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Plant–Microbe Interactions: Wetting of Ivy (*Hedera helix* L.) Leaf Surfaces in Relation to Colonization by Epiphytic Microorganisms

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ABSTRACT

Leaf wettability, cuticular wax composition, and microbial colonization of upper and lower leaf surfaces of ivy (*Hedera helix* L.) was investigated for young and old leaves sampled in June and September. Contact angles of aqueous buffered solutions measured on young leaf surfaces ranged between 76° and 86° and were not dependent on the pH value of the applied droplets. Contact angles measured on old leaf surfaces were up to 32°, significantly lower than on young leaf surfaces. Furthermore, contact angles were significantly lower using aqueous solutions of pH 9.0 compared to pH 3.0, indicating the influence of ionizable functional groups on leaf surface wetting properties. Observed changes in leaf wetting properties did not correlate with different levels of alkanoic acids in cuticular waxes. However, microscopic examination of the leaf surfaces indicated the influence of epiphytic microorganisms on wetting properties of old leaves, since their surfaces were always colonized by epiphytic microorganisms (filamentous fungi, yeasts, and bacteria), whereas surfaces of young leaves were basically clean. In order to analyze the effect of epiphytic microorganisms on leaf surface wetting, surfaces of young and clean ivy leaves were artificially colonized with *Pseudomonas fluorescens*. This resulted in a significant increase and a pH dependence of leaf surface wetting in the same way as it was observed on old ivy leaf surfaces. From these results it can be deduced that the native wetting properties of leaf surfaces can be significantly masked by the presence of epiphytic microorganisms. The ecological implications of altered wetting properties for microorganisms using the leaf/atmosphere interface as habitat are discussed.

Introduction

The interface of leaf surfaces provides a large area that forms the natural habitat of a variety of microorganisms (phyllosphere). Species composition and temporal and spatial dynamics of microbial populations in the phyllosphere depend on immigration, emigration, growth, and death of epiphytic microorganisms [29]. Hence the research focused on abiotic and biotic environmental conditions prevailing in the phyllosphere with special interest in the aerial macro- and microclimate around leaf surfaces [8], specific leaf properties

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such as leaf topography, leaf age, leaf health and leaf position [1, 26, 28, 33], the antagonistic potential of the epiphytic microflora against phytopathogens [2, 16, 35], and activity of the leaf-associated microfauna [46]. Our view on this subject deals with the plant–microbe interactions occurring at the interface of leaf surfaces.

Physicochemical properties of the leaf surface such as wetting are determined by the cuticle covering epidermal cell walls of higher plants [34]. This extracellular membrane is composed of two lipid components, cutin and waxes. Cutin consists of C_{16}- and C_{18}-hydroxylated fatty acids esterified to an inoluble polymer [24]. The cuticular waxes belong to aliphatic and cyclic substance classes, such as long-chained alcohols, fatty acids, alkanes, or triterpenes, which are deposited in the cutin matrix and to the outer cutin surface [3]. Many cuticular functions essential for terrestrial plant life are related to the hydrophobic nature of cutin and waxes. The cuticle forms an effective transport barrier for water [42] and polar substances [46, 47] and it is rarely wetted [23].

These cuticular properties have a significant influence on epiphytic growth, as microorganisms living on leaf surfaces are in direct touch with the cuticle. The aliphatic nature of wax and cutin reduces water supply on the phylloplane [23], which is of crucial importance for germination and growth of epiphytic and pathogenic microorganisms [50]. Reduced leaf wetting also inhibits leaching of substances from the interior of the leaves, and consequently low nutrient levels are available to microorganisms living in the leaf surface. Thus, leaf wetting is of great ecological importance for plants themselves as well as leaf-associated microbial populations. In the past, wettability of leaves was studied intensively, considering endogenous leaf surface properties such as the structure and chemistry of waxes or trichome density [4, 6, 22, 39] and environmental factors such as the atmospheric deposition of air pollutants at the leaf surface [9, 10, 36, 38, 48].

Leaf surface wetting in relation to colonization of the leaf surface by epiphytic microorganisms was only rarely investigated [30, 44]. Based on these recent experiments the hypothesis evolved that wettability of leaf surfaces was highly dependent on the presence of epiphytic microorganisms rather than being primarily influenced by the chemical composition of the cuticular waxes. This paper deals with the wetting properties of naturally and artificially colonized ivy leaf surfaces. It provides convincing evidence that leaf surface wetting properties are strongly dependent on coverage by epiphytic microorganisms.

Methods

Plant Material, Cuticle Isolation, and Wax Extraction

Leaves were sampled in June and September 1995 from ivy (*Hedera helix* L.) plants growing on the ground of a beech forest in the Botanical Garden of the University of Würzburg. Leaves of two different age classes were investigated. Freshly emerged, 4- to 6-week-old leaves of a light-green color were defined as young leaves, whereas 3- to 4-month-old leaves of a dark-green color were defined as old leaves. Cuticular wax extracts were obtained from isolated cuticles of the upper and lower leaf surface of *H. helix* in order to distinguish between the wax composition of both leaf surfaces. Therefore, cuticles of young and old leaf samples were enzymatically isolated according to the method of Schönherr and Riederer [43]. Disks 20 mm in diameter were punched from leaves and vacuum-infiltrated with an enzyme solution containing 2% (v/v) cellulase (Celluclast, Novo Nordisk, Bagsvaerd, Denmark) and 2% (v/v) pectinase (Trenolin Super DF, Erbslöh, Geisenheim) dissolved in 10^{-2} M citrate buffer. Microbial aerobic growth was inhibited by adding 10^{-3} M NaN_3 (sodium azide; Sigma, Deisenhofen, Germany). After several days of incubation at room temperature, cuticles could be completely separated from adhering leaf tissue by washing with deionized water. Subsequent isolated cuticles were air dried. In order to obtain representative means, seven cuticles of each age class and leaf side were randomly combined and extracted together in glass vials containing 3.5 ml chloroform, which were placed for 6 h on a rotating bench (60 rpm) at room temperature. Prior to and after wax extraction cuticles were weighed using a microbalance (Sartorius, Model 4401, Göttingen, Germany). The wax coverage (μg cm^{-2}) could be calculated from the difference of weight between intact cuticular membranes (CM) and dewaxed cuticular membranes (MX) after chloroform extraction. Wax extracts were adjusted to a concentration of 1 mg ml^{-1} and stored at 4°C.

Microbial Strains

Pseudomonas fluorescens DSM 50090^T (T = type strain) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSM, Braunschweig, Germany). *P. fluorescens* was grown in the dark at 25°C in 250-ml Erlenmeyer flasks containing 100 ml glucose yeast extract medium with shaking (180 rpm) on an orbital incubator (Sanyo Gallenkamp PLC, England). Cells were harvested at the late exponential growth phase by centrifugation (30 min at 15°C and 2,120*g*). The cells were washed twice in sterile phosphate-buffered saline (10^{-2} M PBS buffer, pH 7.4, Sigma). The concentration of cells of the suspension was photometrically adjusted to an optical density $O.D._{600}$ of 1.0.

Contact Angle Measurements

Leaf surface wetting was determined quantitatively by measuring contact angles of 2-μl droplets of buffered aqueous solutions on upper and lower leaf surfaces using a goniometer (Krüss, Hamburg, Germany) with an accuracy of ±1° (Fig. 1). Buffered aqueous so-

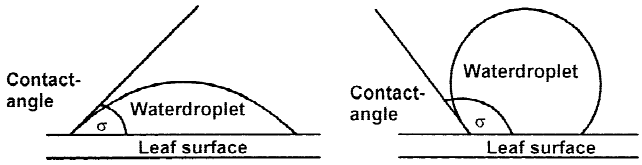

Fig. 1. Diagram of aqueous solutions forming contact angles on leaf surfaces. The contact angle σ is defined by the angle (°) between the flat leaf surface and the line tangent to a water droplet through the point of contact. Contact angles are related to the hydrophobic properties of surfaces. Thus low contact angles indicate easily wettable surfaces (on the left), and high contact angles indicate rarely wettable surfaces (on the right).

lutions had different pH values covering a pH range from 3.0 to 11.0. Acidic solutions with a pH range from 3.0 to 6.0 were prepared with 10^{-2} M citrate buffer (Sigma, Deisenhofen, Germany), while basic solutions covering a pH range from 8.0 to 11.0 were prepared with 10^{-2} M borate buffer (Sigma, Deisenhofen, Germany) and a neutral solution of pH 7.0 was obtained using 10^{-2} M phosphate buffer (50.6 mg L^{-1} K_2HPO_4 and 42.1 mg L^{-1} KH_2PO_4, Merck, Darmstadt, Germany). In order to avoid microbial growth, 10^{-3} M NaN_3 was added and the respective pH values of the buffered solutions were adjusted using either 1 M HCl or 1 M KOH.

Measurements were carried out within 1 h after leaf sampling in order to avoid desiccation of the leaf. Prior to the contact angle measurement, leaves were washed in deionized water for 10 s and carefully blotted with filter paper. This should help to remove any deposits and dust particles weakly adsorbed to the leaf surface, which might dissolve in the aqueous drops used for the contact angle measurements. Leaf strips of 3–4 cm length and 0.4 cm width were cut from the central part of the leaf, avoiding central veins and necrotic lesions. Leaf strips were attached to small microscope slides, with the ends of the strips fixed with tape in order to keep the surface flat. Advancing contact angles were measured immediately after the application of the 2 µl drop on the leaf surface at room temperature. With each sample 12 contact angles were measured on each of six different leaves.

Furthermore, contact angles were measured on young, basically clean ivy leaves artificially colonized by *P. fluorescens*. The leaves were inoculated at 25°C with 100 ml of a washed cell suspension of *P. fluorescens* (PBS-buffer; $O.D._{600}$ = 1.0) in 250-ml Erlenmeyer flasks. After an inoculation period of 24 h, leaves were washed with deionized water in order to remove bacterial cells not adhering to the leaf surface. Leaves were carefully blotted with filter paper. Remaining amounts of water were allowed to evaporate and contact angles were measured as described above. Control experiments were carried out inoculating three ivy leaves with 100 ml of sterile PBS buffer instead of bacterial suspension.

Additionally, the relationship between leaf wetting and coverage by epiphytic microorganisms of the upper leaf surface of *H. helix* was quantified with identical replicates. Firstly, contact angles of 2-µl droplets of citrate buffer, pH 3.0, were measured on small leaf segments. According to the contact angles measured, leaf segments were divided into six groups of different hydrophobicity. The arithmetic means of contact angles belonging to different groups were 90°, 80°, 71°, 64°, 52°, and 40°. Then aerial coverage by epiphytic microorganisms of the leaf segments of each group was determined using epifluorescence microscopy.

Epifluorescence Microscopy

Leaf segments were stained with an aliquot of 1 ml of 0.02% solution of acridine orange (Fluka, Neu-Ulm, Germany) in sterile deionized water for 20 min, washed twice in sterile distilled water, and air dried. A droplet of immersion oil was added to reduce photofading of the fluorescence signal. In order to diminish problems with limited depth of focus, leaf segments were kept as flat as possible by fixing the cover glasses with tape on the slides. All procedures were conducted under protection from daylight. Stained leaf segments of *H. helix* were examined with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with a 50 W mercury high-pressure bulb, a 20× objective (Zeiss, Plan-Neofluar) and a Zeiss filter set No. 09 (excitation: 450–490 nm; dichroic beamsplitter ≥510 nm; emission ≥520 nm).

The image analysis system used consisted of a low-light video camera (Intas, Göttingen, Germany) and an image analyzer (Intas, Göttingen, Germany) digitizing the video image. Digitized image data was transferred to a computer and pixel size of stained microbial cells was determined using Adobe Photoshop 3.0 (Adobe systems Inc., Version DI-3.0.5.). Percentage coverage of epiphytic microorganisms was calculated as follows:

$$\% \text{ coverage} = \frac{\text{No. of pixels of microbial cells of digitized image}}{\text{Total no. of pixels in digitized image}} \times 100 \qquad (1)$$

Digitised images at 200× magnification were recorded at randomly chosen sites from each of the six examined leaf segments of each group.

Cuticular Wax Analysis by Gas Chromatography and Mass Spectrometry

Aliquots of the wax extracts (about 100 µg) were transferred into 1 ml reactivials and 5 µg tetracosane (C_{24}-alkane; Sigma) was added as an internal standard. After evaporation of the chloroform, the solid residue was treated with 10 µl BSTFA (*N,N*-trimethylsilyltrifluoroacetamide; Machery-Nagel, Düren, Germany) and with 10 µl pyridine (Merck, Darmstadt, Germany) at 70°C for 30 min. Free hydroxyl and carboxyl groups occurring in the wax are converted into their corresponding trimethylsilyl ethers and esters by this treatment. Chloroform was added to give a final volume of 100 µl. Of each probe, 1 µl was analyzed by capillary gas chromatography/mass spectrometry (Finnigan-MAT 12S, San Jose, CA USA; Hewlett-Packard, Palo Alto, CA, USA, 5890 Series II gas chromatography with 5971A mass selective detector). Specific correction factors were determined to quantify alkanoic acids.

Table 1. Contact angles of citric buffer (pH 3.0) and borate buffer (pH 9.0) on the upper and lower leaf surface of *H. helix*[a]

	Contact angle (degree) ± ci					
	Upper leaf surface			Lower leaf surface		
	pH 3	pH 9	Δ pH[b]	pH 3	pH 9	Δ pH[b]
June '95						
Young leaf	76.9 ± 2.6	76.3 ± 2.6	0.6	76.8 ± 2.6	80.8 ± 2.4	-4.0
Old leaf	55.1 ± 2.2[c]	50.7 ± 2.2[c,d]	4.5	52.5 ± 2.3[c]	53.7 ± 2.3[c]	-1.2
September '95						
Young leaf	84.7 ± 4.5	86.1 ± 3.9	-1.4	84.8 ± 4.2	79.3 ± 2.2	3.5
Old leaf	65.6 ± 2.8[c]	53. ± 2.3[c,d]	11.7	68.7 ± 2.1[c]	56.8 ± 2.2[c,d]	11.9

[a] Young (4 to 6 weeks old) and old (3 to 4 months old) leaves were sampled in June and September. Values are arithmetic means with 95% confidence intervals (ci) from 72 contact angles measured on 6 leaves.
[b] Δ pH denotes the difference between pH 3.0 and pH 9.0.
[c] indicates significantly lower contact angles on surfaces of old leaves compared to young leaves.
[d] indicates significantly different pH effects on contact angles.

Results

Leaf Wetting

Wetting of the upper and the lower leaf surfaces increased with leaf age (Table 1). Contact angles measured on old leaves were between 16.1 and 32.2° significantly lower than contact angles measured on young leaves. With one exception, there were no significant differences in leaf wetting between upper and lower leaf surfaces. Leaf wetting of young leaf surfaces was not pH-dependent. However, leaf wetting of old leaf surfaces was in all but one case dependent on the pH value of the applied aqueous solutions (Table 1). Contact angle titrations revealed that the inflection points of the titration curves were at pH 8.0 (Fig. 2A,B).

Compared to wetting properties of clean and young leaf, surfaces artificially colonized by *P. fluorescens* showed a pronounced increase and pH dependence of wetting (Table 2). Contact angle titration of artificially colonized leaf surfaces (Fig. 2C) was very similar to results obtained with old leaf surfaces (Figs. 2A and 2B). Contact angles decreased with increasing microbe densities on the upper leaf surface (Fig. 3).

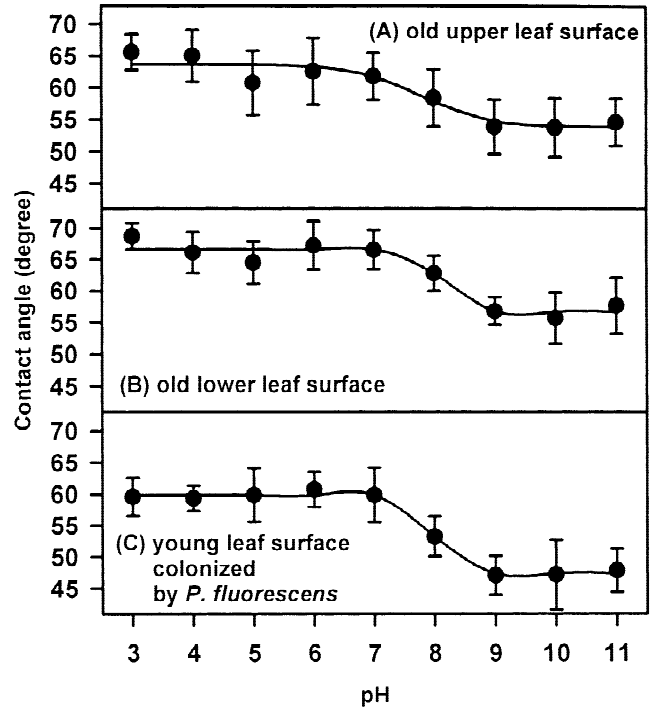

Fig. 2. Contact angles of buffered aqueous solutions of different pH values (pH 3.0 to pH 11.0) measured on leaf surfaces of *H. helix*. (**A**) Contact angle titration on the upper leaf surface of 3- to 4-month-old leaves sampled in September. (**B**) Contact angle titration on the lower leaf surface of 3- to 4-month-old leaves sampled in September. (**C**) Contact angle titration on the upper leaf surface of 4- to 6-week-old ivy leaves sampled in September, which were colonized by *P. fluorescens*. Each point represents the mean value of 12 contact angles with 95% confidence intervals measured on 6 different leaf samples.

Epiphytic Colonization

Epifluorescent micrographs demonstrated that various types of epiphytic microorganisms were present on leaf surfaces, including filamentous fungi and single-celled structures presumably representing yeast and bacteria (Fig. 4). Generally, microbial colonisation was found to be higher on old (Figs. 4B, 4C, and 4D) than on young leaf surfaces (Fig. 4A).

Table 2. Contact angles of citric buffer (pH 3.0) and borate buffer (pH 9.0) on the upper surface of young leaves of *H. helix* colonized by *P. fluorescens*[a]

Upper leaf surface	Contact angle (degree) ± ci			
	pH 3	pH 7	pH 9	Δ pH[b]
+ *P. fluorescens*	59.6 ± 3.1[c]	59.9 ± 4.3[c]	47.1 ± 3.1[c,d]	12.5
Control	77.4 ± 3.8	78.0 ± 3.0	74.3 ± 2.6	3.1

[a] Prior to contact angle measurements, leaves were incubated with cell suspensions of *P. fluorescens* for 24 h. Untreated control surfaces were incubated with sterile PBS.
[b] Δ pH denotes the difference between pH 3.0 and pH 9.0.
[c] indicates significantly lower contact angles on surfaces colonized by *P. fluorescens* compared to untreated control surfaces.
[d] indicates significantly different pH effects on contact angles.

However, the spatial colonization pattern was very heterogeneous, showing highly variable microbial population densities even on single leaves. In contrast to the nonhomogenous natural colonisation, young leaf surfaces were colonized homogeneously after artificial inoculation with a cell suspension of *P. fluorescens* (Fig.4E).

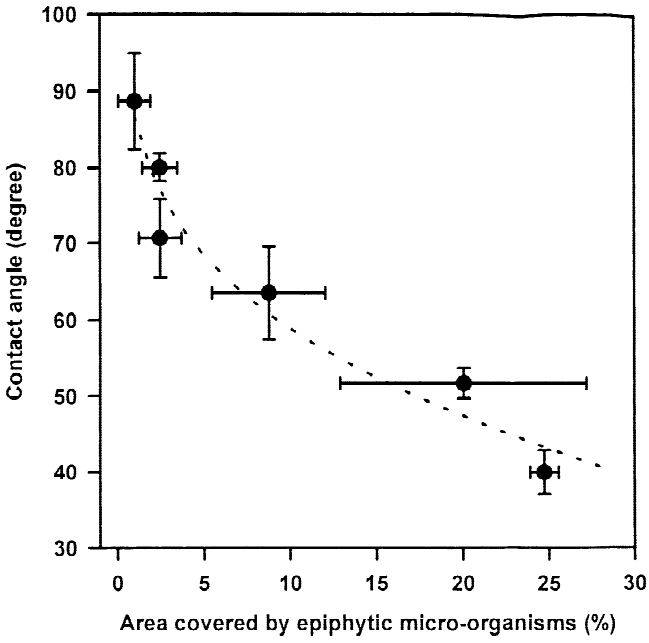

Fig. 3. Relationship between the contact angles and the population density of epiphytic microflora on the upper leaf surface of *H. helix* L. Contact angles with aqueous solutions of pH 3.0 were measured on small leaf segments, and microbial coverage was determined on the identical samples by epifluorescence microscopy. Six contact angles were measured on six leaf segments, respectively. Percentage coverage by microbial cells is given as the mean value of five analyzed digitized images at 200× magnification from randomly chosen sites. Standard errors are the 95% confidence intervals. A curve was fitted to the experimental data corresponding to the equation $y = a/x + b$; $a = 2069.1$, $b = 23.8$.

Cuticular Wax Composition

Alkanols (22.7–41.5%) and alkanoic acids (20.9–44.5%) constituted the major components in wax of *H. helix* (Table 3). Aldehydes (6.8–20.8%), esters (4.0–21.0%), alkanes (1.3–4.5%), and coumaric acid esters (0–2.2%) were minor components. Wax coverage was always higher at the upper than at the lower leaf side.

Discussion

Contact angles measured on young leaf surfaces were high and independent of the pH value (Table 1). This fact perfectly fits to the general concept of a nonpolar, highly water repellent leaf surface whose wetting properties exclusively depend on the long-chain, aliphatic wax constituents and on morphological features of the leaf surface [4, 23]. However, wetting properties fundamentally changed as leaves aged since a significant decrease and a pronounced pH dependence of contact angles could be observed (Table 1, Fig. 2), and the question arises: What are the reasons for the observed age-dependent changes in wetting between young and old leaf surfaces?

Contact angle titration is a powerful tool for analyzing the nature of functional groups occurring at interfaces [25]. Contact angle titrations on upper and lower ivy leaf surfaces revealed significantly higher angles at acidic pH values compared to basic pH values (Fig. 2), which indicates the existence of ionizable functional groups at the leaf surface. Moreover, titration curves showed inflection points at pH 8 (Fig. 2). pKs values around 8 are typical for carboxylic groups located at the interface of solid surfaces [25]. Furthermore, it was recently shown [44] that contact angle titrations measured on pure stearic acid revealed inflection points in the same range of pH values. Thus, contact angle titrations imply the existence of interfacial carboxylic groups in the interface of the leaf surfaces. Carboxylic groups are polar, ionizable functional groups, and thus they help to explain the decrease of contact angles and pH dependence of leaf surface wetting. When solutions of increasing pH values are used, carboxylic groups become more and more ionized. Consequently, the number of negative charges increases, which in turn renders the interface more wettable.

Thus, the altered wetting properties of old leaf surfaces

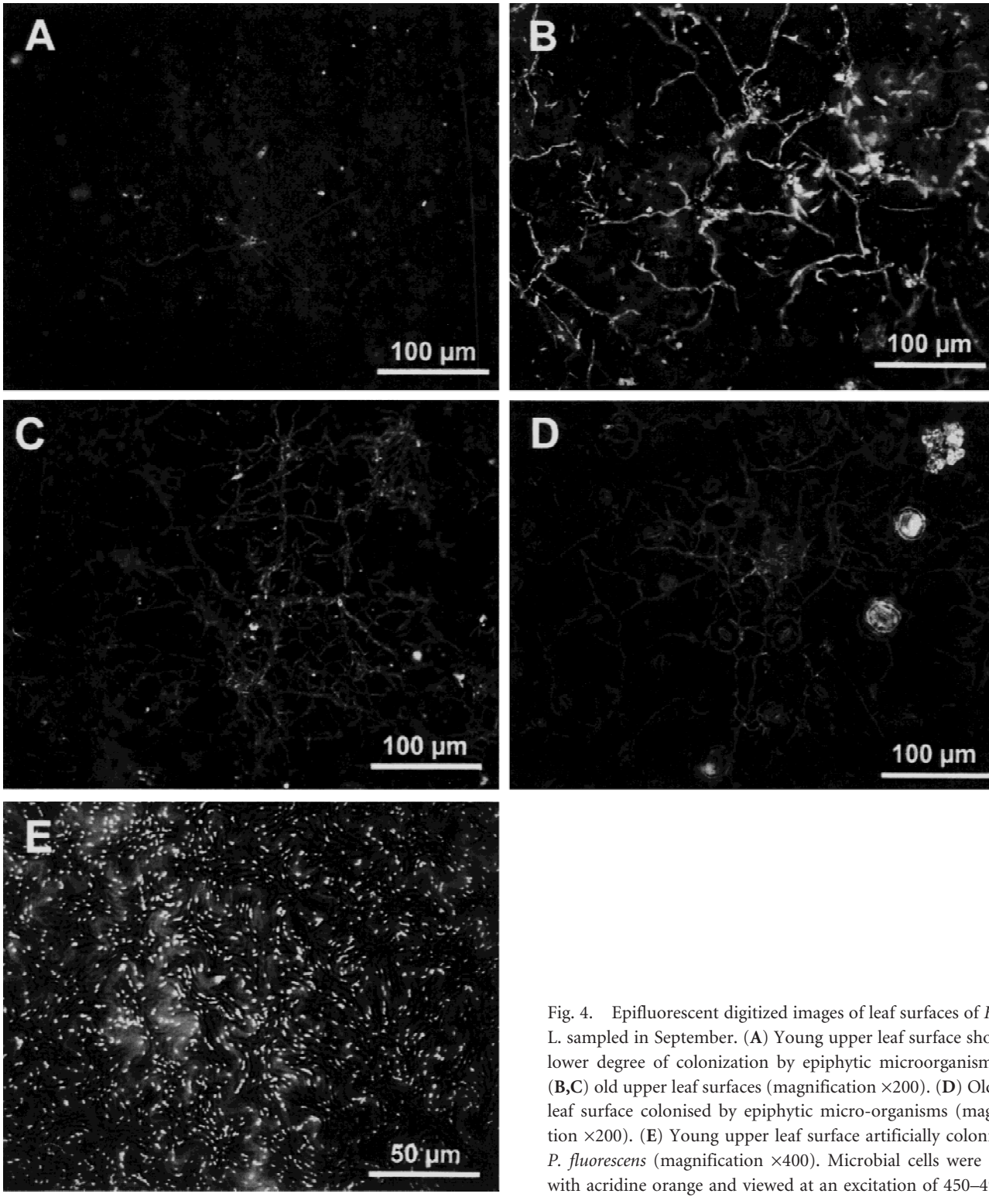

Fig. 4. Epifluorescent digitized images of leaf surfaces of *H. helix* L. sampled in September. (**A**) Young upper leaf surface showing a lower degree of colonization by epiphytic microorganisms than (**B,C**) old upper leaf surfaces (magnification ×200). (**D**) Old lower leaf surface colonised by epiphytic micro-organisms (magnification ×200). (**E**) Young upper leaf surface artificially colonized by *P. fluorescens* (magnification ×400). Microbial cells were stained with acridine orange and viewed at an excitation of 450–490 nm.

can be assigned to interfacial carboxylic groups. What is the origin of these carboxylic groups? Wax analysis of the upper and lower ivy leaf surfaces revealed that alkanoic acids were in fact major wax constituents (Table 3). However, as young and old upper leaf surfaces had similar contents of alkanoic acids between 20 and 30% (Table 3), it is questionable whether observed changes in wetting are due to the alkanoic acids. Moreover, wax of old leaf surfaces sampled in June had the highest amounts of alkanoic acids (45%), but simultaneously leaf wetting was pH independent (Table 1),

Table 3. Relative composition of cuticular wax of *H. helix* and absolute wax coverage of the upper and lower leaf surface

	Mass percent of wax component[a]					
	June young leaves		June old leaves		September old leaves	
	Lower side[b]	Upper side[b]	Lower side[b]	Upper side[b]	Lower side[b]	Upper side[b]
Alkanols	33.0	41.5	22.7	30.1	36.0	38.1
Alkanoic acid[c]	32.3	28.0	44.5	30.4	38.4	20.9
Aldehydes	6.8	17.1	7.6	11.2	11.9	20.8
Esters	21.0	9.5	9.6	14.0	4.0	9.8
Coumaric acid esters	0.5	—	1.1	2.2	—	0.5
Alkanes	2.2	1.3	4.5	4.0	2.1	3.2
Σ[d]	95.8	97.4	90.0	91.9	92.4	93.3
Total wax[e] [$\mu g\ cm^{-2}$]	9.4	16.0	12.4	19.8	8.5	24.2

[a] Wax components as percentage of total wax amount.
[b] Cuticular wax extracts of upper and lower leaf surfaces represent means of seven isolated cuticles.
[c] Specific correction factors were determined for alkanoic acids.
[d] Sum of identified wax components as percentage of the total wax mass.
[e] Total wax coverage of the cuticle determined gravimetrically.

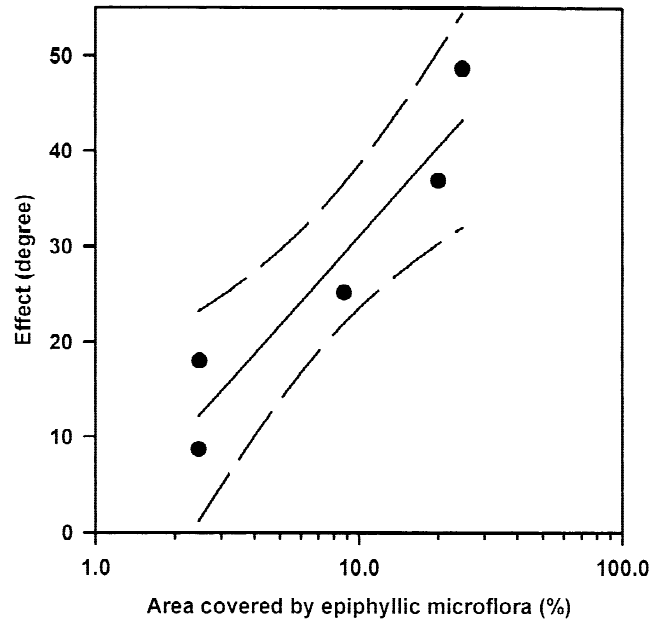

Fig. 5. Relationship between the effects on wetting (decrease of contact angle) and the population density of the epiphytic microflora on the upper leaf surface of *H. helix* L. Effects were calculated from Fig. 3 by subtracting the contact angle measured on colonized leaf surfaces from that measured on leaf surfaces without any detectable colonization. The observed effects were plotted vs the logarithms of percentage area covered by epiphytic microorganisms. A linear regression was fitted to the data: effect = 31.0 (±19.5) × log (area coverage) +0.1 (±19.2); $r^2 = 0.90$. The broken lines show the 95% limits of the confidence intervals.

whereas leaf surfaces with lower contents of alkanoic acids of 21 and 38% showed a significant pH dependence of wetting. Besides alkanoic acids, there are no other wax components of *H. helix* carrying free carboxylic acids, and therefore it is very unlikely that altered leaf wetting properties solely relied on the chemical composition of the cuticular wax.

With increasing age, leaf surfaces were more and more colonized by epiphytic microorganisms (Fig. 4), and the question arises as to whether they contributed to the observed wetting properties of older leaf surfaces. Leaf wettability was strongly correlated with the degree of epiphytic colonization (Fig. 3). When 2.5% of the leaf surface was covered by epiphytic microorganisms, contact angles decreased by 20°, and at a population density of 25%, contact angles decreased by 50° (Fig. 3). The hyperbolic relationship between the population density and the effect on wetting (Fig. 3) can be described by a linear dose–response curve plotting the effects of wetting (decrease of contact angles) as a function of the logarithm of the area covered by epiphytic microorganisms (Fig. 5):

$$\text{Effect} = \log(\text{Area coverage}) + \text{Intercept} \qquad (2)$$

Thus, it is not the absolute, but the relative increase in the area covered by microbial cells that is relevant to the effect. Even a very low surface coverage of 5% already had a significant effect on wetting (Figs. 3 and 5). This demonstrates that low population densities of naturally occurring epiphytic microflora [11] can have strong effects on leaf surface wetting properties.

These results were further confirmed by the observation that surfaces of young and clean ivy leaves that were artificially colonized with *P. fluorescens* exhibited wetting properties similar to those of old leaf surfaces (Fig. 2). *P. fluorescens* was capable of increasing leaf wettability significantly and to introduce a pH dependence to wetting (Table 2). The titration curve of artificially colonized ivy leaf surfaces (Fig. 2C) was very similar to those measured on old leaf surfaces naturally colonized by an epiphytic microflora (Figs. 2A and B). Moreover, the pKs-value was again around 8 (Fig. 2C), which indicates again the presence of carboxylic groups involved in altered leaf surface wetting properties due to leaf surface microorganisms. Here we used *P. fluorescens* as a model bacterium as it is commonly found on leaf surfaces [15, 32]. Besides *P. fluorescens,* a variety of different species typically found on leaf surfaces, including bacteria and fungi,

were found to be capable of triggering a pH dependence of wetting [30].

From our results we deduce that the native wetting properties of leaf surfaces could be significantly masked by the presence of epiphytic microorganisms. Young leaf surfaces are usually less colonized by epiphytic microorganisms than old leaf surfaces. Thus, wetting properties of young and clean leaf surfaces are mainly determined by the hydrophobic nature of the cuticle and the waxes. As leaves age, their surfaces become more and more colonized by microorganisms, and consequently the impact of the micro-organisms on leaf wetting increases. Epiphytic microorganisms create a new interface between leaf and atmosphere. Thus, the chemical composition of the microbial cell surfaces and the population density determine the wetting properties of the colonized leaf surface (Tables 1 and 2, Figs. 2 and 3).

At the moment the exact mechanism at the leaf surface is still unknown. Principally cell wall constituents of microbial cells and extracellular polymeric substances (EPS) such as slimes, capsules, and biosurfactants form microbial surfaces. Acidic lipopolysaccharides of the outer cell membrane of Gram-negative bacteria [41, 49], teichuronic acids of Gram-positive bacteria [49], and acidic polysaccharides of fungal cell walls [45] contain carboxylic groups. The acidic nature of many microbial EPS is due to uronic acids and substituents of sugars such as pyruvate or succinate [13, 19, 37]. Furthermore, major classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, and fatty acids contain free carboxylic groups [17, 40]. EPS and biosurfactants can be secreted as a dispersed soluble slime in the vicinity of the adherent cells [12]. Thus, they could effectively change wetting of the surface in close proximity to the adherent cells. A surface-active strain of *Pseudomonas putida* increased wettability by the production of biosurfactants [7] and a surface-active strain of *P. fluorescens* was shown to wet the waxy surface of broccoli florets [20].

This study and recent investigations [30, 44] demonstrated that wetting properties of leaf surfaces can strongly depend on the degree of epiphytic colonization. Apparently the formation of microhabitats with more favorable microbial living conditions is a common strategy for the survival of epiphytic microorganisms. The abundance of water is the most important abiotic factor limiting microbial growth on interfacial surfaces [50]. Most fungi grow at water activities of their habitat higher than 0.8 [41]. Bacterial growth takes place at even higher water activities of at least 0.98 [41]. The introduction of polar interfacial chemical groups of microbial origin renders the leaf surface more polar and hydrophilic. Consequently, water droplets will spread and water vapor will be more easily adsorbed to the leaf surface. Thus, the formation of water films on the leaf surface is supported, which has important consequences for microbial growth on the leaf surface. The prolonged presence of water increases metabolic activity and microbial growth processes, and it increases rates of foliar leaching of solutes diffusing across the cuticle, which in turn result in a better nutrient supply to leaf surface microorganisms [18]. Free water is also necessary for diffusion of antimicrobial compounds secreted by epiphytic bacteria [31]. Either microorganisms themselves are transported passively in the water film, or motile bacteria move actively by the means of flagella [14, 27]. Thus, new habitats on the leaf surface can be colonized and there is a higher probability of finding entry sites into the leaf interior, such as defects in the cuticle or stomata [21]. In the case of pathogenic microorganisms, endophytic populations finally can cause plant diseases [5]. This illustrates the complex interactions taking place in the phyllosphere, and it stresses the influence of the epiphytic microflora on leaf wetting, posing a series of interesting new questions in the field of the microbial ecology of the leaf surface.

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