 the literature data. The quantitative composition of the mixtures was studied using capillary GC with flame ionisation 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. All quantitative data are given as means and standard errors. Statistical analyses were performed with SPSS 13.0 (SPSS Inc., USA).

4.5. Scanning electron microscopy

Samples consisting of untreated needles, treated needles (see above), and gum arabic films were air-dried ca. 12 h before mounting on stubs using double-sided adhesive tape. Specimens were then sputter coated with ca. 15 nm of Au/Pd (Nanotech, Manchester, UK) and investigated by SEM (Hitachi, Tokyo, Japan).

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