



REVIEW PAPER

# Anti-adhesive effects of plant wax coverage on insect attachment

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Received 9 May 2017; Editorial decision 11 July 2017; Accepted 17 July 2017

Editor: Eva Dominguez, IHSM La Mayora, Spain

## Abstract

**The long period of reciprocal antagonistic coevolution between some insect and plant species has led to the development of plant surface attributes that reduce insect attachment. These features serve as a defence against herbivores, sap-sucking insects and nectar robbers, contribute to a temporary capture of insect pollinators, and prevent the escape of insects from traps of carnivorous plants. This review summarises the literature on attachment-mediated insect–plant interactions. A short introduction to attachment systems of insects is presented and the effect of three-dimensional epicuticular waxes on insect attachment is illustrated by many examples. Special attention is given to the mechanisms of the anti-attachment properties of plant wax structures (the roughness hypothesis, the contamination hypothesis, the fluid-adsorption hypothesis, and the wax-dissolving hypothesis) and their ecological implications.**

**Key words:** Adhesive pad, epicuticular wax, mechanism, reduction, three-dimensional coverage, wax crystals.

## Introduction

The majority of insect species are associated with plants. Such association requires from the insect an ability to efficiently attach to the plant surface. In order to understand the principles of insect attachment to plants, one has to consider the problem of contact and interaction between the insect's adhesive pads and the plant surface (Gorb and Gorb, 2009a). Both adhesive pads and plant surfaces differ in their surface profiles and physico-chemical properties. Furthermore, insect attachment devices produce a fluid secretion into the contact gap. Contact is made more complex by plants exhibiting an extremely broad diversity of surface textures generated by the cuticle or cuticular products.

The plant cuticle represents the interface between the organism and its environment. The structural and chemical features of the cuticle itself and of related structures reflect numerous influences of the particular environment. In the course of

coevolution with insects, flowering plants have evolved both surface chemistry and surface structures that enable proper attachment and locomotion of pollinating insects, whereas in the case of an antagonistic coevolution, plants have developed surfaces specialised in reducing the ability of insects to attach. The latter kind of interactions have resulted, for example, in the development of surface-related defence strategies against herbivorous insects and nectar robbers. In some special cases, insect escape from the highly specialised traps of carnivorous plants is prevented.

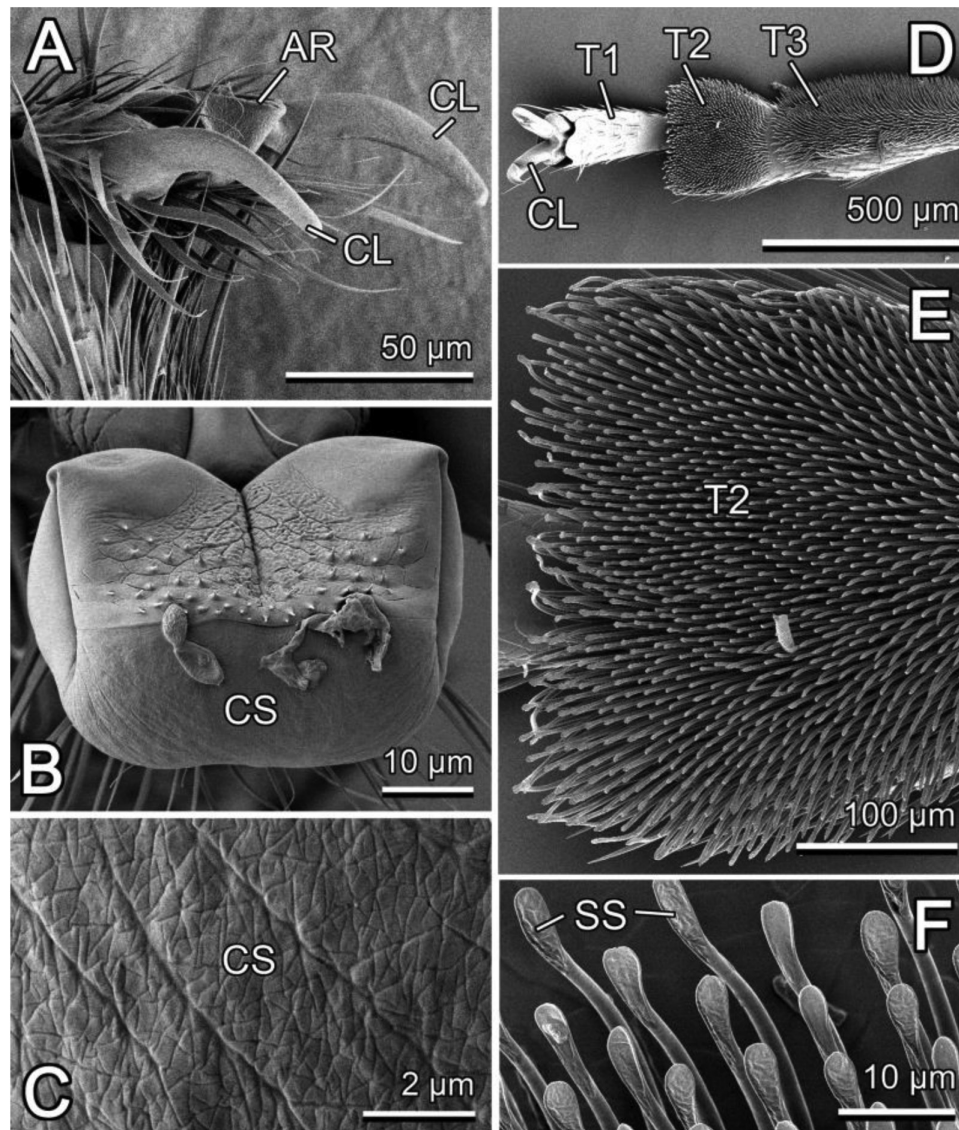
Here we review both the literature and our own previous experimental results on cuticular plant adaptations associated with insect–plant interactions. We demonstrate how plant surfaces can affect insect attachment ability. In particular, the significance of three-dimensional epicuticular waxes for prevention of insect attachment is demonstrated.

The structural and physical background of the anti-adhesive properties of plant cuticles covered by epicuticular waxes is discussed. Since a few physical terms are used in this review, we provide short definitions. We define adhesion (or pull-off force) as the force resisting separation of two contacting bodies, while friction or shear force is the force preventing sliding of two contacting bodies. Attachment is a rather general term referring to holding two bodies together without specifying the exact physical mechanism.

## Insect attachment devices

Mechanisms of insect attachment depend on the particular surface profile of the plant substrate. Usually, insects use their sharp-pointed claws to grip onto the mesoscale

surface asperities (Fig. 1A, D). However, a claw is only able to interlock successfully with surface irregularities when its tip diameter is smaller than the characteristic dimensions of the surface roughness (Dai *et al.*, 2002). Additionally, insects possess two specialised structures, smooth (Fig. 1A–C) and setose or hairy (Fig. 1D–F) adhesive pads, for building tight contact and, in turn, generating strong adhesion on smooth and micro-rough plant surfaces. The considerable softness of smooth pad material and the flexibility of fine hairy cuticular structures (often called tenent setae) of the hairy pads result in an enhancement of the contact area on substrates of different texture (Gorb, 2001). Interestingly, attachment devices are not restricted to one particular part of the insect leg, but may be associated with various leg structures, such as claws, parts of the pretarsus,



**Fig. 1.** Insect attachment devices. Pretarsus of the ant *Lasius niger* (A–C), which has a smooth adhesive pad (arolium), and hairy attachment devices of the beetle *Coccinella septempunctata* (female) (D–F). Scanning electron micrographs showing lateral view of the ant's claws and the folded arolium (A) and frontal view of the spread arolium (B), the arolium surface frozen in contact with a substrate (C), and ventral views of the foreleg tarsus (D), its second proximal tarsomere (E), and tenent setae of the second proximal tarsomere (F). AR, arolium; CL, claw; CS, arolium surface, which contacts a substrate; SS, spatulate setae (with flattened and widened tips); T1–T3, tarsomeres. ((A–C) From Gorb and Gorb (2011b): Gorb E, Gorb S. 2011. *Arthropod–Plant Interactions* 5, 297. With permission of Springer. (D–F) From Gorb *et al.* (2010).)

ventral surfaces of tarsomeres, and the tibio-tarsal joint. Phylogenetic reconstructions of insects have revealed an independent origin of these structures in different insect groups (Beutel and Gorb, 2001).

Attachment devices of insect legs generate fluid secreted into the contact gap. This fluid consists of non-volatile, lipid-like compounds that have been detected in footprints through various histological and chemical techniques (Ishii, 1987; Kosaki and Yamaoka, 1996; Eisner and Aneshansley, 2000; Vötsch *et al.*, 2002; Geiselhardt *et al.*, 2009). Using high-resolution microscopy, it was demonstrated that some insects produce fluids that represent a kind of micro-emulsion composed of two non-mixable groups of substances: water-soluble and lipid-soluble (Gorb, 2001; Vötsch *et al.*, 2002; Federle *et al.*, 2002).

In general, adhesion relies on several basic physical forces. In an adhesion experiment on the bug *Rhodnius prolixus*, Edwards and Tarkanian (1970) detected strong adhesion reduction when tarsi were treated with organic solvents. Stork's (1980a) experiments with leaf beetles led him to the conclusion that cohesive forces, surface tension, and molecular adhesion may contribute to the attachment mediated by the pad fluid. Langer *et al.* (2004) applied atomic force microscopy in order to resolve forces on the tips of individual tenent setae in the fly *Calliphora vicina* and clearly demonstrated that adhesion strongly depends on the presence of the pad fluid surrounded by air. This is strong evidence that insects use attractive capillary interactions mediated by the pad secretion between individual hair tips and the substrate.

## Epicuticular plant waxes

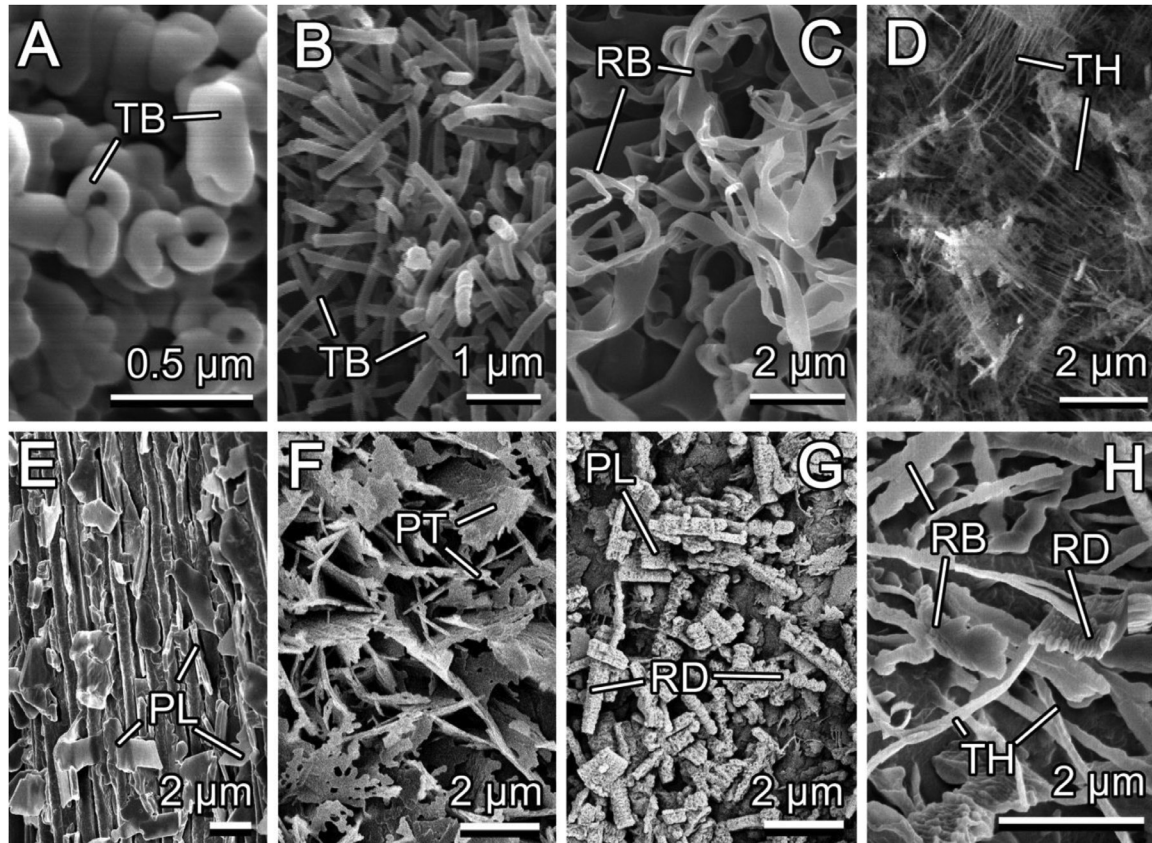
Cuticular waxes are lipids that represent a hydrophobic component of the plant cuticle. The cuticle might be impregnated by intracuticular waxes or waxes can be transported through the cuticle and deposited onto its surface (epicuticular waxes) (Jeffree, 2006). The latter waxes are usually a complex mixture of cyclic (e.g. triterpenoids) and long-chain aliphatic substances, such as primary and secondary alcohols, primary aldehydes, fatty acids, and alkanes (Barthlott *et al.*, 1998; Jetter *et al.*, 2006). In higher plants, they cover all aerial primary surfaces and usually form relatively smooth two-dimensional films. In many plant species, three-dimensional (3D) micro- or nano-scale projections emerge from these two-dimensional (2D) films (Jeffree, 1986). Films vary greatly in thickness from just a few molecular layers on aquatic plant surfaces up to 0.5 mm for some terrestrial plant wax crusts. Wax projections may have a very wide range of dimensions from 0.5 to 100  $\mu\text{m}$  (Barthlott *et al.*, 1998; Koch *et al.*, 2010).

Both 2D and 3D waxes have a crystalline structure that was recently revealed by using electron and X-ray diffraction analyses (Meusel *et al.*, 2000; Ensikat *et al.*, 2006). Wax projections (here called wax crystals) are protrusions of 3D wax coverage with various shapes, such as rodlets, threads, platelets, and tubules (Fig. 2; Barthlott *et al.*, 1998). This complexity of shapes originates from molecular self-assembly on the cuticle surface (Jeffree *et al.*, 1975; Jetter and Riederer, 1994, 1995; Meusel *et al.*, 2000; Koch and Ensikat, 2007). The

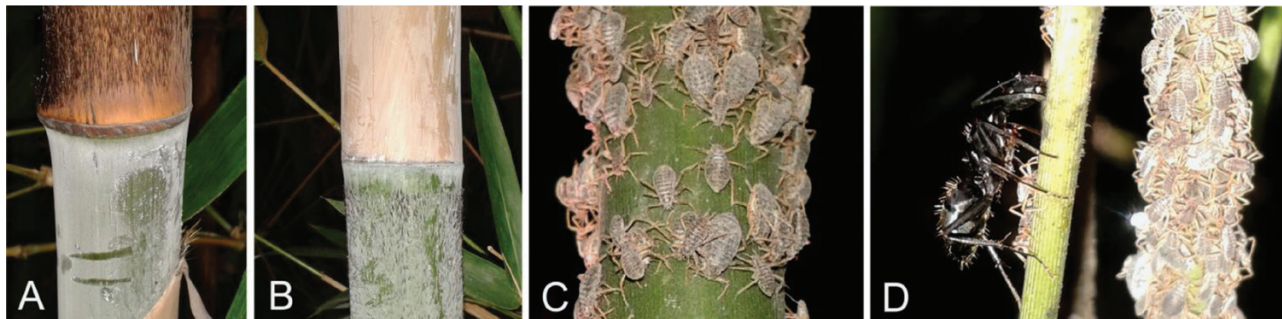
crystal morphology correlates well with the presence of the particular dominant chemical compound in the wax (Jetter and Riederer, 1994, 1995; Barthlott *et al.*, 1998; Meusel *et al.*, 1999, 2000; Bargel *et al.*, 2006).

Since epicuticular waxes are situated at the interface between plant cuticle and environment, they are rather multifunctional. They can contribute to an enhancement of mechanical stability of the surface, they may influence transpiration and gas exchange, and they control surface temperature (Jeffree, 1986). Furthermore, such wax projections were reported to be the structures that reduce the wettability of the cuticle surface, as well as preventing surface contamination by dust particles or pathogenic spores (Barthlott and Neinhuis, 1997; Fürstner *et al.*, 2005). It has also been repeatedly reported and experimentally supported for many plant species (for example, from genera *Eucalyptus*, *Pisum*, *Brassica*, etc.) that the 3D wax coverage has a protective function against herbivorous insects (Anstey and Moore, 1954; Edwards, 1982; Bodnaryk, 1992; Brennan and Weinbaum, 2001a; Chang *et al.*, 2006). Recent observations on young culms of several *Bambusa* species, which often show heavy infection by sap-sucking insects, in particular aphids (Fig. 3), suggest that the waxy culm surface, often in combination with trichomes, hinders the access of aphidicolous ants to aphid colonies, thus indirectly guarding the plants from aphids (Gorb *et al.*, 2017a). This phenomenon is called 'stem guard syndrome' (Gorb *et al.*, 2017a). Plant surfaces with 3D wax coverage may also prevent attachment, locomotion, and foraging behaviour of insect predators and parasitoids (see review by Eigenbrode, 2004). For example, of five coccinellid species studied, the larvae and adults showed significantly better attachment (with the only exception of *Adalia bipunctata* larvae) and higher consumption rate of aphids on *Pisum sativum* leaf surfaces of mutants with strong 3D wax coverage compared with those with reduced wax coverage (Eigenbrode *et al.*, 2009). Plant flowering stems with wax coverage may also be a part of the 'greasy pole syndrome', which prevents nectar robbery and protects other plant resources from ants through the slippery nature of the stems. This syndrome is widespread in numerous plant genera, such as *Salix*, *Hypenia*, and *Eriope* (Harley, 1988, 1991; Juniper, 1995; Gorb and Gorb, 2011b). Some myrmecophilic plant species also bear wax crystals on their stems. In the genus *Macaranga* such wax coverage functions as a kind of selective barrier for insects: in this particular case, it protects the plant against non-specialised ant species, but provides access by ants having a mutualistic association with the plant (Federle *et al.*, 1997, 1998, 2000; Markstädter *et al.*, 2000).

Kettle trap flowers of some plants (e.g. in the genera *Aristolochia* and *Arisaema*) bear slippery wax-covered surfaces that are capable of temporary capture of minute insect pollinators (Vogel and Martens, 2000; Oelschlägel *et al.*, 2009; Poppinga *et al.*, 2010). Some carnivorous plants can even permanently capture insects for digestion using strongly specialised slippery surfaces. The best known examples are certain insect-trapping Bromeliaceae and the inner pitcher surface of the genera *Nepenthes*, *Sarracenia* and *Darlingtonia* (Juniper and Burras, 1962; Juniper *et al.*, 1989; Gaume *et al.*, 2002, 2004; Gorb *et al.*, 2005; Di Giusto *et al.*, 2009; Bauer *et al.*, 2009, 2012;



**Fig. 2.** Epicuticular wax crystals. Scanning electron micrographs of surfaces of *Prunus domestica* fruit (A), adaxial leaf side of *Chelidonium majus* (B), *Acer negundo* young stem (C), abaxial leaf side of *Brassica oleracea* (D), flower stems of *Dahlia pinnata* (E), *Tagetes patula* (F), and *Anethum graveolens* (G), and abaxial leaf side of *Pisum sativum* (H). RB, ribbons; RD, rodlets; TB, tubules; TH, threads; PL, plates; PT, platelets. ((A–D) From Gorb and Gorb (2002): Gorb EV, Gorb SN. 2002. Attachment ability of the beetle *Chrysolina fastuosa* on various plant surfaces. *Entomologia Experimentalis et Applicata* **105**, 13–28. Copyright © 2002 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc. (E–G) From Gorb and Gorb (2011b): Gorb E, Gorb S. 2011. *Arthropod–Plant Interactions* **5**, 297. With permission of Springer. (H) From Gorb et al. (2008).)



**Fig. 3.** Stem guard syndrome. Surface of young culms in *Bambusa chungii* (A) and *B. emeiensis* (B) showing whitish epicuticular wax coverage, and aphid infestation on young culms (C, D). (From Gorb et al. (2017a): Gorb EV, Dai Z, Gorb SN. 2017. Micromorphology of stem surface in three species of *Bambusa* (Poaceae, Bambusoideae) with a focus on its impact on plant–insect interactions. *Flora* **230**, 14–25. Copyright © 2017, with permission from Elsevier.)

Poppinga et al., 2010; Scholz et al., 2010; Wang and Zhou, 2010; Gorb and Gorb, 2011a). These surfaces are usually anisotropic due to the coverage by lunate cells or tiny pointed trichomes, but may be additionally covered by 3D epicuticular waxes.

### Ability of insects to attach to plant cuticles covered with 3D wax projections

As mentioned above, wax crystals can decrease the ability of insects to attach to the plant cuticle surface (reviewed by

Eigenbrode, 1996; Müller, 2006). This effect has been studied with many insect and plant species using a variety of experimental approaches. These studies have shown that on smooth surfaces, either without wax or with the wax coverage removed, the insects generate strong attachment forces; however, on surfaces covered with 3D waxes, weak attachment and multi-fold force reduction were almost always observed (e.g. Way and Murdie, 1965; Stork, 1980b, 1986; Edwards, 1982; Federle et al., 1997, 2000; Eigenbrode and Kabalo, 1999; Eigenbrode et al., 1999a, b, 2000, 2009; Brennan and Weinbaum, 2001b; Eigenbrode and Jetter, 2002;

Gaume *et al.*, 2002, 2004; Gorb and Gorb, 2002, 2011a; Rutledge and Eigenbrode, 2003; Gorb *et al.*, 2005, 2008; Voigt *et al.*, 2007; Prüm *et al.*, 2012a, b; Friedemann *et al.*, 2015). In very few cases was a reverse effect revealed (Federle *et al.*, 2000; Brennan and Weinbaum, 2001a; Eigenbrode, 2004).

Insect attachment depends not just on the presence of 3D wax, but also on the crystal size and density or distribution of individual crystals. For example, attachment forces of the ladybird beetle *Cryptolaemus montrouzieri* walking on *Pisum sativum* plants with wild-type waxes and with reduced waxes caused by the *wel* mutation (a wax eliminator) showed significant correlations with the wax crystal length (negative) and density (positive) (Gorb *et al.*, 2008). Experiments performed with the beetle *Coccinella septempunctata* on bio-inspired wax surfaces formed by four alkanes of varying chain length also demonstrated stronger insect attachment on surfaces having a higher density of the wax coverage formed by smaller crystals (Gorb *et al.*, 2014a).

The comparative study of attachment ability of the chrysomelid beetle *Chrysolina fastuosa* to 12 plant species clearly demonstrated that 3D epicuticular waxes only temporarily reduced attachment (Gorb and Gorb, 2002). Either no influence of the surfaces on the subsequent insect attachment ability was observed or recovery of the attachment ability occurred rather quickly. Also, force tests with the aphid *Acyrtosiphon pisum* showed that it was able to completely restore its attachment ability quickly after walking on wax-covered leaf surfaces of *Trifolium pratense*, *Medicago sativa*, and *Pisum sativum* (Friedemann *et al.*, 2015).

## Mechanisms of insect adhesion reduction on waxy plant surfaces

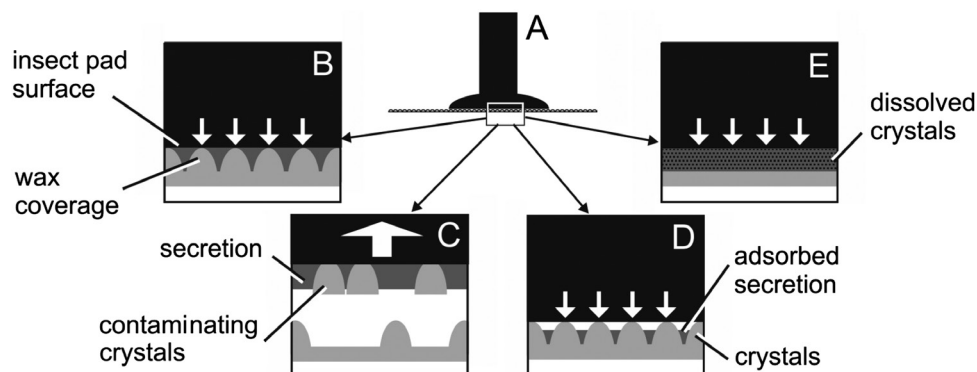
The phenomenon of reduced insect adhesion on plant cuticles covered with 3D epicuticular waxes has no simple explanation. There are several contributing mechanisms and four hypotheses were proposed (Fig. 4): (1) the roughness hypothesis, (2) the contamination hypothesis, (3) the fluid-adsorption hypothesis, and (4) the wax-dissolving hypothesis

(Gorb and Gorb, 2002). Below, we provide a detailed discussion of these hypotheses.

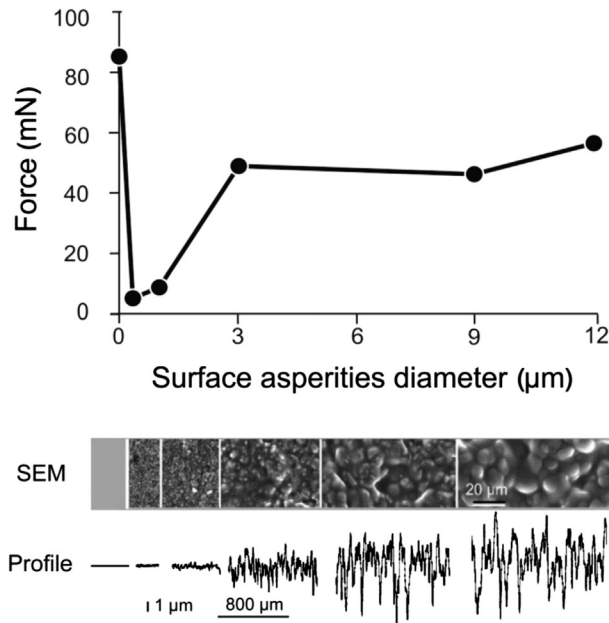
### Roughness hypothesis

Wax crystals on the plant cuticle usually have microscopic to nanoscopic dimensions generating a certain micro-roughness. It is known from the tribological literature that surface roughness causes a decrease of the real contact area between the plant surface and the insect attachment pad (Fig. 4B). Contact forces, such as adhesion and friction, strongly depend on the real contact area, where distances between contacting surfaces are in the range of atomic dimensions (Bowden and Tabor, 2001; Kendall, 2001; Varenberg *et al.*, 2011), and if the area is reduced, an insect would be unable to stick to such micro-rough surfaces caused by wax crystals.

The fact that both micro- and nano-scale roughness affect insect attachment has been experimentally demonstrated for the fly *Musca domestica* and the beetles *Gastrophysa viridula*, *Leptinotarsa decemlineata*, and *Coccinella septempunctata* (Gorb, 2001; Peressadko and Gorb, 2004; Voigt *et al.*, 2008; Bullock and Federle, 2009; Gorb and Gorb, 2009b; England *et al.*, 2016). These insect species showed stronger attachment in centrifugal and traction experiments on smooth artificial substrates in comparison with their performance on the nano- and micro-rough substrates (Fig. 5). Surprisingly, the lowest attachment was revealed on the rough surfaces with the nominal asperity size ranging from 0.3 to 1.0  $\mu\text{m}$  (Gorb, 2001; Peressadko and Gorb, 2004; Voigt *et al.*, 2008; Bullock and Federle, 2009). This range of dimensions corresponds to that of plant wax crystals. In a numerical simulation, the anti-adhesive properties of these surfaces have been explained by a diminution of the real contact area between the rough surface profile and the tips of the individual insect setae responsible for intimate contact with the surface (Peressadko and Gorb, 2004). The model assumed a negative correlation between attractive forces and the average distance between contacting surfaces and demonstrated the maximum distance and, thus, the minimum attractive force between the setal tip of the insect pad and the substrate for surfaces with an asperity size



**Fig. 4.** Schematic presentation of hypotheses explaining anti-adhesive properties of plant cuticle covered with 3D wax crystals. (A) Tenent seta contacting a waxy surface; (B) the roughness hypothesis; (C) the contamination hypothesis; (D) the fluid-adsorption hypothesis; and (E) the wax-dissolving hypothesis. (Adapted from Gorb and Gorb (2002): Gorb EV, Gorb SN. 2002. Attachment ability of the beetle *Chrysolina fastuosa* on various plant surfaces. *Entomologia Experimentalis et Applicata* **105**, 13–28. Copyright © 2002 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.)



**Fig. 5.** Attachment forces of the Colorado potato beetle *Leptinotarsa decemlineata*. Results were obtained in centrifugal friction experiments on artificial substrates with different surface micro-roughness generated by asperities with nominal sizes ranging from 0.3 to 12.0 µm. (From Voigt *et al.* (2008): Voigt D, Schuppert JM, Dattinger S, Gorb SN. 2008. Sexual dimorphism in the attachment ability of the Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) to rough substrates. *Journal of Insect Physiology* **32**, 765–776. Copyright © 2008, with permission from Elsevier.)

of 0.3–1.0 µm. Force tests with insects having hairy attachment pads showed that surfaces with larger asperity sizes (3–12 µm) did not cause such strong force reduction (Gorb, 2001; Peressadko and Gorb, 2004; Voigt *et al.*, 2008; Gorb and Gorb, 2009b). The fact that on the latter substrates insect attachment forces were comparable to those measured on smooth substrates has been explained by the particular dimension of the setae (2–10 µm) and spatula-like geometry of setal tips. Insect tenent setae are presumably able to generate sufficient contact area on substrates with relatively large dimensions of surface asperities that bear relatively large smooth areas on the tops of their elevations (Peressadko and Gorb, 2004; Voigt *et al.*, 2008).

The stick insect *Carausius morosus* demonstrated the lowest attachment ability on the finest roughness (3 µm asperity size) compared with smooth and other micro-rough substrates (10, 25, and 46 µm asperity sizes) (Scholz *et al.*, 2010). The substrate roughness where the attachment force is the lowest is called the critical roughness. The critical roughness in the case of the stick insect was found to display very similar geometrical parameters to the waxy zone of pitchers in the carnivorous plant *Nepenthes alata*. Scholz *et al.* (2010) combined results of their force experiments with a theoretical model and concluded that the surface roughness in a certain range (the critical roughness) prevents insect adhesion by being ‘too rough’ for the dimensions and deformability of adhesive pads (Scholz *et al.*, 2010). Thus, *Nepenthes* wax crystals lead to the reduction of insect attachment due to micro- and nanoscopic surface roughness. A similar effect was previously shown in

traction force experiments with the coccinellid beetle *Adalia bipunctata* walking on the lower wax layer of *N. alata* (Gorb *et al.*, 2005). The adhesion tests performed with *N. alata* wax coverage, where a newly developed method for adhesion (pull-off) force measurements on surfaces having low adhesion capability was applied (Purtov *et al.*, 2013), showed an intermediate force value on the 3 µm micro-rough surface compared with the lower and upper wax layers of *N. alata* (Gorb *et al.*, 2014b). In these experiments, deformable tacky polymer half-spheres with an elasticity modulus similar to that of the smooth adhesive pads in insects (50 kPa) were used as probes and the normal load force (500–1000 µN) resulting in pressure similar to that of an insect (138–174 Pa) was applied.

Recently traction experiments performed with the beetle *Coccinella septempunctata* on eight types of artificial surfaces, each with different chemical properties and topography, clearly demonstrated the dominant effect of surface roughness on the attachment ability of the beetles (England *et al.*, 2016). All four micro-rough samples showed the lowest force, with no statistically significant difference between these samples. Interestingly, chemical surface properties, such as de-wettability of water and oil caused by a specific chemical composition, showed a certain, but non-significant, effect on the attachment of the beetles. These results are in line with those obtained in the attachment experiments previously performed with the beetle *Hippodamia convergens* on several genotypes of *Brassica oleracea* differing in wax characteristics and on wax extracts from these plants applied to glass (Eigenbrode and Jetter, 2002). Beetles showed significantly lower attachment force on the prominent wax coverage compared with the reduced one and smooth layer of wax extracts. As for the effects deriving from chemical composition, none of the correlations examined between the relative composition for each compound class in wax extracts and beetle attachment was significant (Eigenbrode and Jetter, 2002).

#### Contamination hypothesis

In some plants, the wax crystals are rather firmly connected to the underlying layers, whereas in others, they are easily detachable structures that can readily adhere to insect attachment organs and contaminate them (Fig. 4C). Additionally, this effect is strongly enhanced in adhesive pads covered with fluid secretion. Such pad contamination by wax may impair proper functioning of the pads and lead to the reduction of insect adhesion up to the complete disabling of insect locomotion on such plant substrates.

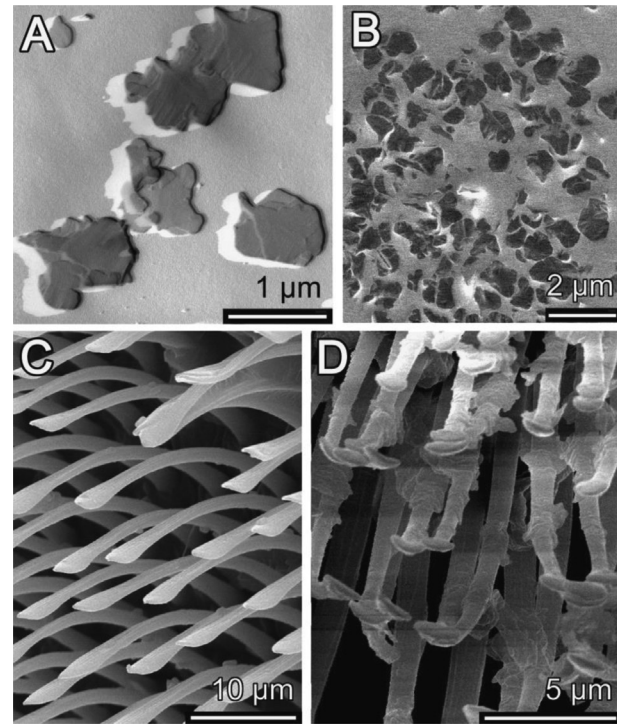
There are some experimental data confirming this hypothesis. Insects usually clean their tarsi after locomotion on waxy plant surfaces. It has been, for example, reported that the beetle *Paropsis charybdis* showed grooming behaviour after walking on young leaves of *Eucalyptus nitens* (Edwards, 1982). Similar behaviour was also demonstrated in two other insect species, the fly *Drosophila melanogaster* and the ant *Iridomyrmex humilis*, after their attempts to climb up the waxy zone of *Nepenthes alata* pitchers (Gaume *et al.*, 2002). Some earlier authors also provided direct evidence that the 3D wax, which

is loosely connected to the cuticle, contaminates insect attachment pads. Using scanning electron microscopy (SEM), the ability of extremely fragile wax crystals of *Brassica* spp. to contaminate attachment organs of the herbivorous beetle *Phaedon cochleariae* and three species of predatory insects has been shown (Stork, 1980b, 1986; Eigenbrode *et al.*, 1999a). The contaminating effect of the upper wax crystals of *Nepenthes* on attachment devices of the fly *Musca domestica* has been verified with the aid of transmission electron microscopy (Juniper and Burras, 1962; Juniper *et al.*, 1989). By SEM, wax platelets of the carnivorous plants *N. ventrata*, *Brocchinia reducta*, and *Catopsis berteroniana* were found to be attached to pads of the fly *Calliphora vomitoria* (Gaume *et al.*, 2004).

Experimental evidence for the contamination hypothesis has been provided for several insect species with the hairy type of pads and a range of plant species. For example, the outermost wax layer of the waxy zone in *Nepenthes alata* pitchers easily contaminates insect pads (Gorb *et al.*, 2005). The upper wax layer consists of stalked crystals having a specific leaf-like shape. These are very brittle and may be easily exfoliated or broken into multiple parts (Fig. 6A). The attachment force of the coccinellid beetle *Adalia bipunctata* was very strongly reduced on this surface in traction force tests. SEM inspection of the pads after this experiment provided clear evidence that the force reduction was due to the contamination of the adhesive pads by the upper wax crystals (Fig. 6C, D). Recent pull-off force tests using tacky polymer probes made of polydimethylsiloxane (Purtov *et al.*, 2013) support the mechanism of strong anti-adhesive properties of the upper wax layer. Also, the ability of these crystals to easily exfoliate under minimal preload and contaminate sticky surfaces was clearly demonstrated (Fig. 6B; Gorb *et al.*, 2014b).

Attachment pads of the chrysomelid beetle *Chrysolina fastuosa* showed different degrees of contamination by wax crystals after walking on 12 plant surfaces covered by 3D wax (Gorb and Gorb, 2006). The nature of the contamination, which is defined as the structure of the contaminating material (Fig. 7A–C), and the degree of contamination, which is defined as the portion of setal tip surface covered with wax particles as well as the proportion of contaminated setae, were considerably different (Fig. 7D). The observed differences were presumably caused by different shapes and dimensions of the crystals. Specifically, the largest aspect ratio of wax crystals had the strongest effect on the degree of the contamination. That is why the rod-like and especially thin platelet-like crystals caused the strongest contamination effect, whereas tube-like crystals had a much lower effect.

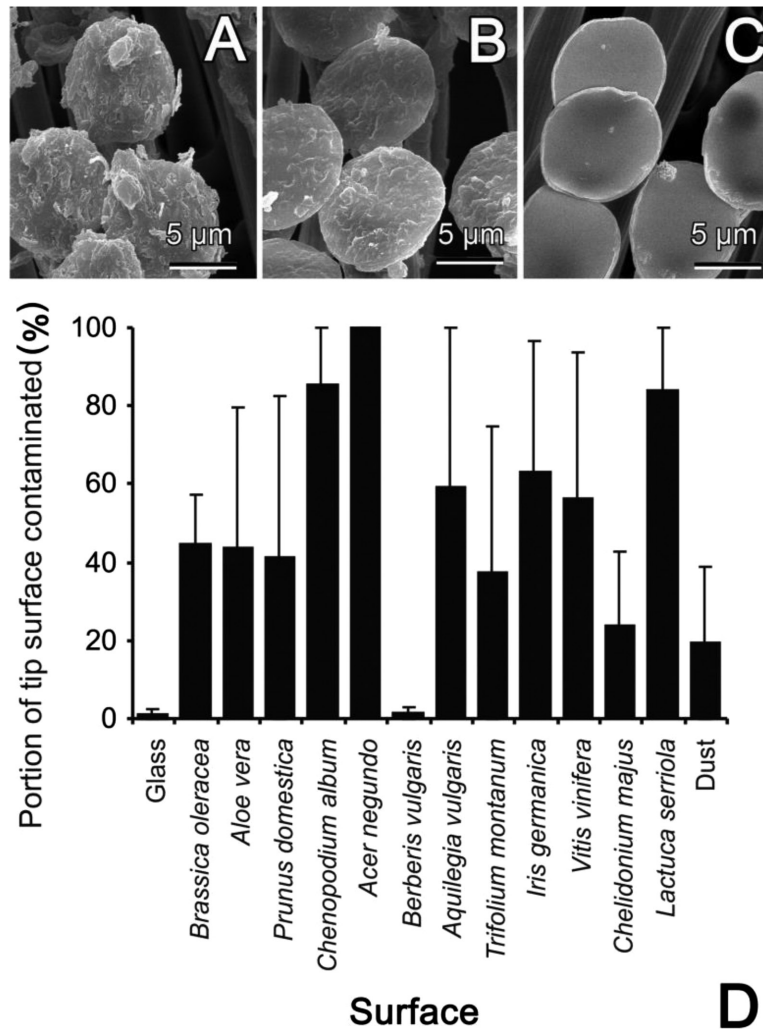
Keeping these experimental results in mind, a theoretical mechanics approach (Borodich *et al.*, 2010) was applied to explain different degrees of insect pad contamination by waxes of different plant species. It was hypothesised that the contamination mainly depends on the wax crystal fracture behaviour, which in turn depends on the geometry of the crystals. Crystals of five plant species (*Aristolochia fimbriata*, *Aquilegia vulgaris*, *Berberis vulgaris*, *Chelidonium majus*, and *Prunus domestica*) were selected and precisely measured



**Fig. 6.** Evidence for the contamination hypothesis. (A) Transmission electron micrograph of isolated wax crystals from the upper wax layer of the waxy zone of the pitcher of the carnivorous plant *Nepenthes alata*. (B) Cryo-scanning electron micrograph of the tacky polymer probe made of polydimethylsiloxane after adhesion test with the *N. alata* upper wax layer. (C, D) Scanning electron micrographs of tarsal tenent setae of the coccinellid beetle *Adalia bipunctata* after walking on the wax-free (treated with warm chloroform) (C) and intact (wax-bearing) waxy zone (D) of the *N. alata* pitcher. ((A, C, D) Adapted from Gorb *et al.* (2005) with permission of *The Journal of Experimental Biology*.)

using SEM images. It was shown that during contact formation between insect adhesive pads and the plant surface, the mechanisms of the wax crystal fracture may be very different, depending on the crystal slenderness ratio. Crystals with a high ratio may buckle elastically or in an elastic–plastic way. For the majority of crystals studied, the calculated values were much lower than the critical ones (26.5 for elastic buckling and 18.7 for elastic–plastic buckling), which means that these crystals could hardly buckle. However, careful examination of these crystals for their resistance to elastic–plastic bending demonstrated that insects are able to fracture crystals of some plant species by such bending. This general approach is applicable for crystals of different shapes, even if the models were initially applied for tubular crystals (Borodich *et al.*, 2010).

Contamination of insect adhesive pads with wax crystals may temporarily affect the ability of insects to attach further. The experimental study with the chrysomelid beetle *Chrysolina fastuosa* demonstrated that the wax coverage of *Acer negundo* stems, showing the highest contamination capability among plant species studied, disabled insect attachment for a certain amount of time (Gorb and Gorb, 2002). Another 11 waxy plant surfaces studied either did not reduce or reduced only for a short while the further attachment ability of beetles.



**Fig. 7.** Degree of contamination of adhesive pads in the chrysomelid beetle *Chrysolina fastuosa* by wax crystals of various plant species. (A–C) Scanning electron micrographs showing beetle adhesive setae after walking on the young stem of *Acer negundo* (A), and adaxial leaf side of *Chenopodium album* (B) and *Berberis vulgaris* (C). (D) The portion of setal tip areas contaminated with wax material after beetles had walked for the same period of time on different plant surfaces. (From Gorb and Gorb (2006): in: Herrel A, Speck T, Rowe NP. 2006. *Ecology and biomechanics: A mechanical approach to the ecology of animals and plants*. Copyright © 2006. Reproduced by permission of Taylor and Francis Group, LLC, a division of Informa plc.)

In traction experiments with the aphid *Acyrtosiphon pisum* and the waxy leaf surfaces of *Trifolium pratense*, *Medicago sativa*, and *Pisum sativum*, immediately after walking over the plant surfaces, insects were repeatedly tested on a clean reference glass surface, where they had successfully performed earlier (Friedemann *et al.*, 2015). Three consecutive pulling events showed very low force values during the first trial. By the third trial, the aphids regained their attachment ability and pulled with the same force as they did before they had walked over the waxy plant surfaces. However, adhesion tests on the upper wax layer in *Nepenthes alata*, where strong contaminating properties of upper wax crystals were confirmed, revealed no subsequent reducing effect of this contamination on adhesion (Gorb *et al.*, 2014b). From these experimental data, it was suggested that in such cases broken off wax crystals act as a separation layer between an insect pad and a plant surface and thus minimise both capillary adhesion and adhesion caused by van der Waals forces.

#### Fluid-adsorption hypothesis

This hypothesis predicts that the 3D waxes may adsorb the adhesive fluid from the insect pad surface (Fig. 4D). This effect is caused by the high porosity of the wax coverage and by its affinity to lipid-bearing pad secretions. The fluid adsorption leads to a reduction of the fluid thickness between the insect pads and the substrate, and this, in turn, results in the reduction of capillary forces in the contact zone between the pad and the porous surface.

Previous studies have experimentally demonstrated that insect pad secretion is a crucial factor maintaining pad adhesion to smooth surfaces. Pads of the bug *Rhodnius prolixus* treated with lipid solvents demonstrated strong reduction in attachment (Edwards and Tarkanian, 1970). The aphid *Aphis fabae*, after walking on silica gel, almost completely lost its attachment ability (Dixon *et al.*, 1990). The stick insect *Carausius morosus*, walking on smooth polyimide substrates that selectively adsorbed the watery component of the

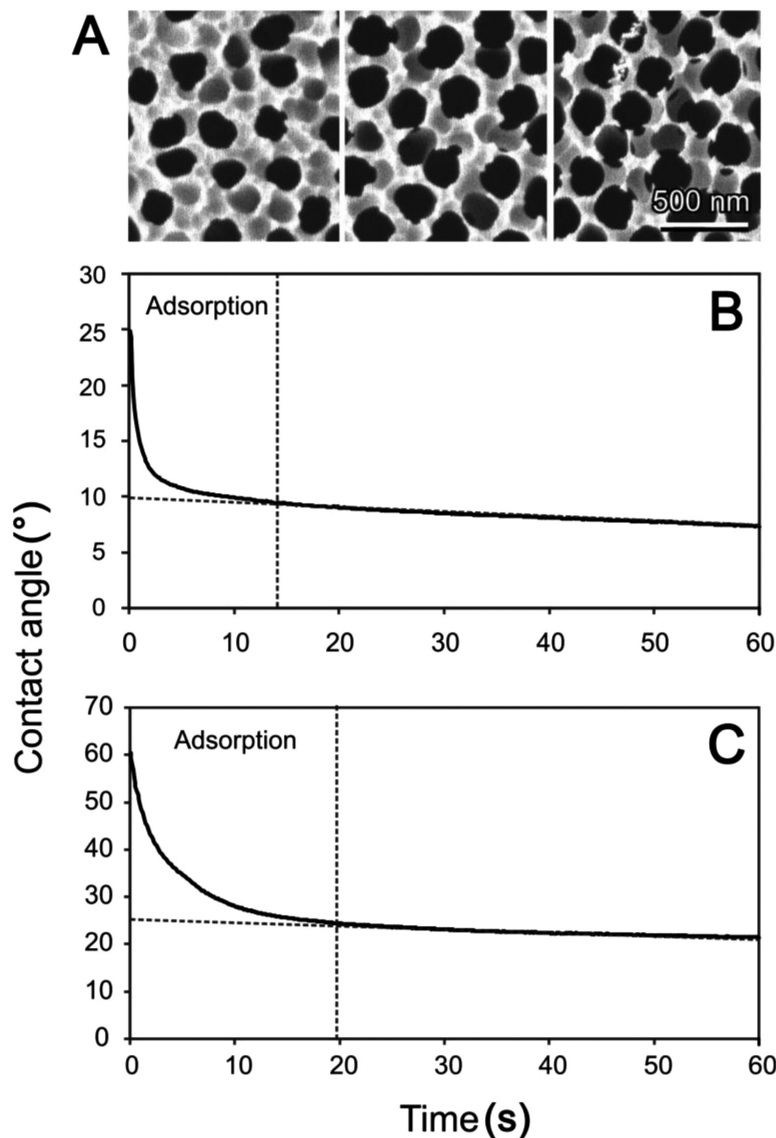


secretion, showed considerable force reduction, which was caused by a decrease in the thickness of the pad secretion (Dirks *et al.*, 2010). Usually the secretion fills the gaps between asperities of rough substrates and this may result in adhesion enhancement due to maximisation of the contact area (Drechsler and Federle, 2006), but when the gaps are endless, as in the case of a porous substrate, the fluid will disappear from the contact area between the pad and the substrate.

The fluid-adsorption hypothesis was tested experimentally using the beetle *Coccinella septempunctata* with hairy pads walking on nano-porous substrates with the same pore diameter (220–250 nm) but different porosity (28, 42, and 51%) (Fig. 8A) and smooth solid surfaces used as the control (Gorb *et al.*, 2010). Using the traction force method, considerably higher attachment forces of beetles could be seen on the solid compared with the porous substrates. Between the porous ones, only surfaces with the highest and lowest porosity

demonstrated a significant difference. The experimental evaluation of changes in the contact angles of water and oil to these surfaces with time revealed that nano-porous surfaces had strong adsorption ability for both polar and non-polar fluids (Fig. 8B, C). Both experiments taken together led to the conclusion that high porosity of nano-porous membranes caused the fluid adsorption from beetle adhesive pads, thereby diminishing capillary interactions and reducing insect attachment ability.

Very recently, the adsorption ability of 3D epicuticular plant waxes was experimentally confirmed (Gorb *et al.*, 2017b). Using the cryo-SEM approach and high-speed video recordings of both water and oil drop behaviour, followed by numerical analysis of the data obtained, it was found that the wax coverage of *Nepenthes alata* is able to strongly adsorb oil. On both intact (two-layered) and treated (one-layered, bearing only the lower wax layer) wax samples, changes in the base, height,



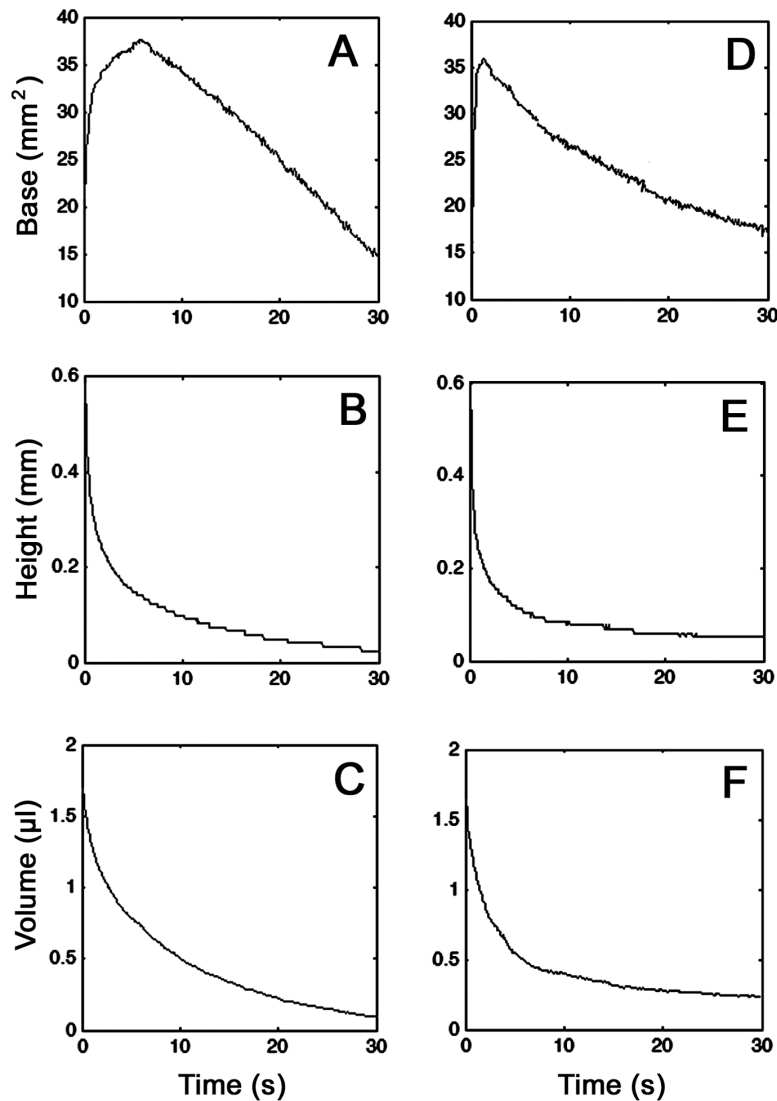
**Fig. 8.** Evidence for fluid-adsorption hypothesis. (A) Scanning electron micrographs of nano-porous substrates with different porosities (28, 42, and 51% from left to right) used in force experiments with *Coccinella septempunctata* beetles. (B, C) Changes in contact angles of water (B) and oil (C) on the nano-porous sample with a porosity of 42%. Dotted lines demarcate parts of curves showing the effect of fluid adsorption. (Adapted from Gorb *et al.* 2010.)

and volume of the oil drops over a period of time were detected (Fig. 9). Although wax plant samples differed in layer thickness, this had no considerable effect on the oil drop behaviour. Based on these results it was concluded that 3D wax coverage on plant surfaces, due to its adsorption capability, is in general anti-adhesive for insects that rely mainly on wet adhesion.

#### Wax-dissolving hypothesis

Since insect pad secretion contains a strong proportion of lipids and lipophilic substances, it may be hypothesised that some components of the fluid are able to dissolve wax crystals (Fig. 4E). This in turn will lead to an increase of fluid layer thickness on the plant surface. The contact forces (friction and adhesion) between pads and the surface decrease and the substrate becomes slippery for insects due to the newly formed thick fluid layer in the contact area.

There is only indirect evidence that pad secretion can dissolve wax. The fly *Calliphora vomitoria*, which walked on wax-bearing surfaces of the carnivorous plants *Nepenthes ventrata* and *Brocchinia reducta*, demonstrated tenent setae glued together with an amorphous substance (Gaume *et al.*, 2004). This substance might be a product of wax crystals that lost their structure due to the chemical interactions with the pad secretion. Similar effects were also observed in the study of the contamination of hairy adhesive pads in the beetle *Chrysolina fastuosa* by the wax coverage of 12 plant surfaces: wax contamination of some plants, such as *Aquilegia vulgaris*, *Trifolium montanum*, *Aloe vera*, and *Berberis vulgaris*, exhibited a relatively homogeneous texture, where wax crystals were not recognisable (Gorb and Gorb, 2006). This almost unstructured homogeneous contamination was also explained by a possible dissolving of wax crystals caused by insect secretory fluid.

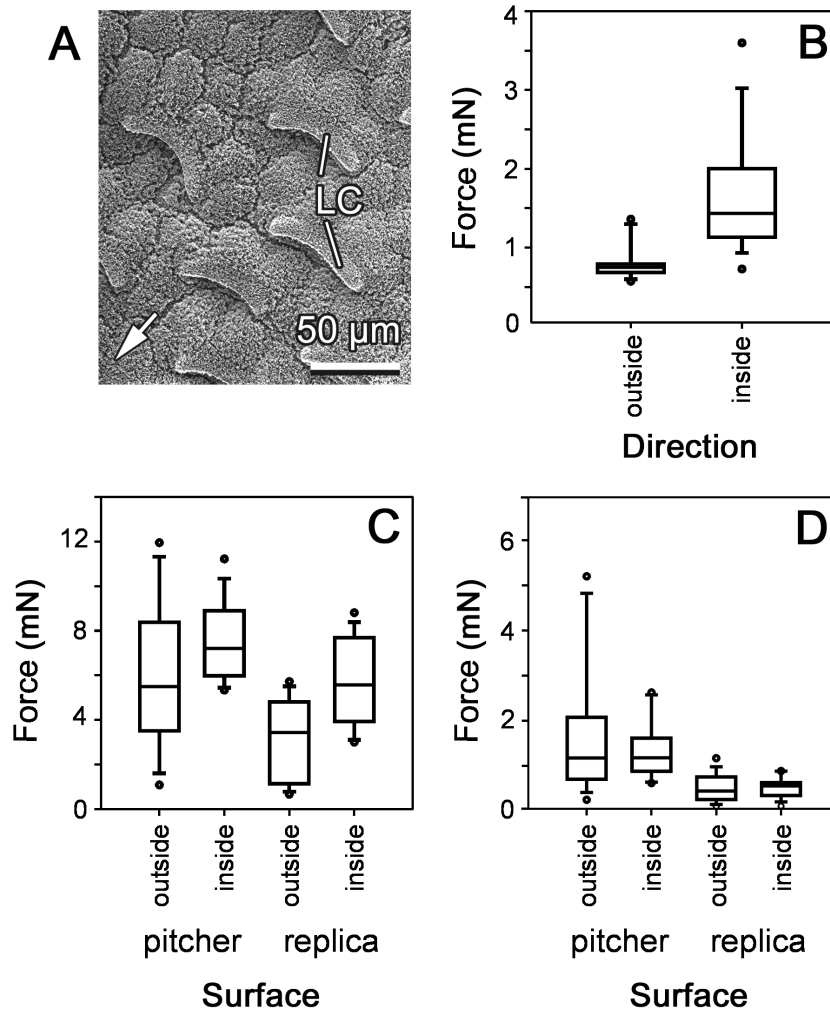


**Fig. 9.** Behaviour of oil drops on wax-covered surfaces of the *Nepenthes alata* pitcher. Time-dependant values of the base (A, D), height (B, E), and volume of the drop (C, F) on one-layered (A–C) and two-layered (D–F) wax samples. (From Gorb *et al.* (2017b): Gorb EV *et al.* 2017. Oil adsorption ability of three-dimensional epicuticular wax coverages in plants. *Scientific Reports* 7, 45483; doi:10.1038/srep45483. Copyright © 2017. Reprinted by permission from Macmillan Publishers Ltd.)

## Combination of plant cuticle sculpture and wax coverage

Many plant species are covered not just by waxes, but rather a combination of cuticle sculpture and 3D wax. Both types of structure can differ in shape, size, and orientation (Koch, 2010). Hierarchy often starts at the macro-scale with a dimension of several millimetres causing, for example, a particular waviness of a leaf plane. The next hierarchical level may consist of much smaller features, such as multicellular structures (trichomes) and curved outlines of cells. The third hierarchical level is built by cuticular folds and wax crystals with dimensions ranging from hundreds of nanometres to a few micrometres (Barthlott and Ehler, 1977; Koch, 2010). This level might contain an additional complexity due to the occurrence of hierarchical sub-levels, because (i) cuticular folds are often covered with wax crystals and (ii) wax crystals may be hierarchically superimposed, building sublayers with crystals of different size, shape, and orientation (Gorb *et al.*, 2005; Gorb and Gorb, 2009a, c; Prüm *et al.*, 2012a; Benz *et al.*, 2012).

The functional significance of such additional structural complexity has only recently been systematically studied, though hierarchically structured plant surfaces have been repeatedly reported, especially in plants having the capability of trapping insects (e.g. Oelschlägel *et al.*, 2009). It seems logical to assume that insect attachment is further reduced by such a hierarchical surface architecture. A comparative SEM study of insect pitfall traps in 53 plant species (57 surfaces) revealed a variety of combinations of epidermal cell curvatures, trichomes, cuticular folds, and epicuticular wax crystals (Poppinga *et al.*, 2010). This diversity was classified into 12 types of anti-adhesive surfaces depending on the composition of their structural elements and observed hierarchy. Twenty-eight species, which was the largest group of plants examined, had only single-level structured surfaces, e.g. convex cell shapes or tabular cells with superimposed waxes. Twenty-four species displayed a two-level hierarchy and only five species showed a three-level hierarchy. These surfaces, for example, have papillate epidermal cells covered with waxes or cuticular folds (two-levelled), or papillate cells with cuticle folds and



**Fig. 10.** The waxy zone of the *Nepenthes alata* pitcher and its effect on the attachment ability of the beetle *Coccinella septempunctata*. (A) Scanning electron micrograph of three-levelled waxy zone with lunate cells and epicuticular wax coverage. LC, lunate cells; arrowhead indicates the direction toward the pitcher bottom. (B) The results of force measurements with intact insects and intact pitcher surfaces. (C, D) Intact (C) and clawless insects (D) were also tested on de-waxed (treated with warm chloroform) pitchers and their polymer replicas. Inside: insects move in the direction toward the pitcher bottom; outside: insects move in the direction outside the pitcher. (From Gorb and Gorb (2011a).)

superimposed waxes (three-levelled). It was assumed that such hierarchical surfaces represent a combination of two or more strategies for inducing surface slipperiness, which might enhance efficiency of insect trapping. Based on the literature data on insect attachment, the authors proposed an original hypothesis that the slipperiness of a plant surface correlates with its hierarchical complexity, i.e. with an increased number of strategies implemented in a specific surface.

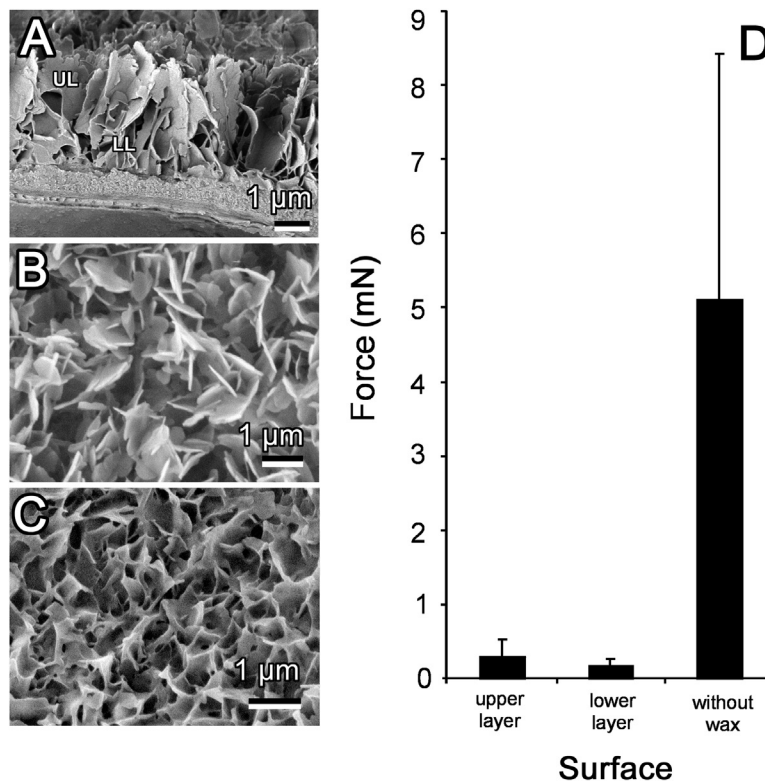
Previous studies on carnivorous *Nepenthes* plants have provided experimental evidence regarding the effect of hierarchical plant surface structure on insect attachment. The intact *N. alata* surface with both lunate cells and 3D wax coverage completely disabled attachment of the ant *Iridomyrmex humilis*, whereas chemically de-waxed pitchers having only lunate cells were much less effective and allowed ants to escape (Gaume *et al.*, 2002). The beetle *Coccinella septempunctata* displayed significantly lower traction forces on chemically de-waxed pitchers and their replicas than on smooth glass. This effect was strongly expressed, especially when insects walked in the direction out of the pitcher. In this case, their claws could not properly grip downward-pointed asymmetric lunate cells (Fig. 10C, D; Gorb and Gorb, 2011a). The intact waxy zone of the pitcher with a three-levelled hierarchical structure, combining lunate cells and two levels of wax crystals (Fig. 10A), drastically reduced beetle attachment forces in both pulling directions, when compared with the forces measured on glass and wax-free pitcher surfaces (Fig. 10B–D). The large reduction in beetle adhesion on the

waxy pitcher surface can be explained by the contamination of adhesive pads with wax crystals and by the further contact reduction between contaminated insect attachment pads and lunate cells due to an additional anisotropic surface micro-roughness (Gaume *et al.*, 2002; Gorb *et al.*, 2005; Bauer *et al.*, 2009; Wang and Zhou, 2010).

In this and some other *Nepenthes* species (Benz *et al.*, 2012), the wax coverage consists of two layers of wax crystals (Fig. 11A–C). Wax crystals from these layers show differences in their morphology, chemical composition, as well as mechanical material properties (Gorb *et al.*, 2005). The coccinellid beetle *Adalia bipunctata* demonstrated a similar degree of adhesion reduction on upper and lower wax layers (Fig. 11D). This result is quite surprising and can only be explained by the assumption that different wax layers reach a similar anti-adhesive effect through two different mechanisms. Wax crystals of the upper wax layer are easily erodible and cause contamination of the beetle adhesive pads, whereas the lower wax layer is mechanically more stable and reduces adhesion mainly by the reduction of the real contact area between the feet and plant surface.

### Some perspectives

In spite of a great number of publications on the effects of the plant cuticle micromorphology on insect adhesion, there are a number of interesting problems that still remain unresolved.



**Fig. 11.** The waxy zone of the *Nepenthes alata* pitcher and its effect on attachment of the coccinellid beetle *Adalia bipunctata*. (A) Cryo-scanning electron micrograph of the side view of the wax coverage (fractured pitcher sample). LL, lower wax layer; UL, upper wax layer. (B, C) Scanning electron micrographs (upper aspects) of the intact upper wax layer (B) and the lower wax layer after treatment of the intact pitcher with the fluid silicone elastomer and removal of the upper wax layer by peeling off (C). (D) Traction forces produced by beetles on the different exposed wax layers and on the completely de-waxed pitcher surface treated with warm chloroform. ((B–D) Adapted from Gorb *et al.* (2005) with permission of *The Journal of Experimental Biology*.)

One is a direct experimental proof of the wax-dissolving hypothesis. Up until now only very non-polar solvents, such as benzene, hexane, and chloroform, have been reported as being able to dissolve epicuticular waxes of most plants (Hallam 1982). However, experiments should be performed that prove the solubility of various plant wax types in pad secretions of different insects having different chemical compositions.

A further interesting direction for continued research is the question of animal adaptations to overcoming the slipperiness of specialised plant cuticles. There are highly specialised arthropod species that are strongly adapted to the plant surfaces covered by 3D waxes (Federle *et al.*, 1997, 1998, 2000). Usually their adaptations are behavioural, but we cannot completely exclude that these specialist animals possess particular shapes and/or dimensions of their attachment pads and generate a specific adhesive fluid composition, if compared with those of generalists.

Many structural aspects of the self-assembly of wax crystals on the plant surface and its dependence on environmental conditions have still to be experimentally tested for the vast majority of wax crystal types. The effect of the abiotic environment on the insect attachment to plants is almost not considered in the literature. For example, temperature, relative humidity or rainfall should potentially affect the degree of cuticle porosity and wettability. In turn, this would affect some of the mechanisms of insect–plant interaction, in terms not only of day-period and seasonal fluctuations, but also of day/night fluctuations.

The information on mechanical properties of single crystals and complex wax coverage is present only for a very few plant species. This information is crucial for understanding of anti-adhesive mechanisms based on wax detachment and contamination of insect pads. Since insects apply shear forces, while gripping the substrate, direct measurements of frictional properties of various wax coverages would provide further insight into the variability of different waxes in contaminating insect feet. So far, there are only theoretical predictions based on the geometry of individual crystals. Recent developments in force probe microscopy represent a proper toolbox for these kinds of studies. Finally, a further novel direction would be using plant waxes to generate templates for nanofabrication in materials science and nanotechnology (Koch *et al.*, 2008; Niemietz *et al.*, 2009; Schulte *et al.*, 2009). This could lead to development of surface coatings for technical surfaces with various useful properties, but also to the development of sprays preventing insect attachment to plant surfaces that are not naturally covered by 3D wax.

## Acknowledgements

The authors are grateful for permissions to reuse, in this review, previously published figures to the Company of Biologist (Gorb *et al.*, 2005), Taylor and Francis Group, LLC (Gorb and Gorb, 2006), Elsevier (Voigt *et al.*, 2008; Gorb *et al.*, 2017a), John Wiley and Sons (Gorb and Gorb 2002), Macmillan Publishers (Gorb *et al.*, 2017b), the Royal Society (Gorb *et al.*, 2010), and Springer (Gorb *et al.*, 2008). We thank Vicky Kastner (Tübingen, Germany) for linguistic correction of the manuscript. The preparation of this review was partly supported by the Leverhulme Trust (project CARBTRIB ‘Nanophenomena and functionality of modern carbon-based tribo-coatings’).

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