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EPICUTICULAR WAXES AND GLAUCOUSNESS OF *ENCEPHALARTOS* LEAVES

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Abstract—The epicuticular leaf waxes from four glaucous and four non-glaucous species of *Encephalartos* were examined by GC–mass spectrometry and SEM techniques. The four glaucous-leaved species, *E. horridus*, *E. lehmannii*, *E. princeps* and *E. trispinosus*, all occurring in xeric conditions in the Eastern Cape Province of South Africa, were conspicuous in having leaf waxes containing a series of secondary alcohols. In addition to 10-nonacosanol as the principal wax component, minor amounts of C_{25}–C_{31} 10-alkanols, 4,10-, 5,10- and 7,10-nonacosanediol and the ketone, 10-nonacosanone, were detected in these species. The adaxial leaflet surfaces of the glaucous species all showed a distinctive trabecular deposit of wax platelets when viewed microscopically. By contrast, secondary alcohols and ketones were absent in waxes from the non-glaucous species, and the surface of leaflets of these taxa were relatively featureless microscopically. Varying quantities of alkanes, fatty acids, primary alcohols, aldehydes and alkyl esters were present in all samples. Amongst the non-glaucous species, *E. altensteinii*, *E. natalensis* and *E. woodii* were similar in their wax composition, but differed from *E. villosus*. Alkanes were dominant in the latter species, which had high proportions of hentriacontane and tritriacontane in parallel with the most prominent oxygenated wax compounds, C_{32} and C_{34} fatty acids and aldehydes.

INTRODUCTION

The African endemic cycad genus, *Encephalartos*, comprises 54 species, most of which are considered endangered, vulnerable or rare; many are highly sought after for display in public and private living plant collections [1]. Ornamentally, the most popular taxa are those with a glaucous blue or blue-grey 'bloom' to their foliage, including especially a group of four closely related species (*E. horridus*, *E. lehmannii*, *E. princeps* and *E. trispinosus*), all occurring in xeric areas of high solar flux in the Eastern Cape Province of South Africa [2].

The function, morphology, chemical composition and chemotaxonomic usefulness of epicuticular waxes of a great many plant species have been investigated, and the environmental effects on wax composition, ontogenetic variation in wax composition and mechanism for wax biosynthesis are well documented [3–6]. The components of most plant waxes are compounds, such as *n*-alkanes, fatty acids, primary alcohols, esters and aldehydes, each of which usually occurs as a mixture of odd-numbered (for *n*-alkanes, secondary alcohols and ketones) or even-numbered (for other compounds) homologues. Because of the mutual depression of melting points and because of the randomness of orientation of molecules in the solid phase, it is unusual for the epicuticular waxy layer to show order or crystallinity. However, when there is a clear-cut dominance of one compound and especially when that compound does not occur as a mixture of homologues, the waxy layer will be deposited in an ordered microcrystalline structure [7].

The form and spatial distribution of crystalline wax deposits and, hence, the light-scattering effects which result in glaucousness, must thus be determined by the chemical composition of the wax exuded through the cuticle. Glaucousness has been variously correlated with the presence of high concentrations of β-diketones, C_{29} and C_{31} hydrocarbons and primary alcohols [3], while triterpene ketones and esters have also been implicated [8]. The latter observations have been confirmed by one of us (J.F.S.) in detailed studies of epicuticular waxes of the genus *Sedum*, where pruinose and glaucous taxa gave both higher total wax yields and higher proportions of triterpenoid esters than waxes from plants with shiny green leaves [9, 10].

In an earlier investigation of the epicuticular leaf wax

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Table 1. Leaf epicuticular wax composition of some *Encephalartos* species

Species	Leaf colour	Leaf wax composition*						
		Alkanes	Fatty acids	Primary alcohols	Secondary alcohols†	Aldehydes	Alkyl esters	Unidentified
E. altensteinii	Green	21	6	27	—	12	9	25
E. natalensis	Green	10	10	46	—	9	2	23
E. villosus	Green	87	2	1	—	2	1	7
E. woodii	Green	16	19	13	—	6	22	23
E. horridus	Glaucous/blue	11	9	7	50	6	3	14
E. lehmannii	Glaucous/blue	10	5	8	63	5	3	6
E. princeps	Glaucous/blue	2	—	12	63	8	2	13
E. trispinosus	Glaucous/blue	5	—	7	61	8	1	18

*In percentage determined by GC.

†Including 10-nonacosanone and a number of unidentified secondary alcohols/wax ketones (see Table 2).

hydrocarbons of *Encephalartos*, one of us found that the alkane profile typical for higher plants, with well-defined maxima for C_{29}, C_{31} and C_{33} homologues, was uncommon in this genus, and many taxa showed no discrimination between odd and even carbon numbers in their alkane distribution patterns [11]. The four Eastern Cape glaucous species, mentioned above, all had hydrocarbons with a skewed profile centred around C_{20}. This pattern, however, was not confined to glaucous taxa.

We now present the results of a more detailed chemical and ultrastructural investigation of the leaf wax composition of selected glaucous and non-glaucous representatives of the African cycads.

RESULTS AND DISCUSSION

Leaf waxes were obtained by brief immersion in $CHCl_3$ and separated into alkane and oxygenated wax fractions by column chromatography on silica gel. The alkane fraction was directly analysed by GC, while the second fraction was analysed by GC and GC-EI mass spectrometry before and after trimethylsilylation. The results of the chemical analyses are summarized in Tables 1 and 2, while illustrations typical of the SEM views obtained are shown in Fig. 1 (a–d). In evaluating the results, it must be noted that all samples were obtained from mature plants in a fairly small area of the Durban Botanical Gardens. Consequently, the eight taxa inspected are from an identical environment in terms of soil-type, irrigation, nutrition, temperature, light, humidity, etc. The only variable is thus the genetic profile of the individuals sampled.

The most conspicuous results in our analysis relate to the distribution of secondary alcohols, which we found uniformly abundant in all glaucous-leaved samples, but absent in all non-glaucous species. In addition to 10-nonacosanol, the principal wax component of the four glaucous-leaved species (ranging from 37 to 52% of the whole wax), minor amounts of five other 10-alkanols, three nonacosanediols and 10-nonacosanone were detected (Table 2). 10-Nonacosanol and the nonacosanediols are relatively common constituents of plant epicuticular waxes, occurring sporadically in angiosperms [12], but commonly in coniferous gymnosperms [7, 13, 14] and in *Gingko biloba* [7]. The compound 10-nonacosanol has also been reported in the leaf wax of the Japanese cycad, *Cycas revoluta*, where it exists together with nonacosane, 10-nonacosanone and 1-octacosanol, collectively at 4% of the total wax yield [15].

The green-leaved species are characterized by the predominance of homologous series of alkanes (n-C_{17}–n-C_{35}) [11], fatty acids (C_{16}–C_{34}), primary alcohols

Table 2. Composition of secondary alcohols and ketones from epicuticular waxes of *Encephalartos* species*

Compound	*E. horridus*	*E. lehmannii*	*E. princeps*	*E. trisinosus*
10-Pentacosanol	—	—	tr	—
10-Heptacosanol	2	1	1	1
10-Octacosanol	—	tr	—	—
10-Nonacosanone	3	2	4	6
10-Nonacosanol	73	82	71	69
4,10-Nonacosanediol	2	1	1	1
5,10-Nonacosanediol	6	2	4	6
7,10-Nonacosanediol	4	5	1	3
10-Triacontanol	1	1	2	2
10-Hentriacontanol	2	1	2	2
Unidentified†	7(1)	4(1)	15(3)	9(2)

*In percentage determined by GC. Key: — = absent; tr = trace (< 0.5%).

†Number of components in parentheses.

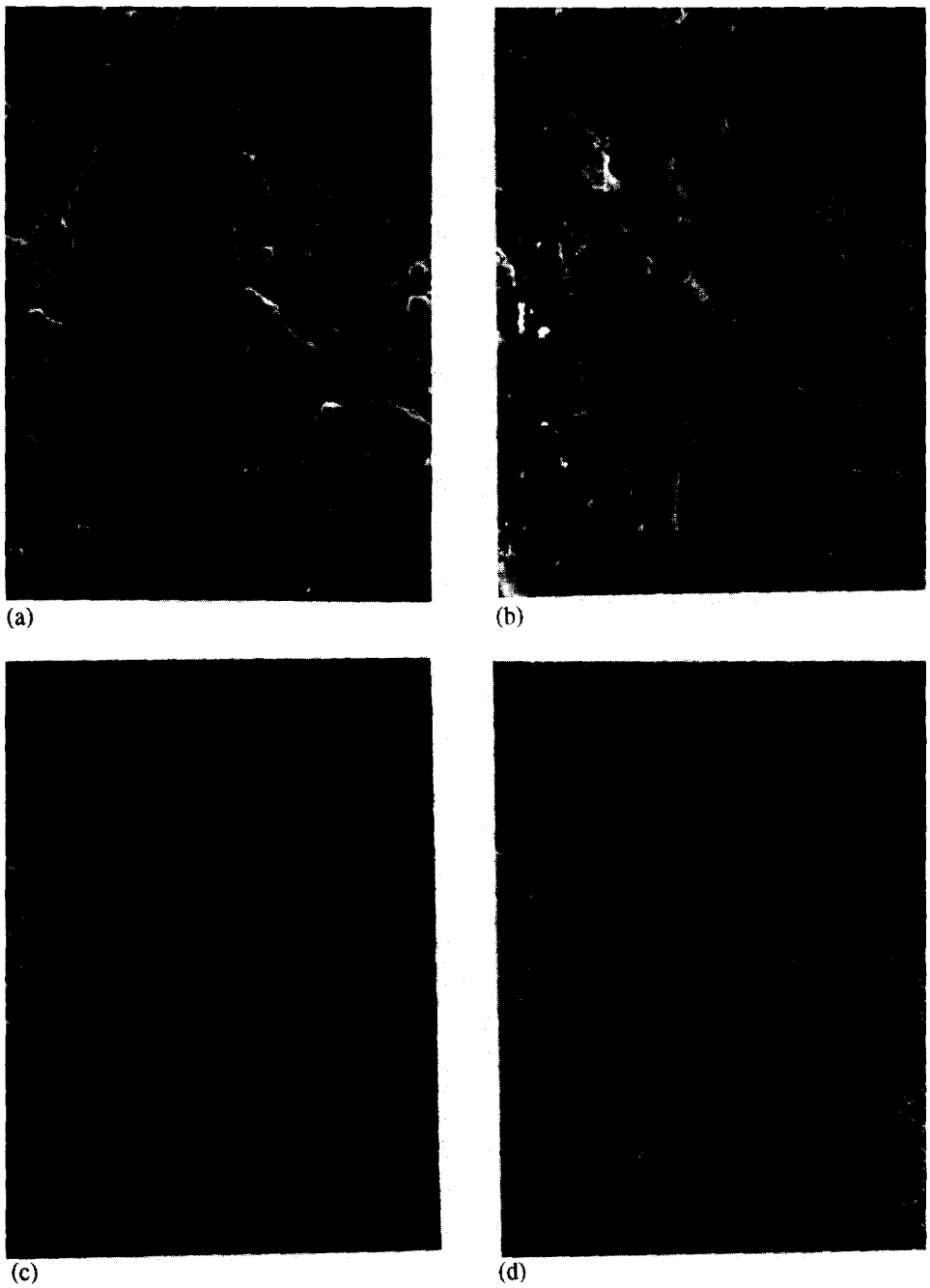

Fig. 1. SEM views of adaxial surfaces of *Encephalartos leaflets* (× 1000): (*a*) *E. altensteinii*; (b) *E. woodii*; (c) *E. lehmannii*; (d) *E. trispinosus*.

(C_{22}–C_{34}), aldehydes (C_{24}–C_{34}) and alkyl esters. The alkyl esters were characterized as esters of C_{20}, C_{22}, C_{24} and C_{26} acids. These homologous series of chain lipids were also present in varying proportions in the glaucous species (Table 1). Biosynthetically, they are formed by elongation reactions and reduction of fatty acids found both in gymnosperms and angiosperms. On the other hand, oxidative reactions leading to bifunctional lipids (α, ω-diols and ω-hydroxy fatty acids) and their esters, the estolides, are typical of gymnosperms [7].

The gymnosperm, *G. biloba*, seems to hold a distant position as its wax contains only metabolites of the 'reductive' type and, hence, shows a closer affinity to angiosperm waxes [7]. The only cycad previously investigated in any detail for its wax composition, *C. revoluta*, was reported to have a wax consisting mainly of acid and neutral estolides [15]. The components now identified in *Encephalartos* are all products of the 'reductive' pathway, but the additional presence or absence of epicuticular estolides remains equivocal. In our analysis, thin-layer chromatograms of the wax

samples showed a few bands in the estolide range (R_f 0.05–0.15, [13]), but these make up only a minor proportion of the total. The secondary alcohol band (R_f 0.61) was by far the most intense band in the chromatograms of the glaucous-leaved samples, whereas primary alcohols (R_f 0.33), fatty aldehydes (R_f 0.71) or alkyl esters variously produced the most intense bands in chromatograms of the green-leaved samples. All these observations are consistent with the GC results (Table 1). However, the alkyl ester bands were more intense than one would anticipate from GC data, possibly indicating that the samples also contain higher alkyl ester homologues not volatile enough for GC detection.

Within *Encephalartos*, wax composition has considerable taxonomic value at the species level. The fatty acids, primary alcohols and fatty aldehydes from waxes of *E. altensteinii*, *E. natalensis* and *E. woodii*, known to be closely related [2], are of similar chain length distribution (maximum centred at C_{26}–C_{30}), but that of *E. villosus* is quite different. In particular, the latter species has high concentrations of C_{31} and C_{33} alkanes, consistent with our previous report [11] and now seen to occur in parallel with chain length maxima at C_{32} and C_{34} for fatty acids and aldehydes. This is consistent with a common biosynthetic pathway terminating in a decarboxylation step to yield the respective hydrocarbons.

The adaxial leaflet surfaces of all glaucous-leaved samples exhibited a regular and distinctive honeycomb-like or trabecular deposit of wax platelets, above an amorphous layer, when viewed at $\times 500$ or greater magnifications [Fig. 1 (c)–(d)]. By contrast, the leaflets from the non-glaucous taxa were relatively featureless when viewed microscopically [Fig. 1 (a)–(b)]. The secondary alcohol, 10-nonacosanol, in the range 10–40% of the total wax, is often associated with tubular structures [7, 12, 16]. The glaucous-leaved samples in our study also exhibit a structured, but not identical, pattern. Since our non-glaucous samples were devoid of 10-nonacosanol, it is our suggestion that 10-nonacosanol is the agent responsible for glaucousness in *Encephalartos*.

EXPERIMENTAL

Plant material. Fully expanded leaves from accessioned mature specimens of 4 green-leaved South African cycads (*E. altensteinii* Lehm. [RO/4171], *E. natalensis* Dyer & Verdoorn [RO/4172], *E. villosus* Lem. [RO/4173] and *E. woodii* Sander [RO/4174]) and 4 Eastern Cape cycads with glaucous foliage (*E. horridus* (Jacq.) Lehm [RO/4175], *E. lehmannii* Lehm. [RO/4176], *E. princeps* Dyer [RO/4177] and *E. trispinosus* (Hook.) Dyer [RO/4178]) were sampled from the living cycad collections of the Durban Botanic Gardens, Durban, South Africa, on 5 January 1995.

Wax extraction and sample preparation. Individual unblemished leaflets were removed from the rachis and stored at −18° prior to analysis. Leaf waxes were removed by immersing *ca* 50 g of sample material twice in $CHCl_3$ for 15 sec at room temp. The extract was filtered to remove detritus, dried with Na_2SO_4 and the solvent removed *in vacuo*. The crude extract was fractionated on a 3.5×1.3 cm silica gel (40 μm particle size, J. T. Baker) column. The hydrocarbon fr. (fr. A) was eluted with 20 ml hexane after which the oxygenated constituents (fr. B) were eluted with 5 ml $CHCl_3$ and 15 ml $CHCl_3$–MeOH (1:1), successively. After addition of 0.3 mg cholesteryl benzoate as int. standard and solvent removal, the residues from both frs were dissolved in a vol. of $CHCl_3$ sufficient to give a suitable GC response. The solns were then analysed by GC and GC-MS. The oxygenated wax frs were re-analysed after trimethylsilylation. TMSi ethers and esters were prepd by reacting aliquots of B frs in 100 μl pyridine with 100 μl bis-(trimethylsilyl)acetamide (Janssen) for 1 hr at 70° before injection into the chromatograph.

Chromatography. B frs were chromatographed on silica gel TLC plates (Merck Art. 5715) with $CHCl_3$ containing 1% EtOH. Bands were visualized by H_2SO_4 charring at 160° for 15 min. For GC, a WCOT fused silica CP Sil 5 CB column, 10 m × 0.32 mm i.d. was used and the column programmed from 150° to 325° at 4° min^{-1}, then held at 325° for 20 min; injector temp. 250°, FID temp. 300°, carrier gas N_2 at 34 cm sec^{-1}, injection vol. 1–5 μl, split ratio 1:60. For GC-MS, 70 eV EIMS were recorded under similar conditions with He as carrier gas. Quantitation of alkanes and oxygenation components (as their TMSi ethers or esters) was by peak area integration of the FID signal without considering differences in detector response for each class of wax compounds. The alkane proportion of the total eluted material was determined on the basis of the int. standard peak areas in chromatograms of both frs.

Identification of wax components. Hydrocarbons, free acids, alcohols and aldehydes were identified by GC and GC-MS comparison with authentic compounds as available [9, 10]. Alkyl esters (RCO_2R') showed prominent fatty acids fragments at *m/z* 313 (C_{20}), 341 (C_{22}), 369 (C_{24}) and 397 (C_{26}) corresponding to the protonated acid ions $[RCO_2H_2]^+$, but intensities of $[RCO_2R']^+$, $[R\text{-}1]^+$ and $[R'CO]^+$ ions were too weak for identification of alcoholic components [17]. Secondary alcohols were identified by examination of their characteristic α-fragment ions in the GC-EI mass spectra of their TMSi ethers [17]. The *m/z* (rel. int.) values were: 10-pentacosanol, 313 (12), 229 (34), 73 (100); 10-heptacosanol, 341 (21), 229 (68), 73 (100); 10-octacosanol, 355 (24), 229 (48), 73 (100); 10-nonacosanol, 496 $[M]^+$ (0.2), 481 $[M\text{-}15]^+$ (3), 369 (74), 229 (70), 73 (100); 4,10-nonacosanediol, 541 (missing), 451 (2), 369 (33), 317 (9), 227 (5), 145 (18), 73 (100); 5,10-nonacosanediol, 527 (2), 369 (32), 317 (9), 159 (18), 73 (100); 7,10-nonacosanediol, 409 (21), 369 (30), 317 (2), 227 (40), 187 (29), 73 (100); 10-triacontanol, 383 (75), 229 (46), 73 (100); 10-hentriacontanol, 397 (66), 229 (43); 73 (100); 10-nonacosanone, 422 $[M]^+$ (0.5), 295 (10), 155 (15), 71

(100). GC-EI MS of the nonacosanediol bis-TMSi ethers were similar to literature reports [13, 18].

SEM. Representative portions of the fresh leaflet samples were coated with gold and viewed under a Hitachi S520 scanning electron microscope.

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