

Mapping leaf surface landscapes

(epicuticular lipids/phyloplane/ecological scale/microtopography/atomic force microscopy)

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ABSTRACT Leaf surfaces provide the ecologically relevant landscapes to those organisms that encounter or colonize the leaf surface. Leaf surface topography directly affects microhabitat availability for colonizing microbes, microhabitat quality and acceptability for insects, and the efficacy of agricultural spray applications. Prior detailed mechanistic studies that examined particular fungi–plant and pollinator–plant interactions have demonstrated the importance of plant surface topography or roughness in determining the outcome of the interactions. Until now, however, it has not been possible to measure accurately the topography—i.e., the three-dimensional structure—of such leaf surfaces or to record precise changes in patterns of leaf surface elevation over time. Using contact mode atomic force microscopy, we measured three-dimensional coordinates of upper leaf surfaces of *Vaccinium macrocarpon* (cranberry), a perennial plant, on leaves of two age classes. We then produced topographic maps of these leaf surfaces, which revealed striking differences between age classes of leaves: old leaves have much rougher surfaces than those of young leaves. Atomic force microscope measurements were analyzed by lag (1) autocorrelation estimates of leaf surfaces by age class. We suggest that the changes in topography result from removal of epicuticular lipids and that the changes in leaf surface topography influence phylloplane ecology. Visualizing and mapping leaf surfaces permit detailed investigations into leaf surface-mediated phenomena, improving our understanding of phylloplane interactions.

Landscape heterogeneity is a major determinant of species composition in ecological communities (1, 2). Although landscape structures are typically measured in meters, landscape heterogeneity occurs at strikingly finer scales, as demonstrated by investigations into plant architecture (3–5) and the fractal dimensions of plants (6, 7). For microbes and insects on plant surfaces, plant surface features represent ecological landscapes (7, 8), and provide essential information about the plant as a resource (8, 9). Until now, this information has been largely inaccessible to researchers because accurate measurements and images of plant surface landscapes, or topography, have been difficult to obtain (10). Using atomic force microscopy (AFM), we measured three-dimensional coordinates for leaf surface landscapes of a perennial plant and produced the corresponding topographic maps. Data analysis demonstrated significant quantitative differences between surface topographies of young and old leaves, revealing the topographical changes occurring in phylloplane habitats. Detailed examination of leaf surface topography is critical for basic and applied phylloplane investigations.

The principles of landscape ecology that link spatial pattern to ecological process, have transformed thinking about the influence of habitat structure on plant and animal populations

(11). Meaningful analysis of spatial pattern requires landscape examination at the appropriate scale (12). With this in mind, the concepts of landscape ecology (11) can be extended to the micron scale and below to consider the influence of surface landscapes on phylloplane organisms. We demonstrate that leaf surface landscapes, examined at the cellular level, sustain significant topographic alterations over time. These changes may be the result of both biotic and abiotic processes and may have important effects on phylloplane ecology.

Microbial community ecology on leaf surfaces has been without unifying concepts to explain or predict the dynamics of these phylloplane communities (13). The leaf surface as a habitat, viewed topographically at the cellular level, has not been routinely considered in evaluating ecological processes on the leaf surface, primarily because of the difficulties in collecting the appropriate data. Nevertheless, the importance of plant surface microtopography to trophic-level interactions has been clearly demonstrated with microbes and plants (7) and insects and plants (14, 15) and has been suggested as a factor in epiphyllous algal and plant interactions (16).

MATERIALS AND METHODS

We examined leaf surface topography of *Vaccinium macrocarpon* Ait. (Ericaceae), the American cranberry, with contact mode AFM. We examined the topography of epicuticular lipids at approximately $\times 1600$, a scale that reveals features relevant to organisms on the leaf surface (7, 14, 15). *V. macrocarpon* is a perennial plant native to North America and is cultivated commercially for the berries. We used the leaves on floral stems and focused on the differences in topography between two age classes: leaves from the current growing season (young leaves) and leaves from the previous growing season (old leaves).

Plant Material. Floral stems from a Massachusetts commercial cranberry bog, variety Early Black, were picked in the fall. Stems were placed in water in florist's water piks immediately upon cutting. Leaves were prepared for imaging by first rinsing the stems in a beaker of deionized water and then exposing the stems, cut ends in water piks, to 10% osmium tetroxide vapors in the dark. Stems remained in osmium tetroxide vapors until all of the leaf material had turned black, approximately 5 hr. Leaves were imaged at TopoMetrix within 72 hr of fixation. All scans included in this analysis were done on the adaxial (upper) leaf surface to the right of the midvein. The age of cranberry leaves is readily determined by leaf position on the stem relative to flower stem placement of the current season's flowers; old leaves are found below flower stem attachment, and new leaves are found above flower stem attachment (17).

AFM Scans and Topographic Maps. Using AFM (TopoMetrix TMX 2000 Explorer) at a magnification of approximately

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Abbreviation: AFM, atomic force microscopy.

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$\times 1600$, we obtained localized scans on leaf surfaces of both old and new leaves. We used silicon nitride tips to scan the leaf surface. The tips are pyramidal in shape, $3 \mu\text{m} \times 3 \mu\text{m}$ at the base, and $3 \mu\text{m}$ in height. The radius of curvature is 500 \AA . The force of the probe is 1 nN , with a force constant of 1 nN . Low

cantilever force limits deformation of the leaf surface. The force was held constant for all scans.

We conducted AFM scans on selected regions of four old leaves and five new leaves. We selected leaves for imaging at approximately the midpoint on the stem of each season's

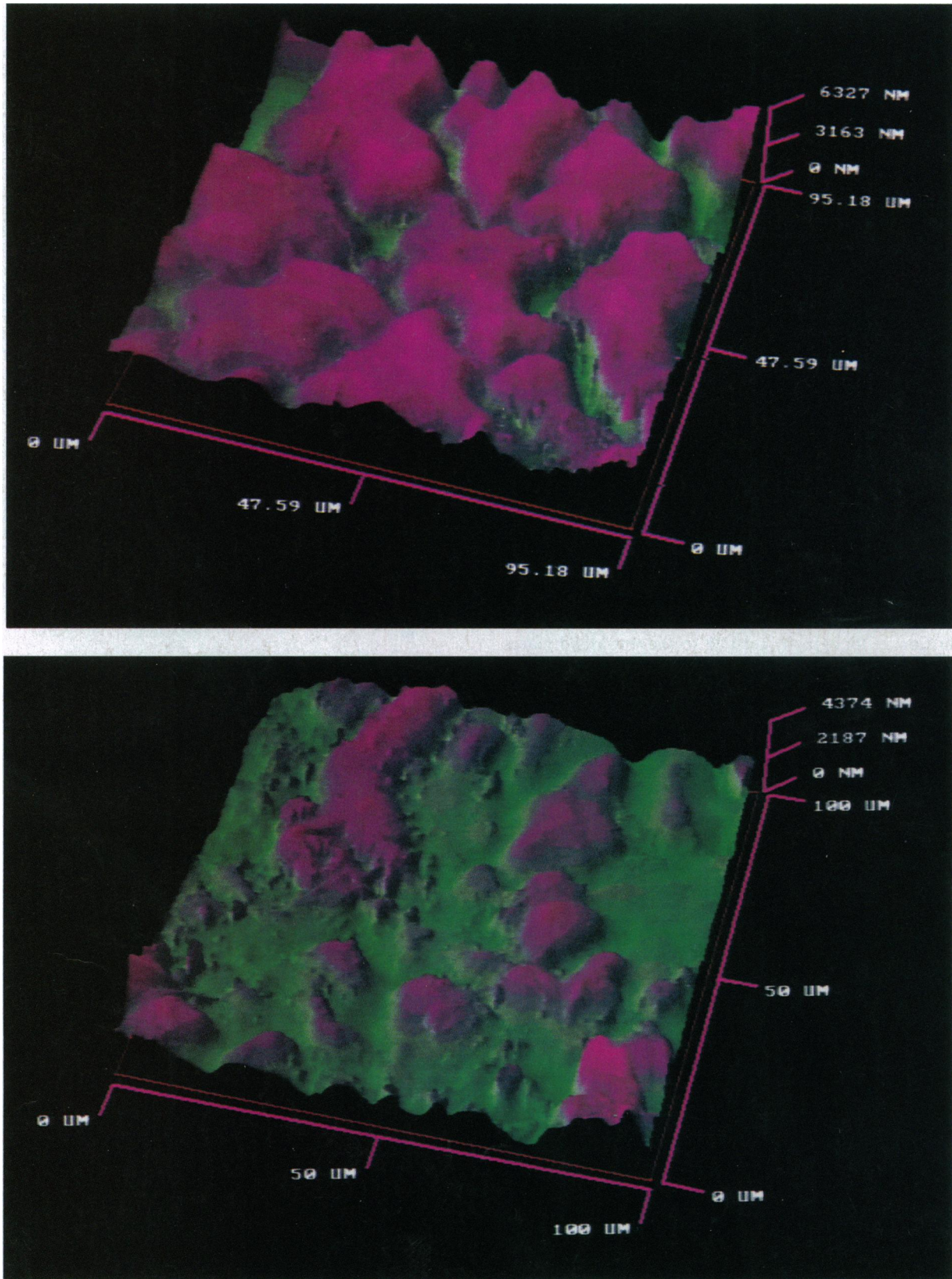


FIG. 1. Localized AFM scans of adaxial (upper) leaf surfaces of *V. macrocarpon*, showing the leaf surface contours of a young leaf (Upper) and an old leaf (Lower); x and y axes are in micrometers and the z axis is in nanometers.

growth. Scanned areas were perfect squares, ranging in size from $95 \mu\text{m} \times 95 \mu\text{m}$ to $100 \mu\text{m} \times 100 \mu\text{m}$. Scan area size was determined by visual examination of leaf surfaces under magnification comparable to that of the scan and included as many clean epidermal cell surfaces as possible. We collected data from one scan per leaf, with the exception of one especially clean old leaf on which we conducted two separate surface scans. (Old leaves, in general, had more debris on the adaxial surface than did young leaves. Because surface debris caused the AFM probe to go out of range during scanning and interfered with the collection of data, we scanned two distinct areas on this particular old leaf, the surface of which was clean.) For the old leaf on which we conducted two scans, we completed one scan at the proximal end and one scan at the distal end of the leaf. Each leaf scan yielded a 200×200 grid of data points for height values. We analyzed data from all 10 scans.

Coordinates obtained from the AFM scans (x , y , and z coordinates) were then mapped topographically as individual quadrants with SURFER for Windows (Golden Software Inc., Golden, CO).

Features Contributing to Topography. We examined leaf surfaces under two types of experimental conditions to determine if epicuticular lipids on epidermal cells contributed to the observed topography. (i) Isolated epidermes of *V. macrocarpon* stained with periodic acid-Schiff reagent (PAS) revealed the outlines of epidermal cell walls. The pattern observed with PAS resembled the morphology observed by AFM scans of young leaf surfaces, suggesting that the leaf surface topography reflected the features of epidermal cells. (ii) Leaf surfaces were washed with 1:1 (vol/vol) chloroform/methanol, 10 ml per leaf, to remove epicuticular lipids. Washed leaves (epicuticular lipids removed) as well as the unwashed leaves (with the lipids intact) were viewed with an environmental scanning electron microscope (Electroscan, Wilmington, MA). Leaf surfaces washed with chloroform/methanol exhibited an overall reduction in topography relative to the unwashed leaves when viewed with an environmental scanning electron microscope. (These photographs are not included in this report.)

Data Analysis. We tested the null hypothesis that there is no difference between young and old leaves in leaf surface topography. Data were analyzed by autocorrelation analysis (18). We calculated lag (1) autocorrelation values for each leaf scan and then combined the values by leaf age class: old leaves versus young leaves (SAS Institute, Cary, NC, version 6.08). The data from the autocorrelation analysis were then tested for distributional equality by the two-sample Kolmogorov-Smirnov (KS) test (SPSS, Chicago, version 4.1).

RESULTS AND DISCUSSION

Young leaf surfaces are characterized by a regular pattern of broad expanses or plateaus on the cell surfaces, with drops in elevation between the cells (Fig. 1 *Upper*). Old leaf surfaces are characterized by the absence of broad elevated surfaces, and in general have a less regular pattern of surface morphology (Fig. 1 *Lower*).

Topographic maps produced from AFM data provide information about leaf surface contours and their dimensions as well as images from which accurate quantitative comparisons can be made. The topographic maps of the young (Fig. 2 *Upper*) and old (Fig. 2 *Lower*) leaves illustrate the striking differences between age classes of leaves; old leaves have much rougher surfaces than those of young leaves.

Autocorrelation analysis considers the two-dimensional grid of surface heights as a series of two-dimensional slices of height versus grid spacing. In each slice a surface profile is represented as a correlated series—i.e., a time series in which time is replaced by a sequential grid number. The autocorrelation coefficients are lag dependent so that the autocorrelation of

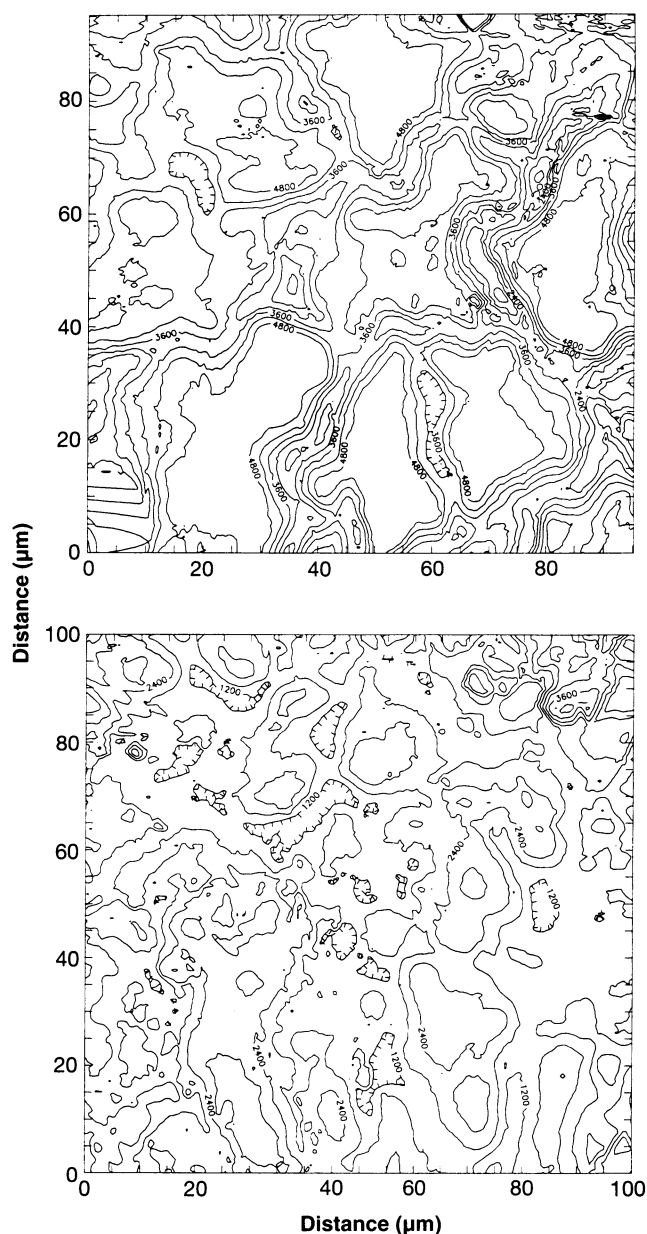


FIG. 2. Topographic maps of the scanned portions of adaxial leaf surfaces of *V. macrocarpon* young leaf (*Upper*) and old leaf (*Lower*). *Upper* and *Lower* correspond to the scans shown in Fig. 1 *Upper* and *Lower* respectively. Distances along the x and y axes are measured in micrometers. The contour interval is 600 nm. Both the AFM scans and the topographic maps show clear differences in surface morphology between the leaves in the two age classes.

lag (1) describes the dependence of the current height on the previous height. Local smoothness corresponds to heights that do not change markedly, but local roughness corresponds to heights that change more rapidly. Lag (1) autocorrelation values close to one suggest sequential heights that are smooth—e.g., correlated—but lags close to zero suggest roughness.

Although fractal analysis (19) has been used commonly in recent mathematical analyses of surface features (20), analysis of the AFM data by fractal dimension, as calculated by the lakes method (21), did not account for the observed differences between the two types of leaf surfaces. Concerns about the lake filling method for analysis of AFM data have been raised (22). We suggest that the lake filling method is not appropriate for AFM leaf surface analysis because it analyzes

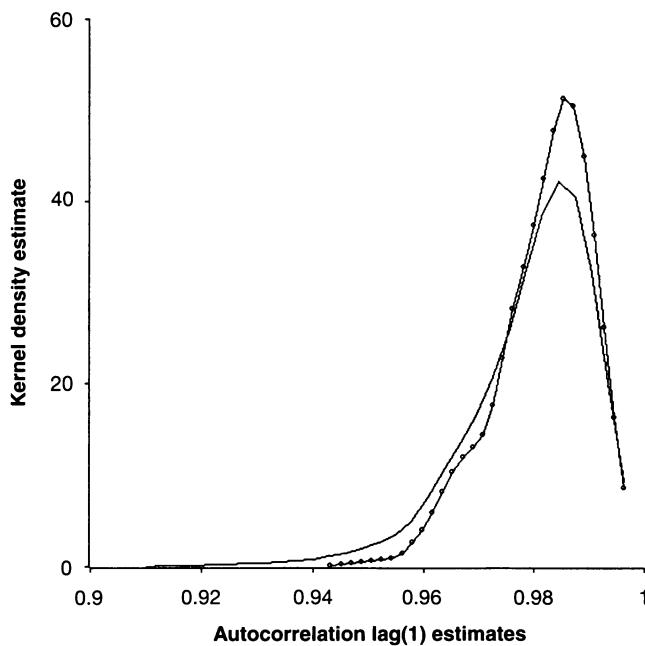


FIG. 3. Kernel density estimate of the distribution of autocorrelation coefficients for the leaf surface data for young leaves (—●—) and old leaves (—). The kernel density estimate, similar to the histogram, is the preferred, nonparametric technique for estimating population distributions.

a small subset of the total surface data, thus omitting much of the data's richness. By contrast, autocorrelation analysis incorporates all of the surface data points in the analysis.

The combined estimates of lag (1) autocorrelation (Fig. 3) indicate significant differences in surface roughness between young and old leaves when the pooled values are tested by the two-sample Kolmogorov–Smirnov test ($P < 0.0001$). The strong significance of the Kolmogorov–Smirnov test is the result of the shift in the distribution of lag (1) autocorrelation values for old leaves versus young leaves, indicating surface roughness present in old leaves that is not present in young leaves. The combined distribution of lag (1) autocorrelation values for young leaves is stochastically larger—i.e., closer to 1—than the combined distribution of values calculated for old leaves. Although some of the old and young leaves have distinctly different distributions of lag (1) autocorrelation values, the pooled data show there is some overlap in the distributions between the age classes of leaves. This distributional overlap in autocorrelation values suggests that portions of young leaf topography are retained through leaf aging, much as geologic erosion preserves some land features while strongly changing others.

Patterns of leaf surface topography have implications for both biological and physical processes on the leaf surface, altering habitat quality and acceptability by organisms. The topographical differences may lead to local differences in leaf surface wettability (23) and agricultural spray effectiveness by determining droplet adhesion or spread (24). Droplet spread affects the likelihood that an organism would encounter a droplet on the leaf surface (23). Microclimatic features such as temperature, humidity, or windspeed may also be modulated by leaf surface topography, as rougher leaf surfaces have thicker boundary layers of air than do smooth surfaces. In addition, changes in surface roughness may affect localization of plant compounds released from leaf cells onto the leaf surface (25), distribution of secondary plant compounds in epicuticular lipids (26, 27), release of plant volatiles, adsorp-

tion of insect semiochemicals by plants (28, 29), or spectral reflectance from the plant surface (30).

Detailed study of leaf surface habitat heterogeneity is made possible by visualizing and mapping changes in leaf surfaces. We have demonstrated an effective method for quantifying and comparing leaf surface landscape patterns and identifying the critical features at a scale relevant to phylloplane organisms.

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