ORIGINAL ARTICLE

Michael Riedel · Anna Eichner · Reinhard Jetter

Slippery surfaces of carnivorous plants: composition of epicuticular wax crystals in *Nepenthes alata* Blanco pitchers

Abstract Plants in the genus *Nepenthes* obtain a substantial nutrient supply by trapping insects in highly modified leaves. A broad zone of the inner surface of these pitchers is densely covered with wax crystals on which most insects lose their footing. This slippery wax surface, capturing prey and preventing its escape from the trap, plays a pivotal role in the carnivorous syndrome. To understand the mechanism of slipperiness, the present investigation aimed at an ultrastructural and physico-chemical characterization of the wax crystals in pitchers of *N. alata* Blanco. Scanning electron microscopy revealed that entire platelets protruded perpendicularly from the surface. Methods were developed that allowed the mechanical removal of wax crystals from the pitcher surface. It could be shown that the sampling was selective for the epicuticular wax, relevant for plant–insect interactions. The crystals consisted of a mixture of aliphatic compounds dominated by very-long-chain aldehydes. Triacontanal, at 43% the most abundant constituent, was largely responsible for crystal formation. Solubility data indicate that the *Nepenthes* crystals contained polymeric forms of this aldehyde. The resulting mechanical properties of the polymer crystals and the mechanism of slipperiness are discussed.

Keywords Carnivorous plants · Epicuticular wax crystals · Insect attachment · Mechanical wax removal · *Nepenthes* · Plant–insect interaction

Abbreviation SEM: scanning electron microscopy

M. Riedel · A. Eichner · R. Jetter (✉)
Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für
Botanik II, Universität Würzburg, Julius-von-Sachs-Platz 3,
97082 Würzburg, Germany
E-mail: jetter@interchange.ubc.ca
Fax: +1-604-8226089

Present address: R. Jetter
Departments of Botany and Chemistry, UBC,
6270 University Blvd, Vancouver, BC, V6T1Z4, Canada

Introduction

Carnivorous plants display a high diversity of specialized morphological features for trapping, retaining and digesting prey (Darwin 1875; Lloyd 1942; Juniper et al. 1989). The use of predominantly insect-derived nutrients is considered as an evolutionary advantage, and hence the organs contributing to the carnivorous syndrome are expected to be highly optimized. In pitcher plants of the genus *Nepenthes*, modified epiascidiade leaves form passive, tube-like traps (Juniper et al. 1989). The opening of these pitchers is lined by a peristome (zone 1, Fig. 1), which bears, within deep depressions underneath the inner rim, nectar glands for prey attraction (Owen and Lennon 1999). Immediately below the peristome the inner wall of the pitcher is covered with epicuticular wax crystals (zone 2, Fig. 1; Juniper 1995; Juniper et al. 1989). Early on, it was recognized that the inner surface of zone 2 plays a major role in the trapping efficiency of the pitchers because the epicuticular wax crystals render this surface extremely slippery for insects (Macfarlane 1893; Knoll 1914). Insect attachment is further hampered by the special arrangement of modified stomata (Gaume et al. 2002). Foraging insects lured to this surface lose their footing and fall into the trap. The slippery walls also prevent the prey from escaping and it eventually drowns in the fluid in zone 3 (Fig. 1) containing digestive solutions and absorptive glands (Schulze et al. 1997; Owen and Lennon 1999; Owen et al. 1999; An et al. 2002a, 2002b).

In recent years, several studies have shown that epicuticular wax crystals on diverse plant species can impede insect attachment (Stork 1980; Eigenbrode et al. 1996; Federle et al. 1997), demonstrating the importance of slippery surfaces for plant–insect interactions. But the mechanism underlying this principal phenomenon has not been understood, mainly because the physico-chemical properties of the involved biomaterials could not be appropriately investigated. For chemical analyses of the plant surface compounds, cuticular waxes were
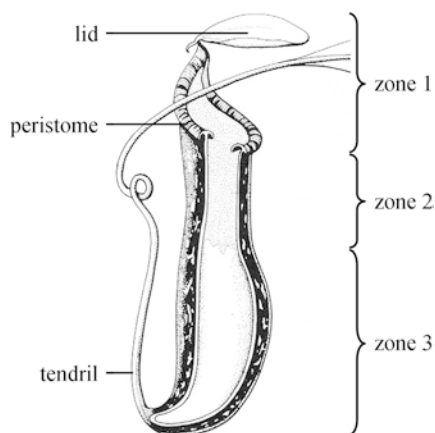
Fig. 1 Longitudinal section through an idealized pitcher of *Nepenthes alata*. In zone 1, nectaries are located underneath the inner rim of the peristome. The inside of the pitcher wall in zone 2 is covered with epicuticular wax crystals. Zone 3 is characterized by glands secreting digestive solutions and absorbing nutrients (modified after Juniper et al. 1989)

generally prepared by extraction of the intact organ with organic solvents. But it was shown that the solvents not only mobilized epicuticular wax crystals, but would also enter into the cuticle and release intracuticular waxes (Jetter et al. 2000). Although extraction experiments lacked the necessary selectivity for surface compounds, they could nonetheless yield indirect evidence for the chemical composition of epicuticular crystals. Certain wax constituents were found to be correlated with characteristic crystal shapes on diverse plant surfaces (reviewed in Jeffree et al. 1976; Baker 1982; Jeffree 1986). Consequently, it was concluded that these compounds, generally predominating in the respective wax mixtures, were responsible for crystal formation. For example, very-long-chain primary alcohols were assumed to form the entire-edged platelets on various plant organs and species.

To corroborate these inferences, and to judge whether minor constituents are present within the epicuticular wax crystals, direct evidence on the crystal composition is necessary. To this end, the extractive methods had to be replaced with mechanical sampling strategies. Ensikat et al. (2000) had shown that cryo-adhesives could be used to remove epicuticular wax crystals from plant surfaces and transfer them onto artificial substrates, perfectly preserving their shape and arrangement. Unfortunately, neither the treated areas, nor the crystal compositions were quantified, and hence the epicuticular crystal coverage could not be judged. A similar cryo-adhesive method was then employed to selectively prepare the smooth epicuticular wax film from *Prunus laurocerasus* leaves (Jetter et al. 2000), and for the first time the coverage of all epi- and intracuticular wax constituents was quantified. It was shown that the chemical composition of the epicuticular wax film differed drastically from the underlying intracuticular wax mixture. Based on these quantitative results, the selectivity of other sampling techniques could be

tested, and finally gum arabic was established as an alternative adhesive for the selective removal of epicuticular waxes (Jetter and Schäffer 2001). Consequently, a variety of methods for mechanically sampling epicuticular waxes have been described, but they have not yet been employed for the chemical analysis of epicuticular wax crystals.

In the present study, adhesive procedures were adapted to characterize the epicuticular crystals in *Nepenthes alata* pitchers. We combined scanning electron microscopy (SEM) and chemical analyses by gas chromatography and mass spectrometry to address the following questions: (i) Which chemical compounds are responsible for crystal formation? (ii) Are other wax constituents present in the crystals? (iii) Which physico-chemical processes lead to crystal formation? (iv) How can epicuticular wax crystals disrupt insect attachment, i. e. what is the mechanism of slipperiness? (v) What is the special evolutionary adaptation of the pitcher cuticle?

Materials and methods

Plant materials

Specimens of *Nepenthes alata* Blanco were taken from a continuous greenhouse culture in the Botanical Garden of the University of Würzburg. Using freshly harvested pitchers, circular leaf discs of 1.5 cm or 1.6 cm in diameter were cut from the slippery zone and used without further preparation.

Mechanical wax removal

For the mechanical removal of epicuticular wax, two different adhesives were used. In one experimental set-up, water was used as cryo-adhesive. To apply liquid water to the surface of the leaf discs, they were placed on an aluminium holder. Cylinders (inner diameter: 1.4 cm) were centred on the leaf discs and filled with water, completely covering the free surface of the leaf discs. The whole arrangement was then cooled in liquid nitrogen. The resulting ice blocks could be taken off the leaf discs by lifting the cylinders, thereby stripping off wax (Fig. 2, right). By rapidly warming the walls of the cylinders, the ice blocks slipped out and were directly collected in a two-phase system of 4 ml each of chloroform and water. The preparations from four to six leaf discs of the same pitcher were pooled and tetracosane was added as internal standard. The two-phase system was warmed to approximately 40 °C in a water bath and agitated repeatedly. Then, the organic phase was removed and the aqueous phase extracted two more times with chloroform. All three chloroform fractions were combined and the solution was prepared for the chemical analysis. The treatment with the cryo-adhesive was repeated five times with the same leaf discs.

In a second experimental set-up, an aqueous solution of gum arabic was applied to circular mesh-wires of stainless steel, 1.6 cm in diameter. Prior to use, the commercially available gum arabic (Roth, Karlsruhe, Germany) powder was soxhlett-extracted in chloroform (99.9%; Roth) for 3 h. The gum arabic-coated mesh-wires were centred on leaf discs of the same diameter and the gum arabic was allowed to dry within 2 h at 25 °C. Subsequently, the mesh-wires were lifted off the leaf discs, thereby stripping off the hardened polymer film together with adhering wax constituents (Fig. 2, left). To separate the wax constituents from the gum arabic, the mesh-wires were transferred into vials containing a two-phase
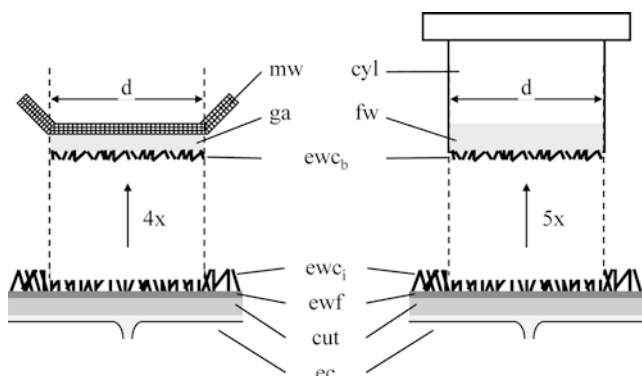
Fig. 2 Set-up for the mechanical removal of epicuticular wax crystals from the slippery zone of *N. alata* pitchers. Gum arabic (*ga*) or frozen water (*fw*) as adhesives are applied to a circular area of defined diameter (*d*). The adhesives with embedded intact epicuticular wax crystals (*ewc$_i$*) are removed with a mesh-wire (*mw*) or a cylinder (*cyl*), respectively. The broken epicuticular wax crystals (*ewc$_b$*) are then prepared for analysis. *cut* Cutin and intracuticular wax, *ec* epidermal cell, *ewf* epicuticular wax film

system of 4 ml each of chloroform and water. The wax was then separated from the water, as described for the treatment with frozen water as adhesive. The preparations from four to six leaf discs of the same pitcher were pooled in one sample. The whole protocol could be repeated four times on the same leaf discs without damaging the cuticle or the underlying tissue, as confirmed by SEM.

Extraction of cuticular wax

After the mechanical removal of epicuticular wax the remaining cuticular wax was finally extracted with warm chloroform (ca. 40 °C). To this end, glass cylinders (1.0 or 1.4 cm in diameter) were gently pressed onto the leaf discs and filled twice with 1 ml of warm chloroform for 30 s. Both chloroform solutions were combined and tetracosane was added as internal standard.

Alternatively, the total wax coverage was assessed by extraction of the whole cuticular wax from untreated leaf discs. The same protocol as described above for the final extraction after mechanical removal was applied five times on the same leaf discs and all five samples were analysed separately.

Chemical analysis

Prior to GC analysis, chloroform was evaporated from all samples under a gentle stream of N_2 while heating the sample-vials to 50 °C. Then all samples were treated with bis-*N,N*-(trimethylsilyl)trifluoroacetamide (BSTFA; Macherey-Nagel, Düren, Germany) in pyridine (30 min at 70 °C) to transform all hydroxyl-containing compounds to the corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was studied by capillary GC (8000Top; Fisons Instruments, Rodano-Milan, Italy) with He carrier gas inlet pressure constant at 30 kPa and a mass-spectrometric detector (70 eV, m/z 50–850, MD1000; Fisons). GC was carried out with temperature-programmed injection at 50 °C, oven 2 min at 50 °C, raised by 40 °C min^{-1} to 200 °C, held for 2 min at 200 °C, raised by 3 °C min^{-1} to 320 °C, held for 30 min at 320 °C. The quantitative composition of the mixtures was studied by capillary GC (5890 II: Hewlett Packard, Avondale, PA, USA; 30 m DB-1, 0.32 mm i.d., d$_f$=1 μm: J & W Scientific, Folsom, CA, USA) and flame ionization detection under the same gas-chromatographic conditions as above, but the H_2 carrier gas inlet pressure was programmed for 50 kPa at injection, held for 5 min, raised at 3 kPa min^{-1} to 150 kPa and held for 40 min at 150 kPa. Single compounds were quantified against the internal standard by manually integrating peak areas.

Aldehyde homologues were quantified using peak areas of individual free aldehydes and corresponding reaction products of the aldehydes with the silylating agent (BSTFA). To this, the molar contribution of corresponding aldehyde homologues to reaction products of the aldehydes with primary alcohols was added. Similarly, peak areas of free primary alcohol homologues (TMSi-ethers) and the molar contribution of the alcohols to the corresponding reaction products of aldehydes with primary alcohols were used to quantify the individual primary alcohol homologues. The homologue distribution of aldehydes and primary alcohols in the dimeric reaction products were calculated based on the distribution of diagnostic fragment-ions. The assignments and relative intensities of these signals were authenticated using reaction products of synthetic standards (docosanal, obtained from C. Vermeer, University of Würzburg) with commercially available primary alcohols (eicosanol, heneicosanol, docosanol, tricosanol or tetracosanol; Sigma-Aldrich, Deisenhofen, Germany).

Scanning electron microscopy

Dried (overnight, 25 °C) samples of untreated leaf discs and of leaf discs from the various treatments were mounted on aluminium holders, sputter-coated with gold-palladium (Bal-Tec SCD005 Sputter Coater; 25 mA, 300 s; Balzers, Switzerland) and examined by SEM (Zeiss DSM 962, 15 kV; Zeiss, Oberkochen, Germany). The sputtering conditions, depositing approximately 20 nm of the alloy on the tissue samples, were optimized for the acceleration voltage used in the SEM (G. Krohne, Center for Electron Microscopy, University of Würzburg, personal communication).

Statistics

To characterize the performance of both mechanical removal methods, a linear regression function (Statistica 6.0; StatSoft, Tulsa, USA) was fitted to the corresponding wax yields of the four and five consecutive removal steps, respectively. For the fitting procedure, the least sum of squared deviations was used as loss function. To characterize the kinetics of the extraction experiment, a nonlinear regression function was fitted to the corresponding wax yields of the five successive extraction steps. The least sum of squared deviations was used as loss function with the quasi-Newton method as the estimation procedure.

Results

To characterize the shape and arrangement of the epicuticular wax crystals, native surfaces of *Nepenthes alata* pitchers were investigated by SEM. The entire area of the slippery zone, comprising epidermal and lunate cells, was covered with a dense network of wax platelets (Fig. 3a). Most crystals had similar height, standing upright and exposing their narrow side towards the lumen of the pitcher. They were ca.1 μm long and 0.1 μm thick, had entire margins and were only slightly curved. Angles between neighbouring or partially connected platelets varied widely, creating a random pattern of small air spaces (Fig. 3c).

In a first experiment, frozen water was used to mechanically remove wax crystals from a defined area within the slippery zone. The cryo-adhesive treatment could be repeated five times on the same area without damaging the plant tissue (confirmed by SEM). Wax yields decreased steadily from 5.4 μg/cm^2 in the first step

Fig. 3a–h SEM images of the slippery zone of *N. alata* pitchers, taken from air-dried samples before and after various treatments. **a, c** Untreated surface, characterized by modified stomata and a dense network of epicuticular wax crystals, forming entire platelets. **b, d** Surface after the first mechanical removal of epicuticular wax crystals with remaining irregularly edged wax structures. **e** Border between an untreated surface (*left*) and an area after the first mechanical removal of epicuticular wax (*right*). **f** After the fifth mechanical removal with frozen water, in places the smooth surface of the cutin matrix becomes visible (*right*). **g** Epicuticular wax crystals mechanically removed in a first preparation step with frozen water and deposited on a cover slide, preserving their shape and three-dimensional arrangement. **h** Underside of a gum arabic film after the first mechanical removal with adhering epicuticular wax crystals. The shape and structure of the proximal parts of the removed crystals are identical to those of the distal parts of the structures remaining on the plant surface. Bars = 50 µm (**a, b**), 1 µm (**c–h**)
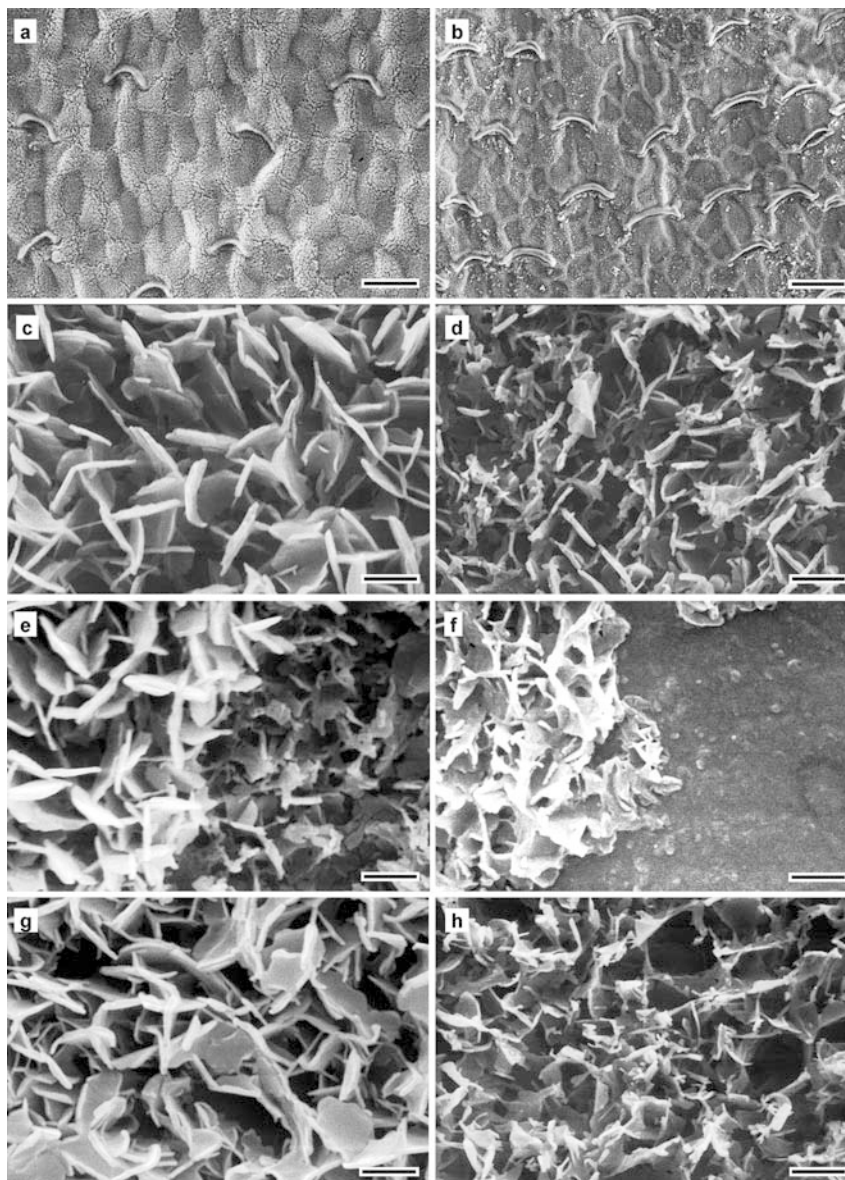
to 1.8 µg/cm² in the fifth mechanical removal, adding up to a total amount of 17.0 µg/cm² (Fig. 4a). A linear regression function ($y = -0.94x + 6.22$) was fitted to the wax yields of the consecutive removal steps. Accordingly, only 0.6 µg/cm² of wax could have been expected from a sixth preparation step and further mechanical treatments would not have yielded additional wax. Instead, the extraction of the same surface with chloroform mobilized further wax material (9.5 µg/cm²), bringing the overall wax yield to 26.5 µg/cm² (Fig. 4a).

In a second experiment, gum arabic was used as adhesive for the mechanical removal of the wax. Due to the drying intervals between consecutive steps, the treatment could be repeated only four times without damaging the gradually drying plant tissue. The yields of repeated treatments with gum arabic decreased linearly (from 4.1 µg/cm² to 2.1 µg/cm²) and totalled 11.0 µg/cm² (Fig. 4a). From a linear regression function

($y = -0.60x + 4.27$), it was extrapolated that three additional removal steps would have released ≤ 1.9 µg/cm² of wax. With a successive chloroform treatment 20.4 µg/cm² of wax was extracted and the overall yield accumulated to 31.4 µg/cm², not differing significantly (Mann–Whitney U-Test: $P > 0.05$) from the overall yield of the first experiment.

In parallel to the chemical analyses, the effects of successive steps of mechanical wax removal from the pitcher surface were monitored by SEM. Already in the first treatment with frozen water or gum arabic the entire-edged platelets were removed from extensive areas, instead exposing wax platelets with irregular margins (Fig. 3b, d). At the borderline between native and treated areas it became obvious that the original crystals had been much higher than the newly exposed structures (Fig. 3e). This suggests that only the outermost parts of the crystals had been broken off by the first adhesive
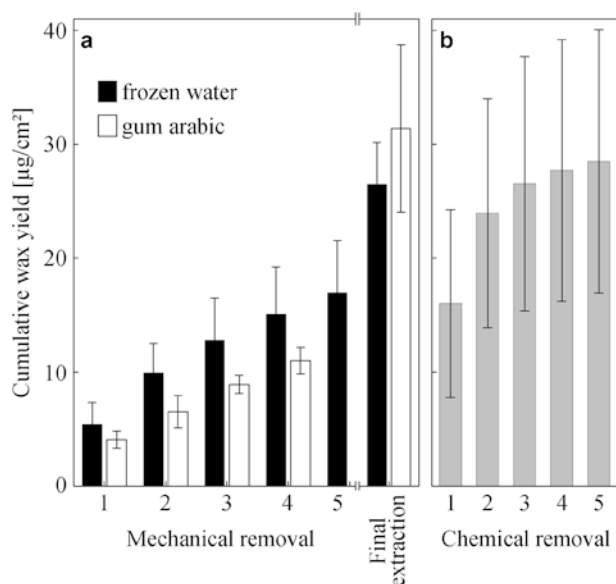
Fig. 4a, b Cumulative yield of wax from the slippery zone of *N. alata* pitchers. Various preparation methods were combined in three parallel approaches. **a** Epicuticular wax was first mechanically removed treating the surface either five times with frozen water ($n = 5$) or four times with gum arabic ($n = 4$) as adhesives. After the mechanical removal, a final extraction with warm chloroform followed. **b** For the assessment of total wax coverage, the wax was chemically removed by five successive surface extractions with warm chloroform ($n = 4$). Data are means $\pm$ SD

treatment (c.f. Fig. 3c and d) and the irregular margins of the remaining platelets represented the breakage lines. Additionally, after the fifth treatment with frozen water a smooth surface became visible in places (Fig. 3f). The sharp delineation between the two areas highlighted two layers, in this case reflecting the sharp contrast between the fringed platelets and the underlying smooth surface of the cuticle itself.

SEM was also employed to characterize the structure of the removed material. To this end, the cryo-adhesive preparations were transferred onto cover slides, thus allowing the microscopic inspection of the distal parts of the platelets after removal (Fig. 3g). Both their shape and arrangement were largely preserved, only the orientation of a few crystals being disturbed (c.f. Fig. 3c and g). Alternatively, the proximal parts of the removed crystals could be viewed on the underside of stripped pieces of gum arabic (Fig. 3h). This part of the removed material closely resembled the remaining crystal counterparts on the pitcher surface (c.f. Fig. 3d and h).

In the first preparation step, both frozen water and gum arabic yielded identical patterns of compound classes (Fig. 5a, b). With the exception of trace amounts of pentacyclic triterpenoids, only unbranched very-long-chain aliphatic components were present. Homologous series of C_{20}–C_{34} primary alcohols, C_{20}–C_{32} fatty acids, C_{38}–C_{58} alkyl esters, and C_{26}–C_{36} *n*-alkanes were detected. The most prominent compound class were C_{20} to C_{34} *n*-aldehydes, comprising 60% and 62% of the first cryo-adhesive and gum arabic preparation, respectively (Fig. 5a, b). Except for the alkanes, even-numbered

carbon backbones dominated all of the aliphatic compound classes. The first cryo-adhesive preparation contained especially high portions of the C_{24} fatty acid homologue, while the alcohols showed a broad chain length distribution with maxima for C_{24} and C_{30} (Fig. 6a, b). In contrast, the homologous series of aldehydes was characterized by a pronounced maximum, triacontanal (C_{30}) prevailing with 72% of the fraction (Fig. 6c). This single aldehyde homologue therefore constituted 43% of the prepared crystal mass. The first gum arabic treatment yielded very similar homologue patterns (data not shown).

After exhaustive mechanical wax removal with frozen water, the remaining wax was sampled with chloroform. The resulting extract contained only 28% of aldehydes and relatively high quantities of alcohols (31%, Fig. 5d). In addition, alkyl esters and pentacyclic triterpenoids had higher concentrations in the extracts than in the mechanically sampled wax portions. The homologue distribution of the extracted aldehydes was shifted towards chain lengths below C_{28} (Fig. 6c), as compared to the surface-stripped wax, with a local maximum at C_{24} (28% of the fraction). Although triacontanal was the major homologue (34% of the fraction), it constituted only 9% of the extract. Within the fatty acid fraction, there were no differences in the distribution patterns between extracted and stripped wax compartments, whereas the homologue pattern of the alcohol fraction was slightly shifted towards chain lengths below C_{28} in the extracted wax (Fig. 6a, b). After repeated treatments with gum arabic, the corresponding chloroform extract contained 40% of aldehydes (Fig. 5e). The concentrations of the other compound classes were similar in the final extractions of both experiments. The chain length distribution of aldehydes in the extract diverted from that of the corresponding gum arabic treatment. The shift towards chain lengths below C_{28} with a local maximum at C_{24} (22% of the fraction) and the absolute maximum at C_{30} (39% of the fraction) was less pronounced than after the cryo-adhesive treatment. Hence, the wax mixture obtained after gum arabic treatment (Fig. 5e) shows an intermediate composition between that of the first cryo-adhesive treatment (Fig. 5a) and the corresponding final extraction (Fig. 5d).

In a third experiment, the total wax coverage on the slippery zone of *N. alata* was assessed by chemical extraction. But as preliminary experiments had shown that the epicuticular crystals themselves were not readily soluble in chloroform at room temperature (Fig. 7a, c), the extraction was performed at approximately 40 °C. In the first extraction step 16.0 μg/cm^2 of wax was obtained, while with four more steps a total yield of 28.5 μg/cm^2 was achieved (Fig. 4b). To the individual wax yield of the five extraction steps a nonlinear regression function was fitted ($y = -0.40 + e^{-0.75 * x + 3.56}$). From this it was estimated that an additional extraction would not have yielded considerable amounts of wax and it can be assumed that the cuticular wax was extracted exhaustively. This result was confirmed by SEM

Fig. 5a–f Relative amounts of compound classes in wax from the slippery zone of *N. alata* pitchers. For three parallel approaches, the first and the last preparation steps are compared. **a** First mechanical removal with frozen water as adhesive and **d** corresponding final extraction with warm chloroform (*n* = 5). **b** First mechanical removal with gum arabic as adhesive and **e** corresponding final extraction with warm chloroform (*n* = 4). **c** First and **f** fifth of five successive chemical removals by surface extraction with warm chloroform (*n* = 4). Median; 25th, 75th percentile
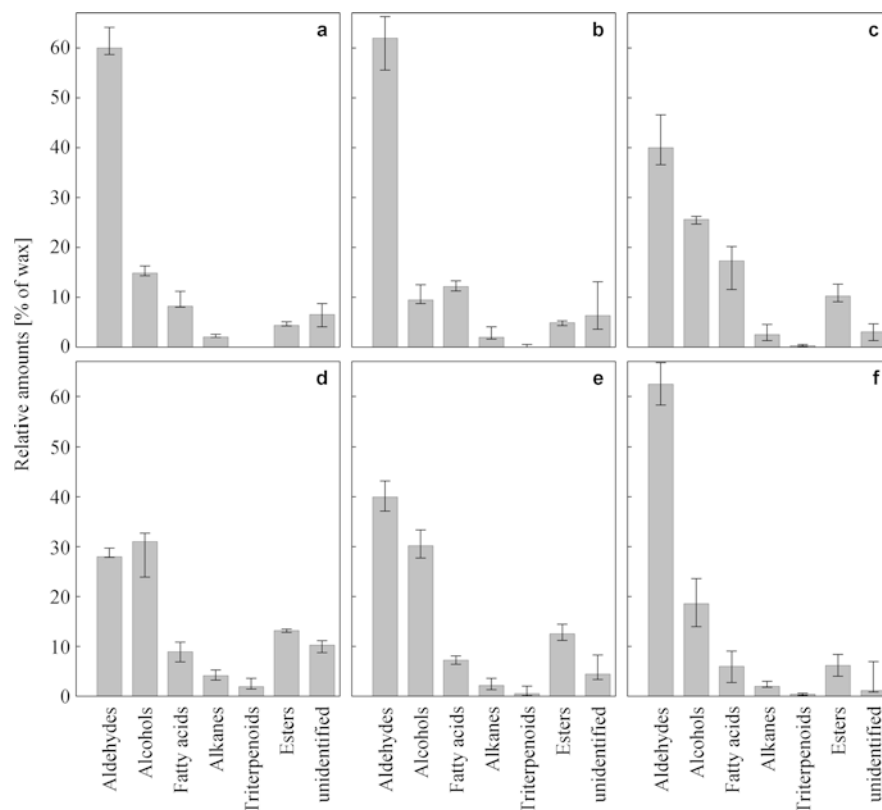
of the pitcher surface after five extraction steps (Fig. 7b, d). On the other hand, the presence of marker compounds for the underlying tissue (e.g. chlorophylls or carotenoids) in the extracts could be excluded both by visual inspection and by chemical analyses, hence indicating that the extraction protocol was selective for soluble cuticular material. The cumulative extraction yield closely matched the total wax yields assessed in the first two experiments (Kruskal-Wallis-Test: H (2, *n* = 13) = 1.74; *P* = 0.42).

The results of the first extraction step (Fig. 5c) showed striking similarities to those from the final extraction after gum arabic treatment (c.f. Fig. 5e). While aldehydes predominated in the mixture, substantial amounts of alcohols, fatty acids and esters also were obtained. In the course of consecutive extraction steps, the relative amounts of compound classes shifted gradually, portions of aldehydes increasing to 62% and alcohols decreasing to 19% instead (Fig. 5f). Therefore, the wax mixture of the fifth extraction step (Fig. 5f) closely resembled that of the first adhesive treatments (Fig. 5a, b).

Discussion

The chemical composition of epicuticular wax crystals has been the topic of many studies (Jeffree 1986; Walton 1990; Bianchi 1995) and it is generally accepted that single compounds or compound classes are responsible for the formation of the crystals (Baker 1982). This was first inferred from comparative studies of diverse plant species, showing that the predominance of certain wax constituents was correlated with characteristic crystal shapes (Holloway et al. 1976). Conversely, crystals of various characteristic shapes could be generated in vitro by recrystallization of selected neat wax constituents (Jetter and Riederer 1994, 1995). Based on this indirect evidence it was hypothesized that epicuticular wax crystals spontaneously self-assemble as soon as a single compound is concentrated beyond a threshold value. Consequently, the separation of a crystalline phase from the complex amorphous wax mixture should cause differences between the chemical composition of the crystals and the surrounding mixture. In order to detect these gradients within the cuticular wax mixture, the exact percentages of individual compounds either in the epicuticular crystals or in the underlying (partially) amorphous intracuticular wax have to be assessed. But unfortunately, methods that would give direct chemical evidence by selectively probing both layers were not available previously.

Selectivity of mechanical wax sampling techniques

In the present investigation, the cuticular wax mixtures on the slippery zone of *Nepenthes alata* pitchers were sampled and analyzed in three independent experiments. By repeating and combining various methods for wax removal from the tissue surface, both the absolute amounts and the relative composition of epicuticular

Fig. 6a–c Homologue composition [% of fraction] of fatty acids (**a**), primary alcohols (**b**) and *n*-aldehydes (**c**) from the wax of the slippery zone of *N. alata* pitchers. The wax was sampled by a single mechanical removal step with frozen water as adhesive (*n* = 5) or extracted with warm chloroform after five successive removal steps with frozen water (*n* = 4). Median; 25th, 75th percentile
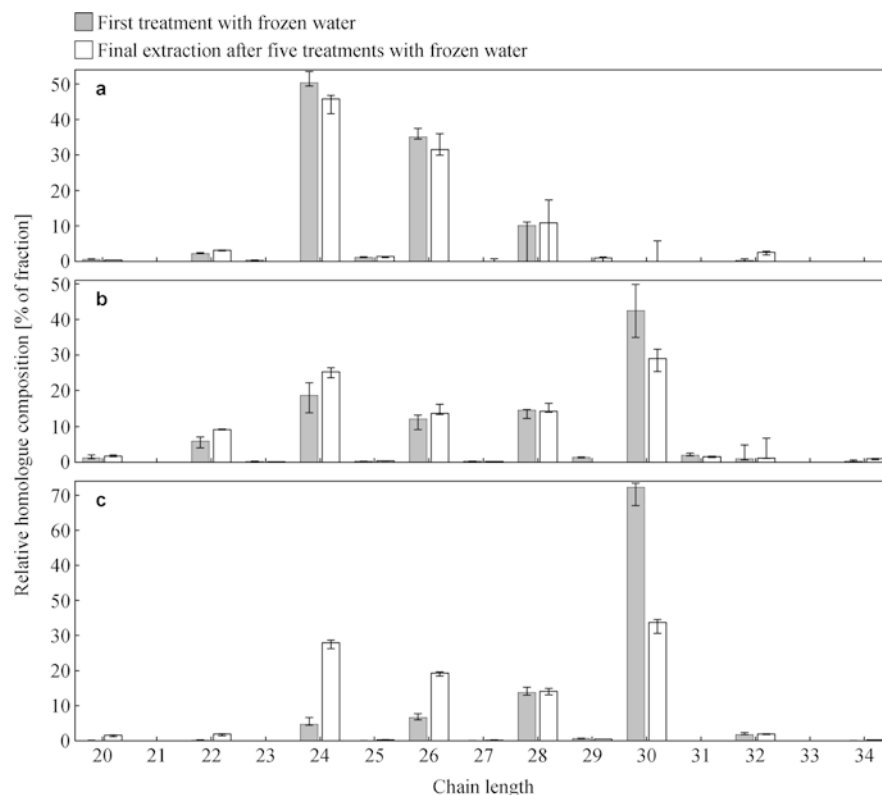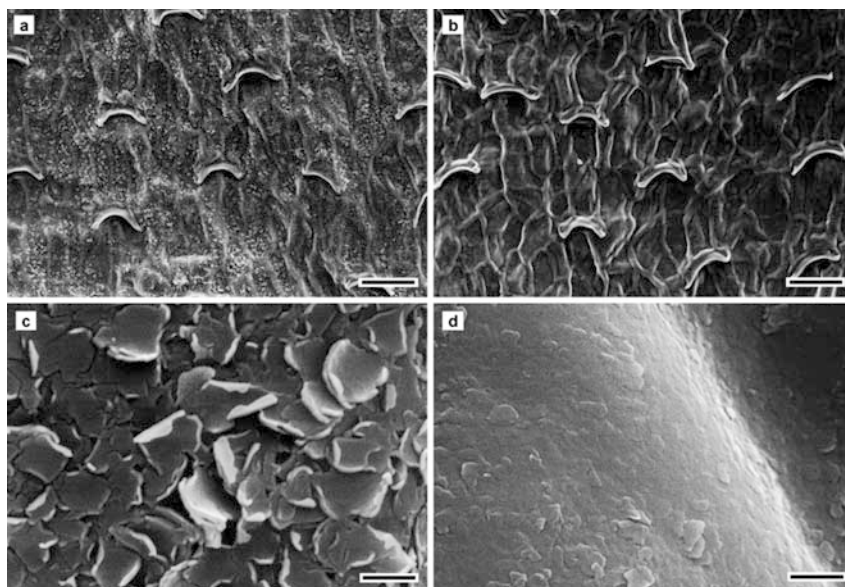

Fig. 7a–d SEM images of the slippery zone of *N. alata* pitcher, taken from air-dried samples. **a, c** After one surface extraction with chloroform at room temperature, leaving substantial amounts of crystals lying flat on the surface. **b, d** After five successive surface extractions with warmed chloroform. All epicuticular wax structures are removed. Bars = 50 µm (**a, b**), 1 µm (**c, d**)

crystals and intracuticular waxes were assessed. The overall wax yield of approx. 30 µg/cm² determined in all three experiments did not differ significantly, and therefore represents the total coverage of epi- plus intracuticular waxes.

Taking all results from the chemical analyses and SEM into account, the selectivity of the employed methods can be judged. Repeated treatments with frozen water, sampling surface material exclusively by adhesion, yielded steadily decreasing amounts of wax

(c.f. Fig. 4a). Hence, in this experiment a mechanically stable boundary was reached, which is most likely defined by the outer limit of the cutin polymer matrix (Jetter et al. 2000). Wax constituents beyond this boundary were only accessible by chemical extraction, shown by the increased wax yield of the final extraction. Consequently, the mechanically prepared material can be designated as epicuticular wax, whereas the extracted compounds represent intracuticular wax. The regression analysis showed that only small amounts of

epicuticular wax, not sampled with the cryo-adhesive, contaminated the intracuticular sample. Frozen water, hence, proved to be a very efficient adhesive for a sharp distinction between the two *Nepenthes* wax compartments. Of the total wax coverage, 64% was found to be located in the epicuticular wax compartment.

The results of the gum arabic treatments differed in some major aspects from the cryo-adhesive experiment: (i) in the mechanical wax removal steps, gum arabic yielded significantly less wax than frozen water (35% of the total wax coverage; c.f. Fig. 4a); (ii) in the consecutive chloroform extraction, relatively large portions of aldehydes were detected (c.f. Fig. 5); and (iii) all compound classes showed intermediate homologue patterns between those of the first cryo-adhesive treatment and the corresponding chloroform extract (data not shown). These findings indicate that the repeated mechanical treatment with gum arabic did not suffice to remove all epicuticular wax from the pitcher surface. The remaining portion of the epicuticular material was released together with the intracuticular wax in the consecutive extraction step and, consequently, the intracuticular wax composition can only be qualitatively evaluated by the gum arabic data. Nevertheless, the first gum arabic treatment was highly selective for the epicuticular wax crystals and could serve to corroborate corresponding results from the cryo-adhesive method.

Additionally, sublayers could be distinguished within the epicuticular wax crystals on *Nepenthes* pitcher surfaces. With both adhesives, all platelets were broken at equal height above their bases, leaving fringed stubs. Hence, differences in the mechanical properties create a weak zone between the entire-edged distal part of the platelets and their proximal end at (or underneath) the cuticle surface. Wax preparations from the first adhesive treatment contained only the outer sublayer of the crystal network and were therefore specifically reflecting the chemical composition of the plant–insect interface. The relatively small wax yields together with the intact structure of the removed crystals indicate that both adhesives were equally selective for the distal portions of the platelets.

Composition of epicuticular crystals and intracuticular wax

Both methods, employing frozen water or gum arabic to mechanically sample waxes, proved to be very selective for the outermost part of the epicuticular wax platelets on pitchers of *N. alata* (see above). The results of the first adhesive treatment therefore allow the crystal composition to be inferred directly. Both experiments independently showed that the epicuticular wax platelets consisted mainly of very-long-chain aldehydes (c.f. Figs. 5, 6). Triacontanal was a crucial factor in the formation of the characteristic *Nepenthes* crystals, as this single aldehyde homologue contributed 43% of the entire crystal mass. This result, for the first time quantifying the major compound within epicuticular crystals, confirms the original hypothesis that described crystal formation as a spontaneous phase separation of one highly concentrated constituent.

Triacontanal had been detected in the cuticular waxes of diverse plant species, usually being a minor component together with shorter-chain aldehydes (Walton 1990). It had also been reported that aldehydes can be localized in epicuticular wax platelets on rice and sugar cane leaves (Haas et al. 2001). In general, this characteristic crystal shape had been associated with the presence of primary alcohols on diverse plant species (Baker 1982). Together with these previous reports, our results demonstrate that this crystal type can be formed on various plant surfaces by pure alcohols, aldehydes, or possibly by mixtures of both compound classes. This raises the question whether platelets consisting of aldehydes or of alcohols differ in their physical properties and ecological functions.

It has been a long-standing question whether minor constituents can contribute to the epicuticular wax crystals. The present analyses showed that more than half of the crystal mass consisted of compounds other than triacontanal. Homologous series of primary alcohols, fatty acids, alkyl esters, and alkanes were detected. It cannot be decided whether these compounds are randomly distributed throughout the crystal network, or whether they segregate into distinct platelets. But interestingly, compounds with similar polarity, e.g. homologous C_{24}–C_{30} aldehydes, and compounds with similar molecular geometry, e.g. C_{30} aldehyde and C_{30} alcohol, were found at especially high concentrations in the epicuticular wax of *N. alata*. These strong similarities between the crystallizing molecules, together with the homogeneous appearance of the crystals, might indicate that the compounds co-crystallize.

The final chloroform extraction in the cryo-adhesive experiment allowed a direct analysis of the intracuticular wax of the slippery zone in *N. alata* pitchers. This wax layer contained only moderate amounts of aldehydes, but relatively high portions of alcohols, alkyl esters and triterpenoids. Hence, distinct gradients between epi- and intracuticular layers were found for individual wax constituents of *N. alata* pitchers. The aldehydes, being the major fraction in the epicuticular crystals, showed the most drastic differences between the two layers. Less pronounced effects were detected for the other epicuticular compounds. Interestingly, aldehydes and alcohols with especially long chains were accumulating in the epicuticular crystals.

The third experiment, assessing the total wax coverage of *N. alata* by repeated chloroform extractions, showed that relatively large solvent volumes and elevated temperatures are necessary to entirely dissolve the triacontanal-dominated epicuticular crystals. The results of this experiment are in apparent conflict with the good solubility of most plant cuticular waxes in organic solvents at room temperature (Holloway 1984;

Walton 1990; Bianchi 1995). Although synthetic triacontanal has a solubility of 50 mg/ml in chloroform at room temperature (P. Schmid, University of Würzburg, personal communication), only approx. 6 µg/ml of the same compound dissolved under identical conditions from the *N. alata* crystals. This indicates that very-long-chain aldehydes can exist in two forms. Accordingly, earlier studies had described both monomeric and polymeric forms of aldehydes in the cuticular wax of sugarcane (Lamberton and Redcliffe 1960). As the polymers were insoluble in organic solvents at room temperature, their structure could not be fully elucidated (Lamberton 1965). Haas et al. (2001) reported that the wax platelets on the leaf surfaces of sugar cane and rice were insoluble at room temperature, while hot chloroform dissolved them and released high portions of aldehydes. The authors inferred that the previously described polymeric aldehydes were (at least in part) localized in the epicuticular crystals. In accordance with these earlier reports, we conclude that the *N. alata* crystals contained aldehydes in a polymeric and, hence, insoluble form.

The presence of polymeric aldehydes in the epicuticular crystals of *Nepenthes* pitchers is further supported by the composition of the chloroform extracts in the third experiment. The first and the fifth extraction yielded wax mixtures (c.f. Fig. 5c and f) similar to the chemically extracted and the mechanically stripped samples from the cryo-adhesive experiment (c.f. Fig. 5d and a), respectively. In the first step mainly the intracuticular wax constituents were extracted, while little material from the epicuticular crystals could be dissolved. In the course of the consecutive extractions all readily soluble wax constituents were removed and only the chloroform-resistant crystals remained on the surface. Consequently, the later extraction steps selectively yielded constituents of the epicuticular wax crystals.

To our knowledge, there is only one literature report on the chemical composition of epicuticular wax crystals of *Nepenthes* pitchers. Juniper et al. (1989), after wiping the pitcher surface of *Nepenthes* × *williamsii* with chloroform-soaked cotton wool, found that aldehydes, alcohols and fatty acids contributed almost equally to the composition and therefore could not infer a single crystal-forming compound (or compound class). But the authors also mentioned that the wax crystals could not be removed completely by washing the surface with chloroform, hence casting doubt on the epicuticular origin of their material. As the present findings for the intracuticular wax of *N. alata* are very similar to the reported composition of the *N.* × *williamsii* wax, it is possible that mainly the intracuticular lipids of the latter species were analysed. The corresponding epicuticular crystals might contain polymeric aldehydes and would, hence, differ significantly from the intracuticular wax. This hypothesis can now be tested employing the present sampling techniques on *N.* × *williamsii* pitchers.

Mechanism of slippery epicuticular crystals in *Nepenthes* pitchers

Insects have evolved a wide variety of structures for attachment to different substrates, involving either smooth pads or structures with setose or hairy surfaces (summarized by Beutel and Gorb 2001). Both types of attachment device employ flexible material to optimize the area of contact to the substrate (Gorb 2001). Contact can be further enhanced by surface-active secretions on the insect feet mediating forces by molecular adhesion and surface tension of a thin layer of liquid (Eisner and Aneshansley 2000, Federle et al. 2001). On the other hand, plant surfaces covered with epicuticular wax crystals can be extremely slippery for walking insects (Juniper 1995; Juniper et al. 1989; Eigenbrode 1996). The slippery zone of *Nepenthes* pitchers, playing a pivotal role in the capture of insect prey, was the first instance where a distinct ecological function could be attributed to epicuticular wax crystals (Knoll 1914). In recent years, various aspects of slippery wax crystals have been investigated in the context of symbiotic ant–plant interaction (Federle et al. 1997, 2000) and of direct (Stork 1980; Brennan and Weinbaum 2001) or indirect (Eigenbrode et al. 1996, 1999) defence against herbivores. But it is an open question how the crystals make plant surfaces slippery for insects. The interaction between the insect attachment devices and various plant surfaces strongly depends on the physico-chemical characteristics of the involved materials. But to date there is only little knowledge about the properties, e.g. of insect adhesive secretions (Attagalle et al. 2000; Vötsch et al. 2002).

For the plant side of the interaction, Knoll (1914) postulated that epicuticular wax crystals are easily detachable from the plant surface. Insects tried to clean their feet after walking on *Nepenthes* surfaces, indicating that crystals broke under the weight load of an insect foot and contaminated the insect attachment devices. This interpretation was supported by transmission electron micrographs showing carbon replicas of *Nepenthes* wax platelets (Juniper and Burras 1962). They led to a model describing these crystals as especially slippery: the parallel orientation to the pitcher surface, similar to roof tiles, would allow perfect contact between foot and crystal. As the crystals are fastened by thin hooks to the underlying cuticle surface, they would easily break, detaching both foot and crystal from the pitcher surface. The present findings for the inner surfaces of *N. alata* pitchers are inconsistent with these hypotheses: (i) the narrow edges of the platelets are directed to the lumen of the pitcher; (ii) the crystals are not singular, isolated aggregates but networks of platelets partly penetrating each other; (iii) even after being separated from the underlying tissue, the network of crystals shows high structural integrity; (iv) the platelets consist of polymeric aldehydes that probably reinforce them chemically and mechanically. Based on these results, we hypothesize that the crystals are mechanically

stable in the force ranges applied by an insect attachment device (albeit not in the force range applied in our adhesive treatments). Other hypothetical mechanisms, possibly in combination, would then cause the slipperiness of the inner surface of *N. alata* pitchers. The orientation of the crystals, exposing only small areas to the insect foot, will limit the number of attachment points, possibly reducing applicable forces below a critical threshold. Furthermore, the reduced surface energy of the substrate might impair wetting by the adhesive secretion on the attachment devices and, hence, cause slipperiness. It had previously been shown that microscopic surface roughness could be a very important factor limiting insect attachment (Stork 1980).

The data presented here for the composition and structure of the outermost surface of the slippery zone in *N. alata* pitchers provide the basis for mechanistic investigations of the interaction between this plant surface and insect attachment devices. The adhesion properties of insects on different surfaces were shown to be measurable variables (Eigenbrode and Kabalo 1999; Eigenbrode et al. 1999; Federle et al. 2000; Scherge and Gorb 2001). However, the mechanism of slipperiness can only be understood if also the physical properties of the crystals are known. The question will have to be addressed of how the chemical composition of the crystals is related to their mechanical capacity. The methods developed in the present investigation can be employed to analyse the slippery surfaces of other carnivorous plant taxa, e.g. *Brocchinia reducta*, to compare diverse crystal compositions, shapes and mechanical properties.

Conclusions

In the present study, the composition of epicuticular wax crystals was for the first time probed and analysed directly. Based on a comparison between surface crystals and the underlying intracuticular wax layer, the physicochemical processes leading to crystal formation could be judged. Platelet-shaped crystals on the inner walls of *Nepenthes* pitchers, creating slippery surfaces that are crucial for the capture of insect prey, consisted of aldehydes (predominantly triacontanal). Hence, the special ecological function of these cuticular structures is exerted by a few compounds that are ubiquitous surface constituents of most plant species. Crystal formation was found to be a spontaneous phase separation, the only prerequisite being the accumulation of single compounds beyond a threshold concentration. Hence, the evolution of the slippery pitcher surface involved only the enhanced biosynthesis of triacontanal, likely by up-regulation of a pre-existing gene product. It is interesting to see that similar crystals on various other plant surfaces are formed by wax alcohols instead of the aldehydes. These crystal-forming aldehydes were found to be in a polymeric form and are likely to reinforce the platelets chemically and mechanically. The present results contradict previous

models that described the *Nepenthes* crystals as especially fragile, breaking under the load of insect feet and, hence, causing slipperiness. Conversely, we hypothesize that the evolutionary optimization of *Nepenthes* pitcher surfaces led to the formation of polymeric crystals that are especially stable, perfectly resisting erosion by insect feet and thus initiating the fall of prey and defeating repeated escape efforts.

References

An C-I, Fukusaki E, Kobayashi A (2002a) Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. Planta 214:661–667

An C-I, Takekawa S, Okazawa A, Fukusaki E, Kobayashi A (2002b) Degradation of a peptide in pitcher fluid of the carnivorous plant *Nepenthes alata* Blanco. Planta 215:472–477

Attagalle AB, Aneshansley DJ, Meinwald J, Eisner T (2000) Defence by foot adhesion in a chrysomelid beetle (*Hemisphaerota cyanea*): characterisation of the adhesive oil. Zoology 103:1–6

Baker EA (1982) Chemistry and morphology of plant epicuticular waxes. In: Cutler DF, Alvin KL, Price CE (eds) The plant cuticle. Academic Press, London, pp 139–166

Beutel RG, Gorb SN (2001) Ultrastructure of attachment specializations of hexapods (Arthropoda): evolutionary patterns inferred from a revised ordinal phylogeny. J Zool Syst Evol Res 39:177–207

Bianchi G (1995) Plant waxes. In: Hamilton RJ (ed) Waxes: chemistry, molecular biology and functions. Oily Press, Dundee, UK, pp 177–222

Brennan EB, Weinbaum SA (2001) Effect of epicuticular wax on adhesion of psyllids to glaucous juvenile and glossy adult leaves of *Eucalyptus globulus* Labillargière. Aust J Entomol 40:270–277

Darwin C (1875) Insectivorous plants. John Murray, London

Eigenbrode SD (1996) Plant surface waxes and insect behaviour. In: Kersteins G (ed) Plant cuticles. BIOS, Oxford, pp 201–221

Eigenbrode SD, Kabalo NN (1999) Effects of *Brassica oleracea* waxblooms on predation and attachment by *Hippodamia convergens*. Entomol Exp Appl 91:125–130

Eigenbrode SD, Castagnola T, Roux M-B, Steljes L (1996) Mobility of three generalist predators is greater on cabbage with glossy leaf wax than on cabbage with a wax bloom. Entomol Exp Appl 81:335–343

Eigenbrode SD, Kabalo NN, Stoner KA (1999) Predation, behaviour, and attachment by *Chrysoperla ploribunda* larvae on *Brassica oleracea* with different surface waxblooms. Entomol Exp Appl 90:225–235

Eisner T, Aneshansley DJ (2000) Defence by foot adhesion in a beetle (*Hemisphaerota cyanea*). Proc Natl Acad Sci USA 97:6568–6573

Ensikat HJ, Neinhuis C, Barthlott W (2000) Direct access to plant epicuticular wax crystals by a new mechanical isolation method. Int J Plant Sci 161:143–148

Federle W, Maschwitz U, Fiala B, Riederer M, Hölldobler B (1997) Slippery ant-plants and skilful climbers: selection and protection of specific ant partners by epicuticular wax blooms in *Macaranga* (Euphorbiaceae). Oecologia 112:217–224

Federle W, Rohrseitz K, Hölldobler B (2000) Attachment forces of ants measured with a centrifuge: better 'wax-runners' have poorer attachment to a smooth surface. J Exp Biol 203:505–512

Federle W, Brainerd EL, McMahon TA, Hölldobler B (2001) Biomechanics of the movable pretarsal adhesive organ in ants and bees. Proc Natl Acad Sci USA 98:6215–6220

Gaume L, Gorb SN, Rowe N (2002) Function of epidermal surfaces in the trapping efficiency of *Nepenthes alata* pitchers. New Phytol 156:479–489

Gorb S (2001) Attachment devices of insect cuticle. Kluwer, Dordrecht

Haas K, Brune T, Rücker E (2001) Epicuticular wax crystalloids in rice and sugar cane leaves are reinforced by polymeric aldehydes. J Appl Bot 75:178–187

Holloway PJ (1984) Surface lipids of plants and animals. In: Mangold HK, Zweig G, Sherma J (eds) Handbook of chromatography, Lipids vol 1. CRC Press, Boca Raton, pp 347–380

Holloway PJ, Jeffree CE, Baker EA (1976) Structural determination of secondary alcohols from plant epicuticular waxes. Phytochemistry 15:1768–1770

Jeffree CE (1986) The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution. In: Juniper BE, Southwood SR (eds) Insects and the plant surface. Arnold, London, pp 23–63

Jeffree CE, Baker EA, Holloway PJ (1976) Origins of the fine structure of plant epicuticular waxes. In: Dickinson CH, Preece TF (eds) Microbiology of aerial plant surfaces. Academic Press, London, pp 199–158

Jetter R, Riederer M (1994) Epicuticular crystals of nonacosan-10-ol: in-vitro reconstitution and factors influencing crystal habits. Planta 195:257–270

Jetter R, Riederer M (1995) In vitro reconstitution of epicuticular wax crystals: formation of tubular aggregates by long-chain secondary alcohols. Bot Acta 108:111–120

Jetter R, Schäffer S (2001) Chemical composition of the *Prunus laurocerasus* leaf surface. Dynamic changes of the epicuticular wax film during leaf development. Plant Physiol 126:1–13

Jetter R, Schäffer S, Riederer M (2000) Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. Plant Cell Environ 23:619–628

Juniper BE (1995) Waxes on plant surfaces and their interactions with insects. In: Hamilton RJ (ed) Waxes: chemistry, molecular biology and functions. Oily Press, Dundee, UK, pp 157–174

Juniper BE, Burras JK (1962) How pitcher plants trap insects. New Sci 13:75–77

Juniper BE, Robins RJ, Joel DM (1989) The carnivorous plants. Academic Press, London

Knoll F (1914) Über die Ursache des Ausgleitens der Insektenbeine an wachsbedeckten Pflanzenteilen. Jahrb Wiss Bot 54:448–497

Lamberton JA (1965) The long-chain aldehydes of sugar-cane wax. Aust J Chem 18:911–913

Lamberton JA, Redcliffe AH (1960) The chemistry of sugar-cane wax. Aust J Chem 13:261–268

Lloyd FE (1942) The carnivorous plants. Chronica Botanica Co., Waltham, MA

Mcfarlane JM (1893) Observations on pitchered insectivorous plants, part II. Ann Bot 7:401–458

Owen TP Jr, Lennon KA (1999) Structure and development of the pitchers from the carnivorous plant *Nepenthes alata* (Nepenthaceae). Am J Bot 86:1382–1390

Owen TP Jr, Lennon KA, Santo MJ, Anderson AN (1999) Pathways for nutrient transport in the pitcher of the carnivorous plant *Nepenthes alata*. Ann Bot 84:459–466

Scherge M, Gorb SN (2001) Biological micro- and nanotribology. Springer, Berlin Heidelberg New York

Schulze W, Schulze ED, Pate JS, Gillison AN (1997) The nitrogen supply from soils and insects during growth of the pitcher plants *Nepenthes mirabilis*, *Cephalotus follicularis* and *Darlingtonia californica*. Oecologia 112:464–471

Stork NE (1980) Role of wax blooms in preventing attachment to brassicas by the mustard beetle, *Phaedon cochleariae*. Entomol Exp Appl 28:100–107

Vötsch W, Nicholson G, Müller R, Stierhof Y-D, Gorb S, Schwarz U (2002) Chemical composition of the attachment pad secretion of the locust *Locusta migratoria*. Insect Biochem Mol Biol 32:1605–1613

Walton TJ (1990) Waxes, cutin and suberin. In: Harwood JL, Boyer J (eds) Methods in plant biochemistry, vol 4. Academic Press, London, pp 106–158