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# Attachment to Plant Surface Waxes by an Insect Predator[1]

SANFORD D. EIGENBRODE[2,*] AND REINHARD JETTER†

*Department of Plant, Soil and Entomological Sciences, Agric. Sci. 242, University of Idaho, Moscow, Idaho 83844-2339
†Lehrstuhl für Botanik II, University of Würzburg, Julius-von-Sachs-Platz 3, D-97082 Würzburg, Germany

**SYNOPSIS. Insects foraging on plant surfaces must attach to the layer of lipophilic materials known as epicuticular waxes (EW) that cover these surfaces. In this paper, we briefly review the evidence that variation in EW morphology can influence the ecology of herbivorous insects directly, by affecting their attachment to plant surfaces, and indirectly by affecting attachment by actively foraging predatory insects to plant surfaces. We then present new data examining how EW micromorphology and chemical composition of *Brassica oleracea* influence attachment by the predatory beetle, *Hippodamia convergens* (Coccinellidae). Bioassays with genotypes of *B. oleracea* differing in wax characteristics, and with EW extracts from these plants applied to glass, show that wax crystals disrupt attachment. In addition, bioassays show that attachment by *H. convergens* differs among EW extracts prepared to have smooth surfaces without crystals. The differences in attachment under these conditions are evidently due to the chemical composition of the waxes. Bioassays with two pure wax constituents show that wax composition can significantly affect attachment by *H. convergens*. The study opens the way for using a similar approach to understand attachment by insects to waxy plant surfaces.**

## CONTEXT OF THE STUDY: ATTACHMENT TO PLANT SURFACE WAXES BY INSECTS

Both insect herbivores and predators that attack them must attach effectively to plant surfaces (Southwood, 1986; Eigenbrode, 1996), which implies that they must attach to the waxy materials that cover these surfaces. The cuticle of the primary organs of higher plants consists of a polymeric cutin matrix and cuticular waxes soluble in organic solvents such as hexane or dichloromethane. The waxes are complex mixtures of very-long-chain aliphatics including primary alcohols (*n*-alkan-1-ols), aldehydes, fatty acids (*n*-alkanoic acids) and alkyl esters, all of which occur predominantly with even-numbered chain lengths, and hydrocarbons, secondary alcohols and ketones with predominance of odd-numbered chain lengths (Walton, 1990). Small proportions of these materials may have branched carbon chains. Cuticular waxes from certain species and organs also contain relatively large proportions of triterpenoids, including oleananes and ursanes, and phytosterols (Walton, 1990). Although these patterns are robust, the majority of plant species has yet to be examined and our current information is based on an overrepresentation of temperate species (Riederer and Markstädter, 1996).

A portion of plant cuticular waxes is located outside the cuticular matrix and, hence, is exposed on the immediate surface of the plant. These ''epicuticular waxes'' (EW) generally form a thin, continuous film but can also be decorated with protruding microscopic crystals occurring as filaments, rods, platelets, tubes, and complex dendritic structures (Jeffree, 1986; Barthlott *et al.*, 1998). Grossly, such crystals give the plant surface a whitish (''glaucous'') appearance or waxy bloom.

Comparative chemical and micromorphological investigations have shown that special wax constituents form the EW crystals on diverse plant surfaces (Jeffree *et al.*, 1975; Jetter and Riederer, 1994, 1999; Meusel *et al.*, 2000) and the composition of these crystals sometimes appears to differ from the underlying intracuticular waxes. This has been determined by very brief extraction (Silva Fernandes *et al.*, 1964) or mechanically stripping the EW from the cuticle (Baker *et al.*, 1983; Jetter *et al.*, 2000; Jetter and Schäffer, 2001).

Although it is clear that the bulk wax mixture is important for waterproofing (Schönherr, 1976), the necessity of EW for this function of the cuticles is not certain (Baur, 1998). Meanwhile, EW may have other ecological functions including shielding of UV-B (Mulroy, 1979; Day *et al.*, 1992), protection against pathogen invasion (Blakeman, 1973; Carver and Thomas, 1990; Nielsen *et al.*, 2000), and influencing insect behavior by functioning as allelochemicals (Eigenbrode and Espelie, 1995; Spencer, 1996; Udayagiri and Mason, 1997; Cervantes *et al.*, 2002).

In addition to their demonstrable allelochemical effects on insects, EW also can influence insect adhesion or attachment to the plant surfaces, as was first demonstrated by Kerner von Marilaun (1898), Haberlandt (1909) and Knoll (1914). The ecological implications of this phenomenon have been documented in four contexts: (i) EW crystals in pitchers of the carnivorous genera *Nepenthes* and *Darlingtonia* generate slippery surfaces, which are pivotal in prey capture (Juniper, 1995); (ii) EW crystals on stems of the paleotropical ant-plant genus *Macaranga* generate slippery surfaces that protect mutualistic partner ants against generalist competitors (Federle *et al.*, 1997); (iii) EW crystals on floral stems of certain plants prevent ants from removing pollen (Harley, 1991); (iv) differences in adhesion

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[2] E-mail: sanforde@uidaho.edu

or attachment to EW crystals mediate interactions involving herbivorous insects and their insect natural enemies. This last aspect is the context of the present report, and will be reviewed in more detail.

*EW effects on attachment by herbivorous insects*

Crop species with typically glaucous appearance, caused by EW crystals, sometimes produce so-called ''glossy'' mutations in which the waxy bloom is greatly reduced (Eigenbrode and Espelie, 1995). The glossy forms are often more susceptible to certain insect pests (Tsumuki *et al.*, 1987; Stoner, 1990; Bodnaryk, 1992) and this has been shown in some cases to be related to an increased ability of these insects to attach or adhere to plants with reduced waxy blooms. A glossy variety of *Brassica oleracea* provided better adhesion for the chrysomelid beetle, *Phaedon cochleariae*, than a variety with typical waxy bloom (Stork, 1980*a*). In the field, damage by flea beetles, *Phyllotreta cruciferae*, was greater on *Brassica* spp. with reduced waxy bloom (Stoner, 1990; Eigenbrode *et al.*, 2000). Bodnaryk (1992) showed that waxy bloom was the responsible factor in *B. napus* by removing the wax crystals mechanically, rendering the plants more susceptible to beetle damage. For similar reasons, reduced waxy bloom peas, *Pisum sativum*, are more susceptible to damage by the pea leaf weevil *Sitona lineata* (unpublished data, SDE).

Similar effects have been documented in natural systems. Juvenile leaves of some *Eucalyptus* spp. possess a waxy bloom that prevents attachment and feeding by beetles (Edwards, 1982). The distribution and abundance of leaf feeding beetle species on *Eucalyptus* spp. is related to the waxy bloom on the trees and the attachment abilities of the individual beetle species (Edwards and Wanjura, 1991).

*EW effects on attachment by predatory insects*

Contrary to expectations based on the foregoing examples, agricultural crop varieties with reduced waxy blooms typically are relatively resistant to insects as compared with normal waxy bloom varieties (75% of the reported occurrences) (Eigenbrode and Espelie, 1995). This has been reported for soybeans (Baker *et al.*, 1985), onions (Molenaar, 1984), sorghum (Starks and Weibel, 1981), wheat (Lowe *et al.*, 1985) and *Brassica* crops (*e.g.*, Way and Murdie, 1965; Dickson and Eckenrode, 1980; Stoner, 1990).

Evidence is accumulating that at least a partial explanation for the apparent resistance of reduced waxy bloom crop varieties is enhanced action by insect predators on these plants, as first suggested by Way and Murdie (1965). Three taxonomically diverse species of generalist predators (*Chrysoperla plorabunda*, *Orius insidiosus*, and *Hippodamia convergens*) more effectively reduce populations of the diamondback moth, *Plutella xylostella*, on a glossy cabbage (Eigenbrode *et al.*, 1995). A similar pattern occurs for *H. convergens* attacking aphids on glossy peas (Eigenbrode *et al.*, 1998; White and Eigenbrode, 2000). In the field, glossy *Brassica napus* has lower densities of aphids but higher densities of the aphid predator *H. convergens* than normal EW *B. napus* (Eigenbrode *et al.*, 2000).

EW blooms in general appear to interfere with predator mobility (von Arzet, 1973; Shah, 1982; Grevstad and Klepetka, 1992). Observations on glossy and glaucous plants show that the predators are more active, cover more of the plant surface, and fall less from the reduced EW *Brassica* and *Pisum* (Eigenbrode *et al.*, 1996, 1998; White and Eigenbrode, 2000). These behavioral differences are related to substantially greater attachment forces achievable by predators on glossy leaf surfaces than on glaucous ones (Eigenbrode *et al.*, 1998). Indeed, differences in attachment force generated by two predator species to *B. oleracea* differing in EW bloom are correlated with effectiveness of these predators at taking prey on the plants (Eigenbrode and Kabalo, 1999; Eigenbrode *et al.*, 1999).

Although it is clear that EW blooms interfere with attachment by predatory and phytophagous insects, the EW properties responsible for the effects are not yet understood. It is not known to what extent the morphology of plant wax crystals, their specific composition, the density of wax crystals, and the composition of underlying waxy layers of the cuticle influence insect attachment. These potential factors are confounded in EW mutants because composition and crystal morphology and density are all changed to some degree by the mutations (Macey, 1970; Macey and Barber, 1970; von Netting and Wettstein-Knowles, 1972; Holloway *et al.*, 1977; Eigenbrode *et al.*, 1991*a*, *b*; Eigenbrode, 1998). In addition, the work thus far has focused on plants that differ obviously in waxy bloom, but has left unexamined the effects on insect attachment of amorphous waxes that differ only in composition, despite the fact that such variation is widespread in nature (Walton, 1990).

## EXPERIMENTS: ATTACHMENT TO PLANT WAXES BY A PREDATORY INSECT, *HIPPODAMIA CONVERGENS*

To develop a better understanding of how plant EW affect insect attachment, we conducted experiments with *H. convergens*, a predator known to forage on diverse plants, including grasses, forbs, and perennial shrubs (Beers *et al.*, 1993; Hoffmann and Frodsham, 1993). We first examined our previously reported data (Eigenbrode and Kabalo, 1999) on attachment by *H. convergens* to eight *Brassica oleracea* genotypes differing in waxy bloom. We then measured attachment by *H. convergens* to these plants with EW altered mechanically and to EW extracted from the plants for *in vitro* tests.

### *Study 1: attachment to* B. oleracea *leaf surfaces with varying amounts and shapes of epicuticular wax crystals*

*Materials and methods.* We used four mutations affecting EW in different genetic backgrounds of *Brassica oleracea*. The mutants and wildtype counterparts

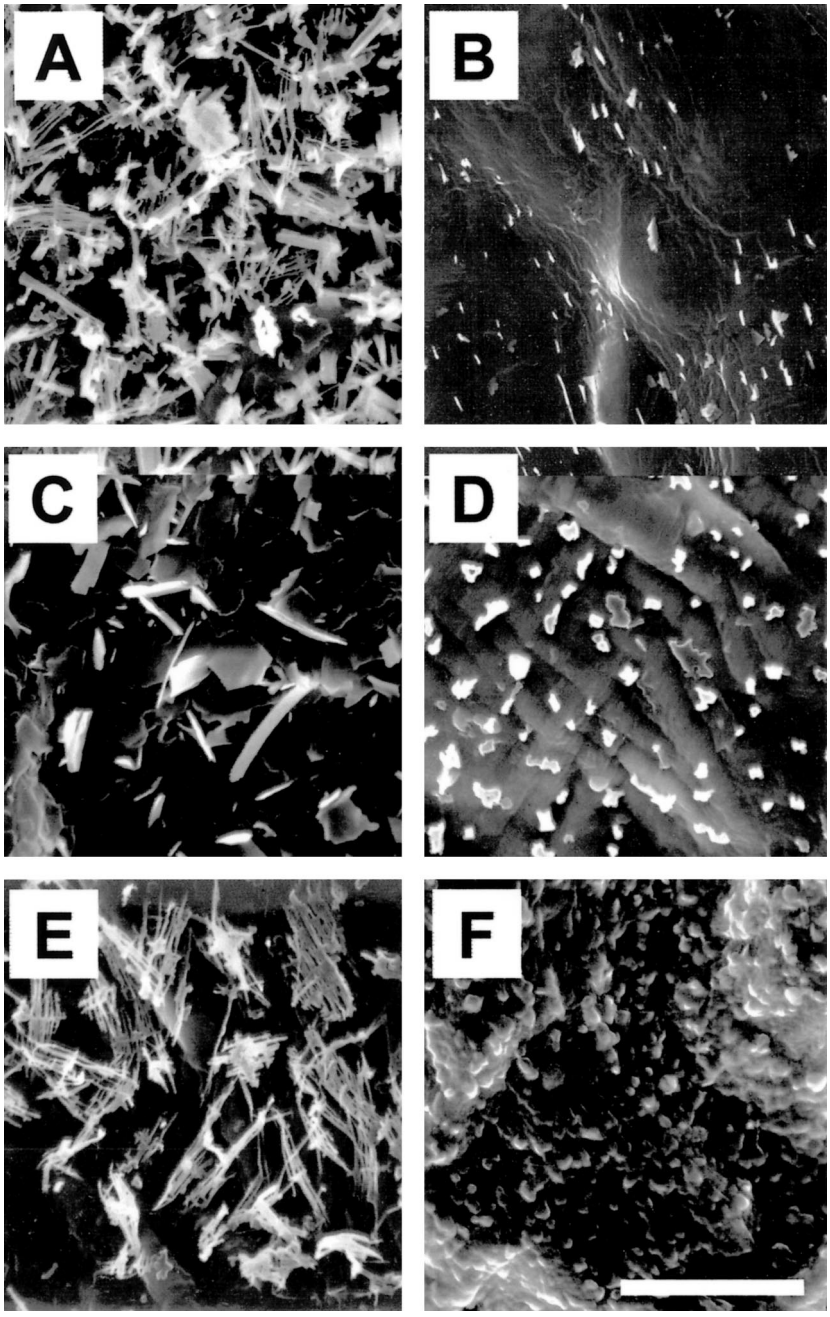


FIG. 1. Scanning electron micrographs of the epicuticular waxes on *Brassica oleracea* types used in the bioassays: (A) Round-Up cabbage, representative of normal-wax bloom types, which have similar crystalline morphology, (B) Round-Up with wax crystals removed by mechanical polishing, (C) Broc 5 × Packman $F_2$ glossy, (D) Glossy Andes, (E) KCR4 × Packman $F_2$ glossy, (F) NY 1406. Scale bar = 10 μm.

provide a range of EW phenotypes as shown by SEM (Fig. 1A, C–F). With their normal counterparts, these produce 9 EW types referred to in this paper as *Brassica* types. The mutant allele $gl_a$ was tested in glossy cabbage NY1406 (obtained from M. H. Dickson, Cornell University) and compared with its normal waxy bloom counterpart ''Round-Up'' cabbage. The mutant allele $gl_d$ was tested in the inbred cauliflower line ''Glossy Andes'' (obtained from K. A. Stoner, Connecticut Agricultural Experiment Station) and compared with its normal waxy bloom counterpart ''Andes.'' The two other glossy mutations were compared to glaucous phenotypes within segregating $F_2$ populations (obtained from K. A. Stoner). KCR4 × ''Packman'' $F_2$ population segregates for $gl_{a-1}$ and the Broc5 × ''Packman'' $F_2$ population segregates for $gl_c$. The KCR4 × ''Packman'' $F_2$ populations include an intermediate EW phenotype (Eigenbrode *et al.*, 1999), which was not included in our experiments. Plants were grown in the greenhouse under supplemental metal halide lighting (L18:D6) (500 μmol photosynthetically active radiation/$m^2$), 18°C and 25°C (night,

day), and average approx. ambient RH of 60%, in 10-cm pots with greenhouse potting soil (Sunshine Mix 1, SunGro Horticulture, Bellevue, WA). Six to 10 weeks after germination, leaves from all eight *B. oleracea* types were harvested for adhesion experiments.

Field-collected *Hippodamia convergens* of undetermined age were maintained for several weeks on a diet of pea aphids (*Acyrthosiphon pisum*). Only female insects that had been provided water but no prey for 24 hours were used in the experiments.

Lateral attachment forces by *H. convergens* were measured with a centrifugal device as described by Eigenbrode *et al.* (1999) and Federle *et al.* (2000). Leaf pieces (or wax mixtures deposited on glass, see below) were fastened to a horizontal aluminum turntable (30 cm diam). An individual insect was placed on the test material within a clear plastic canopy to eliminate effects of air resistance. The preparation was then attached to the turntable, which was accelerated gradually until the insect was separated from the leaf surface by centrifugal force. To aid observation of the insect during the test, a stroboscopic light directed at the insect was triggered to flash on each rotation of the turntable. The turntable revolutions per second $f$ [Hz] required to detach the insect and the radius $r$ [m] of the insect location on the turntable were used to calculate the effective velocity $v$ [m sec$^{-1}$]. Together with the individual insect mass m [g] this gave the attachment force F [milli-Newtons] according to:

$$F = m\frac{v^2}{r} = m\frac{(2\pi r \cdot f^2)}{r} = 4\pi^2 m r f^2$$

Typical forces were more conveniently expressed in μN. Attachment by each insect was measured 2 to 3 times and the average of these values was used as a single observation. Ten to 12 insects were tested on the upper leaf surface of each of the *Brassica* wax types. Each insect was tested on a fresh leaf piece and there were two leaf pieces from each of six plants from each *Brassica* type.

Following a similar protocol, at least 20 insects were tested on leaf pieces of each of the same *Brassica* types mechanically polished by gently wiping them with a lint-free cloth. This procedure eliminated the waxy bloom and removed most of the EW crystals or incorporated them into the wax surface, or both, as revealed by scanning electron microscopy (SEM) (*e.g.*, in Fig. 1B). The gross appearances of the glossy phenotypes were not visibly affected by this polishing.

For statistical analysis, attachment forces to plant surfaces were transformed [*ln*(x)] and compared with analysis of variance (ANOVA). Planned contrasts compared attachment to each wild-type and its respective reduced wax counterpart; Pearson's correlation coefficient was calculated between *H. convergens* attachment and the previously reported (Eigenbrode *et al.*, 1991*b*) mean EW crystal density (crystals per μm$^2$) for the eight *Brassica* types.

*Results.* While glaucous types had surfaces with dense networks of rods and filaments (Fig. 1A), the EW of glossy phenotypes had less dense vestures of filaments, rods, angular and irregular plates, polygons and globules (Fig. 1C–F). Attachment by *H. convergens* to untreated leaf surfaces of the *B. oleracea* genotypes spanned a 30-fold range (Fig. 2A), differing significantly among the types ($P \leq 0.0001$). With one exception (Broc 5 × Packman $F_2$ Normal *vs.* Broc 5 × Packman $F_2$ Glossy), the reduced waxy bloom line in each pair allowed significantly greater attachment force by *H. convergens*. Crystal counts on the untreated plant surfaces and attachment by *H. convergens* were negatively correlated ($r = -0.930$, $P = 0.0001$). Mechanical polishing did not alter attachment to the glossy types from the KCR4 × Packman $F_2$ and NY1406, as expected from their unchanged surface appearance. The low adhesion force on Broc 5 × Packman $F_2$ Glossy and the high adhesion force on Glossy Andes were both brought closer to the average for all types by polishing.

### *Study 2: attachment by* H. convergens *to reconstituted* Brassica *wax extracts*

In order to investigate the effect of different EW compositions and morphologies, the influence of epidermis topography and other *in situ* parameters had to be eliminated. To this end, leaf cuticular waxes of the eight *Brassica* types were extracted, analyzed, and reconstituted on glass to measure attachment force by *H. convergens*. We prepared the waxes for testing either so as to allow semi-natural crystals to form, which should influence attachment as on intact leaf surfaces, or as amorphous films to eliminate the complex effects of crystal morphology and allow assessment of chemical composition alone on attachment by this insect.

*Materials and methods.* Previous SEM studies had shown nearly identical EW crystals on both leaf sides for all eight *Brassica* types (unpublished data, RJ). Hence, it could be expected that the qualitative wax compositions would be similar on both sides and a total leaf extract could be used to simulate adaxial cuticles. Wax extracts of each *Brassica* line were prepared by immersing leaves for 10 sec in *n*-hexane at room temperature. Extracts were concentrated to dryness under a stream of nitrogen, weighed, taken up in *n*-hexane and applied to glass coverslips. Wax coverage on the intact plants ranged 10-fold from *ca.* 2 to 20 μg/cm$^2$. For the present experiments, we did not test the effects of wax coverage, but used a standard superabundant deposit of 100 μg/cm$^2$ for all the wax mixtures.

Wax extract solutions were standardized to 2.42 mg/ml and 200 μl of this solution was dispensed onto a 4.84-cm$^2$ glass cover slip to deposit a 100 μg/cm$^2$ coating. The solvent was allowed to evaporate slowly to produce a bed of crystals. Two methods were employed to generate amorphous surfaces. One method was to melt the crystals of waxes deposited onto glass from solvent by warming the cover slip to approxi-

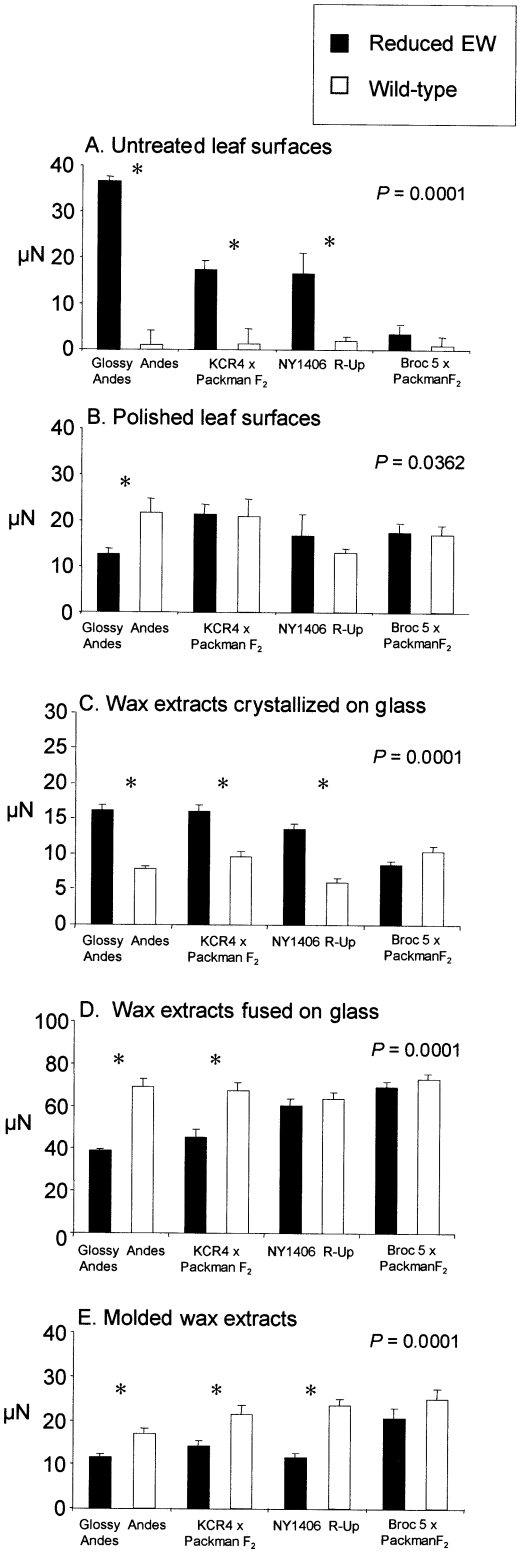


FIG. 2. Attachment force by *H. convergens* to different substrates: (A) the intact leaf surfaces of the eight *Brassica oleracea* types, (B) leaves of these same genotypes after polishing to remove wax crystals, EW extracts of the *B. oleracea* types presented as (C) crystallized films on glass, (D) films thermally fused to reduce crystals, (E) amorphous solids created by melting waxes into polystyrene molds to achieve similar surfaces. *P* values are for overall ANOVA F tests; asterisks indicate significance ($<0.05$) of the comparison between each reduced waxbloom line and its normal-wax type in the same genetic background.

mately 70°C and then cooling it rapidly by placing the cover slip onto ice, whereupon the waxes solidified as a smooth film. The other method was to extract the natural waxes from bulked plant samples in sufficient quantity to melt them in small aluminum boats (0.3 ml volume with a 1-cm$^2$ surface area) floating in 80°C water. The boat was removed from the water bath and immediately a polystyrene plastic weighing pan (VWR Scientific, West Chester, Pennsylvania, USA) was pressed into the molten wax. When the waxes cooled and solidified, the polystyrene boat was removed, leaving a relatively smooth, molded surface. The topography of different wax mixtures prepared in this way are indistinguishable under SEM at >5,000× (Gorb and Eigenbrode, unpublished data). GC/MS of the waxes in these preparations showed that the procedure does not detectably alter the chemical composition of the waxes, nor was there any evidence that molded waxes became contaminated by polystyrene from the weigh boat used to mold their surfaces. *Hippodamia convergens* attachment was measured as in Study 1.

Waxes were extracted by clamping the adaxial surface of a leaf over the opening of a 30-ml scintillation vial (diam. 1.6 cm) containing approx. 15 ml of *n*-hexane and repeatedly inverting the leaf and vial for 30 sec. A defined amount of tetracosane was added as an internal standard to the pooled extracts of 10 leaf surfaces from each line. The resulting solutions of cuticular waxes were filtered and the solvent removed under reduced pressure. Compounds containing free hydroxyl groups were transformed into TMSi ethers by reaction with bis-N,N-trimethylsilyltrifluoroacetamide in pyridine. Gas chromatographic analyses were carried out as described previously (Jetter and Schäffer, 2001). At least four replicate extractions were analyzed for each variety.

*Statistical analysis.* Analysis of attachment data was similar to that employed for examining attachment to intact plants. Results of plant wax analysis were not analyzed statistically, but standard errors were calculated from the data. Pearson's correlation coefficients were calculated between wax component class concentrations and attachment to wax extracts using PROC CORR in SAS (SAS, 2000).

*Results.* Attachment forces generated by *H. convergens* to *B. oleracea* wax extracts depended on how they were prepared for bioassay, being greatest for wax extracts presented as amorphous films on glass slides (mean = 0.061 ± 0.004 mN), lower for waxes prepared with molded smooth surfaces (0.018 ± 0.002 mN), and lowest for waxes applied to glass and allowed to crystallize (0.012 ± 0.002 mN). Attachment forces to EW preparations were greater than the average attachment force to intact plant surfaces (0.009 ± 0.004 mN). The pattern of relative attachment to EW extracts prepared as crystals was similar to that to intact plant surfaces (*cf.*, Fig. 2A and C). As for intact plant surfaces, the effect of plant type was significant and attachment was greater to crystalline EW extracts from the glossy type in each pair, except those from

TABLE 1. *Chemical of the epicuticular wax on upper leaf surfaces of eight* Brassica oleracea *types.*

| Comparison wax bloom | Andes vs. Andes G | | Broc5 × Packman $F_2$ | | KCR4 × Packman $F_2$ | | Round-Up vs NY1406 | |
|---|---|---|---|---|---|---|---|---|
| | Normal | Reduced | Normal | Reduced | Normal | Reduced | Normal | Reduced |
| Compound Class [%] | | | | | | | | |
| Alkanes | 39 | 44 | 42 | 57 | 65 | 17 | 37 | 3 |
| *sec.* Alcohols | 12 | 8 | 10 | 3 | 4 | 3 | 14 | 1 |
| Ketones | 27 | 8 | 24 | 30 | 9 | 8 | 27 | 5 |
| Ketols | 3 | tr | 2 | tr | 1 | 1 | 5 | tr |
| *prim.* Alcohols | 5 | 14 | 3 | 1 | 3 | 16 | 5 | 21 |
| Aldehydes | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 1 |
| Alkanoic acids | tr | 1 | 1 | tr | tr | 1 | tr | 2 |
| Alkyl esters | 7 | 5 | 9 | 4 | 11 | 37 | 6 | 49 |
| Triterpenoids | 1 | 9 | 1 | tr | 1 | 2 | 1 | 1 |
| Not identified | 4 | 10 | 8 | 4 | 4 | 14 | 4 | 13 |
| Coverage [$\mu g\ cm^{-2}$] | 12.0 | 2.3 | 12.7 | 20.2 | 14.9 | 5.0 | 19.0 | 10.2 |
| S.D. [$\mu g\ cm^{-2}$] | 10.7 | 1.1 | 7.6 | 9.6 | 5.9 | 1.5 | 6.4 | 2.8 |

the Broc-5 × $F_2$ population (*cf.*, Fig. 2A and C). On EW extracts prepared with smooth surfaces the effect of plant type was significant, but attachment tended to be greater to normal-type waxes, significantly so for two comparisons (Fig. 2D and E). In addition, the general pattern of attachment to waxes applied as smooth films tended to resemble those to plant surfaces mechanically polished to reduce EW crystals (Fig. 2B, D, and E).

Fifty-four different compounds were identified in EW mixtures from the *Brassica* types (data not shown) and these were grouped into homologous series representing eight classes of aliphatics (Table 1). Secondary alcohols and ketones were found to carry the functional group mainly on the central carbon atom (15-isomers). The EW extracts from glossy and glaucous *Brassica* types were similar qualitatively, but differed quantitatively. The glaucous *Brassica* types had wax coverages between 12 and 19 $\mu g\ cm^{-2}$ (Table 1). Three of these normal-wax types (Andes, Broc5 × Packman $F_2$, and Round-Up) had similar composition, containing 3–5% primary alcohols, 10–14% secondary alcohols, 37–42% alkanes, 24–27% ketones, 6–9% esters and 1% aldehydes. Normal-wax line KCR4 × Packman $F_2$ wax differed from the others in its lower concentration of secondary alcohols (4%) and ketones (9%), and correspondingly higher concentration of alkanes (65%) and esters (11%). The normal-wax types had similar chain-length distributions within major compound classes (data not shown). Predominant homologs were $C_{26}$ for the unbranched alcohols, $C_{27}$ for the branched alcohols, and $C_{29}$ for the secondary alcohols, ketones, ketols and alkanes, respectively.

A group of three glossy *Brassica* types (Glossy Andes, KCR4 × Packman $F_2$ glossy, and NY1406 glossy) had drastically decreased wax coverages ranging from 2 to 10 $\mu g\ cm^{-2}$ (Table 1), relative to their normal-wax counterparts. In KCR4 × Packman $F_2$ glossy and NY1406 the overall drop in wax loads was due to reduced amounts of alkanes, secondary alcohols, ketones and ketols, with resulting dominance of primary alcohols, fatty acids and alkyl esters. These two EW types also were characterized by shifts towards smaller chain lengths ($C_{27}$ more abundant than $C_{29}$ alkane, secondary alcohol and ketone, respectively, data not shown). In Glossy Andes the drop in overall coverages was due to reduced amounts of all aliphatic compound classes, with slightly increased portions of alkanes and primary alcohols, and decreased percentages of esters, secondary alcohols, ketones and ketols. Broc5 × Packman $F_2$ glossy showed a unique increase in the wax load as compared with Broc5 × Packman $F_2$ normal-wax plants. The overall increase was based on higher levels of ketones and alkanes, over-compensating for the reduced amounts of primary and secondary alcohols.

None of the correlations examined between the relative composition for each compound class in EW extracts and attachment by *H. convergens* were significant. There was a marginally significant negative correlation between attachment and the relative proportion of primary alcohols ($r = -0.691$, $P = 0.057$).

### *Study 3: attachment by* H. convergens *to pure wax constituents*

To overcome the difficulty in interpreting attachment to complex mixtures of wax components, we used similar methods to examine *H. convergens* attachment to pure $C_{22}$ primary alcohol and $C_{22}$ fatty acid. Although not predominant homologues in natural *Brassica* waxes, these compounds represent two important classes of EW components in many natural waxes.

*Materials and methods.* Standard $C_{22}$ *n*-alkanoic acid and $C_{22}$ primary alcohol were obtained commercially (Sigma-Aldrich, Deisenhofen, Germany) and dissolved in chloroform (app. 10 mg $ml^{-1}$). Carefully pre-rinsed 2 × 2 $cm^2$ glass slides were mounted on aluminum spacers and placed on a heating block (35°C). Up to 1 ml of the standard solution was placed onto the glass and evaporated under a gentle stream of $N_2$ within approx. 1 min, forming an homogenous bed of crystals. SEM confirmed that loads of 1–2.5 mg $cm^{-2}$ could guarantee continuous coverage of the glass substratum with platelet-shaped crystals. Individ-

TABLE 2. *Attachment force [μN] by* H. convergens *to artificial surfaces, reconstituted* in vitro *as continuous layers of representative was constituents.*

| | Crystallized on glass | Molded, with tarsal claws intact | Molded, with claws removed |
|---|---|---|---|
| $C_{22}$ primary alcohol | 5.9 ± 0.3 | 14.3 ± 0.8 | 15.3 ± 0.8 |
| $C_{22}$ *n*-alkanoic acid | 4.9 ± 0.3 | 12.2 ± 0.9 | 11.4 ± 1.1 |
| *P* value for *t*-test | 0.012 | 0.12 | 0.006 |

ual crystal preparations were transformed into amorphous surfaces using the molding procedure described above.

Attachment to these materials by *H. convergens* was measured as described above. In addition, to isolate the effects of attachment to pure compounds using tarsal adhesion from attachment using pretarsal claws as anchors, an experiment was conducted using insects with the pretarsal claws removed surgically. Tarsal claws were removed under a microscope using fine scissors. After the surgery, the animals were fed aphids *ad libidum* and allowed to recover for 48 hr. Those exhibiting normal activity levels after 48 hours were used in the attachment bioassay.

Students *t*-test was used to compare *Hippodamia convergens* attachment to $C_{22}$ primary alcohol *vs.* $C_{22}$ *n*-alkanoic acid in each of the three experiments. Attachment data were log transformed before the analysis.

*Results.* Attachment to $C_{22}$ primary alcohol tended to be greater than attachment to $C_{22}$ *n*-alkanoic acid regardless of the method of preparation and whether the tarsal claws were removed from the insects before testing (Table 2). Attachment to both of these compounds was approximately 3-fold greater when they were presented as molded solids than as beds of crystals, although this difference could not be evaluated statistically because the experiments were conducted separately. Removal of pretarsal claws from *H. convergens* enhanced the trend towards greater attachment to the primary alcohol than to the fatty acid, and resulted in a significant difference between attachment to these two materials presented as amorphous films (Table 2).

## DISCUSSION

Here we have reviewed the published evidence that reductions in the amount of epicuticular wax crystals allow insects to achieve greater forces of attachment to plants, thereby affecting their trophic interactions. The published work has not attempted a systematic experimental approach to deciphering what EW attributes influence insect attachment. We present definitive experimental evidence that crystallization of EW is a principal factor determining attachment by *H. convergens.* Specifically, attachment by *H. convergens* to leaves from eight *Brassica* types is correlated with the density of wax crystals on these leaves. Polishing *B. oleracea* with prominent waxy blooms to remove crystals increases attachment by this insect, but polishing has little effect on *B. oleracea* with genetically reduced waxy blooms. Crystalline deposits of the extracted EW from eight *B. oleracea* types reproduce the relative attachment potential of intact plants. These crystalline deposits allow lower attachment forces by *H. convergens* than EW deposited as smooth films on glass or as solids with smooth, molded surfaces. Finally, two pure wax components crystallized on glass allowed lower attachment forces than the same compounds tested as smooth solids.

We also show that waxes differing in chemical composition but prepared with identical amorphous surfaces allow significantly different attachment by *H. convergens. Brassica* EW extracts as smooth films on glass or as molded solids yielded qualitatively similar patterns of attachment (Fig. 2D and E) characterized by greater attachment to extracts from wildtype (normal) waxes than to extracts from mutants. A similar but weaker pattern appears to be emerging on intact leaf surfaces wiped to remove EW crystals. This procedure does not eliminate crystals (Fig. 1), but may reduce their influence on attachment, allowing stronger influence of chemical composition on *H. convergens* attachment. Although our result shows that chemical composition of waxes influences *H. convergens* attachment, the effects of specific EW components or component classes remain to be determined. Correlation analysis suggests that an increased proportion of primary alcohols, as occurs in EW from reduced EW Glossy Andes, NY1406, and in reduced waxy bloom KCR4 × Packman $F_2$ individuals, contributes to lower attachment by *H. convergens* to extracts of these waxes. The lack of significant correlations and the complex composition of the EW extracts, in which many other components could be influential, prevent drawing a firm conclusion about chemical composition and attachment.

Nonetheless, our bioassay method will be useful for determining attachment effects of defined EW components and mixtures. Here we have shown that this approach is feasible. *Hippodamia convergens* attachment forces to $C_{22}$ *n*-alkan-l-ol was greater than to $C_{22}$ *n*-alkanoic acid, significantly so when the materials were presented crystallized on glass or molded and tested using insects from which tarsal claws had been removed. Because this difference is strongest for animals without tarsal claws, adhesion rather than friction by the claws apparently contributes importantly to the effect. The result with pure compounds also suggests a mechanism: *n*-alkanoic acids are less wettable than primary alcohols (the former have larger contact angles) (Holloway, 1969). *Hippodamia convergens* almost certainly employs a wetting agent, as do all insects so far examined (Stork, 1980*b*; Walker *et al.*, 1985; Ishii, 1987; Eisner and Aneshansley, 2000) (Gorb, 2002; Federle, 2002). If so, this agent may be hydrophilic, spreading more effectively on the hydrophilic *n*-alcohol (but see Ishii [1987] for evidence that another coccinellid secretes a hydrophobic wetting

agent). Our result suggests that the wetting agent employed by *H. convergens* needs to be characterized as part of continued efforts to understand the basis of its attachment to EW.

## CONCLUSION

Using a new approach, we have demonstrated the effect of wax crystals on plant surfaces in reducing attachment forces obtainable by an insect. This confirms the importance of wax crystals for influencing the ecology of insects that must adhere to plants to feed or forage for prey. We additionally show that composition of natural waxes prepared as smooth surfaces influences insect attachment. Thus, plant epicuticular waxes have the dual potential for influencing insect ecology through their allelochemical activity and through their influence on insect attachment to plant surfaces.

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