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Wettability and morphology of the leaf surface in cashew tree from the Amazon, Northern Brazil

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ABSTRACT. Leaves surfaces, which represent an interface with plants and the environment, have several structures with specific functions. Some foliar properties, including wettability and mechanical containment, are inferred in terms of cellular adaptation and the presence or absence of cuticular wax. Various morphological parameters, ranging from macro- to nano scales, are analyzed and contribute to the study of taxonomy, pharmacognosy, and ecology of plants. The aim of this paper was to analyze the effect and influence of epicuticular wax granules on the hydrophobicity of *Anacardium occidentale* L. leaf surfaces. Leaf specimens were directly examined with an environmental scanning electron microscope without metal coating. Images revealed epidermis ornament, stomata type, was, and trichomes. Static contact angle between water and the surface was also measured on both sides. On the adaxial side, an angle of 104.09° ± 0.95° was found, suggesting that adaxial surface is hydrophobic. On the abaxial side, the angle was 62.20° ± 1.60°, which indicates a hydrophilic nature, probably because of the greater amount of epicuticular wax on the adaxial leaf surface. The present investigation provided an important contribution to morphological and ultrastructural characterization of leaves of cashew tree, which is a plant of great medicinal and economic importance.

Keywords: hydrophobic, hydrophilic, *Anacardium occidentale* L., wax.

Molhabilidade e morfologia da superfície da folha em cajueiro da Amazônia na Região Norte do Brasil

RESUMO. Superfícies de folhas têm diversas estruturas com funções específicas e contribuem para a relação delas com o meio ambiente. Algumas propriedades foliares, incluindo molhabilidade e contenção mecânica, são inferidas em termos de adaptação celular e da presença ou ausência de cera cuticular. Diversos parâmetros morfológicos, variando da macroescala até a nanoescala, são analisados e contribuem para o estudo de taxonomia, farmacognosia e ecologia de plantas. O objetivo deste trabalho foi analisar o efeito e a influência de grãos de cera epicuticular na hidrofobicidade da superfície de folhas de *Anacardium occidentale* L. Amostras de folha foram examinadas com um microscópio eletrônico de varredura ambiental sem recobrimento metálico. As imagens evidenciaram algumas características (epiderme, tipo de estômato, cera e tricomas). O ângulo de contato estático entre a água e a superfície também foi medido em ambos os lados. No lado adaxial, foi encontrado um ângulo de 104,09° ± 0,95°, sugerindo que esta é hidrofóbica. No lado abaxial, o ângulo foi de 62,20° ± 1,60°, que indica uma natureza hidrofílica, provavelmente devido à grande quantidade de cera epicuticular na superfície abaxial da folha. A presente investigação forneceu uma contribuição importante para a caracterização morfológica e ultra-estrutural de folhas de cajueiro, que é uma planta de grande importância econômica.

Palavras-chave: hidrofóbico, hidrofílico, *Anacardium occidentale* L., cera.

Introduction

Wetting (hydrophilic property) can be defined as the ability to spread a liquid on a given surface (Bhushan & Jung, 2006). When a drop of water can spread easily on the surface of a material, this material is hydrophilic and is characterized by the static contact angle (angle that a liquid makes with a solid surface) when $\theta < 90°$, as shown in Figure 1.

This happen when the material has high surface energy, formed by polar molecules and intermolecular bonds, which emerge between the liquid and material. Atoms or molecules on the bulk surface have fewer chemical bonds with their neighborhood than similar atoms or molecules located inside the bulk material, thus the outermost atoms and molecules have higher energy. This additional energy is quantitatively characterized by surface tension or free surface energy. When a solid is in contact with a liquid, the molecular attraction reduces the energy to lower levels than that observed for the two separate surfaces. On high-energy surfaces, most liquid droplets spread out on the surface in the form of a thin liquid film, while on a low-energy surface, partial wetting occurs and most droplets form a spherical cap on the surface. Therefore, when the surface of the material repels water droplets, the surface is hydrophobic and the contact angle is greater than 90°. If the contact angle approaches 0°, it is said that the material is superhydrophilic. On the other hand, if the contact angle exceeds 150°, as in the case of water on a lotus leaf, the material is considered superhydrophobic (Bhushan & Jung, 2011; Koch, Bhushan, & Barthlott, 2008) (Figure 1A-D). The contact angle depends on several factors, such as surface energy, surface roughness and cleanliness (Israelachvili, 1992; Bhushan, 2002).

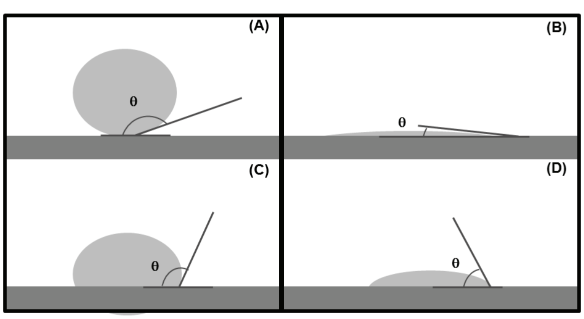

Figure 1. (A-D) Schematic figures of four classes of surface wettability and respective static contact angle. Superhydrophobic (CA > 150^0), superhydrophilic (CA < 10^0), hydrophobic (90^0< CA < 150^0) and hydrophilic (10^0< CA < 90^0) surfaces, respectively.

Since the 1970s, scanning electron microscope (SEM) studies have shown that the hydrophobicity of leaf surface is related to its microstructure. Three general levels of structuring are observed in leaf surfaces: the general shape of the cell, cuticular folds and epicuticular waxes. Wetting is important for many biological processes, like germination of seed, fungi, reproduction of bacteria, being essential for water uptake from soils (McHale, Newton, & Shirtcliffe, 2005). The plant cuticle with its integrated and exposed waxes is, in general, a hydrophobic material, but structural and chemical modifications induce variations in surface wetting, ranging from superhydrophilic to superhydrophobic. Architecture of the cells, the presence of hairs and the fine structure of the surfaces, e.g., folds of the cuticle or existing epicuticular waxes (formation of three dimensional wax crystals on the plant surface) have a strong influence on surface wettability. Shape, size and fine structure of cells greatly influence several functional approaches of the plant boundary layer. Wetting properties of surfaces have been the subject of extensive studies in biology, as well as in physics and chemistry. Studies related to bioinspiration (Bixler & Bhushan, 2012; Wong et al., 2011), hysteresis (Liu & Choi, 2013), thermogenesis (Ramachandran & Nosonovsky, 2014), precipitation (Holder, 2012) and penetration of external agents, are well reported in the literature.

A previous knowledge of the nature of leaf surfaces is of great interest to agrochemical companies since it governs both the amount of spray that is retained by plants during spraying by the farmer. Physical tests, such as dynamic surface tension or contact angle, are essential to implement appropriate chemical formulations, and the nature of the species must take into consideration how the sprayed droplets behave on the leaf surface. Considering the importance of detailed studies in providing a better understanding and characterization of leaf morphology, this work analyzed the ultrastructural differences and compared the water repellency of the adaxial and abaxial sides of fresh leaves of *Anacardium occidentale* L. by measuring the static contact angle, and examining images from an environmental scanning electron microscope (ESEM). Reports were not found regarding the wettability of leaves used in this work. The hypothesis of this study was that abaxial surface is more hydrophilic than the adaxial side, given the presence of some structures, such as stomata, which increase the roughness on the lower side. In this way, this work intended to characterize the leaf surface at the microscale, investigating the effect and influence of epicuticular wax granules on the hydrophobicity of these surfaces.

Material and methods

Leaves were collected from a cashew tree at the campus of the *Universidade Federal do Amapá*, northern Brazil, close to French Guiana. A voucher specimen was deposited in the Amapaense Herbarium (HAMAB) located at IEPA (*Instituto de Pesquisa Científica e Tecnológica do Estado do Amapá*), under registration nº 018684. Samples of fresh leaves were cleaned with deionized water to remove any residues on the surface.

Adaxial and abaxial surfaces were examined. Sections of 5 x 5 mm^2 of five fresh leaves were fragmented using a blade, avoiding the midrib areas, in order to obtain a relatively consistent surface. Leaf specimens were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape. Without metal coating, surfaces of leaf specimens were directly examined with an environmental scanning electron microscope (SEI-quanta 250, Hitachi High Technologies America Inc, USA) at an accelerating voltage of 5 kV.

For further characterization of the surfaces, the static contact angle between water and the surface was determined. For this, five different leaves were used to estimate the average contact angle on both adaxial and abaxial surfaces, using small sections and a contact angle goniometer (model 100, Ramé-hart Instrument Co, Succasunna, USA) and droplets of deionized water. For these measurements, the droplet size should be smaller than the capillary length, but larger than the dimension of the structures present on the surfaces. Droplets of 2.5μL were gently placed on the substrate using a microsyringe to measure the static contact angle. All measurements were made at three different points of each leaf side at 25 ± 1ºC and 50 ± 5% RH. The measurements were reproduced within ± 2º.

Results

SEM images

Scanning electron microscopy analysis revealed micromorphological diversity in leaf surfaces. There were great differences in leaf morphology between the adaxial and abaxial surfaces. The upper side was formed by a highly undulated cuticle or ridged with depressions in some regions, and no stomata (Figure 2A). Epicuticular wax granules were observed in the upper side (Figure 2B), which varied in diameter (ca. 1-4 μm) and dispersed on the undulated cuticle along the adaxial surface.

On the abaxial leaf surface, stomata were observed (Figure 3) with a slightly ridged cuticle, which was denser in the surroundings. Stomata are ellipsoidal and inserted at the epidermal level, with random distribution, and therefore, the leaf was hypostomatic. The stomata were accompanied by subsidiary cells, which had a ridged cuticle. No epicuticular wax granules were observed on the abaxial surface.

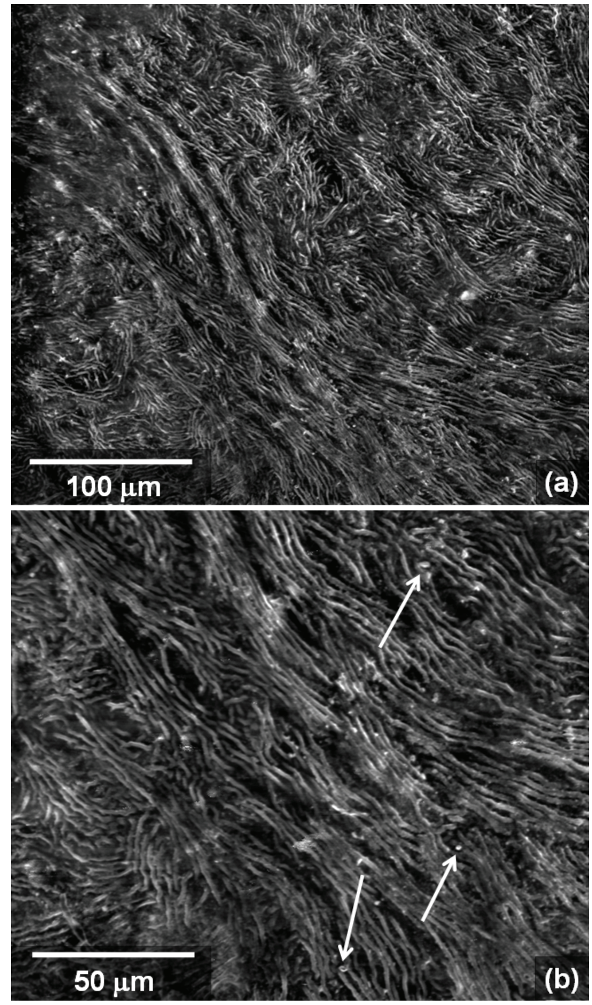

Figure 2. Scanning electron micrograph of (A) adaxial leaf surface showing epidermis features; (B) Higher magnification of (A) where arrows are indicating wax granules.

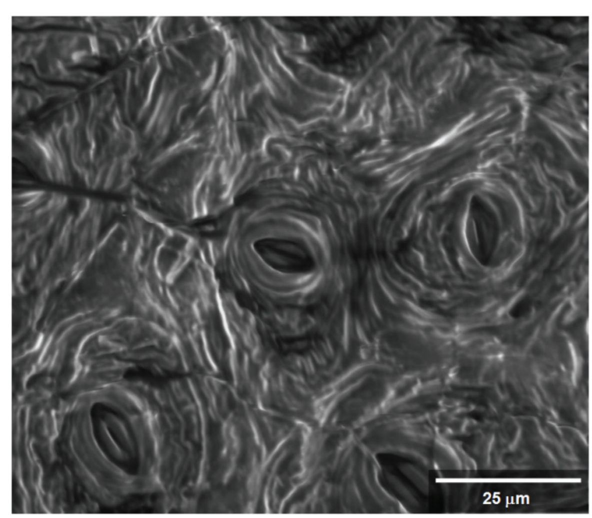

Figure 3. Scanning electron micrographs of abaxial leaf surface. Stomata distribution, where subsidiary cells are surrounded by a ridged cuticle.

In a recent study of *A. occidentale* L. leaf morphology, Ramos, Cotta, and Fonseca-Filho (2015) have shown that different types of trichomes were present on both leaf surfaces. Non-glandular trichomes with one basal cell branched into four arms were found on the adaxial surface and unicellular glandular trichomes were only verified on the abaxial surface.

Static contact angle

Values of the average contact angle (θ) measured and the respective images after water was placed onto the adaxial and abaxial surfaces of *A. occidentale* leaves are presented in Figure 4.

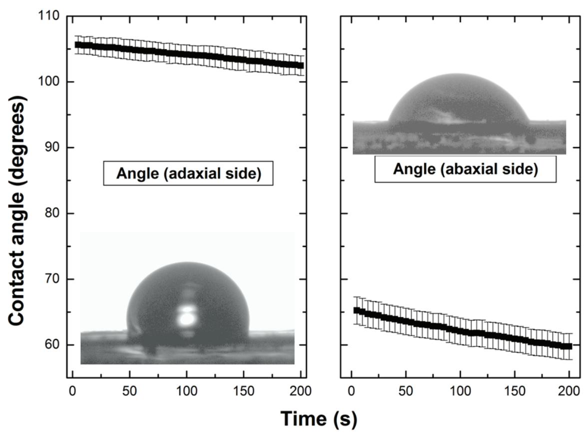

Figure 4. Plots of static contact angle as a function of time and images of droplets of water on adaxial and abaxial surfaces of *Anacardium occidentale* leaves.

The measured static contact angle of water drops placed on the fresh leaf was 104.09° ± 0.95° on the adaxial side, suggesting that the cashew leaf is hydrophobic. On the other hand, the static contact angle on the abaxial side was 62.20° ± 1.60°, which indicates a hydrophilic nature.

Discussion

The starting point of the investigations was the observations of the wetting properties presented by cashew tree leaves. With the aim of gaining insight into epidermal structure, composition, epicuticular wax, and function in relation to water-leaf surface interactions, we analyzed the adaxial and abaxial leaf surface of *Anacardium occidentale* L. There was a different performance of the adaxial (unwettable and water-repellent) versus the abaxial (wettable and retaining water drops) surface when in contact with water drops.

Leaf epidermis exhibits a thin extracellular membrane, called cuticle, which consists of cutin and epicuticular waxes, which are in general hydrophobic materials, whose primary function is to create a barrier against water loss. Koch et al. (2008) presented possible surface structures based on wetting behavior of plant leaves. Differences at the nano- and/or microscale levels of plant surface sculpturing have been observed by scanning electron microscopy, generally in relation to the topography of a wide range of different structures, ranging from glands, trichomes, stomata, smooth wax or highly crystalline wax (Koch & Barthlott, 2009). Such features together with their chemical composition (Khayet & Fernández, 2012) may lead to a high degree of roughness and hydrophobicity, controlling the wetting of these surfaces (Koch & Barthlott, 2009).

Surfaces of leaves vary greatly between different species, from relatively smooth surfaces of amorphous wax to highly rough surfaces, in which the leaf surface is covered by a coating of very fine epicuticular wax crystals, such as on the lotus plant or even cabbage (Jeffree, 1996). Ringelmann, Riedel, Riederer, and Hildebrandt (2009) have studied the adaxial and abaxial surface of perennial ryegrass (*Lolium perenne*). This is an interesting species, in which the two leaf sides show very different wettability; the adaxial side is covered with microcrystalline wax (predominantly primary alcohols) while the abaxial side is smooth (primarily alkanes and aldehydes). *A. occidentale* leaf surface showed these features, even though the abaxial surface was not entirely smooth, due to the high number of stomata and an undulated cuticle (Ramos et al., 2015). Metcalfe and Chalk (1957) and Jaiswal, Naik, Tatke, Gabhe, and Vaidya (2012) had already described the presence of paracytic stomata connected with irregular subsidiary cells in *Anacardium occidentale* leaves. Barthlott and Neinhuis (1997) verified that leaves exhibiting permanent water-repellency always displayed a very conspicuous layer of epicuticular wax crystals. The waxes are complex mixtures of relatively non-polar aliphatic and cyclic compounds (Baker, 1982; Bianchi, 1995; Jetter, Kunst, & Samuels, 2006).

Brewer and Smith (1997) reported microscopic features that reduce water capture on leaf surfaces among plants in open-field habitats, where dew formation is more frequent than in the drier forest understory. The selective pressure for reducing the wettability of leaf surfaces is usually thought to be physiologically driven (Brewer, Smith, & Vogelmann, 1991; Bradley, Gilbert, & Parker, 2003). On the other hand, plants in wet environments are thought to have been selected for water shedding capabilities, aiming to prevent the blockage of stomata pores, thereby enhancing photosynthesis rates during and immediately after fog interception. Adaptations that reduce water retention in leaves may also reduce disease incidence, but the selective advantage of these traits may vary among habitats (Huber & Gillespie, 1992).

The importance of surface roughness and heterogeneity has long been recognized as a key parameter in the wettability of hydrophobic surfaces by aqueous systems (Neinhuis & Barthlott, 1997). From a physical point of view, the roughening, which is caused by different structural elements (trichomes, cell walls and epicuticular waxes) is not necessarily a pre-requisite for an unwettable leaf. Metcalfe and Chalk (1957) described several types of trichomes in *Anacardium occidentale* leaves, indicating the presence of glandular trichomes with thin layers of secretion and Jaiswal et al. (2012) observed the presence of tector trichomes on adaxial face. According to Belhadj et al. (2007), differences in density of trichomes in Anacardiaceae are related to different climatic conditions.

Theoretical considerations on wettability reveal that a hydrophobic leaf becomes water-repellent if air is enclosed between surface structures and an applied water droplet but, if a roughened surface loses its waxes, the water-repellent characteristic may be reversed and the leaf becomes wettable (Holloway, 1970). According to Neinhuis and Barthlott (1997), the main causes of water-repellency are epicuticular waxes, which have a low mechanical stability and are easily removed by erosion through rainfall or mechanical abrasion.

In the case of *A. occidentale* leaf surface, the hypothesis that the abaxial surface is more hydrophilic than the adaxial side, due to the presence of some structures, such as stomata, which increase the roughness on the lower side, is confirmed. The hydrophobic nature of the adaxial surface is due to a combination of a great amount of microcrystalline epicuticular waxes and a highly undulated cuticle with depressions in some regions. On the other hand, the abaxial side is hydrophilic because of the absence of epicuticular wax and micromorphological diversity between both sides of leaves, leading to a difference in the contact angle between the surfaces of approximately 40°.

Conclusion

Contact angle measurements in *Anacardium occidentale* leaves are important to know the wettability on the leaf surface. The abaxial surface is more hydrophilic than the adaxial side due to the presence of some structures, such as stomata and ridged cuticle in the surroundings and lack of epicuticular wax, increasing the roughness on the lower side. Granules of wax influence the hydrophobicity on the adaxial surface.

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