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Ultrastructure of the *Anacardium occidentale* L. leaves from Amazon in Northern Brazil

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Resumo

A superfície das plantas possui diversas estruturas com funções específicas e que contribuem para a relação delas com o meio ambiente. Em estudos morfológicos são analisados diversos parâmetros variando da escala macro, passando pela micro e atualmente chegando à escala nanométrica, contribuindo assim com o estudo da taxonomia, farmacognosia, ecologia, entre outras. Embora o *Anacardium occidentale* L., conhecido também como cajueiro, seja uma planta popular e amplamente distribuída no mundo, estudos morfológicosde suas folhas são raros. Considerando a ausência de trabalhos realizados com a folha de *Anacardium occidental*e L., conhecido como cajueiro, este artigo apresenta resultados sobre a ultraestrutura dos lados adaxial e abaxial da folha com imagens realizadas em um microscópio eletrônico de varredura ambiental, destacando a presença de ceras epicuticulares apenas na epiderme superior e analisando sua cristalinidade através de medidas de difração de raios-x.

Palavras-Chave: Caju, DRX, MEV, Cera epicuticular, Epiderme.

Ultrastructure of the *Anacardium occidentale* L. leaves from Amazon in Northern Brazil. Leaves surfaces have various structures with specific functions and contribute to the relationship with the environment. On morphological studies are analyzed several parameters, ranging from macroscale through the microscale to the nanoscale, which contribute to the study of taxonomy, pharmacognosy and ecology, among others. Although the *Anacardium occidentale* L., also known as cajueiro, is a popular plant and widely distributed in the world, morphological studies of their leaves are rarely. This study reports results about the ultrastructural features of adaxial and abaxial sides using a set of environment scanning electron microscope (ESEM) images, highlighting the presence of epicuticular waxes only over the upper epidermis and analyzing its crystallinity by X-rays diffraction (XRD) measurements.

Key-words: Cashew, XRD, ESEM, Epicuticular wax, Epidermis.

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1. Introdução

A million years of land plants evolution led to a large diversity of functional biological surface structures. In this way, plants have an interface, consisting of an extracellular layers of polymeric cutin matrix and soluble cuticular lipids, that is able to share between primary plant tissues and the atmosphere. This interface, so-called cuticle, has been of great interest to many researchers for more than a century (MEUSEL et al. 2000), due to its functionality and properties such as light reflection or wettability, a barrier against UV radiation and bacterial and fungal attacks, and so on (MEUSEL et al. 2000; HOLMES & KEILLER 2002).

The micro- and nanostructures of plant surfaces have a great influence on their attributes as interfaces. Even in a cursory look at different plant surfaces they show different optical appearances, which arise from the surface structures in the micro- and nanoscale dimension (KOCH et al. 2009). The plant cuticle is technically a composite material mainly built up by a network of cutin and hydrophobic waxes. Based on structural characteristics, the cuticle can be divided into the cuticle proper and the thicker underlying cuticle (cuticular layer). In both layers the cuticle network is formed by cutin, a polyester like biopolymer, composed of hydroxyl and hydroxyepoxy fatty acids, and sometimes also by cutan, which consists of polymethylene chains, or by another polymer called lignin (JEFFREE et al. 2006).

The waxes are important to the structure and function of cuticle and are either deposited within the cutin matrix (intracuticular wax) or accumulate on its surface as epicuticular wax crystals, or films (YEATS & ROSE 2013; Jeffree et al. 2006). In most cases, the majority of compounds comprising the cuticular wax are derived from very-long-chain fatty acids, including alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. In some species, various lipophilic secondary metabolites, such as pentacyclictriterpenoids, flavonoids, and tocopherols, can also be substantial components (TULLOCH, 1976; BACKER et al. 1982; BIANCHI, 1995).

Epicuticular waxes create a macroscopic effect as reduction wettability, leading to self-cleaning leaves surfaces (NEINHUIS et al. 1997). They also reduce the uptake of molecules from the environment, which might become a crucial factor in agriculture, when the uptake of nutrients or fungicides is desired (KOCH et al. 2009). With respect to epicuticular waxes classification, the most common wax morphologies are thin films and several three-dimensional structures such as massive crusts, granules, plates, platelets, filaments, rods, and tubules with a hollow center (KOCHET al. 2009). According to Barthlott et al. (1998), the environment conditions could influence on the amount and how the waxes would be distributed along the leaf surface.

X-Ray Diffraction (XRD) is one of several fundamental techniques used for materials characterization as well as Differential Scanning Calorimetry (DSC) (DASSANAYAKE et al. 2009), Fourier Transform Infrared Spectroscopy (*FTIR*) and Scanning Electron Microscopy (SEM) (Sigoli et al. 2000). When the SEM arose, new possibilities to study plant structures, ranging from the micro- up to nanoscale, were established. For a conventional SEM, which works in high vacuum and at room temperature, it is widely accepted that biological samples must be dehydrated and require a deposition of conductive material on the surface. As an alternative, ESEM can make images on non-conducting samples, being a powerful tool to analyze biological material at room temperature (SHAH & BECKETT 1979; DANILATOS & POSTLE 1982).

On the other hand, XRD enables the determination of composite structural patterns, being possible to characterize factors such as crystallinity, amorphicity, residual stress, texture, as well as to get analysis of the tension behavior as a function of temperature (GOLDSMITH et al. 2000). XRD investigations in waxes exhibit a relationship between chemical composition and morphology with a diversity of molecular order and crystalline structure (ENSIKAT et al. 2006; KOCH & ENSIKAT 2008).

Considering the importance of detailed studies to provide a best comprehension and characterization about leaf morphology, this work examined the ultrastructural differences comparing adaxial and abaxial surfaces of fresh *Anacardium occidental*e L. leaves using an environmental scanning electron microscope (ESEM). The purpose of this work is also to characterize the cristallinity of reconstituted epicuticular waxes onto a glass substrate surface.

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2. Methods and materials

2.1. Plant material

Leaves of *Anacardium occidentale* L. were collected in Macapá, into the Universidade Federal do Amapá campus. Voucher material was deposited at the Herbário Amapaense (HEMAB) of the Instituto de Estudos e Pesquisas do Estado do Amapá (IEPA), under number 018684.

2.2. Isolation and purification of wax

This process consisted of collecting the leaves, followed by washing and drying at room temperature. For the extraction of epicuticular wax single carefully handled leaves were immersed and gently shaken for 30s in $CHCl_3$, at room temperature (Hamilton, 1996). The resulting solution of wax was dried, filtered and the excess of solvent removed using a rotary evaporation.

2.3. Environmental Scanning Electron Microscopy

Fresh leaf pieces of 5 x 5 mm^2 were cut using a razor blade, avoiding the midrib areas to give a relatively consistent surface. Adaxial and abaxial surfaces were examined. 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) at an accelerating voltage of 5 kV.

2.4. X-ray diffraction

In order to evaluate the presence of crystalline compounds in the epicuticular wax it was used a diffractometer model Rigaku Miniflex II using CuK_α (λ=1,5404 Å) radiation. The diffractogram was recorded between 2 and 50º (2θ) in 0,02º steps, counting 4 s per point at 30 kV and at 15 mA. The samples were deposited on glass substrate.

3. Results

The study of the macroscopic characters of fresh leaves reveals on the upper side a dark green in color and on the lower one a light green, lamina 15-18 cm long and 8-10 cm wide, with obovate shape, pinnately veined, symmetric base and obtuse apex, entire margin and the smooth surface has a straight petiole, as depicted in figure 1.

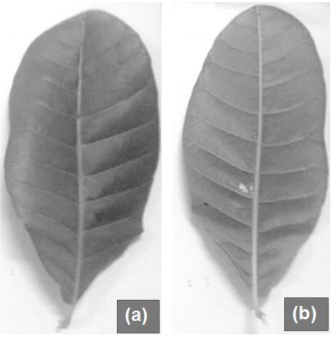

Figure 1 Leaf of A. occidentale. (a) Adaxial and (b) abaxial sides.

Scanning electron microscopy revealed micromorphological diversity in the leaf surface. There were significant differences between the adaxial and abaxial surfaces of the leaf. The adaxial surface is recovered by a cuticle highly undulated or ridged with depressions in some regions, and had no stomata (Figure 2a). The presence of epicuticular wax granules that varied in diameter (ca. 1-4 μm) and are scattered over the undulated cells along adaxial surface can be noted (Figure 2b).

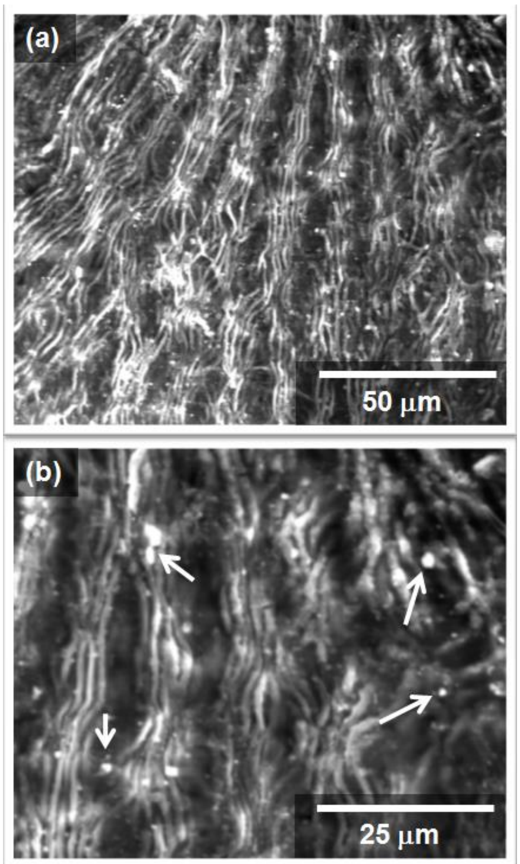

Figure 2 Scanning electron micrograph of (a) adaxial leaf surface showing epidermis features. (b) Higher magnification where arrows indicate wax granules.

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In the abaxial surface was noted a large quantity of stomata (Figure 3a) with a slightly ridged cuticle that is denser in their surroundings. The leaf is hypostomatic. Stomata are reniforms and inserted at the same level as the epidermis, with a random distribution. The stomata are accompanied by subsidiary cells, with a ridged cuticle (Figures 3a, b). It was not observed the presence of epicuticular wax granules on this surface.

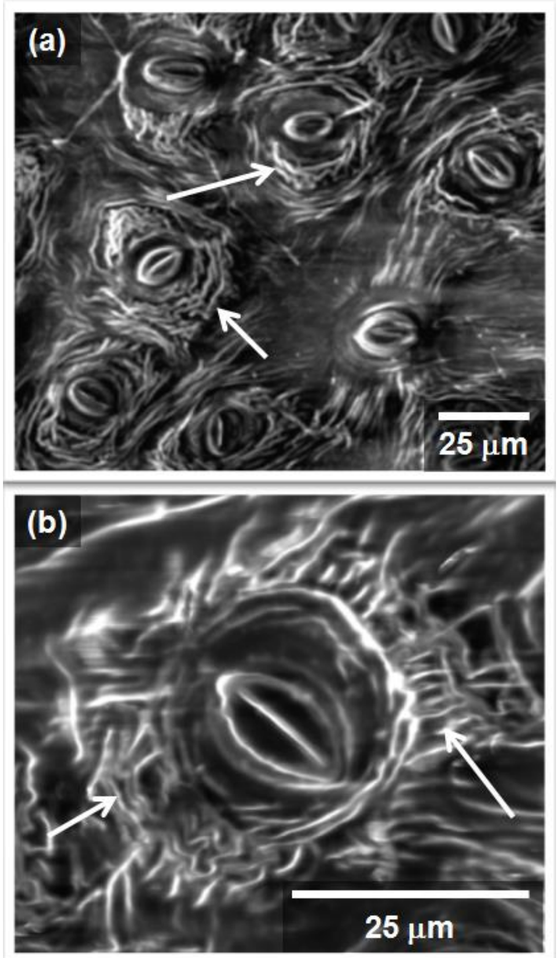

Figure 3 Scanning electron micrograph of (a) Abaxial leaf surface. The appearance and distribution of stomata. Arrows indicate subsidiary cells surrounded by a ridged cuticle. (b) Higher magnification of stomata.

The X-ray pattern of the reconstituted epicuticular wax is shown in figure 4. The X-ray diffractogram presents only diffraction peaks from lattice planes of the crystalline wax. The amorphous band, a less ordered component of the wax, contributed only to a broad band characteristic of diffraction from non-crystalline material, ranging from 10 to 40º.

1. Discussion

Carpenter (2005) reported the importance dedicated to the study of the stomata based on the anatomical architecture, quantity, shape and the organization of epidermis cells associated with guardians cells, because it provides subsidies to the taxonomy of plants (STACE,1965). Stomata are composed of a pore, the ostiole and a pair of guardians' cell that are surrounded by subsidiary cells that differ from the others in size, shape and orientation (Esau, 1953; Pant, 1964). The position of the subsidiary cells is an important factor to classify the type of stomata on the leaf epidermis (CARPENTER, 2005). This morphology of the stomatal complex is according to Metcalfe & Chalk (1957) and Jaiswal (2012), which described the presence of paracytic stomata cells that are connected to the irregular subsidiary cells in the *Anacardium occidentale* leaves. This irregularity is due to the presence of a ridged cuticle.

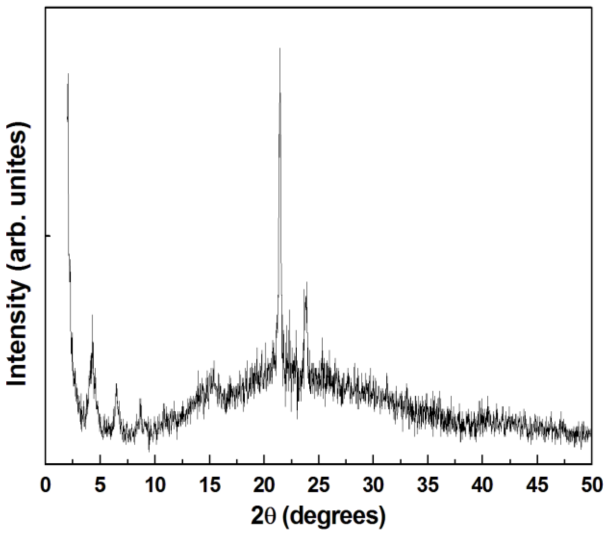

Figure 4 X-ray diffraction pattern of epicuticular wax of cashew**.**

The epidermis present a thin extracellular membrane, called cuticle, which is composed by cutin and epicuticular waxes, that is, in general, a hydrophobic material, whose primary function is to create a barrier against water loss. Kock et al. (2008) presented possible surfaces structures based on wetting behavior of plant leaves. The sculptures of the cells, the presence of hairs and the fine structure of the surfaces, e.g., folding of the cuticle or presence of epicuticular waxes, have a strong influence on surface wettability. Beside this, structural and chemical modifications can

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induce variations in surface wetting, ranging from superhydrophilic to superhydrophobic (HOLLOWAY, 1982; BUKOVAC, 1995; KOCH, 2008).

The crystalline order can be analyzed in detail by X-ray diffraction (XRD) and electron diffraction (ED). XRD has frequently been used in the early studies of waxes as a tool for the identification of compounds isolated from natural waxes. The XRD powder diffraction diagrams contain information about lattice parameters, the chain length of the molecules and the position of oxygen-containing functional groups (MALKIN, 1952; MAZLIAK, 1968). Previous XRD studies of plant waxes were performed on solvent-extracted, recrystallized waxes, but crystal structure analyses of the natural, mechanically isolated epicuticular waxes are published rarely (MEUSEL et al. 2000).

The two narrow peaks of high intensity, superimposed on the broad peak in figure 3, can be attributed to 010 and 100 reflections, according to Casado et al. (1999) for the reconstituted cuticular waxes of grape berry cuticle. In that study, a comparison of the n-hexacosanol peaks diffractions was donewith grape berry cuticle and epicuticular wax results. These peaks appeared on a very broad band of high intensity at ~21°, and were mainly attributed to the amorphous network that constitutes the cutin of the plant cuticle.

2. Conclusion

In this work it was studied the *Anacardium occidental*e L. leaf micro morphology on both adaxial and abaxial sides by ESEM, showing ultrastructural details that not found yet, enhancing distinct differences on leaf epidermis. This research also analyzed the reconstituted epicuticular waxes deposited on a glass substrate by X-ray diffraction, presenting a crystalline order.

Divulgação

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