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Composite structure of the crystalline epicuticular wax layer of the slippery zone in the pitchers of the carnivorous plant *Nepenthes alata* and its effect on insect attachment

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Summary

The slippery zone situated below the peristome inside pitchers of most carnivorous plants from the genus *Nepenthes* is covered with a thick layer of epicuticular wax. This slippery zone is reported to play a crucial role in animal trapping and prey retention. In *N. alata*, the wax coverage consists of two clearly distinguished layers. These layers differ in their structure, chemical composition and mechanical properties, and they reduce the insect attachment in different ways. The lower layer resembles foam, composed of interconnected membraneous platelets protruding from the surface at acute angles. The upper layer consists of densely placed separate irregular platelets, located perpendicular to the subjacent layer. Crystals of the upper layer bear small stalks, directed downwards and providing connections to the lower layer. These morphological distinctions correlate with differences in the chemical composition of waxes. The compound classes of alkanes, aldehydes, primary alcohols, free fatty acids, esters and triterpenoids occurred in extracts from both wax layers, but in different proportions. Chain length distributions in aliphatics were different in extracts from the lower and the upper wax layers. Waxes of the upper and lower layers exhibited different mechanical properties: wax of the lower layer is harder and stiffer than that of the upper layer. Moreover, crystals of the upper layer are brittle and may be easily exfoliated or broken to tiny pieces. Laboratory experiments using tethered insects showed that both wax layers reduce the attachment force of insects. It is assumed that a decrease in insect attachment on the two distinct wax layers is provided by the two different mechanisms: (1) crystals of the upper wax layer contaminate insects' adhesive pads; (2) the lower wax layer leads to a reduction of the real contact area of insects' feet with the plant surface.

Key words: trapping function, wax layer, microstructure, platelet, compound class, chain length distribution, hardness, elasticity modulus, friction force, pad contamination, real contact area, *Nepenthes alata*.

Introduction

Carnivorous plants, though rather restricted in number of taxa and habitats, have stimulated research to date because of their considerable accumulation of highly specialized adaptations related to arthropod capture, digestion and uptake of nutrients. The selection pressure directed to such adaptations arises from growth and survival of these plants in habitats deprived of such nutrients as nitrogen and phosphorus. The plants subsist by relying on digested arthropods to provide these nutrients, either partly or almost completely (Thum, 1988; Juniper et al., 1989; Schulze and Schulze, 1990; Schulze et al., 1997; Ellison and Gotelli, 2001).

To trap insects, pitcher-like leaves or analogous leaf arrangements have been evolved in carnivorous plants. Traps of this type developed repeatedly in the evolution of angiosperms and occur in rather unrelated taxa (Juniper, 1986; Barthlott et al., 2004). The pitchers are passive 'pitfall' traps, but may also contain devices for insect restraint and impedance. In representatives of Sarraceniaceae, for example, long hairs directed downward on the inner surface of pitchers enable such traps to act like lobster pots (or weir baskets), preventing insects from moving back once they have entered (Adams and Smith, 1977; Slack, 1983). Anti-adhesive surfaces within the pitchers covered by epicuticular wax (EW) crystals are another special adaptation. Such wax layers occur in *Brocchinia reducta* Baker, *Darlingtonia californica* Torr. (combined with downward-pointed hairs) and other carnivorous plant species, and are probably most developed in *Nepenthes* spp. (Juniper and Burras, 1962; Juniper, 1995; Gaume et al., 2002, 2004).

Within *Nepenthes* pitchers, surfaces having different

functions are arranged in distinct zones serving for insect attraction, retention, digestion and uptake of released nutrients (Juniper et al., 1989; Gaume et al., 2002). The slippery zone, located above the digestive one, is obviously crucial for successful trapping (Lloyd, 1942; Juniper and Burras, 1962; Juniper et al., 1989; Gaume at al., 2002, 2004). It is covered by a layer of crystalline EW. Insects are not able to get a foothold on this layer, they glide in the digestive fluid and are restrained from further escape. This function of the wax covering was first observed by McFarlane (1893) and then confirmed by others (e.g. Knoll, 1914).

The micromorphology of EW from the slippery (waxy) zone in *N. rafflesiana* was studied by Juniper and Burras (1962), using the carbon replica technique and inspection by transmission electron microscopy (TEM). The wax covering was shown to consist of two superimposed layers of EW crystals, with the upper layer composed of wax platelets arranged parallel to the surface and overlapping each other. The layer beneath was a complex network of partially fused crystals. Since micrographs of detached upper platelets often showed thin stalk-like structures, it was concluded that the platelets are arranged like roof tiles and fastened to the lower layer only by thin 'stalks' (Martin and Juniper, 1980). Insects moving on the upper layer would therefore readily cause detachment of the platelets and, because of this, would lose their footing.

Recent studies on *N. alata* pitchers using scanning electron microscopy (SEM) show the upper platelets oriented perpendicular to the surface (Gaume et al., 2002; Riedel et al., 2003). In wax isolation sequences employing both mechanical and dissolution steps, gradually changing wax compositions and polymeric aldehydes were found in the arrangement of EW crystals (Riedel et al., 2003). It is known that polymeric aldehydes are responsible for partial resistance of crystals to solvent attack (Haas et al., 2001). They probably also enhance the mechanical stability of EW microstructures. These components may therefore play an important role in the interaction of insect feet with the underlying wax.

The results presented so far are focused either on wax structure or on insect behaviour, and a convincing explanation of the anti-adhesive mechanism in the waxy zone is still lacking. The goal of this study was to combine investigations on wax micromorphology, chemical composition and mechanical properties with behavioural experiments using insects in order to explain the function of the EW layers in insect capture.

Materials and methods

Plant and insect species

The pitcher plant *Nepenthes alata* Blanco (Nepenthaceae) originates from mountain mossy forests in the Philippines (Jebb and Cheek, 1997). The plant specimens (pitchers) were obtained from plants grown in the greenhouse of the Botanical Garden at the University of Hohenheim (Stuttgart, Germany).

The two-spotted ladybird beetle *Adalia bipunctata* L. (Coleoptera, Coccinellidae) was chosen as a model insect species for friction tests because of its availability and appropriate size (body length=4.8±0.3 mm, mean ± S.D., *N*=20). Capture of beetles by the pitchers of several *Nepenthes* species has previously been reported (Kato et al., 1993; Moran, 1996; Moran et al., 1999).

The beetles bear adhesive pads, belonging to the hairy type of locomotory attachment devices in insects (Gorb and Beutel, 2001; Beutel and Gorb, 2001; Gorb, 2001). Adult insects were obtained from a commercial supplier (Katz Biotech Services, Welzheim, Germany) and kept in a small terrarium at 22–24°C, 40–60% humidity. Beetles were fed with a weak solution of honey in tapwater.

Micromorphological studies

Epicuticular wax layers

The micromorphology of the waxy zone was studied using SEM and TEM.

For SEM, small pieces (ca. 1 cm^2) of the pitcher wall were cut out with a razor blade from the middle part of the waxy zone, mounted on holders by means of conductive carbon double-sided adhesive tape, air-dried for several days, and sputter-coated with gold (10 nm). Pitcher samples were observed either intact or after treatment with chloroform for 20 s at room temperature, or at 60°C. Solvent-free removal of the upper EW layer was achieved by application of a two-component dental wax Coltène President light body (Coltène Whaledent Dentalvertriebs GmbH., Konstanz, Germany) for 5 min, peeled off after polymerisation. Detachment of the upper EW layer was similarly carried out using collodion films (see below). Intact and treated samples were examined in a Scanning Electron Microscope LEO 1530 VP at 3–7 kV. Crystal dimensions were quantified from digital images using SigmaScan software (SPSS).

Isolated wax crystals of the upper wax layer

Mechanically detached crystals were also examined by the carbon–platinum coating technique (Schwarz and Gorb, 2003). Wax crystals were collected by wiping off the inner surface of the pitcher using a soft paintbrush. The crystals were transferred onto the copper TEM slit, and covered with thin transparent pioloform film, by shaking the brush over the film. The crystals were coated with carbon–platinum at a shallow angle (about 15°). This method resulted in an increase of image contrast and better appearance of surface structures. The preparations were observed and photographed in a Philips CM10 TEM. This method enabled observations of wax crystals from the upper layer only.

Attachment organs of insects

To obtain detailed information about the attachment organs of the insect species used in the experiments, tarsi were cut from legs, fixed in 70% ethanol, dehydrated in an increasing series of ethanol, critical-point dried, mounted on holders, sputter-coated with gold–palladium (10 nm) and examined in

a Hitachi S-800 SEM at 20 kV. The density of hairs per 1 mm^2 of the pad area was quantified from digital images using SigmaScan software (SPSS).

To study the effect of wax surfaces on attachment pads, insects were allowed to walk for 1 min on each surface used in the friction experiments (see below). The beetle was transferred to a clean glass plate, and its legs were immediately cut off with a sharp razor blade and mounted on SEM-holders using conductive carbon double-sided adhesive tape. On each plant surface, at least three beetles were tested. Insects demonstrating cleaning behaviour of the tarsi were excluded from the further microscopic procedure. Preparations were air-dried, sputter-coated with gold–palladium, and examined in the SEM.

Epicuticular wax analysis

Prior to extraction of EW from the waxy zone, intact pitchers were immersed in hot chloroform (60°C, 1 min) with the solvent level kept carefully below the peristome in order to remove wax material from the upper surface of pitchers only. Afterwards, cylinders containing only the waxy zone were excised out of pitchers. To obtain EW from this zone, a two-step extraction procedure was employed by immersing cylinders first in cold (room temperature, 20 s) and then in hot (60°C, 20 s) chloroform. In other samples, the upper EW layer was obtained by application of highly viscous solution of collodion in acetone/*n*-amyl acetate (2:1, v:v). After evaporation of the solvent, resulting films were peeled off and wax attached to the films was extracted with cold chloroform (Haas and Rentschler, 1984). Areas from which the EW was extracted, were determined on cylinders dissected in longitudinal strips. The strips were taken by a CCD camera, and the areas were quantified from digital images using WinDIAS software (Delta-T Devices Ltd., Cambridge, UK).

Wax extracts were analysed by thin-layer chromatography (TLC) and gas chromatography (GC) as already described (Haas et al., 2003). The individual extracts were fractionated into compound classes by preparative TLC on silica gel G (Riedel-deHaen) with the two solvents: (1) *n*-hexane and (2) chloroform/*n*-hexane (75:25, v:v). Spray reagent for detection (UV 360 nm) was 0.005% primuline in acetone/water (80:20, v:v). Individual fractions were analyzed by GC using a Shimadzu GC-17A gas chromatograph (Kyoto, Japan) equipped with a CP-Sil 8 CB capillary column (25 m×0.32 mm, Varian-Chrompack, Middelburg, The Netherlands), on-column injector and FID. Operating conditions of the chromatograph were as follows: detector temperature, 360°C, linear velocity of helium carrier gas, 30 cm s^{-1}. The column temperature was initially set to 160°C for 2 min and then increased by 8°C min^{-1} up to 340°C (or 360°C for esters). The fractions of primary alcohols were analysed as trimethylsilyl ethers after derivatization with N,O-bis-(trimethylsilyl)-acetamide/pyridine (1:1, v:v). Free fatty acids were converted to methyl esters using BF_3/methanol (14%, w:w). Appropriate internal standards were employed for each fraction.

Table 1. *Composition of epicuticular waxes obtained by successive extraction steps or attached to collodion (upper layer) and then extracted with cold chloroform*

Compound class	Extraction steps 1	Extraction steps 2	Attached to collodion
Alkanes	0.7	0.4	0.6
Aldehydes	50.6	60.1	49.8
Primary alcohols	33.3	30.7	34.2
Free fatty acids	9.4	6.8	8.1
Esters	4.4	1.5	5.7
Triterpenoids	1.6	0.5	1.6

Composition is given as % compound class.
Extraction steps: (1) with cold chloroform, (2) with hot chloroform.

Data shown in Table 1 and Fig. 3 represent mean values of at least three independent samples, each analysed in duplicate. A fraction presumably containing triterpenoids was further analysed by gas chromatography and mass spectrometry (GC/MS).

Nanoindentation experiments with wax samples

Preparation of wax samples

For the first set of nanoindentation experiments, the samples of the waxy zone of the pitcher were prepared in the same way as for SEM study but without coating. Two types of samples were studied: (1) intact pitchers and (2) pitchers after solvent-free removal of the upper EW layer by application of the two-component dental wax (see above).

In the second set of experiments, waxes transferred onto a glass substrate were tested. (1) Wax of the upper EW layer was extracted with cold chloroform as described above. The solvent was evaporated and the residue applied onto glass coverslips (10 mm in diameter). (2) Wax from the lower layer was extracted with hot chloroform and transferred to the glass substrate as described above. Coverslips with waxes were attached to holders by means of double-sided adhesive tabs.

Nanoindentation experiments

Nanoindentation is widely used to determine material properties of a variety of materials and material systems. The layers under investigation were expected to be highly structured and compliant. In addition, the exact layer thickness and the properties of the substrate (for the measurements on the fresh plant) were unknown. Therefore, it was necessary to perform very sensitive tests to determine the first point of contact of the indenter tip with the surface and to select a method which provides information about the contact behaviour according to the indentation depth.

All tests were carried out using the Nano Indenter SA2 (MTS Systems Corporation, Oak Ridge, TN, USA). As an indenter tip, a three-sided diamond pyramid (Berkovich-shape) was used. During the indentation process, the tip was pushed

into the surface at a constant strain rate of 0.05 s^{-1} until the programmed depth limit of 1 μm was reached. Time, load and displacement were continuously recorded using the CSM (Continuous Stiffness Measurement) method, which allowed further determination of mechanical properties as a function of indentation depth in each single indentation test (Enders at al., 2004). With this extension, the system applies a load to the indenter tip in order to push the tip into the surface. Simultaneously, an oscillating force was superimposed with force amplitude several orders of magnitude smaller than the nominal load. Hardness and elasticity modulus were calculated after Oliver and Pharr (1992). For each sample type, 10 different samples were used, and on each sample an array of 20 indents was performed. In all, 800 indentation experiments were carried out.

Friction experiments with insects

To study the influence of waxy surfaces on the attachment ability of the insects, friction experiments were carried out using tethered walking beetles. Force measurements were performed using a load cell force transducer (10 g capacity, Biopac Systems Ltd., Santa Barbara, CA, USA; Gorb et al., 2004). Both males and females were used for tests. Prior to the experiments, the elytra were glued together with a small droplet of molten wax to make the insects incapable of flying. Each beetle was attached to a force sensor by means of a human hair (10–15 cm long) glued to the dorsal side of the insect thorax with a drop of molten wax. Plant samples (ca. 15 cm^2) were cut out of the waxy zone of fresh pitchers using a razor blade and then intact or treated samples were glued with double-sided tape to a glass plate. In the experiments, insects walked from the lower to the upper part of the zone, simulating the situation of escaping from the pitcher. The force produced by the insect moving on a horizontal test substrate was recorded. Force–time curves were used to estimate the maximal friction force generated by beetles. The behaviour of insect individuals during force experiments was observed and registered.

Three substrate types were tested: (1) intact waxy surface, (2) waxy surface treated with the dental wax, and (3) waxy surface rinsed in hot chloroform for 20 s. In addition, prior to each experiment on the test substrate, a control measurement was conducted on a glass plate used as a reference surface. Three pitchers from three plants were used. For each substrate type, experiments with 14–15 individual beetles were performed. In all, 44 insects were used and 88 individual tests (the test on a glass plate followed by the test on a plant substrate per insect) were conducted.

Results

Morphology of epicuticular wax layers and wax crystals

The EW coverage of the waxy zone is relatively dense and uniform. It consists of two clearly distinguishable superimposed layers of waxes, the upper and the lower, which differ essentially in their morphology (Fig. 1). The upper layer is composed of separate, clearly discernible, irregular platelets (terminology after Barthlott et al., 1998) covering the surface extremely densely and mostly contacting adjacent ones so that gaps are hardly distinguishable (Fig. 1A). Crystals are located more or less perpendicular to the subjacent layer and to the surface of the pitcher wall. Orientation of their planes along the axis perpendicular to the surface is rather random, and they form no clear pattern on the surface. Platelets vary greatly in size (the largest dimension=1.01±0.19 μm, mean ± S.D., N=20; thickness= 34.95±8.70 nm, N=20) and shape (from almost round to irregular-shaped with indistinct margins, e.g. oval, leaf-like, triangular, rhombic etc.; Fig. 1B).

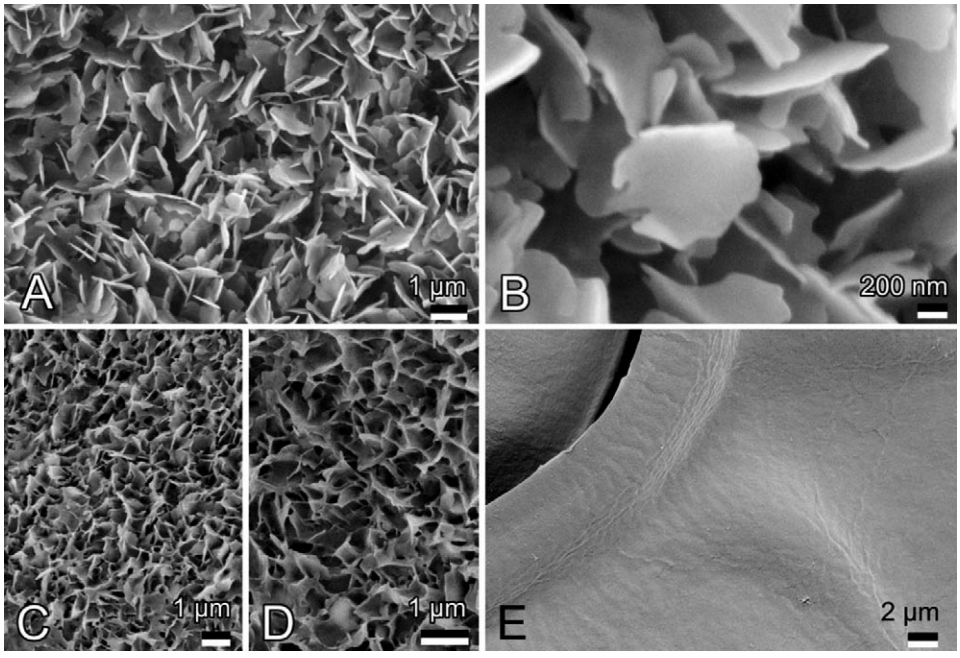

Fig. 1. SEM micrographs of the waxy zone. (A) The upper wax layer (intact surface). (B) Wax crystals of the upper wax layer (intact surface). (C) The lower wax layer (surface after treatment with cold chloroform; the upper wax layer is removed). (D) The lower wax layer (surface after application of the dental wax; the upper wax layer is removed). (E) Waxy zone after complete wax removal (surface after treatment with hot chloroform).

On TEM preparations of isolated wax crystals from the upper layer, platelets are often stuck together (Fig. 2A). Crystals consist of many layers orientated parallel to each other and to the crystal plane (Fig. 2C). Each platelet bears a ‘stalk’ situated in

the same plane as the crystal plate (Fig. 2A,C). In many cases, tiny pieces had broken off the platelets (Fig. 2B,C). Some crystals are partly exfoliated or even broken into several parts. This damage was the result of the preparation process, since a certain force was applied to brush the crystals mechanically from the plant surface.

The platelets of the upper layer were readily removed either by dissolving in cold chloroform or by application of the dental wax, which was stripped off with the platelets adhered to it. This was also achieved by use of collodion films. The lower layer resembles foam composed interconnected membraneous platelets (Fig. 1C,D). Cavities between platelets forming the cells of the foam are usually irregular in shape and vary considerably in size: length=0.65±0.17 μm, mean ± S.D., *N*=20; width=0.42±0.11 μm, *N*=20 (measured in the plane parallel to the pitcher wall surface). Crystals protrude from the surface at acute angles and show no clear orientation. They are mostly irregular in shape with pointed extensions, although almost oval or rectangular crystals also occur. These platelets are smaller that those of the upper layer: length=0.82±0.11 μm, *N*=20; width=0.53 ±0.11 μm, *N*=20; thickness=36.54±7.87 nm, *N*=20; all means ± S.D.). Complete removal of this layer may be achieved by the use of hot chloroform, resulting in a wax-free cuticle surface (Fig. 1E).

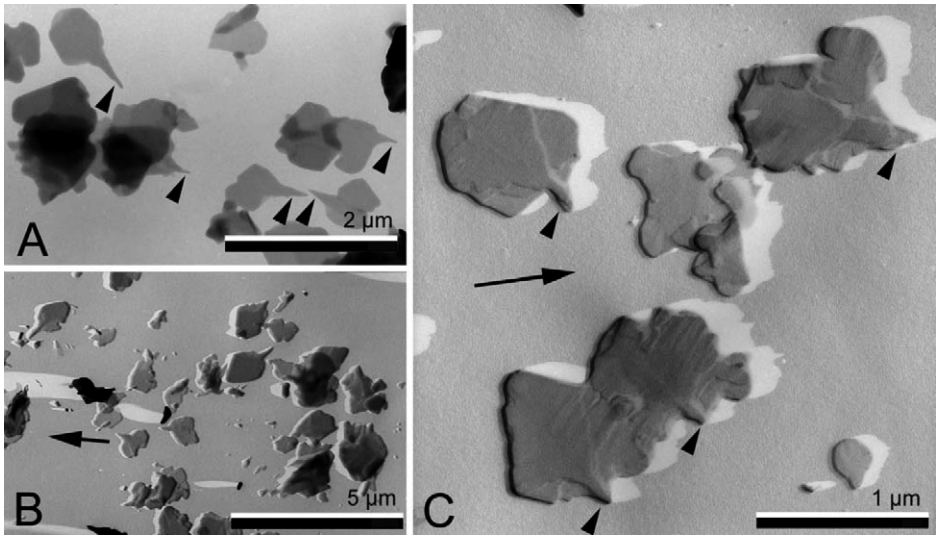

Fig. 2. TEM micrographs of the isolated wax crystals from the upper wax layer without coating (A) and after sputter-coating with carbon–platinum (B,C). Arrows in B and C show the direction of coating. Arrowheads in A and C indicate 'stalks' of wax crystals.

Chemical composition of epicuticular waxes

Treatment with cold chloroform dissolved the upper platelets; this extract amounted to 7.8 μg cm^{-2}. The second extraction step using hot chloroform removed the remaining lower wax layer, yielding an amount of wax=31.6 μg cm^{-2}.

The compound classes of alkanes, aldehydes, primary alcohols, free fatty acids, esters and triterpenoids occur in both wax extracts, but in different percentages (Table 1). Wax detached by collodion films (i.e. from the upper layer) showed relative amounts of fractions rather similar to those of the first extract. Aldehydes are the predominating compound class, especially in the second extract with hot solvent, where they represent about 60% of total wax. Primary alcohols contribute about 30% of

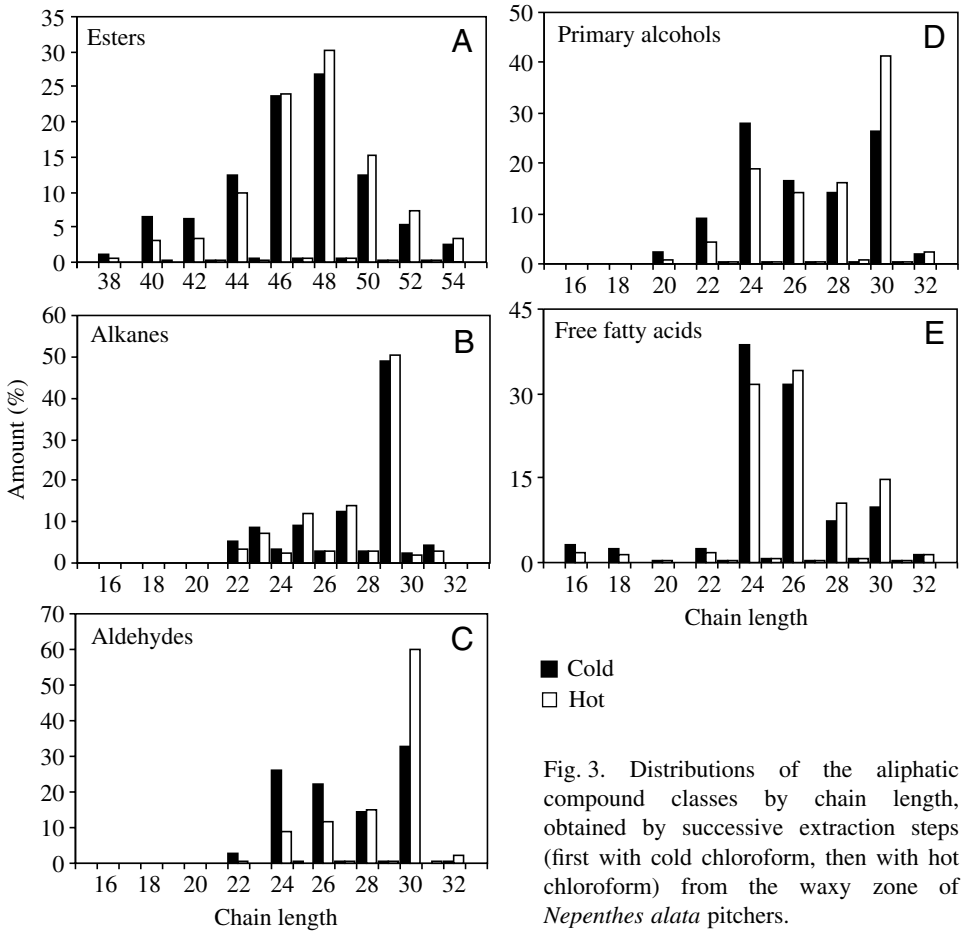

Fig. 3. Distributions of the aliphatic compound classes by chain length, obtained by successive extraction steps (first with cold chloroform, then with hot chloroform) from the waxy zone of *Nepenthes alata* pitchers.

Table 2. *Results of the* t*-tests for hardness and elasticity modulus of different wax samples obtained in nanoindentation experiments*

Comparison	d.f.	*t*	*P*
Hardness			
100 nm indentation depth			
Upper plant *vs* lower plant	38	–10.882	<0.001
Upper extract *vs* lower extract	38	0.286	0.776
500 nm indentation depth			
Upper plant *vs* lower plant	38	–15.649	<0.001
Upper extract *vs* lower extract	38	–2.255	0.030
1000 nm indentation depth			
Upper plant *vs* lower plant	38	–19.721	<0.001
Upper extract *vs* lower extract	38	–2.578	0.014
Elasticity modulus			
100 nm indentation depth			
Upper plant *vs* lower plant	38	–16.529	<0.001
Upper extract *vs* lower extract	38	0.096	0.924
500 nm indentation depth			
Upper plant *vs* lower plant	38	–29.491	<0.001
Upper extract *vs* lower extract	38	0.479	0.634
1000 nm indentation depth			
Upper plant *vs* lower plant	38	–36.764	<0.001
Upper extract *vs* lower extract	38	2.268	0.029

Upper plant, the upper wax layer of a fresh plant; lower plant, the lower wax layer of a fresh plant; upper extract, extracted wax of the upper layer; lower extract, extracted wax of the lower layer.

d.f., degrees of freedom; *t*, the *t*-test statistics; *P*, probability value.

Table 3. *Results of the one-way ANOVA tests for hardness and elasticity modulus of different wax samples obtained in nanoindentation experiments*

Indentation depth	d.f.	SS	MS	*F*	*P*
Hardness					
100 nm	3,79	146.820	48.940	116.632	<0.001
500 nm	3,79	23.112	7.704	232.706	<0.001
1000 nm	3,79	13.745	4.582	355.190	<0.001
Elasticity modulus					
100 nm	3,79	249.059	83.020	87.651	<0.001
500 nm	3,79	75.573	25.191	31.440	<0.001
1000 nm	3,79	165.928	55.309	39.798	<0.001

d.f., degrees of freedom; *F*, the ANOVA test statistics; MS, mean square; *P*, probability value; SS, sum of squares.

the wax mass. Free fatty acids occur in relative amounts of less than 10% with some deviation in the second extract. The other fractions are minor constituents. Triterpenoids consist of triterpenones (α- and β-amyrenone, taraxerone, ψ-taraxasterone) and probably methoxylated triterpenols.

Chain length distributions of the aliphatics do not differ from those usually found in plant waxes, i.e. odd-numbered homologues predominate in alkanes, with even-numbered ones in the other fractions (Fig. 3). Alkane profiles from the two subsequent extracts are relatively similar, having C_{29} as main constituent (Fig. 3B). The homologue distributions of both aldehydes and primary alcohols from the first extract are bimodal with maxima at C_{24} and C_{30} (Fig. 3C,D). In their profiles from the second extract, however, only the peak at C_{30} is pronounced. For the aldehydes, the relative amount of C_{30} represents about 60% of all homologues, being twice as high as that from the first extract (Fig. 3C). For the primary alcohols, the increase of C_{30} in the second extract is less marked (Fig. 3D). Free fatty acids also show bimodal profiles with the first maximum at C_{24} or C_{26} (Fig. 3E). In the second extract, a slight tendency towards higher chain lengths is seen. The same tendency was also found with esters (Fig. 3, compare A with E).

Taking into account the different proportions of compound classes and diverging profiles of the corresponding fractions, the two layers of EW from consecutive extraction steps are clearly distinguishable by their chemical composition.

Mechanical properties of the waxes

The nanoindentation data revealed significant differences between the deformation behaviour of the fresh intact plant surface and samples with the upper wax layer removed (Fig. 4A,B; Table 2). The data on hardness (H) and elasticity modulus (E) differed in these samples by more than one order of magnitude. The intact surface appeared much softer and more compliant than when the upper wax layer was removed. The hardness data scatter more widely for the intact surface, implying several 'breaking' processes during the deformation (Fig. 4A). After removal of the upper layer, such deformation behaviour was no longer seen. The curves shown in Fig. 4A,B represent the mean values of 200 indentation experiments performed on each sample type. However, for the intact surface, only the first 3–5 experiments on each sample could be used (30–50 indents), since after that the data showed an unusual deformation behaviour. We believe that this was due to a gradual contamination of the diamond tip with broken wax crystals. Reference measurements on fused silica (SiO_2), used as a calibration material in nanoindentation tests, showed that for the very first indent (Fig. 4C, Indent 1) on the silica after the series of tests on the wax surface, an atypical load–displacement curve was obtained due to the contaminated tip. Only after a deep imprint in silica did data represent typical values for the reference material (Fig. 4C, Indent 2). This effect might be explained by the tip cleaning during the first indentation on the reference material.

Comparison of the deformation behaviour in all measured samples showed significant differences between the extracted waxes applied on the glass substrate, and the samples from fresh plant surfaces (Fig. 5; Table 3). However, no difference was observed between the extracts from the upper and the lower layers (Table 2). Since the H and E values of wax samples were lower than those of the glass substrate (H_{glass}=4.3 GPa), the influence of the underlying material on indentation results can be excluded.

Table 4. *Results of the paired* t*-tests for maximal friction force generated by beetles* Adalia bipunctata *on experimental substrates*

Comparison	d.f.	*t*	*P*
Intact *vs* glass	13	7.208	<0.001
Treated with chloroform *vs* glass	14	3.166	0.007
Treated with dental wax *vs* glass	14	9.914	<0.001

d.f., degrees of freedom; *t*, the *t*-test statistics; *P*, probability value.

Substrates were glass, intact waxy surface, waxy surface after the dental wax application and waxy surface treated with hot chloroform.

Morphology of the attachment devices of the beetle Adalia bipunctata

The tarsus of *A. bipunctata* females consists of three segments (Fig. 6A). The pretarsus bears two claws curved ventrally. Attachment pads are of the hairy type. The ventral side of the two first proximal tarsomeres (1–2) is densely covered by tiny setae (1st tarsomere: ca. 11.5×10^3 setae mm^{-2}; 2nd tarsomere: ca. 22.5×10^3 setae mm^{-2}; Fig. 6B). The setae are more or less uniform, with curved flattened and widened tips (width=2.08±0.36 μm, mean ± S.D., *N*=35) called spatulae (Fig. 6C,D).

Attachment of insects on the waxy surface

Friction force generated by insects

For all three plant substrates tested, values of the maximal friction force generated by beetles differed significantly from those produced on the glass plate (Table 4). On the intact waxy surface and the surface treated with the dental wax (upper wax layer removed, lower wax layer remaining), the force was considerably lower than that on glass. On the surface treated with chloroform (wax removed completely), the measured force was higher than that on glass (Fig. 7A).

Since the friction force generated by different insect individuals varied greatly, even on glass (mean=2.635±1.314 mN, *N*=44), we used data, normalised to values obtained on glass, for comparison of different surfaces. For each individual, the force, obtained on a test plant surface, was compared to that on glass (considered as 100%).

The maximal friction force was significantly different for the substrates tested (Kruskal–Wallis one-way ANOVA on Ranks, $H_{3,56}$=47.105, *P*<0.001). The highest force was measured on the plant surface lacking wax (Fig. 7B). On the plant substrates covered with wax, insects generated the lowest force. There was no significant difference in the beetles' performances on waxy plant surfaces either with only one lower wax layer, or with both lower and upper wax layers. In comparison with glass, the force on the plant surface with wax completely removed was significantly higher, whereas on waxy surfaces, insects performed much worse.

Behaviour of insects on tested surfaces

Experimental insects performed well and showed normal locomotion on the glass plate and on the plant surface with wax removed. On both types of waxy surfaces, beetles usually were not able to get a grip and slid over the surfaces, refused to walk and came to a standstill or even turned over on their backs.

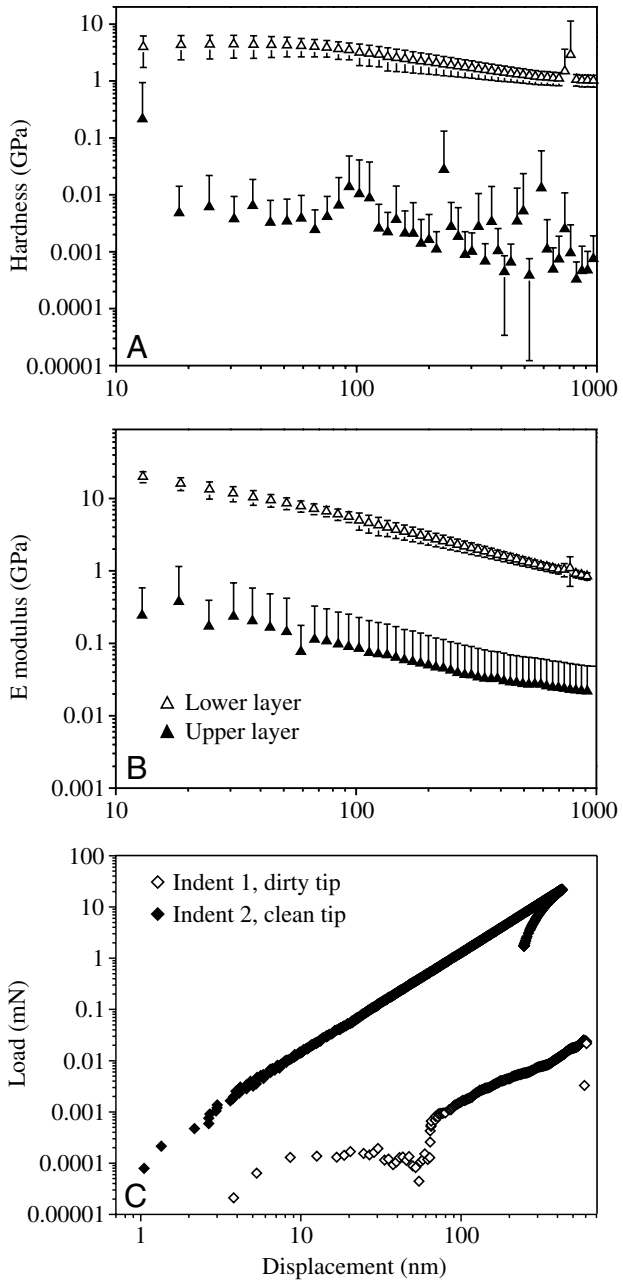

Fig. 4. Dependence of (A) hardness and (B) elasticity (E) modulus on indentation depth in the intact waxy surface containing both wax layers (filled triangles) and the surface treated with dental wax and bearing the lower layer only (open triangles). (C) The effect of contamination of the diamond tip. The curves in A and B represent mean values of 200 indentation tests performed on each sample type.

Adhesive pads of insects walked on different surfaces

SEM observation of adhesive pads in insects revealed the difference between insects walking on different surfaces. After experiments on both treated waxy substrates (when only the

upper wax layer or both wax layers were removed), the pads remained clean (Fig. 8A,B). In insects used in tests on the intact waxy surface, contamination of the tenent setae with wax crystals was observed (Fig. 8C–E).

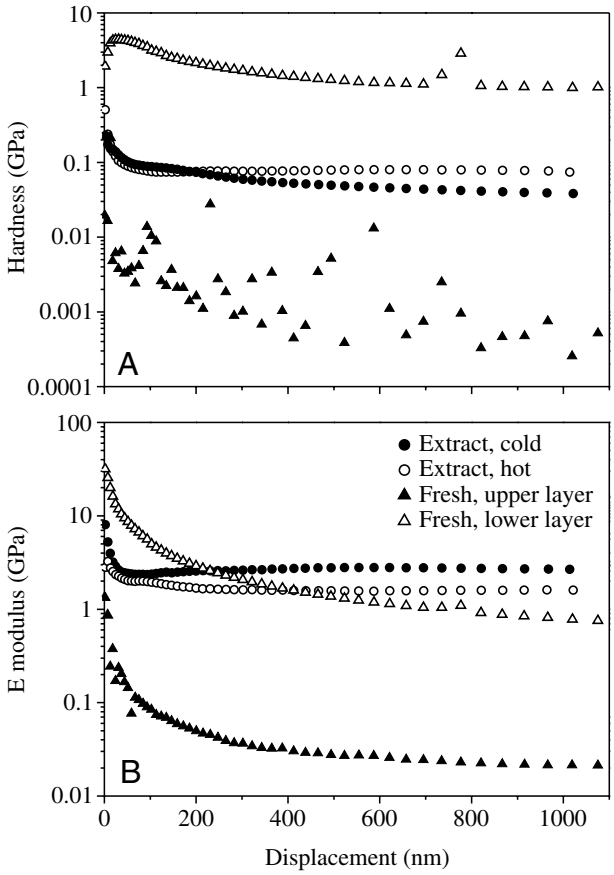

Fig. 5. Dependence of (A) hardness and (B) elasticity (E) modulus on indentation depth in the intact waxy surface (filled triangles), the surface treated with the dental wax (open triangles), and samples covered with wax material extracted with cold (filled circles) and hot (open circles) chloroform. The curves represent mean values of 200 indentation tests performed on each sample type.

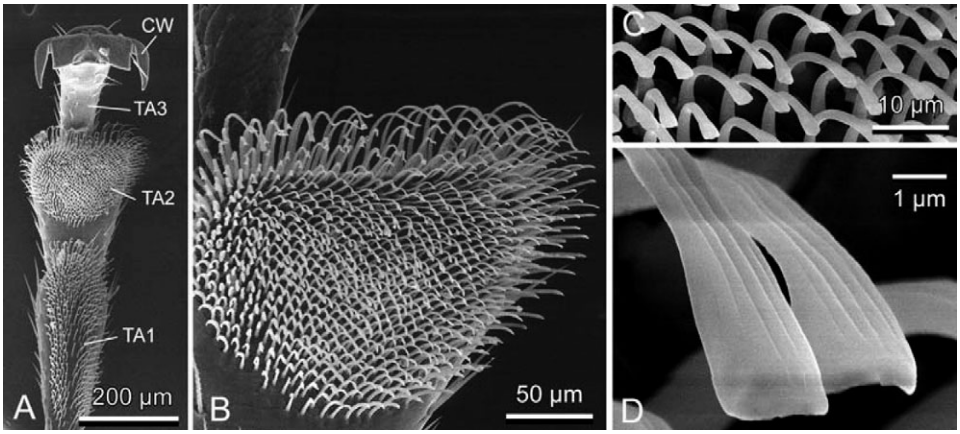

Fig. 6. Attachment organs of the female of *Adalia bipunctata* beetle, visualised by SEM. (A) Ventral aspect of the tarsus. (B) Setal covering of the second tarsomere. (C) Setae of the second tarsomere. (D) Setal tips (spatulae). CW, claws; TA1–TA3, tarsomeres.

Discussion

On the basis of our data concerning micromorphology, chemical composition and mechanical properties of the EW inside the pitcher of *N. alata*, and the results of friction tests with insects, the two superimposed layers of waxes can be clearly distinguished. These layers differ in their structure, chemical composition, hardness and elasticity, and they decrease the attachment force of insects in two different ways.

Micromorphology of the two superimposed wax layers

The upper EW layer comprises densely arranged platelets oriented more or less perpendicular to the inner pitcher surface. These platelets show, after mechanical detachment and examination in the TEM, particular characteristics of crystallinity. They are very fragile and break easily into tiny pieces. This fragility is rather uncommon among plant waxes, since EW crystals exposed to mechanical stress are usually rendered to a smear with almost completely disintegrated microstructure rather than to fragments (Neinhuis and Barthlott, 1997). The brittleness of the platelets, in contrast, seemingly reflects their high crystallinity. Further signs of crystallinity are provided by the microstructure. The platelets are built up by many layers oriented parallel to each other, which could indicate strata of crystal growth. Such crystallization of wax constituents as subsequent layers has recently been demonstrated in wax crystals of e.g. *Galanthus nivalis* (Koch et al., 2004).

The connection of the upper platelets with the subjacent EW layer remains uncertain. Due to their spatial orientation, points of contact are not visible in SEM images. ‘Stalks’ were never found projecting from the upper (i.e. visible) rims of the platelets. This implies that the ‘stalks’ should be directed downward and could well provide the connection to the lower layer. Our results from *N. alata* do not agree with earlier interpretations of the arrangement of these platelets (Martin and Juniper, 1970; Juniper et al., 1989). The upper platelets are not situated parallel to the surface like overlapping roof tiles. Additionally, earlier interpretations imply bending of the ‘stalks’ at right angles to provide contact with the lower layer. Signs of such bending, however, were never observed in the TEM images of isolated platelets.

On the other hand, the length of the ‘stalks’ (up to 0.5 µm), as found in some examples, would not be sufficient to extend downward to the very cuticle surface. However, since the ‘stalks’ probably represent the most fragile part of the platelets, they may act like preformed sites of breakage and would usually not

be preserved in full length after mechanical detachment. Therefore, the upper platelets often show only parts of the 'stalks', broken at different lengths.

The lower EW layer is composed of interconnected platelets, which do not have a preferred orientation and project from the surface at different angles. These crystals resisted low applied forces, e.g. brushing well. Complete removal of this layer may be achieved mechanically by applying higher shear forces or by the use of hot chloroform.

These morphological distinctions in the two wax layers correlated with differences in chemical composition of waxes.

Comparison of the chemical composition of waxes

The lower EW layer is not soluble in cold chloroform. Chloroform at this temperature would be sufficient for complete dissolution of wax crystals if only monomeric constituents are present (Holloway, 1984; Walton, 1990). Our results show that this treatment offers the opportunity to expose the lower layer, since its micromorphology is indistinguishable from that disclosed by removal of the upper platelets, peeled off adhering to the dental wax or collodion. Hence, wax of the two layers can be isolated selectively by subsequent dissolution steps using chloroform at different temperatures. The upper platelets are dissolved in cold chloroform and the lower layer in hot chloroform. Furthermore, the composition of the first extract closely resembles that of the upper layer adhered to the collodion film. The reduced solubility of the lower layer coincides with the presence of polymeric aldehydes (Haas et al., 2001). The polymeric aldehydes are suspected to be responsible for dissolution resistance against cold chloroform and the structural integrity of crystals exposed to this treatment. The depolymerization of polymeric aldehydes using hot chloroform usually leads to increased aldehyde contents in the extracts obtained. This holds true for the subsequent extraction of wax from *N. alata* pitchers, since the

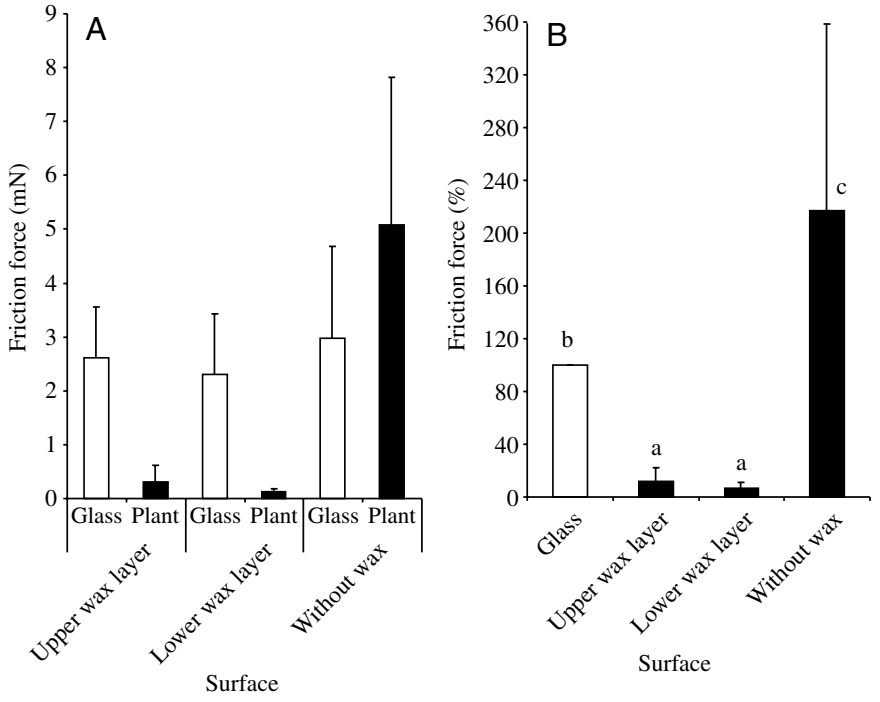

Fig. 7. Maximal friction force generated by beetles *Adalia bipunctata* on test surfaces. (A) Comparison of raw data. (B) Comparison of normalised data. For each insect individual, the force produced on a plant substrate was compared with the force on glass, considered to be 100%. According to Dunn's test of multiple comparisons of means, performed after ANOVA, means with different letters differ significantly from each other.

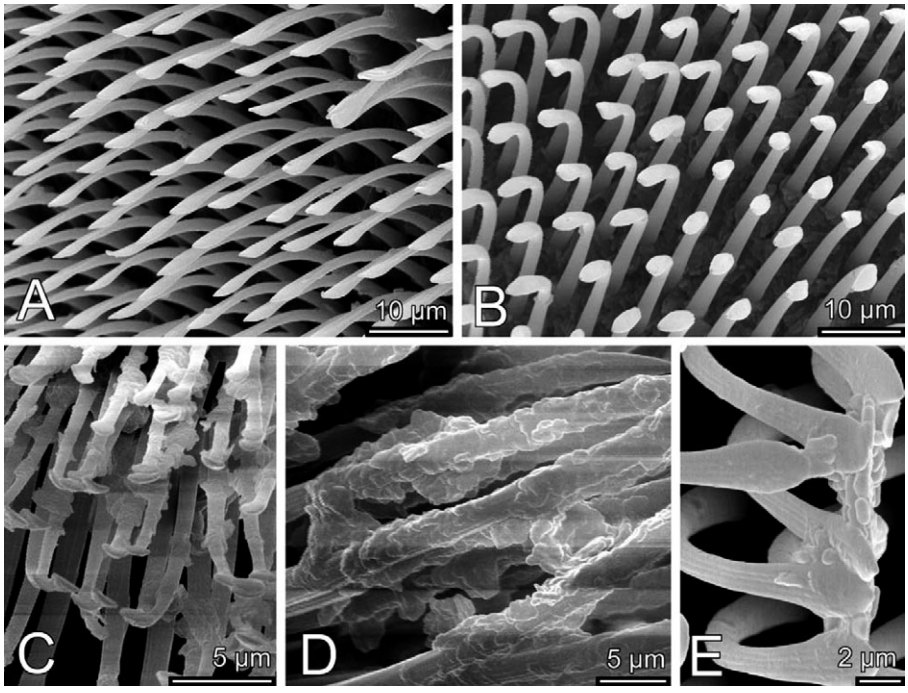

Fig. 8. Adhesive pads of *Adalia bipunctata* beetles after walking on different surfaces: waxy surface after treatment with hot chloroform (A); waxy surface after dental wax treatment (B); and the intact waxy surface of the pitcher (C–E).

aldehyde content of the second extract is clearly increased. As indicated by different solubility and aldehyde content, the lower layer is obviously reinforced by polymeric aldehydes whilst crystals of the upper layer are not. The fourfold increase in wax yield from the lower layer is probably due to the higher density of interconnected wax crystals composing this layer.

Primary alcohols are also present in appreciable amounts in the extracts and could well influence crystal habits, since waxes rich in primary alcohols often tend to crystallize as platelets (Barthlott et al., 1998). High percentages of aldehydes and primary alcohols in the *Nepenthes* wax have also been reported by Riedel et al. (2003), and co-crystallization of alcohols and aldehydes was deduced from this fact. The two EW layers are also discernible by diverging homologue profiles of the most prominent compound classes. The bimodal profiles of the upper layer show two separate main constituents (C_{24}, C_{30}). This profile characteristic is not present in the aldehydes from the lower layer and primary alcohols are also rather inconspicuous. Aldehydes are markedly restricted in the lower layer to one single compound (C_{30}), as are primary alcohols to a lesser extent.

The different chemical compositions of the two layers, concerning both percentages of compound classes and homologue distributions, indicate their independent formation. Recent findings on the transport of wax constituents to the cuticle surface and subsequent crystallization would hardly allow a differentiation of wax compounds and crystallization in two modifications at the same time (Neinhuis et al., 2001), implying that the formation of the two layers occurs at somewhat different stages of pitcher development. However, detailed studies on EW in developing pitchers are lacking, and it is not known whether the two layers develop independently at the same time, or in sequence.

Thus, the two EW layers clearly differ in micromorphology and chemical composition, and this may influence their mechanical properties.

Differences in mechanical properties of waxes

The mechanical properties of plant waxes were measured using nanoindentation experiments for the first time. Our nanoindentation tests with intact and treated pitcher surfaces and wax extracts solidified on a hard substrate showed that waxes of the upper and lower layers exhibit different mechanical properties: wax of the lower layer is harder and stiffer than that of the upper layer. The results of the mechanical tests on the fresh plant surfaces lead to the conclusion that the wax layers studied may affect insect attachment in different ways.

The hardness values obtained on the pitcher surfaces give information about the mean pressure the crystals can withstand. For the whole span of the penetration depth, this pressure is in the MPa range. A rough calculation of the pressure that one seta of an insect applies onto the surface leads to values of several kPa (Enders et al., 2004). Thus, both wax layers appear to insects as mechanically stable surfaces.

Regarding the upper wax layer, an indentation test performed using a pyramidal indenter tip gave additional information. Because of the angled sides of the tip, the crystals experienced a lateral force during further displacement, which led to a shear motion. Starting at the displacement of ca. 100 nm, hardness values began to scatter, and a tendency to lower values was observed. As described above, this implied several ‘breaking’ processes during loading. It can be assumed that the crystals are mechanically relatively stable under loading in the normal direction, but more unstable when a lateral force is applied. However, to verify this assumption, more quantitative nanotribological tests are needed.

Moreover, the fast contamination of the indenter tip in the experiments with the upper wax layer (Fig. 4C) indicates that crystals, once broken or otherwise detached from surface tended to stick strongly to the tip. This result explains why wax crystals of the upper layer adhere well to the attachment organs of insects, and thus impede proper attachment. Such contamination was not observed in all other the samples studied here. During the indentation tests on the lower layer, as well as in the samples with extracted waxes, no adherence of wax material on the tip was revealed in reference tests on fused silica even after a lengthy series of measurements. These effects may be explained by the weak detaching ability of the crystals of the lower layer. In the case of extracts, it may be assumed that the extraction and solidifying processes caused the changes in the structure and chemical composition of waxes, and these changes led to differences in mechanical properties, both the hardness and elasticity moduli, of both wax layers, and in the behaviour of wax samples under load.

Effect of different wax layers on insect attachment

Laboratory experiments with *Adalia bipunctata* beetles showed that waxes significantly reduce the attachment force of insects compared with reference surfaces, such as glass and wax-free pitcher surface. Interestingly, there was no difference in the behaviour and forces generated by beetles on the substrates covered with wax from either the upper or lower layers. Thus, both wax layers contribute to the trapping and retention functions of the pitcher by means of a reduction in insect attachment. However, the morphological study of pads in insects, walked on different surfaces, revealed the essential difference between effects of each wax layer on insect pads. The upper layer caused pad contamination with wax material, whereas the lower layer did not contaminate the pad surface.

To explain the anti-adhesive properties of plant waxes, four hypotheses have recently been proposed: (1) roughness hypothesis, (2) contamination hypothesis, (3) wax-dissolving hypothesis and (4) fluid-absorption hypothesis (Gorb and Gorb, 2002). According to the first hypothesis, the crystalline wax results in micro-roughness, leading to a considerable decrease in the real contact area between the surface and setal tips of insect adhesive pads. The contamination hypothesis states that wax crystals are easily detachable structures, which may contaminate pads. The third and the fourth ones deal with the insect pad secretion, which either dissolve the wax or is absorbed from the setal surface by the wax layer, respectively. Previous studies provided data confirming the first (Stork, 1980;

Gorb, 2001) and the second hypotheses (Juniper and Burras, 1962; Martin and Juniper, 1970; Dixon et al., 1990; Gaume et al., 2004; Gorb and Gorb, in press). It was found that surface roughness in the range from 0.3 to 3 μm reduces strongly the attachment force (Gorb, 2001; Peressadko and Gorb, 2004), and the increased size and aspect ratio of wax crystals causes stronger pad contamination (Gorb and Gorb, in press).

For *Nepenthes* plants, contamination of insect feet with wax crystals has been previously suggested by Knoll (1914) and was later supported in TEM observations by Juniper and Burras (1962). According to these studies, wax crystals are easily detachable structures that are unable to withstand loads applied by insects. According to Juniper and Burras (1962), the weak mechanical connection of crystals with underlying layers through small 'stalks' results in detachment of crystals together with insect feet from the waxy pitcher surface. Recent experimental studies performed with flies *Calliphora vomitoria* and three carnivorous plants, confirmed the pad contamination in the case of *N. ventrata* wax (Gaume et al., 2004). Other authors have rejected the contamination hypothesis and suggested the possible combination of several other mechanisms, such as high mechanical stability of wax crystals, the decrease in the number of attachment points, and low surface energy of the waxy surface in *N. alata* (Riedel et al., 2003). However, the latter study on microstructure and chemical composition of the pitcher wax did not provide experimental evidence to confirm the latter hypotheses.

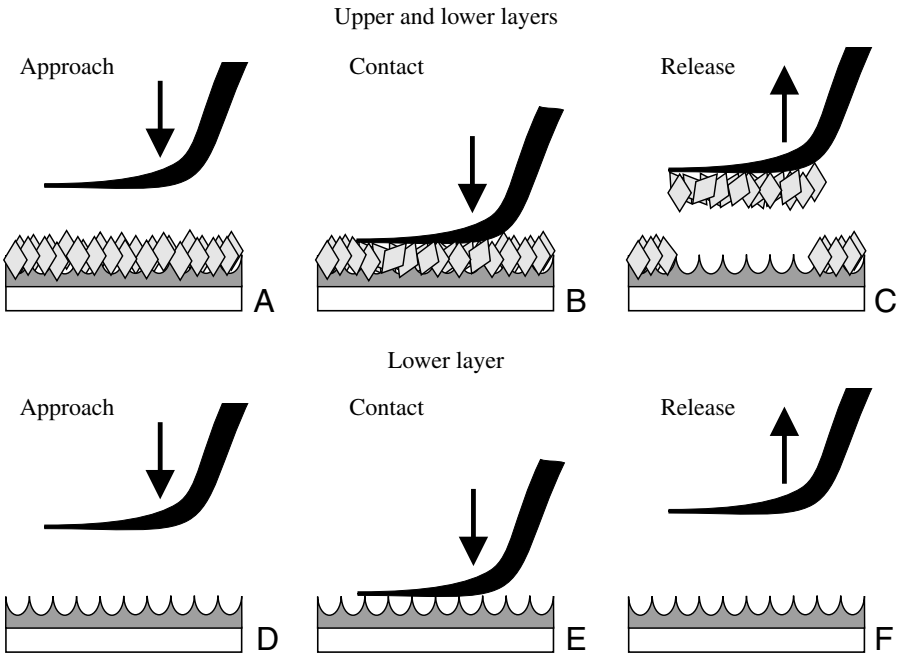

Fig. 9. Scheme explaining the role of the two wax layers in the reduction of the insect attachment force. (A–C) The intact surface with both wax layers. (D–F) The pitcher surface with the lower wax layer only.

On the basis of our data on the micromorphology of wax layers and wax crystals, the chemical composition of the waxes, results of nanoindentation experiments on different wax samples and friction tests with insects, a scheme of the functioning of the waxy zone in the *N. alata* pitcher and the role of the two waxy layers in prevention of insect attachment may be proposed.

Although the crystals of the upper layer are densely arranged, they remain separate and form no network. Their planes are oriented perpendicular to the pitcher surface, and they are attached to the adjacent substrates through small 'stalks'. Therefore, the contact area between a platelet and a substrate is rather small. This causes the ease breakage of 'stalks' and thus the facile removal of crystals of the upper layer, especially if a lateral force is applied. Moreover, these platelets are very brittle and may be easily exfoliated or broken to tiny pieces. Both entire crystals and small fragments may adhere well to insect feet and contaminate attachment organs (Fig. 9C). This prevents proper contact formation between adhesive pads and the surface, and leads to an essential reduction in the attachment force.

Platelets of the lower layer are highly interconnected and form a relatively stable network of crystals, oriented at acute angles to a substrate. The crystals do not detach easily and may remain intact even if the upper layer is removed. This network would be able to resist lateral forces applied by climbing insects, but due to its high micro-roughness, causes a considerable decrease in the real contact area with insects' adhesive organs (Fig. 9E). This provides the further reduction of insect attachment. Hence, the lower layer ensures the effectiveness of the whole system of the waxy zone of the pitcher for its trapping function.

Thus, our results on the waxy surface of the *N. alata* pitcher lead to the conclusion that a decrease in the attachment force of insects is provided by the two different mechanisms: (1) the contamination of insects' attachment organs (the upper wax layer) and (2) the reduction of the real contact area (the lower layer). However, effects of wax dissolving by the insect pad secretion and the secretion adsorption by wax layer cannot be entirely excluded. These hypotheses need to be additionally proven for the case of *N. alata*.

List of abbreviations

CSM	continuous stiffening measurement
E	elasticity modulus
EW	epicuticular wax
GC	gas chromatography
H	hardness
MS	mass spectrometry
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TLC	thin-layer chromatography

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