Epicuticular Leaf Waxes of the Hop *(Humulus lupulus)*. Chemical Composition and Surface Structures

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The epicuticular wax layer of *Humulus lupulus* contains homologous series of hydrocarbons, wax esters, benzyl acyl esters, aldehydes, primary alcohols, fatty acids and the triterpenoids β-amyrin, α-amyrin both free and esterified with long chain fatty acids and also friedelanone. At 54%, primary alcohols form the largest component. No single class of lipids forms a predominant component (*i.e.* more than 80%) of the wax layer. Under scanning microscopical examination, both upper and lower surfaces of *H. lupulus* leaves appear covered by a continuous way layer which is devoid of any sculpturing or crystalloids. Epidermal cells are arranged in dense lamellate or undulate cuticular folds. Four different types of trichomes were identified on the surfaces of hop leaves and tendrils. The adaxial leaf surface bears numerous single, unicellular, silicified, pointed hairs as well as many glands. On the abaxial leaf surface there are very long pointed hairs arising only from the veins. Hairs on the hop tendrils arise from the veins but are completely different in shape having two sharp points extending at right angles to the hair-base.

Introduction

Humulus lupulus (L.) belongs to the family Cannabaceae of which *Cannabis sativa* is also a member. Both are dioecious plants [1, 2]. The hop is a climbing plant and the fruits of the female plant are an important product in beer brewing. Due to its commercial importance there is a vast literature covering hop cultivars, morphology and anatomy of hop leaves, fruits and seeds [3–7] while for *Cannabis sativa,* these have been described by Mahlberg *et al.* [8]. Also extensively described are: hop essential oils and resins [9–12] as well as its bitter and tannic substances [9]. The composition of hop leaf "headspace" volatiles and the role of certain components thereof in determining arthropod pest behaviour has recently been investigated [13].

The epicuticular waxes of plants differ in their yield and composition both between plant species and between organs of the same plant (flowers, seeds, fruits and leaves). Despite considerable diversity in the composition of epicuticular wax components, their common, primary function is maintaining the cuticle as a nearly impermeable membrane. This is a precondition for controlling the processes of transpiration and gaseous exchange *via* the stomata. In addition the epicuticular waxes form a defensive barrier against atmospheric influences, substances from within cells of the plant as well as from microorganismal pathogens and predatory animals of all sorts.

To date the epicuticular waxes of the hop plant have not been studied. In this paper the composition of the wax layer and the morphology of leaf surface structures of hops will be investigated.

Materials and Methods

Male plants of *Humulus lupulus* were cultivated in the garden of the Botanical Institute of the University of Cologne and were harvested in June 1992. Female plants were grown in the greenhouse (Bayer AG, Plant Protection Centre, Monheim).

Epicuticular waxes were extracted from male leaves (63 g) by immersing them in chloroform ($CHCl_3$) at room temperature (twice for max 1 min

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each). The wax extract (55 mg) was then separated into three fractions on a silica gel column, using the solvents *n*-pentane, 2-chloropropane and methanol (fractions 1, 2 and 3 respectively). Subsequently the constituents were analyzed by thin layer chromatography (TLC) with toluene as the solvent. Gas chromatography with a Flame Ionization Detector (FID-GC) was carried out on an OV-1 (10 m) fused silica capillary column [14, 15]. The column temperature was programmed from 140–320 °C as required.

Fraction 1 contains hydrocarbons, fraction 2 triterpenol esters, wax esters, benzyl acyl esters and aldehydes, fraction 3 triterpenoids, primary alcohols and fatty acids. The quantitative composition of the hop-wax is listed in Table I, the composition of the homologous series in Table II. The triterpenoids were identified by GC-MS spectra and are identical with fragmentation patterns found in earlier studies [14–16].

Leaves were found to comprise 18.95% dry-matter after heating at 105 °C for 3 h.

Air dried male leaves as well as female leaves and tendrils were prepared for scanning electron microscopical (SEM) examination by sputter-coating with gold using an Emscope sputter coater and were examined under a Hitachi S-405A scanning electron microscope at 25 kV.

Results

Wax composition

Hop leaves (63 g) contained extractable epicuticular wax (55 mg) accounting for 0.46% of dry weight. From this value 62 μg wax per cm^2 of leaf surface area or an average of 5229 μg for the whole leaf may be calculated. The wax of mature, male leaves consisted of homologous series of wax lipids (78%) and also triterpenoids (22%). The main classes of lipids are primary alcohols (54% wax), followed by fatty acids (13%), wax esters (6%) hydrocarbons (4%), aldehydes (1%) and traces of benzyl acyl esters (see Table I).

The hydrocarbon fraction consisted of *n*-alka-

Table I. Yield and composition of epicuticular leaf wax of *Humulus lupulus*.

	rf.	mg	% Wax	% Dry wt.
Hydrocarbons	0.75	2.0	4	0.002
Triterpenol esters	0.70	0.8	2	0.007
Wax esters	0.60	3.6	6	0.003
Benzyl acyl esters	0.55	0.1	0.2	0.0001
Aldehydes	0.45	0.5	1	0.004
Triterpenoids	0.06	11.0	20	0.09
Alcohols	0.06	30.0	54	0.25
Fatty acids	0.02	7.0	13	0.06
Wax extract		55.0	100.2	0.46

Table II. Composition of hydrocarbons, aldehydes, alcohols, fatty acids and wax esters of the epicuticular leaf wax of *Humulus lupulus* (peak area per cent).

Carbon No.	Hydrocarbons	Aldehydes	Alcohols	Fatty acids	Carbon No.	Wax esters
14					36	+
16				16.0	38	1.9
17				+	39	+
18				4.2	40	4.2
19				+	41	+
20		+	+	4.2	42	10.4
21	+				43	+
22	+	+	+	+	44	20.1
23	4.0				45	2.1
24	1.4	+	15.1	6.9	46	24.3
25	17.0			+	47	+
26	+	2.0	52.8	42.4	48	14.7
27	15.6				49	+
28	1.8	43.0	10.4	13.9	50	11.3
29	47.8			+	51	+
30	2.0	35.0	16.9	0.7	52	11.0
31	10.5				54	+
32	+	20.0	4.2	11.8		
33	+					
34			+	+		

nes with chain lengths ranging from C21 to C33 and nonacosane as the main component (47.8%) followed by pentacosane (17%), heptacosane (15.6%) and hextriacontane (10.5%).

Homologous wax esters (6%) of long chain fatty acids with long chain primary alcohols were present with chain lengths from C36 to C54. Esters with carbon numbers C42, C44, C46, C48, C50, C52 are present at concentrations of about 10 to 25%.

Traces of benzyl alcohol esters with long chain fatty acids of C20 to C30 were also found in hopwax. These esters were identified by their mass spectra showing characteristic fragments *m/z* 91 and 108. Similar homologous series have already been described, first in leaf waxes of Jojoba [17] and also in waxes of Fagus [18], Acer [19], Ginkgo, Magnolia and Liriodendran [16].

Aldehydes were found in smaller amounts (1%) with chain lengths ranging from C20 to C32, without a single component being clearly dominant.

Primary alcohols (54%), the main lipid class of hop wax, formed homologous series with chain lengths of C20 to C34. The main component is the alcohol hexacosanol (52.8%) followed by C24, C28 and C30 which in comparison, were present in far smaller amounts.

Fatty acids (13%) were present in homologous series from C16 to C34 with no main component dominating. C26 amounted to 42.4% of this fraction.

Triterpenoids accounted for up to 22% of the

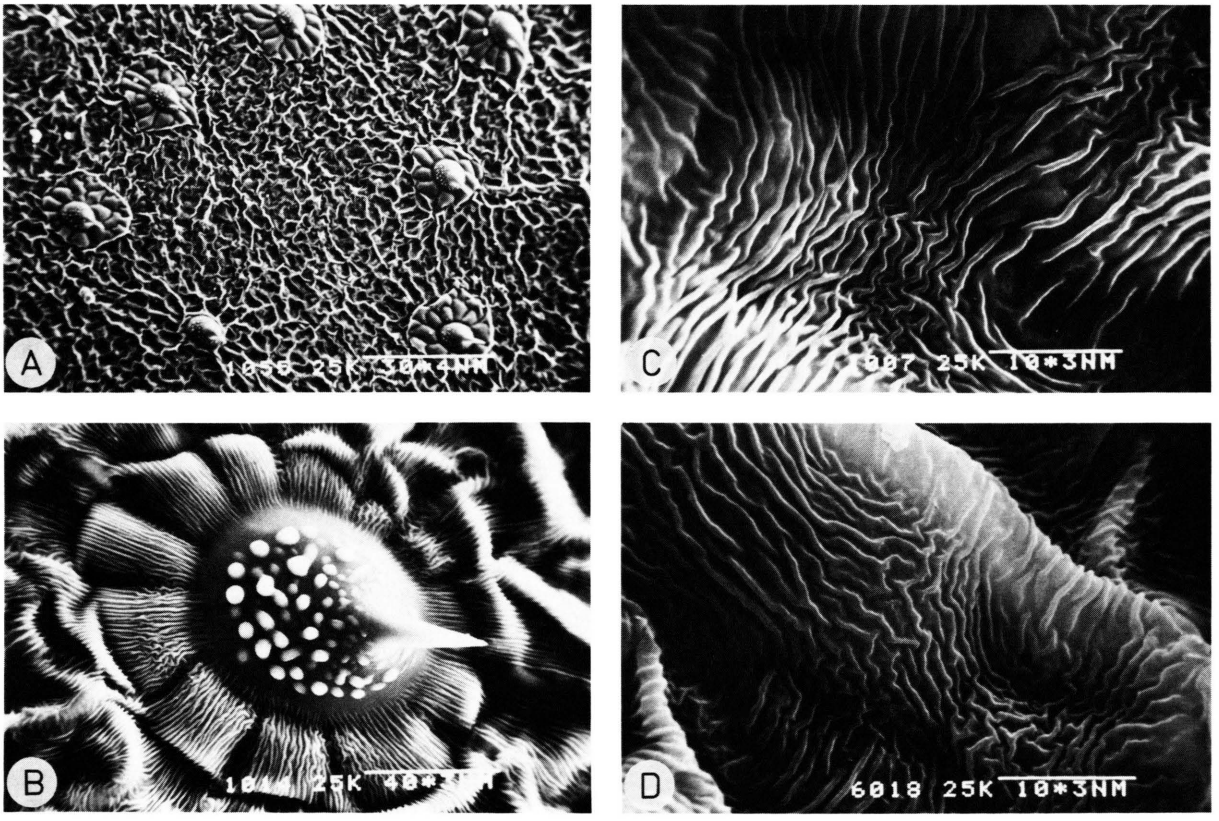

Fig. 1. Upper epidermal surface structures of a mature *H. lupulus* leaf.
A. Adaxial epidermis covered with numerous single sharply pointed hairs and glandular trichomes. Bar = 300 µm.
B. A single sharply pointed hair covered with micropapillae and surrounded by 10 to 12 basis cells. Bar = 40 µm.
C. The epidermal cells show numerous cuticular folds and a continuous wax layer without any sculpturing or crystalloids. Bar = 10 µm.
D. After washing the leaf with $CHCl_3$, the cuticular folds remained unaltered. Bar = 10 µm.

wax, a remarkably large fraction. β-amyrin and α-amyrin were identified by a positive colour reaction with carbazol, discrete R.T. values in the G.C. and characteristic fragments in the MS spectra. Both of these triterpenols were present in equal amounts making up about 4% of the wax each. They were also found in an esterified form with long chain fatty acids. These esters of α- and β-amyrin also gave a positive colour reaction with carbazol. An additional component accounting for some 12% of the wax mass was identified as friedelanone. This triterpenone shows no positive colour reaction with carbazol but was identified from its discrete G.C. retention time (R.T.) value and from characteristic fragments in its M.S. spectrum (M^+ 426) [14–16].

Surface structures

The upper and lower epidermal surfaces of mature hop leaves, harvested in June, show numerous cuticular folds covered with a continuous layer of wax. The surface wax layer showed neither sculpturing nor crystalloids when harvested at this time (Fig. 1 and 3). The wax layer could be completely extracted in $CHCl_3$ and analyzed as described above. Removal of the wax layer with $CHCl_3$ had no effect on the cuticular foldings, demonstrating that these lamellate or undulate folds consist not of wax but of cutin (Fig. 1D and 3D). The two surfaces of the hop leaf bear different kinds of trichomes. On the upper leaf surface there are numerous unicellular, sharply pointed, simple hairs

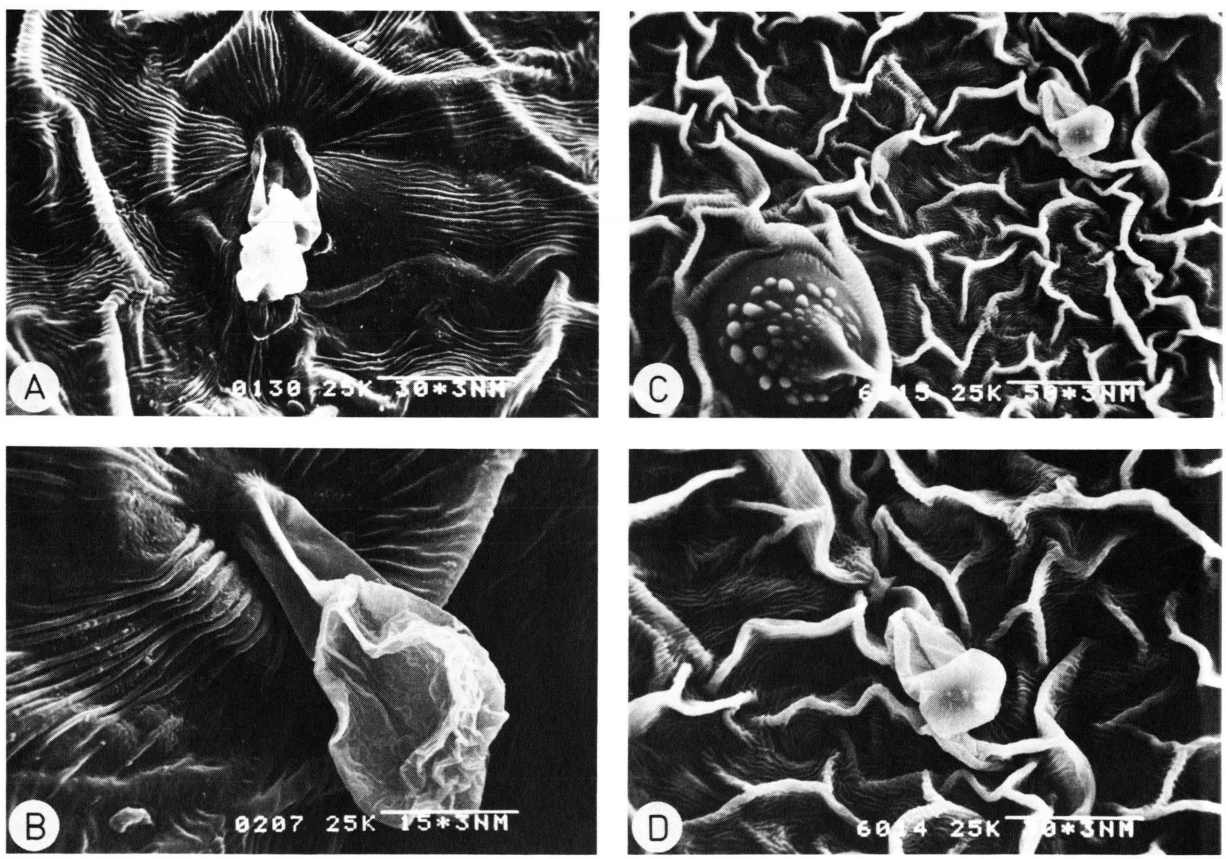

Fig. 2. Epidermal surface structures from the upper surface of a mature *H. lupulus* leaf.
A. Numerous glandular trichomes are also present on the upper leaf surface. This figure shows an empty gland. Bar = 30 μm.
B. Detail of a gland and its base on an epidermal cell. Wax sculpturing is superimposed on the wax layer. Bar = 15 μm.
C. After washing the leaf with $CHCl_3$, glandular trichomes are clearly visible and remained intact. Bar = 50 μm.
D. $CHCl_3$ washed leaf, glands at a greater magnification. Bar = 30 μm.

which are covered with micropapillae. These hairs are silicified (lithocysts) [3–5, 7] and are 80–100 µm in length. The "peaks" of these hair are 40 to 50 µm long and composed of a single cell. At its base this cell is surrounded by a ring of 10 to 12 basis cells (Fig. 1 A and B). Also present on the upper leaf surface are numerous glandular trichomes. These were much smaller than the pointed hairs, measuring 30 to 40 µm in length with a diameter of 10 to 15 µm (Fig. 2).

The pointed hairs of the lower leaf surface differ from those of the upper surface in their greater length (200–300 µm) and in that they arise exclusively from the morphologically distinct vein tissues (Fig. 3 A).

An entirely different type of hair was found on the surfaces of hop tendrils. These hairs also arise from the veins but, have two sharp points giving the hair the shape of an anvil (Fig. 4), presumably an adaptation to aid climbing during growth.

Discussion

Wax composition

Hop wax contains homologous series of wax lipids (78%) and also triterpenoids (22%). The former is to a large extent (54%) composed of the lipid class of primary alcohols. Of these hexacosanol is the dominant constituent, making up some 53% of the class. This proportion is however in-

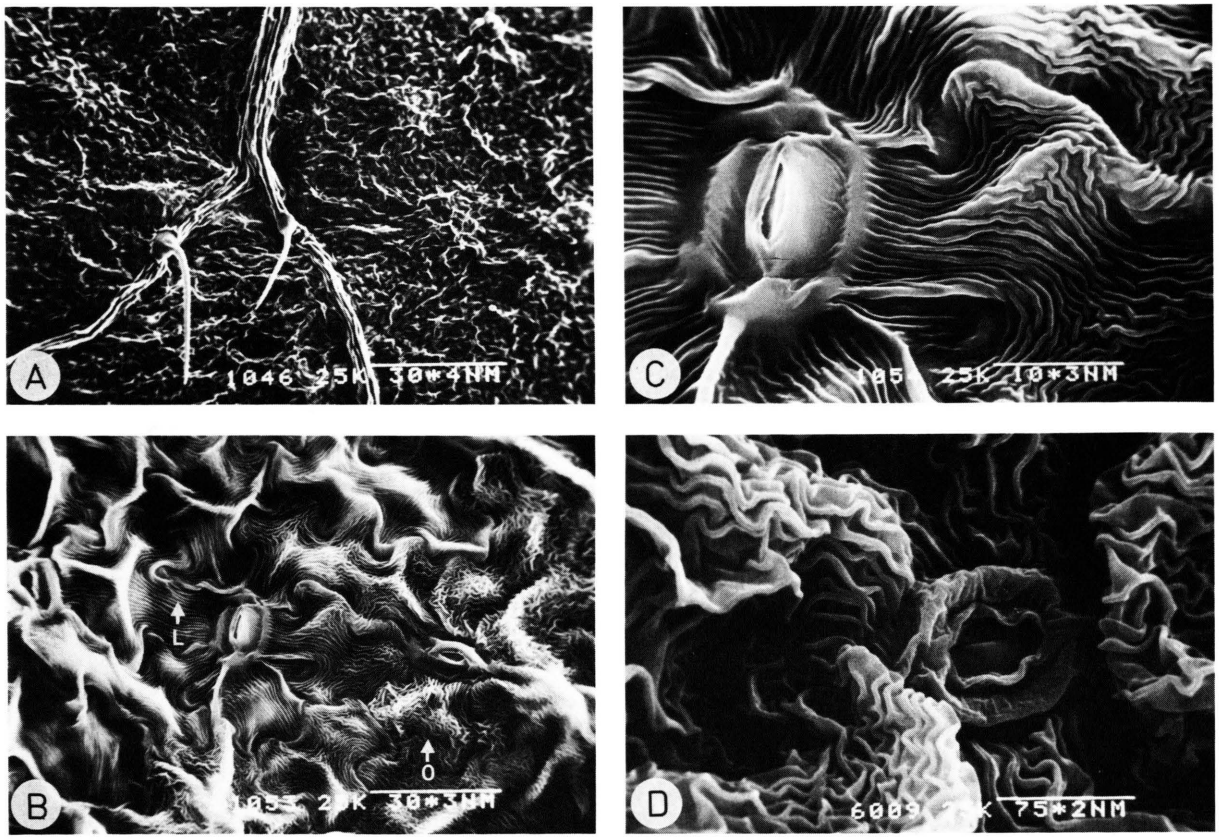

Fig. 3. Epidermal surface structures on the lower surface of a *H. lupulus* leaf.
A. Abaxial epidermis is rich in stomata; veins from which long pointed hairs arise are clearly visible. Bar = 300 µm.
B. Abaxial epidermal cells are covered with many cuticular folds of a lamellate (L) or undulate (O) type. Bar = 30 µm.
C. A stoma surrounded by many cuticular folds is visible. The structures are covered by a continuous wax layer, which is free from any sculpturing or crystalloids. Bar = 10 µm.
D. After washing the leaf with $CHCl_3$, the cuticular foldings remained unaltered. Bar = 7.5 µm.

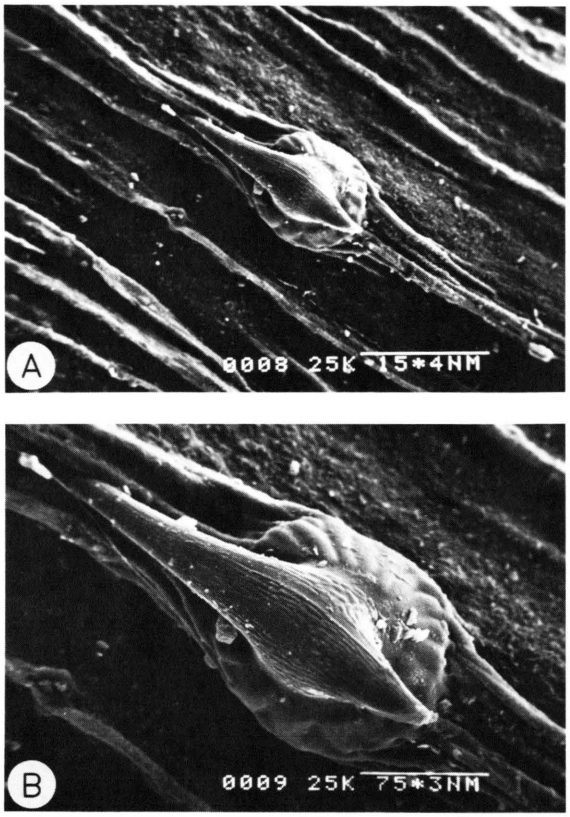

Fig. 4. Surface structures of a tendril from *H. lupulus*.
A. Veins bear hairs with two very sharp points. Bar = 150 μm.
B. A wax covered tendril hair at higher magnification. Bar = 75 μm.

sufficient to form wax crystalloids as has been shown in *Quercus robur* where alcohols account for 40% of the leaf wax and these are to 90% composed of tetracosanol leading to the formation of numerous plateletts [20, 21]. With respect to alcohols, the composition of hop wax has similarities with that of *Fagus sylvatica*. The waxes of this plant contain about 42% alcohols but no individual alcohol dominates and therefore no wax crystalloids were found on the leaf surface of *Fagus sylvatica* [22, 23]. All other lipid classes are present in smaller amounts with no single, main component dominating. This leaf wax composition suggests that no wax crystalloids could be expected on the hop-leaf surface.

Since hops are dioecious plants, we have studied the epicuticular waxes of both male and female leaves, but found no significant differences in wax composition between the sexes. The same results were found in a study of male and female leaves of Jojoba [17]. This also appears to be the case in Ginkgo [16].

Wax surface structure

The SEM figures of mature hop leaves harvested in June, show epidermal cells with numerous cuticular folds covered with a continuous wax layer devoid of any wax sculpturing or crystalloids. These observations are in agreement with the analytical results of the chemical wax composition. There is no substantial difference in the surface wax structures of upper and lower surfaces of the leaf (Fig. 1 and 3). Leaves harvested in July have a similar surface-wax structure to those harvested in June although there is a trend towards more sculpturing in the former (Fig. 2 B). This indicates continued synthesis of hexacosanol in rippening leaves and corresponds with similar observations made for *F. sylvatica* [22, 23]. Similarly numerous lamellate and undulate cuticular folds were found on the leaf surfaces of *Cannabis sativa* by [8] and also in *Aesculus hippocastanum* [24].

Trichomes

The various epidermal appendages borne on the surface of the hop leaf are collectively known as trichomes. From SEM figures it is clear that the hair-like trichomes are of three different types. Present on the upper leaf surface are silicified, unicellular, 80–100 μm-long, sharply pointed hairs which are covered with micropapillae. These hairs are surrounded by 10 to 12 basis cells.

On the lower leaf surface there are long pointed hairs of 200 to 300 μm in length. These hairs arise only from leaf veins.

A third kind of hair is found on hop tendrils. Veins bear a hair with two sharp points. These hairs are up to 300 μm in length.

The fact that all three hair types are sharply pointed may be an adaptation to the climbing mode of growth adopted by this plant. Additionally they may serve a defensive function for warding off animal predators.

Another type of trichome structure, which occurs only on the upper surface of the leaf, is the glandular trichome. These are small glands meas-

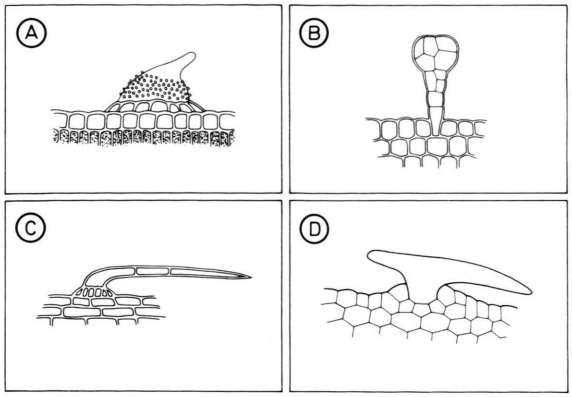

Fig. 5. Drawings of four types of hop leaf trichomes based on those of Holzner and Lermer [3].
(A) A lithocist hair.
(B) A "head-like" gland.
(C) A sharply pointed long hair (climbing hair).
(D) An "anvil"-shaped hair, with two points (climbing hair).

uring 30–40 µm in length. Mahlberg and co-workers [8] described similar glands for *Cannabis sativa*. These glands contain essential oils, the composition of which has been investigated in a number of studies [9–12].

All the above classes of trichome revealed by the present SEM examination of the hop-leaf, may be correlated with light-microscopical observations and drawings (Fig. 5) on hop-leaves and tendrils made by Holzner and Lermer [3, 4].

The cuticle of the hop plant forms the interface between the plant and the biotic and abiotic influences of its environment.

From the properties of hop epicuticular waxes described here, their primary, physiological function of enabling the control of evaporative water loss and gaseous exchange though stomatal opening and closing is clearly fulfilled. The secondary function as a defensive barrier is more difficult to quantify. The form that such a defence will take depends on the nature of the attack (biotic or abiotic influences) and on its source, *i.e.* whether from within the plant or from outside it. The rapid discolouration, presumably as a result of run-away oxidative processes, which is observed on dissolving the epicuticular wax layer from fresh hop-leaves illustrates the important role of the wax in regulating the intracellular environment of the leaf. From the outside, the hop plant must withstand environmental influences (*e.g.* precipitation, wind and intense solar radiation) as well as predation and parasitism from organisms ranging from viruses to higher animals. In commercially cultivated hops, the hop aphid *Phorodon humuli* and the red spider mite *Tetranychus urticae* are the main economically important animal pests, while the principal fungal diseases are mildew, caused by *Sphaerotheca humuli,* and false mildew *(Pseudoperonospora humuli)* [25, 26]. Both against the ingress of fungal spores and of arthropod mouthparts the epicuticular wax layer will provide a degree of physical defence. The complex chemical composition of the wax layer may however have a more specific role in protection. Terpenoids which as stated form a large fraction of the hop-leaf waxes, frequently have a behaviour-modifying (semiochemical) function on phytophagous arthropods; indeed the main component of the aphid alarm pheromone is β-farnesene [27] furthermore oviparous hop aphids secrete the cyclopentanoid monoterpine cis, cis Nepetalactol as a male attractant pheromone [28]. A role for terpenoids and other fractions of the wax as arthropod repellents and antifeedants is an interesting possibility. Likewise, a fungistatic function for leaf-wax components could also be considered.

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