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Artigo de Pesquisa

Rachis Lignification of *Couroupita guianensis* Aubl.

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ABSTRACT

The aim of this study was to substantiate the possible influence of gravity on the lignin content in the rachis of *Couroupita guianensis* Aubl., since it grows in favor of gravitational forces. Chemical analysis, medium infrared (FTIR) spectroscopy and microspectroscopy were conducted, coupled with supporting images obtained from an optical microscope. The rachis lignin content was 5.56%, composed of both syringyl and guaiacyl units; the latter being predominant, showing that the gravity force influenced the lignin process when compared with lignin content of main stem, 17%.

Keywords: gravity, lignin, infrared spectroscopy.

Lignificação da raque de *Couroupita guianensis* Aubl.

RESUMO

O objetivo deste estudo foi substanciar a possível influência da gravidade no teor da lignina em raque de *Couroupita guianensis* Aubl., visto que esta tem seu crescimento a favor da força gravitacional. Análise química, microespectroscopia e espectroscopia no infravermelho médio foram realizadas com apoio das imagens obtidas no microscópio óptico. O teor de lignina das raques foi 5,56%, composto por duas unidades siringil e guaiacil; este último mostrou-se predominante, indicando que a força da gravidade influenciou o processo de lignificação quando comparado com o teor de lignina da tronco principal de 17%.

Palavras-chave: gravidade, lignina, espectroscopia no infravermelho.

1. INTRODUCTION

The advent of lignin on the Earth's surface has been a theoretically speculated subject of evolutionary nature, which explains the existence of lignin owing to the necessity for plants to grow vertically. Lewis & Sarkanen (1998) described the evolutionary process beginning with green algae. However, a recent divergent dichotomy has arisen, giving way to heightened discussion as a result of the discovery of lignin, chiefly consisting of syringyl, in red algae. (Martone et al., 2009).

The occurrence of lignin in plant cell walls rests in the explanation of upward growth, water conduction, resistance and defense against microorganisms, amongst other functions. The influence of gravity in the lignification process was reported from experiments conducted under microgravity conditions (Stutter et al., 2006). Some laboratory experiments portraying conditions that simulate the gravity effect demonstrated the consequent accumulation of lignin in cell walls (Allen et al., 2009).

The formation process of a plant is determined by genetics and it is influenced by environmental pressures, such as gravity. In response to these environmental stimuli, trees have the ability to reorient their axes, branches, or trunk in order to reestablish the equilibrium by developing a special type of tissue. (Du & Yamamoto, 2007; Dérjardin et al., 2010).

The characteristics and location of such tissue differ between groups of plants. In gymnosperms, the tissue is called compression wood, developing in the undersides of branches or twigs. In angiosperms, it is called tension wood, occurring in the upper region. An increase in lignification occurs in the lower stem or branch in both groups (Wardrop, 1965). Plants growing under the influence of gravity usually develop tissues with chemical compositions and physical properties separate from tissues encountered on the main stem. The medium infrared (FTIR) spectroscopy allows for a rapid and non-destructive analysis. It has the ability to show interactions between macromolecules and it is a tool which may be applied to identify the composition of plant cell walls, as lignin and polysaccharide compounds (Dence, 1992). Lignin analysis by infrared has been a tool for identification of lignin content and its composition in wood and other lignocellulosics materials. The experimental technique of Diffuse Reflectance seems to be a precise spectrum for content determination. Computer software technique, such as convolution, was used for β-O-4 bond determination in tropical wood (Abreu, 1997).

The study of plant parts with growth in the direction of gravitational forces showed the production of chemical changes, principally concerning lignin content. Gravity stimulates the accumulation of lignin, and the formation of other substances in the supporting tissues. In this regard, the purpose of this study was to fully substantiate the expedient influence of gravity on the rachis lignification of the *Couroupita guianensis* Aubl. tree.

2. MATERIAL AND METHODS

2.1. Sampling

Rachises of *Couroupita guianensis* Aubl., popularly known as "abricó de macaco", were collected at the campus of 'Universidade Federal Rural do Rio de Janeiro' in Seropedica, State of Rio de Janeiro. The samples were used as anatomic cuts and for chemical analysis. A whole rachis was used, divided into four distinct regions, namely: the upper and lower regions, near the stem and fruit (Figure 1), in a total of five samples per region.

2.2. Optical microscopy

Cross-sections of 10 μm thickness were obtained in a microtome (MICROM HM450), fixed with FAA (50 mL 40% formaldehyde, 50 mL glacial acetic acid, 90 mL 50% ethanol) for two days and subjected to two methods of staining.

The first consisted of staining with an alcoholic solution of Safranin-O (1 g/65 mL 100% ethanol and 30 mL distilled water) for two hours, washed three times in 85% ethanol for 5 minutes, stained once more using an alcoholic solution of Astra Blue (1 g.100 mL^{-1} 100% ethanol and 5ml distilled water) for 3 minutes, then quickly washed in absolute ethanol (95%). Safranin was used to identify lignin and Astra blue, polysaccharides (Vasquez Cooz & Meyer, 2002).

For the second method, the sections were stained with 2% Safranin-O for 24 hours, and then quickly washed in distilled water and 70% ethanol as recommended by Jensen (1962).

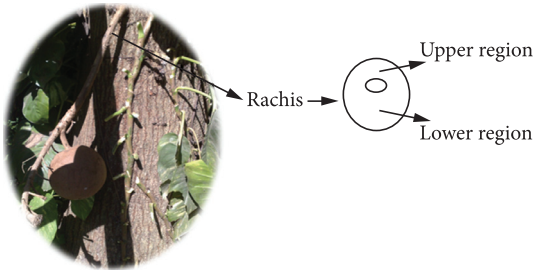

Figure 1. Disposition of the rachis of *Couroupita guianensis* Aubl. The studied areas are in highlight.
Figura 1. Disposição das raques de *Couroupita guianensis* Aubl. As áreas estudadas estão em destaque.

The sections were observed in an optical microscope (Olympus BX51) coupled to a digital image analysis system (Cell * Family).

2.3. Medium infrared (FTIR) microspectroscopy

Anatomical cross-sections of 14 µm thickness were obtained using a microtome (MICROM HM450), they were used to generate, in loco, IR spectra through a microscope coupled to an IR spectrometer (Varian 640-IR FT-IR), in order to characterize and localize the presence of lignin in the tissues.

To this end, natural cuts treated with 1% NaOH; %; which allow better visualization of the organic groups, were frozen in liquid nitrogen for 30 seconds, and then dried between two glass slides for 12 hours in a lyophilizer (Terroni - Enterprise Model). Once dried, the samples were placed on KBr microscope slides and analyzed, by diffuse reflectance, at a wavenumber range of 700-4000 cm^{-1}, resolution of 2 cm^{-1} and 128 scans.

2.4. Chemical analysis

For chemical analysis, the rachis samples acquired from the stems were air dried and ground to sawdust using a Willey mill. The resulting powder was extracted with cyclohexane, ethyl acetate and methanol solvents in a soxhlet extractor, and each extraction was completed over an uninterrupted 24 hours period for each of the solvents (Abreu et al., 2006). The same was performed with the sample wood of diameter at breast height (DBH) of main trunk from the same tree where the rachis was collected. The extractive-free material was used to determine the lignin content and for the infrared analysis.

The Klason method was applied for the determination of the lignin content. To this end, 300mg of extractive-free dried material was transferred to a test tube, gradually adding 3 mL of 72% sulfuric acid, homogenized by continuous stirring for one minute, and placed in a water bath at a temperate ranging between 25-30 °C for 1 hour. Subsequently, the material was transferred to a volumetric flask, to which 84 mL of distilled water was added. The solution was brought to boil at reflux for 4 hours. The residue was then filtered, washed with hot water, and dried in oven at a temperature of 103 ± 2 °C (Dence, 1992).

2.5. Infrared spectroscopy

The extractive-free material was ground in a stainless steel mill (Marconi) and 2 mg of this sample was lyophilized for 12 hours. The sample was subsequently homogenized in 100 mg of KBr into a mortar, and then molded into a pellet which was used to record the spectra data in a Varian 640-IR FT-IR Spectrometer, in transmittance mode with a resolution of 4 cm^{-1}, 128 scans, and wavenumber between 4,000-400 cm^{-1}.

3. RESULTS AND DISCUSSION

In the cross-sections, it was observed that fibers showed varying shapes and compositions, differing between tension wood and the tissue of the lower rachis region (Figures 2a and 2b). A markedly red fibrous zone can be observed in Figure 2b; and in Figure 2d, with this area magnified, it is possible to observe less organized fibers than in the tension wood (Figure 2c), now visually narrower.

Tension wood tends to possess high quantities of cellulose, being less lignified than normal wood (Pilate et al., 2004), demonstrating an increase in the proportion of fibers containing thick walls, as seen in Figures 2d and 3d .

By double staining with Safranin-O/Astra Blue, it was possible to observe a thick layer in the inner secondary wall, suggesting the presence of gelatinous fibers developed mainly in the tension wood, as indicated by the arrows in Figure 3d. According to Pilate et al. (2004), this layer presents itself as less lignified and/or with a higher content of syringyl units, which is consistent with what is essentially observed (violet). In contrast, layers S1 and S2 of the secondary wall are generally narrower, having a higher lignin content, observed in Figure 3d with a pinkish color (Timell, 1969; Joseleau et al., 2004).

Studies indicated that there is an increased Syringyl/Guaiacyl (S/G) ratio in tension wood and, therefore, visually, the blue-violet layer of gelatinous

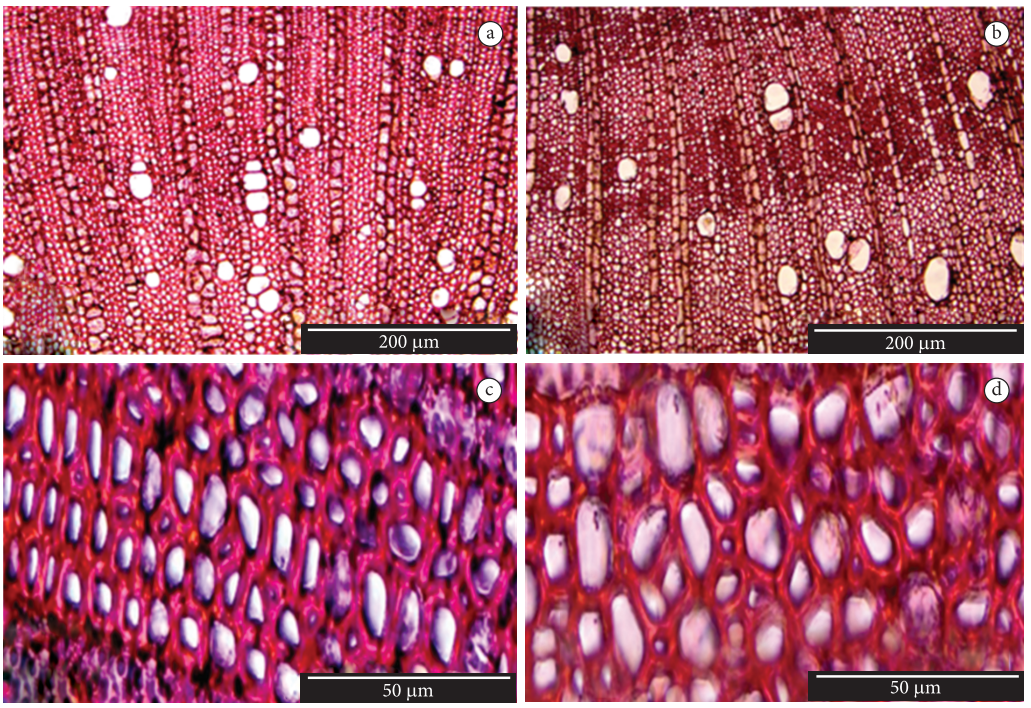

Figure 2. Cross-sections of the rachis of *Couroupita guianensis* Aubl., stained with Safranin-O: a, c) Upper region or tension wood, b, d) Lower or opposing region to the tension wood.
Figura 2. Seções transversais das raques de *Couroupita guianensis* Aubl., coradas com Safranina-O: a,c) Região superior ou tecido de tensão, b, d) Região inferior ou oposta ao tecido de tensão.

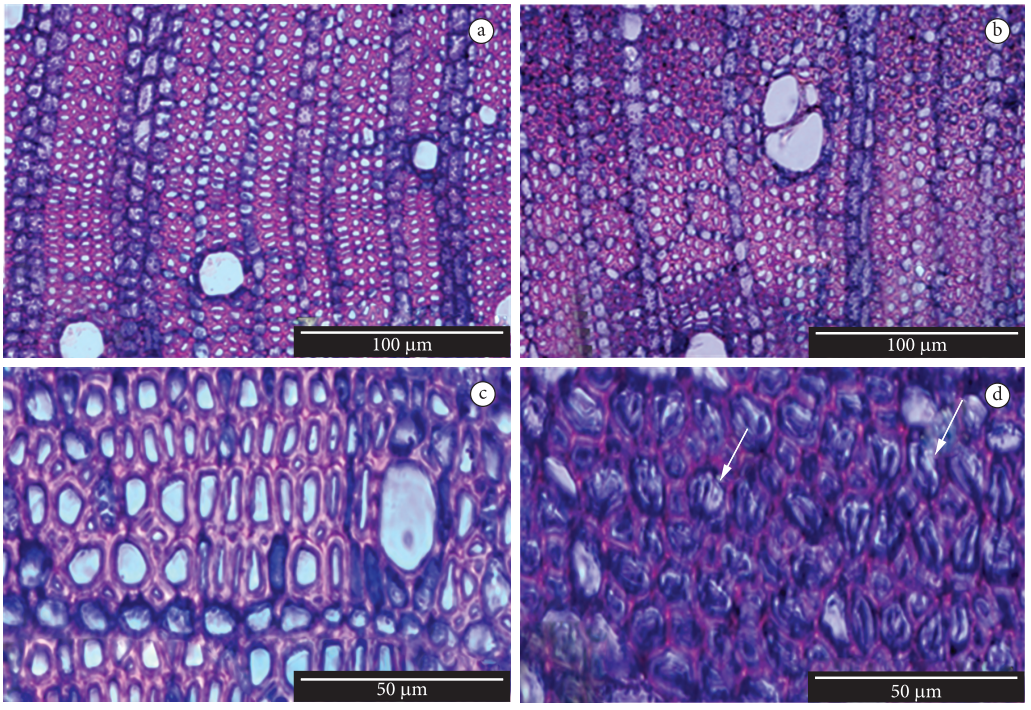

Figure 3. Cross-sections of the rachis of *Couroupita guianensis* Aubl. stained with Safranin-O/Astra Blue: a, c) Upper region or tension wood, b, d) Lower or opposing region to the tension wood.
Figura 3. Seções transversais das raques de *Couroupita guianensis* Aubl. coradas com Safranina-O/ Azul de Astra: a, c) Região superior ou tecido de tensão, b, d) Região inferior ou oposta ao tecido de tensão.

fibers indicates a greater amount of syringyl units (Yoshida et al., 2002; Aoyama et al., 2001).

The secondary wall of fibers usually contains lignin of the guaiacyl and syringyl types. Astra Blue (affinity to the syringyl unit) staining promoted a violet hue, as demonstrated in Figure 3c. Figure 3b provides strong evidence of syringyl lignin in the darker band.

In all five sampled regions of rachis of *Couroupita guianensis* Aubl, the infrared spectra of the lignocellulosic material (ground material) indicated signs of guaiacyl/syringyl lignins (1329-1252 cm^{-1}), as shown in Figure 4.

Figure 4 illustrates signals corresponding to the guaiacyl ring, (with contributions from the C = O stretching), 1252 to 1254 cm^{-1}. The signals corresponding to the presence of the syringyl unit, with assistance from the C = O stretching, and condensed structures were: 1328 to 1330 cm^{-1} (Abreu, 1994). It was noted that guaiacyl lignin was prevalent in all samples, where the spectra showed signals in 1252 cm^{-1} higher than 1328 cm^{-1}.

The spectra of the cross-sections in the upper rachis region of the natural wood indicate the complexities and non-possibility to clearly identify the presence of lignin, as shown in Figure 5. In Figures 5b, c and d, the signals corresponding to the lignin presence faintly demonstrate their intensity, in smaller proportions, when compared to the spectra of the ground material illustrated in Figure 4.

The lignin content of the entire rachis, upper region near the trunk and the near the fruit, lower region near the trunk and near the fruit was 5.40, 5.79, 5.58, 5.89, 5.66%, respectively – each treatment repeated five times. The low lignin content of the rachis, averaging 5.56%, may have contributed to a lower level of absorption associated with a microscopy technique of low resolution. Transmittance mode (Figure 4) was used in the conventional spectrum. These spectra showed more evident signs than the spectra in reflectance mode (FTIR microscopy) (Figure 5), because the analyzed homogenized material in powder form showed presence of lignin in the material diluted in KBr, while tissue showed deposition of lignin on discontinuous layer of KBr.

It is also important to remark that the techniques used to obtain the spectra illustrated in Figures 4 and 5 were different. The techniques used for microscopy can cause distortions in the spectra due to the direct use of the cross section of rachis tissues. In the case of rachis in powder form, as it is diluted in KBr, the bands provided are more reliable, both in wavelength as well as in time.

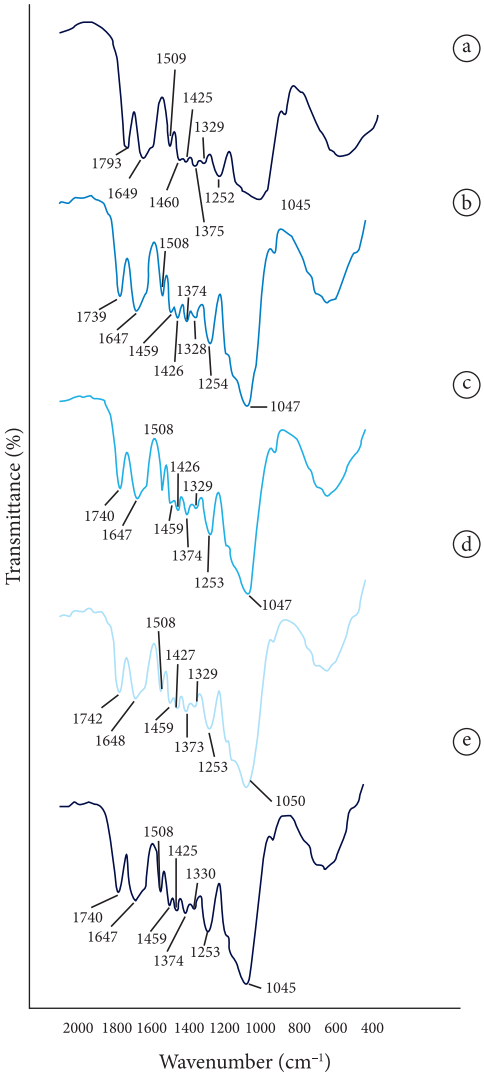

Figure 4. Infrared spectra of (powder) woody material of the rachis of *Couroupita guianensis* Aubl., extracted with cyclohexane, ethyl acetate and methanol solvents. a) entire rachis, b) upper region near the trunk, c) upper region near the fruit, d) lower region near the trunk, e) lower region near the fruit.

Figura 4. Espectros no infravermelho do material na forma de pó da raque de *Couroupita guianensis* Aubl., extraída com ciclohexano, acetato de etila e metanol. a) Raque inteira, b) Região superior próxima ao tronco, c) Região superior próxima ao fruto, d) Região inferior próxima ao tronco, e) Região inferior próxima ao fruto.

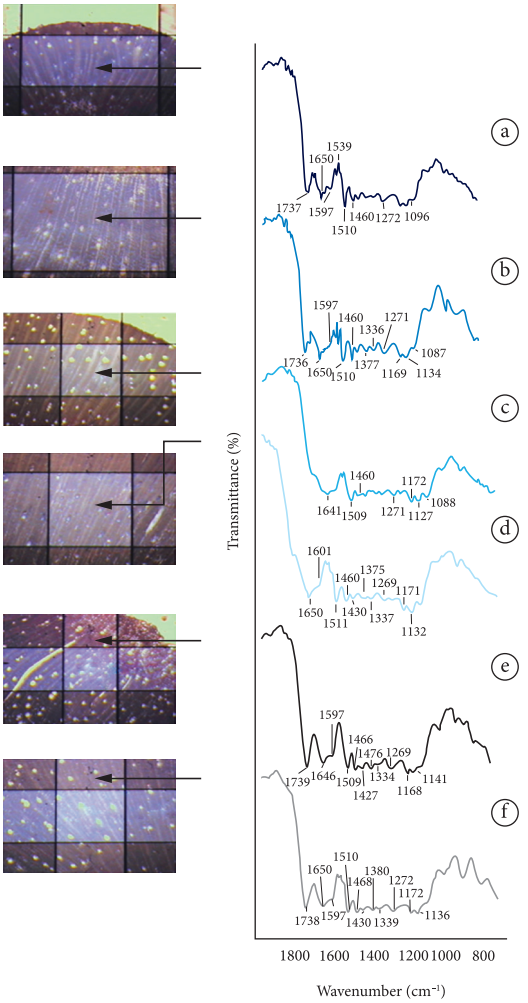

Figure 5. Photos and infrared spectra of the cross-section regions of the rachis of *Couroupita guianensis* Aubl. obtained with a microscope, a) Natural section of upper region, b) Natural section of lower region, c) Section treated with NaOH 1% of upper region, d) Section treated with NaOH 1% of lower region, e) Section treated with acetone of upper region, f) Section treated with acetone of lower region.
Figura 5. Fotos e espectros obtidos por infravermelho através de um microscópio nas regiões da seção transversal da raque de *Couroupita guianensis* Aubl., a) Corte ao natural da região superior, b) Corte ao natural da região inferior, c) Corte tratados com NaOH 1% da região superior, d) Corte tratados com NaOH 1% da região inferior, e) Cortes tratados com acetona da região superior, f) Cortes tratados com acetona da região inferior.

The low lignin content in the rachis, comparable to the wood of main trunk DBH, 17%, may be due to its vertical negative growth (for the force of gravity). It may have caused the deactivation of the enzyme complex activity that regulates the rachis lignification. This was observed in plants which grow in microgravity systems, showing changes in the lignification process (Allen et al., 2009).

The recorded signals at 1737 and 1736 cm^{-1} did not appear in the sections treated with 1% NaOH. These signals correspond to the stretching of ester or carboxylic acid carbonyls, existent in hemicelluloses (Orton et al., 2004). This treatment is recommended to eliminate impurities (phenols and hemiceluoses) in order to reduce the interference of other absorptions in the analysis of lignin (Dence, 1992).

4. CONCLUSION

The rachis tissue of *Couroupita guianensis* Aubl. presented characteristics of tension wood, which is indicative of gelatinous fibers. The infrared spectra recorded in the conventional way revealed the existence of guaiacyl and syringyl lignin, predominantly comprising guaiacyl units, although the spectra recorded in reflectance only provided some evidence.

The total lignin content was 5.56%, demonstrating a minor effect of gravitational forces on the tissue, seemingly discouraging the actions of hormones and enzymes in the lignification process.

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