Chemical Composition and Morphology of Epicuticular Waxes from Leaves of *Solanum tuberosum*

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The chemical composition and morphology of leaf surface waxes of *Solanum tuberosum* were analysed using FT-IR, GLC and MS studies. There is a predominance of saturated and long chained components resulting in a crystalline wax layer in the form of ribbons. *n*-alkanes are a major constituent with C_{31} being prominent. Wax esters and *s*-alcohols form the other major components. Wax composition of five other solanaceous plants, viz., *Solanum eleagnifolium*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Datura stromonium* and *Solanum nigrum* were compared with those of potato using TLC techniques and do not show any qualitative differences.

Introduction

Aerial surfaces of all plants are covered by a thin impervious layer of epicuticular wax occurring mainly on leaves and fruits. A variety of functions have been attributed to this lipid layer which emphasizes its involvement in both physical and physiological processes occurring within the primary surface tissue [1−3].

While extensive studies are being carried out on insect-host plant relationships, there have been few attempts to study in detail the importance of leaf surface waxes to host selection behavior of insects. It has been shown in locusts and grasshoppers that leaf surface waxes influence host plant selection and that sensilla on the maxillary palpi perceive the stimulus. Leaf surface waxes of *Poa annua* promote biting activity in nymphs of *Locusta gregaria* [4]. Adults of the same species could also differentiate between waxes of *Poa annua* and *Bellis perennis* [5]. Certain plants are rejected by the grasshoppers, *Chorthippus parallelus* and *Chortoicetes terminifera*, following contact with the leaf surface by the terminal sensilla on the maxillary palpi [6, 7]. In locusts *(Schistocerca gregaria)*, the tarsal sensilla may play a similar role [8]. In *Locusta migratoria*, resistance to seedlings of sorghum is partly due to the presence of an antifeedant *p*-hydroxybenzaldehyde in the leaf surface waxes [9].

The ability to perceive and respond to leaf surface waxes has also been recorded in *Acyrthosiphon pisum* [10]; *Manduca sexta* [11]; *Choristoneura fumiferana* [12] and *Psila rosae* [13]. Leaf surface waxes also influence the searching behavior of a predaceous beetle, *Adalia bipunctata* where movement is restricted along the leaf edges or protruding veins [14].

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), feeds on potato and some other solanaceous species. The adults have a characteristic pattern of feeding behavior in which they tap the leaf surface extensively with their maxillary palpi before taking a bite [15]. The present study was undertaken to partially determine the chemical composition of leaf surface waxes of potato in order to identify components which are involved in host recognition by the adult beetle [15]. In addition, qualitative analysis was done on leaf surface waxes from five other solanaceous species, which have been used in behavioral studies [15], viz., *Solanum elaegnifolium*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Datura stromonium* and *Solanum nigrum* in order to identify differences in their chemical composition.

Materials and Methods

Plant material

Potato plants (variety: Norland) were grown in the field under normal agronomic conditions without the application of pesticides. Other plants viz., *Nicotiana tabacum*, *Solanum elaegnifolium*, *Lycopersicon esculentum*, *Datura stromonium* and *Solanum nigrum* were grown in 10 inch pots in the greenhouse. Leaves

were collected from 10 week old plants, put in polythene bags and brought to the laboratory for extraction of leaf surface waxes.

Scanning electron microscopy

Fresh leaves, and those from which wax had been removed by dipping in chloroform, were fixed in osmium vapour by placing leaves overnight in petri dishes containing 1% osmium tetroxide in phosphate buffer (pH 7.0). The leaves were air dried for 3 days, sputter coated with gold in a Nano-Semprep 2 and observed under a Cambridge SEM 250 scanning electron microscope operating at 20 Kv.

Extraction of wax

Leaf surface waxes were extracted by dipping leaves in chloroform for 10 seconds. Care was taken to that damaged leaves or any cut portion was not immersed in chloroform. Anhydrous MgSO4 was added to dry the crude extract which was then filtered. The extract was evaporated in a flash point evaporator (Buchi, Rotavapor R) and the crude was collected and weighed.

Fractionation

Detailed analysis were carried out only with potato leaf surface waxes. The procedure for separating wax into its constituents was done following the method described by Kolattukudy [16]. The crude wax (1.3745 g) was redissolved in a small volume of chloroform. A silical gel column (2 × 40 cm) was made using silicic acid (Biosil A, 100−200 mesh) dissolved in heptane. The redissolved wax was loaded on top of the column. The following solvents were then passed through the column:

n-hexane (redistilled)	200 ml
Benzene	400 ml
Chloroform	480 ml
Methanol	520 ml.

25 ml aliquots were collected using a fraction collector and concentrated to about 2−3 ml. TLC was done using glass microscopic slides. Benzene was used as the developing solvent and spots were obtained after heating slides sprayed with 50% sulphuric acid. Aliquots with the same R_f value were pooled together. Semipreparative TLC was done using silica gel coated plates (containing an indicator so that spots could be visualized under UV light).

Bands were eluted by scraping off the silica gel, dissolving extracts in chloroform, filtering and then spotting them on TLC plates. Using solvent systems like benzene:chloroform (7:3) and subsequently by chloroform:hexane (9:1), TLC was done till a single spot was obtained. Such samples were then analyzed by FT-IR, GLC and MS.

Fourier transformation infra-red spectroscopy (FT-IR)

FT-IR spectra were obtained with a Nicolet MX-1 spectrophotometer using a KCl cell.

Gas-liquid chromatography

The crude wax extracts from potato leaves and those from *S. elaegnifolium*, *S. nigrum*, *N. tabacum*, *D. stromonium* and *L. esculentum* were redissolved in chloroform and analysed by gas-liquid chromatography (GLC). The individual components of leaf surface waxes of potato, as obtained through semipreparative TLC, were also analyzed by GLC following the method described in Flore and Bukovac [17] and Tulloch [18]. 1 μl (10 μg/ml) of each sample was chromatographed using a Hewlett Packard Model 5830H gas chromatograph. Flow rate of the helium carrier gas was 50 ml/min. The column used was a 3 mm × 1.2 m stainless steel column containing 1% Dexil 300. The oven temperature was programmed to increase from 120 °C to 330 °C at a rate of 3 °C/min. The injection port and the hydrogen flame ionization detector temperatures were 325 °C and 330 °C, respectively. Chain lengths of *n*-alkanes were determined by comparisions of peak retention times with retention times of known hydrocarbon analytical standards (Analabs-New England Nuclear, Polychem Corp). Identification of other compounds were made specifically by analysis of the MS data as has been to elucidate the structure of a number of wax components [16, 19, 20].

Results and Discussion

Leaf surface waxes of potato are covered by a thick continuous layer of amorphous wax superimposed by a layer of crystalline wax in the form of ribbons (Fig. 1). Ribbons are the most common form of crystalline wax having been reported from a number of plants including *Fragaria* and *Rosa* sp. [21]. A ten second extraction of the leaves in
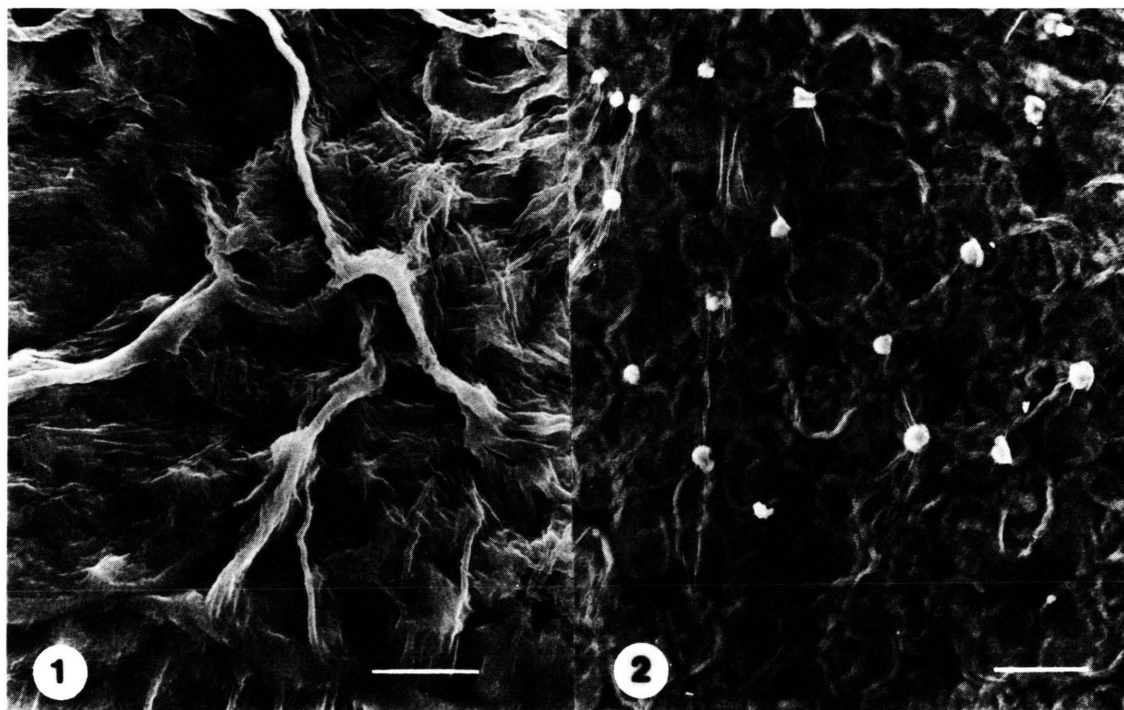
Fig. 1. SEM micrograph of the leaf cuticle of *S. tuberosum* showing distribution of crystalline wax in the form of ribbons (Scale = 10 μm).

Fig. 2. SEM micrograph of the leaf surface of *S. tuberosum* with the wax removed after treating with chloroform for 10 seconds (Scale = 40 μm).

chloroform was sufficient to remove epicuticular waxes (Fig. 2). Longer durations resulted in extraction of more than just the epicuticular waxes and the extract had a greenish tinge.

Wax yields from the six solanaceous plant species show sparse wax deposits. Table I lists the amounts of leaf epicuticular waxes extracted from the six solanaceous plants varying from 2.1 μg cm^{-2} in *S. nigrum* to 10.6 μg cm^{-2} in *S. elaegnifolium*. The leaves of many herbaceous plants like *Lactuca sativa*, *Spinacea oleracea* and *Beta vulgaris* have a thin wax layer with only 5−10 μg cm^{-2} [21] in contrast to leaves of *Allium porrum*, *A. cepa* and *Brassica* sp. which have a heavier deposit of 30−60 μg cm^{-2} [21]. Thicker deposits ranging from 60−300 μg cm^{-2} have been reported from leaves of *Ceratonia siliqua*, *Pistacia lentiscus* and *Olea europaea* grown under arid conditions [22].

The composition of epicuticular waxes from potato and those of other solanaceous species were analysed by TLC and compared with those of cabbage (Table II). The waxes were separated into bands representing 8 component classes which were identified with the help of FT-IR spectra and from comparison of data presented in Kolattukudy [16], Bukovac *et al.* [23] and Knowles and Flore [24]. In order of elution on TLC plates, the classes of constituents included *n*-alkanes, wax esters, ketones, *s*-alcohols, aldehydes, *p*-alcohols, ketols and fatty acids. There were no marked differences in the presence of major classes

Table I. Amounts of leaf epicuticular waxes from six solanaceous species.

Species	Amount [μg cm^{-2}]
S. tuberosum	5.4
S. elaegnifolium	10.6
L. esculentum	7.3
S. nigrum	2.1
N. tabacum	8.5
D. stromonium	3.9

Table II. R_f values of epicuticular wax components of six solanaceous plants compared with those of cabbage. Benzene was used as the developing solvent.

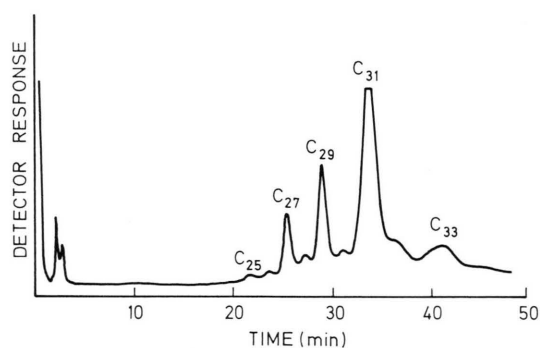| Component Class	B. oleracea	S. tuberosum	S. eleagnifolium	N. tabacum	L. esculentum	S. nigrum	D. stromonium
Alkanes	0.98	0.97	0.97	0.97	0.97	0.97	0.96
Esters	0.63	0.72	0.70	0.77	0.74	0.74	0.74
Ketones	0.54	0.55	0.55	0.55	0.70	0.63	0.54
Aldehydes	0.34	0.32	0.31	0.33	0.36	0.30	0.30
s-alcohols	0.28	0.27	0.25	0.25	0.26	0.27	0.27
Ketols	0.17	–	–	–	–	–	–
p-alcohols	0.11	0.11	0.13	0.11	0.12	0.11	0.11
Fatty acids	0.05	0.05	0.06	0.04	0.06	0.04	0.05

of compounds except for the absence of ketols in the solanaceous species investigated. The percent composition of the major classes of compounds in *S. tuberosum* is listed in Table III. *n*-alkanes form the principal component with about 39% of the total wax. Aldehydes esters and *s*-alcohols form the other major components.

Table III. Percent composition of component classes from leaf surface waxes of *S. tuberosum*.

Component Class	Per cent
n-alkanes	38.96
Ketones	5.91
Esters	19.63
s-alcohols	14.24
Aldehydes	8.55
p-alcohols	10.23
Acids	2.15

Fig. 3. GLC profile of *n*-alkanes isolated from leaves of *S. tuberosum*.

Hydrocarbons

The hydrocarbon fraction consisted of *n*-alkanes ranging from nC_{23}–nC_{33} with only odd carbon numbered homologues. FT-IR spectra of this fraction showed absorbance at 1460, 1470 cm^{-1} and at 2920–2841 cm^{-1} indicating the presence of hydrocarbons. No branched chain alkanes were detected. GLC analysis revealed the presence of nC_{23}–nC_{33} straight chain alkanes when compared with the retention times of *n*-alkane standards (Fig. 3). *n*-C_{31} was the predominant compound with C_{25}, C_{27}, C_{29} and C_{33} forming the other major components. MS data confirmed the presence of C_{25}–C_{33} *n*-alkanes with peaks corresponding to *n*-C_{25} (M$^+$ *m/z* 352), *n*-C_{27} (M$^+$ *m/z* 380), *n*-C_{29} (M$^+$ *m/z* 408), *n*-C_{31} (M$^+$ *m/z*

436) and *n*-C_{33} (M$^+$ *m/z* 464). A major component of surface waxes of potato is *n*-alkanes (32.96%) of which C_{31} is predominant. *n*-alkanes are often the major component of leaf surface waxes. In *Solandra grandiflora*, they account for 92% of the total wax with the highest molecular concentration per unit area [25]. Of the *n*-alkanes, C_{31} is the most common reported so far, being almost the only one in candelilla [3] and peas [16]. In tobacco, it is the predominant *n*-alkane along with odd chain *iso* (2-methyl) and even chain *anteiso* (3-methyl) compounds [26].

Primary alcohols

The primary alcohol fraction consisted entirely of even chain length homologues (C_{18}–C_{34}). FT-IR spectra of this fraction indicate absorbance at 1260–1000 cm^{-1} and at 1420–1330 cm^{-1}. Mass spectrum of this fraction gave diagnostic ions (M$^+$ −18) corresponding to C_{18} (*m/z* 252), C_{20} (*m/z* 280), C_{22} (*m/z* 308), C_{24} (*m/z* 336), C_{26} (*m/z* 364), C_{28} (*m/z* 392), C_{30} (*m/z* 420), C_{32} (*m/z* 448) and C_{34} (*m/z* 470).

Wax esters

FT-IR spectra of the unhydrolyzed fraction showed absorbance at 1735 cm^{-1}. Fragments corresponding to n-C_{42} (M^+ m/z 538) and n-C_{44} (M^+ m/z 566) wax esters could be identified. Peaks corresponding with R-OH fragments were observed at n-C_{22} (M^+ m/z 326), n-C_{24} (M^+ m/z 354), n-C_{26} (M^+ m/z 382), while those for $RCOOH_2$ were obtained for n-C_{18} (M^+ m/z 285), n-C_{20} (M^+ m/z 313).

Ketones

The ketone fraction separated by semipreparative TLC gave an FT-IR spectrum with absorbance at 1718 cm^{-1}. Fragments corresponding with n-C_{25} (M^+ -15 m/z 351) and n-C_{23} (M^+ -15 m/z 323) were obtained.

Secondary alcohols

FT-IR spectra showed absorption at 1195 cm^{-1} confirming the presence of s-alcohols. MS identification revealed fragments of n-C_{25} (M^+ -18 m/z 322) and n-C_{23} (M^+ -18 m/z 350).

Aldehydes

Absorbance at 1724 cm^{-1} of the aldehyde fraction confirmed the presence of aldehydes. Peaks corresponding with n-C_{22} (M^+ -28 m/z 324), n-C_{24} (M^+ -28 m/z 352), n-C_{26} (M^+ -28 m/z 380) and n-C_{28} (M^+ -28 m/z 408) were identified in the MS data.

Acids

The acid fraction consisted of even chainlength homologues ranging from C_{18}–C_{34}. FT-IR spectra revealed absorbance at 1760 cm^{-1}. Fragments corresponding to M^+−COOH were identified for n-C_{18} (m/z 239), n-C_{20} (m/z 267), n-C_{22} (m/z 295), n-C_{24} (m/z 323), n-C_{26} (m/z 351), n-C_{28} (m/z 379), n-C_{30} (m/z 407), n-C_{32} (m/z 435), n-C_{34} (m/z 463) and n-C_{36} (m/z 491).

In the present study, it appears that leaf surface waxes of potato consist of a mixture of compounds commonly seen in waxes of other plant species and which have been shown to be important in host selection in the few species of insects investigated [4, 9, 13, 15]. Although wax morphology is under genetic control [27, 28], the configuration, size and distribution of the crystalline waxes can be significantly modified by prevailing environmental conditions. In addition, wax morphology is closely associated with chemical composition.

Current concepts suggest that wax components are biosynthetically related. An elongation-decarboxylation hypothesis has been suggested for the biosynthesis of hydrocarbons in plants [29]. According to this hypothesis, the usual end product of fatty acid synthetase, palmitic acid (C_{16}) is the substrate for an elongation-decarboxylation system to which acetate (C_2) units are added until the chain length reaches C_{30} or C_{32}. Decarboxylation of this acid results in forming the major alkane. This is then further oxidised to form the major secondary alcohol and ketones. In *Brassica oleracea*, the principal n-alkane is C_{31} and the corresponding ketones and s-alcohols are also found [29]. However, in peas, with C_{31} as the major alkane only the corresponding alcohol is seen [16]. It has been shown however, that where there are mixtures of alkanes, the corresponding mixtures of s-alcohols are also present [30].

In the present investigation on leaf surface waxes of potato, the s-alcohols and ketones were observed to have identical chain lengths of C_{23} and C_{25}. The n-alkanes ranged from C_{23} to C_{33} in chain length. In addition, since the aldehydes are intermediates in the conversion of acids to alcohols [16], the aldehydes would be expected to resemble the alcohols. In leaf surface waxes of potato, the aldehydes (C_{22}–C_{28}) and the p-alcohols (C_{18}–C_{34}) are of sufficient length to have been derived by the reduction of fatty acids *via* the fatty acid elongation pathway. The p-alcohol chain length is similar to that of the alcohol moiety in wax esters thereby suggesting a common origin in which they participate in the sterification process. Variation in the fatty acid moiety of the ester indicates an origin from different sources – probably enzyme mediated as has been suggested in a number of plants [1].

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