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BOTANY

Histochemical analysis of stem and fiber of ramie (*Boehmeria nivea* (L.) Gaud var. Murakami)

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ABSTRACT. Analyzes of samples of stem and fiber *Boehmeria nivea* var. Murakami were performed to increase information on the plant and disseminate its multiple possibilities of use. The histochemical analyzes with dyes and reagents showed the presence of lignified, suberous and cutinized cell walls with Safranin in the xylem region. The presence of pectin-cellulose with Astra Blue was confirmed in the regions of fiber bundles (cortex). In all regions of the stem were identified substances such as fat and mucilage with Methylene Blue evidencing high concentrations in the regions of fiber bundles and bark (violet blue). By means of Lugol (dark brown) starches were identified in several parts of the stem with concentrations in the xylem and epidermal regions. Through analyzes by EDS it was possible to identify F, Mg, Al, Si, K and Ca with predominance of K. In the chemical analyzes of the fibers were found 71.75 of cellulose, 12.11 of hemicelluloses, 1.06 of lignin and 1.70% of ashes. The percentages of extractives soluble in hot and cold water were 5.28 and 3.12% respectively, for Ethanol-Toluene was 8.55 and NaOH (1%) 27.27%. Histochemical analyzes revealed important characteristics of the fiber and stem contributing with better knowledge of the species.

Keywords: ramie; chemical composition; morphological structure.

Análises histoquímicas do caule e das fibras do rami (*Boehmeria nivea* (L.) Gaud var. Murakami)

RESUMO. Análises de amostras de caule e de fibras *Boehmeria nivea* var. 'Murakami' foram realizadas para aumentar o conhecimento da planta e difundir suas múltiplas possibilidades de uso. As análises histoquímicas com corantes e reagentes mostraram paredes celulares lignificadas, suberosas e cutinizadas com Safranina na região do xilema. Confirmou-se a presença de pectocelulose com Azul de Astra nas regiões de feixes de fibras (córtex). Em todas as regiões do caule, foram identificadas substâncias como gordura e mucilagem com Azul de Metileno, evidenciando elevadas concentrações nas regiões dos feixes de fibras e da casca (azul violeta). Por meio de Lugol (marrom escuro), foram identificados amidos em várias partes do caule com concentrações nas regiões do xilema e da epiderme. Por meio de análises de EDS, foram identificados F, Mg, Al, Si, K e Ca, com predominância do K. Nas análises químicas das fibras, foram encontrados 71,09 de celulose, 12,11 de hemiceluloses, 1,06 de lignina e 1,70% de cinzas. As porcentagens de extrativos solúveis em água quente e fria foram de 5,28 e 3,12%, respectivamente. Para o Etanol-Tolueno, foi 8,55 e para o NaOH (1%), 27,27%. As análises histoquímicas revelaram importantes características das fibras e do caule, contribuindo com melhores conhecimentos da espécie.

Palavras-chave: rami; composição química; estrutura morfológica.

Introduction

Nature provides large amounts of fibrous plant materials that are considered renewable, sustainable and biodegradable and can be cultivated on a large scale to obtain fibers to be used in several economic activities. According to Townsend and Sette (2016), 33 million tons of natural fibers were harvested in the world, from a great variety of plants, in 2013.

Ramie (*Boehmeria nivea*) is a fibrous plant monoecious used in diverse areas such as the animal feed, yarns, special paper, pharmaceutical and textile sectors, among others products (Sen & Reddy, 2011). It has high levels of α and β-cellulose I and high concentrations of pure cellulose, according to Hazra and Karmakar (2008), there is some evidence that may explain its superior mechanical strength among the fibers extracted from stems. It also has high degree of crystallinity. Its fiber is durable and resistant to attacks of bacteria and insects (Jose, Rajna, & Ghosh, 2017).

Young ramie stems are green and more or less pilous, while mature ones are brown and glabrous, cylindrical and rarely branched, with average length between 1 and 3 meters (Jose, Rajna, & Ghosh, 2017). It is possible to identify three well-defined structures from the outside in, namely: cortex (cortical zone or bark); phloem; xylem (cambial zone); and pithy core (medullar zone). The epidermis is formed by thin layers of cells containing modified cellulose, coloring materials, minerals and organic salts. It is formed by several layers of cells with very thick membranes (Benatti Jr., 1988) which adhere strongly as a membrane on the collenchyma due to high concentration of gums (20~40%), composed by pectin and hemicelluloses (Duan et al., 2012). Below the epidermis is the parenchyma, which consist of irregularly distributed thin-walled cells, with a few inlays of calcium oxalate salt crystals (Benatti Jr., 1988). These inclusions are common and come from the combination of oxalic acid resulted from the metabolism with the calcium ions absorbed by the plants (Oliveira & Akisue, 2009). In the insoluble form, these elements are formed by Ca^{+2}, Mg^{+2} and Fe^{+2} (Savage, Vanhanen, Mason, & Ross, 2000).

The fibrous or pericyclic region contains the bast fibers, isolated or gathered in groups of three or more strongly adherent layers. These fibers differ from others by their larger diameter and thicker cell walls, more or less polygonal shape, and lumen of varying size (Batra, 2007). The liberian layer, connected to the cortex and xylem, is characterized by having sieve tubes located in a soft parenchyma (Benatti Jr., 1988)

Plant fibers are generally composed of three major structural polymers: cellulose, hemicelluloses (polysaccharides) and lignin (aromatic polymer), along with a few minor nonstructural components, such as proteins, extractives and minerals (ashes) (Marques, Rencoret, Gutiérrez, Alfonso, & Río, 2010). The individual cells of ramie stems are linked by bundles of fibers wrapped in waxes, lignin, cellulose and pectin, among other components, which are difficult to remove (Ray, Banerjee, Satya, Ghosh, & Biswas, 2017). According to Smole, Hribernik, Kleinschek, and Kreze (2013) and Jose, Rajna, and Ghosh (2017) mechanical, chemical and biological processes or combinations of two or more of these are required for extraction of the fibers. Unlike other bast fiber plants, ramie phloem has excessive amounts of colloidal substances such as pectin and gums (Batra, 2007).

Ramie plants have different chemical compositions in their various parts, as reported by Contò, Carfì, and Pace (2011) and Pérez, Wencomo, Armengol, and Reyes (2013), who analyzed the chemical composition and nutritional values of different parts of the ramie plant as livestock and poultry feed additives.

Rami cultivation in Brazil has been decreasing drastically in the last decades, putting the extinction of the crop at risk (Oliveira, 1996). Concentrated in the state of Paraná, its production was 153 tons in 2016 (Food and Agriculture Organization Corporate Statistical Database [Faostat], 2016).

Thus, studying the histochemical structure of stem and fibers of *Boehmeria nivea* (L) Gaud. var. Murakami is of great relevance once it contributes with significant information on the properties and potential of this species and may assist in future studies.

Material and methods

The plant material was collected from specimens cultivated by farmers in the Cumbica region of the municipality of Guarulhos, São Paulo State, Brazil, in November 2016. Samples near the base of the stem were removed and fixed in FAA70 solution at a ratio of 90 mL of 70% ethanol, 5 mL of formaldehyde and 5 mL of acetic acid (Johansen, 1940) for seven days and then stored in 70% ethanol (Berlyn & Miksche, 1976). The stems were then cross sectioned with a razor. For morphological analysis, the botanical materials were subjected to double staining with Astra blue and Safranin (Bukatsch, 1972 modified), and then incorporated into the historesin (Glycol Methacrylate-GMA). The material was prepared and analyzed in the Botanical Department of UFPR (*Universidade Federal do Paraná*, Brazil). Histochemical tests were carried out to identify the occurrence of elements in the stem regions of the structure, with the following reagents: Lugol (Berlyn & Miksche, 1976) to detect starch; and methylene blue (Oliveira & Akisue, 1989) for mucilage, greases and gums. These occurrences were documented by photomicrographs taken with a Bioval L2000 optical microscope coupled to a Canon Powershot A2400 digital camera. The analyzes were conducted in the Laboratory of Anatomy and Wood Quality of UFPR (Brazil).

The Scanning Electron Microscopic (SEM) images of the ultrastructure were obtained by a Carl Zeiss EVO MA15 microscope and the composition of micro and macro and microelements was determined by EDS/EDX with an Oxford Instruments X-Max silicon drift detector from the Microscopy Laboratory of the *Universidade Tecnológica Federal do Paraná* (UTFPR, Brazil). For this analysis, cross sections of fresh stems were obtained freehand with a razor. The sections were dehydrated and sputtered with gold (Au) and affixed with double-sided copper tape (Cu) (Dedavid, Gomes, & Machado, 2007). To acquire the SEM images of the bark fibers, chips were macerated in test tubes with solutions of hydrogen peroxide and acetic acid (1:1) and then were placed in an incubator (60°C) for 24 hour, as described by Franklin and modified by Kraus and Arduin (1997). The fibers were washed in distilled water and the material was affixed in the holder with double-sided carbon tape.

The chemical analysis of the fibers was carried out in the Paper Pulp Laboratory of UFPR (Brazil). The analyzes were conducted in triplicate using manufactured yarn, based on procedures recommended by the Technological Association of the Pulp and Paper Industry (Tappi), for extractives in hot and cold water - T 207 cm-08 (Tappi, 2008a), NaOH extractives - T 212 om-12 (Tappi, 2008b), ethanol-toluene extractives - T 204 cm-97 (Tappi, 1997), total extractives - T 204 om-88 (Tappi, 1996), ash content - T 211 cm-02 (Tappi, 2002a) and lignin content - T 222 om-02 (Tappi, 2002b).

Results and discussion

Stems characterization

Morphological characterization indicated the presence of pubescent trichomes in the epidermis, in other words, the form of thin and short hair (Figure 1). They are micro papillate non-glandular and occur all families of Urticales (Tobe & Takaso, 1996). In botanical studies of the *Boehmeria nivea* species in state of São Paulo, Brazil, Gagliotti and Romaniuc Neto (2012) verified the occurrence of smooth simple and verrucous trichomeas.

When mature, the tissue structure of the cortex decreases significantly, while the fibers form and the cell walls thicken. In the adult stage, the tissues contain a predominance of Xylem (X) and Medulla (M) (Figure 2A). The region of the cortex (Figure 2B) consisting of the epidermis, collenchymas, parenchyma, fibrous layer and liberian layer become denser. It is also possible to identify the presence of sieve tubes (CT) beside the phloem cells, responsible for conducting to the sap (Figure 2B).

Figure 1. Detail of trichomes in epidermis region of the stem.

Through histochemical tests, it was possible to identify different substances in the stem. Detailed analysis of Figure 3A and B shows Safranin (red color) reacted only in the xylem region, highlighting the lignified, suberous and cutinized cell walls and the blue of Astra (blue color) reacted evidenced pecto-cellulosic substances.

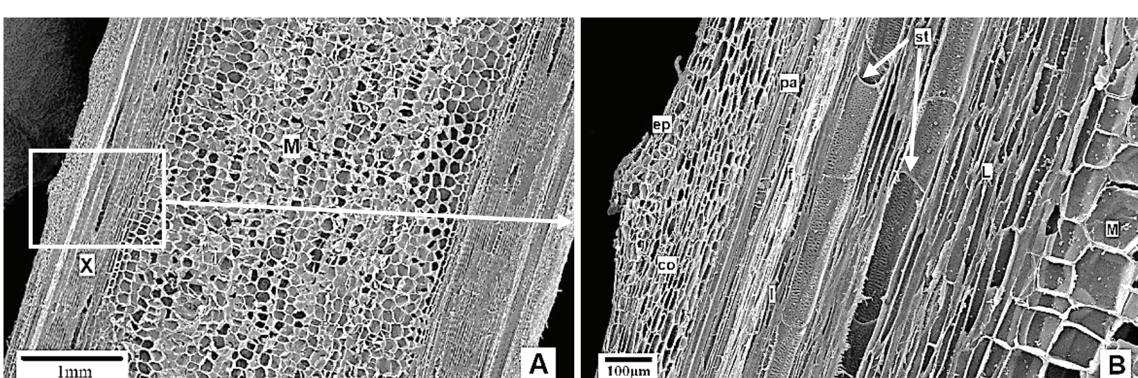

Figure 2. Longitudinal section of ramie stem. A) Predominance of xylem tissues (X) and Medullary zone (M); B) Detail of the outermost region, showing the epidermis (ep), collenchyma (co), parenchyma (pa), fibrous region (f), liberian layer (l), sieve tubes (st).

Chemical analyses carried out by Contò et al. (2011) of different ramie plant parts indicated higher lignin concentration in both the cortex (bark) and xylem. The Astra staining (bluish color) indicated the presence of pectin and cellulose in the region of the fiber bundles and the liberian layer (cortex) and in smaller concentration in the xylem region (Figure 3B). Angelini and Tavarini (2013) analyzed different parts of the stems of ramie at different stages of development, finding that more mature plants showed higher concentration of elements such as cellulose, hemicelluloses and lignin.

According to Batra (2007), the average overall chemical composition of ramie stems varies from 68.6-76.2% for cellulose, 13.1-14.6% hemicelluloses, 0.6-0.7% lignin, 1.9-2.1% pectin, 5.5-6.4% water-soluble extractives and around 0.3% fats and greases. In turn, Contò et al. (2011) obtained percentages of 44.34 cellulose, 0.64 hemicelluloses, 17.98 lignin and 7.97% ash in the pith, while the contents of the bark were 44.44 cellulose, 10.73 hemicelluloses, 23.20 lignin and 12.86% ash.

Figure 4A and B (methylene blue) show stem sections indicating the presence of greases and mucilage in all regions and in high concentrations of gums in the fibrous region and bark (blue-violet color). Figures 4C and D suggested the presence of starch in the concentrated region of the xylem and epidermis, highlighted in dark brown.

Ramie fibers stand out from other bast fibers for their high amounts of colloidal substances such as gum and pectin (Batra, 2007). According to Souza et al. (2005), gums and mucilage are related to carbon hydrates, specifically pectic compounds, which swell in the presence of water or appear because of functional disturbances or pathological disorders.

To assess the quality of the fibers after the degumming process, Thakur, Sarkar, and Sarmah (1999) analyzed the chemical constituents of the stem from three varieties of *Boehmeria nivea* and obtained 0.28-0.32-0.31% fat and greases, 22.93-23.23-25.75% gum, 1.72-1.94-1.97% pectin, 1.23-1.38-1.41% ash, 73.76-71.55-71.15% cellulose, respectively.

The structural details of the cortex (Figure 5A), cortex and xylem regions (Figure 5B) and the transition region between the cortex (C) and the xylem (X) (Figure 6) were obtained by scanning electron microscopy (SEM). The chemical composition of the macro and micro mineral elements (ashes) of the stem was analyzed by EDS/EDX and the results are shown in Table 1 and 2, respectively.

Table 1. Chemical composition of macro and micro mineral elements determined by EDS/EDX in the cross section of the ramie stem in different regions.

Macro and Micro Mineral Elements	(Cortex) Fibrous regions (f); Parenchyma (pa); Xylem (X) (%)	Cortex (C) and Xylem (X) (%)
Mg	0.11	0.31
Al	0.19	0.37
Si	-	0.46
K	0.59	0.87
Ca	0.22	1.19

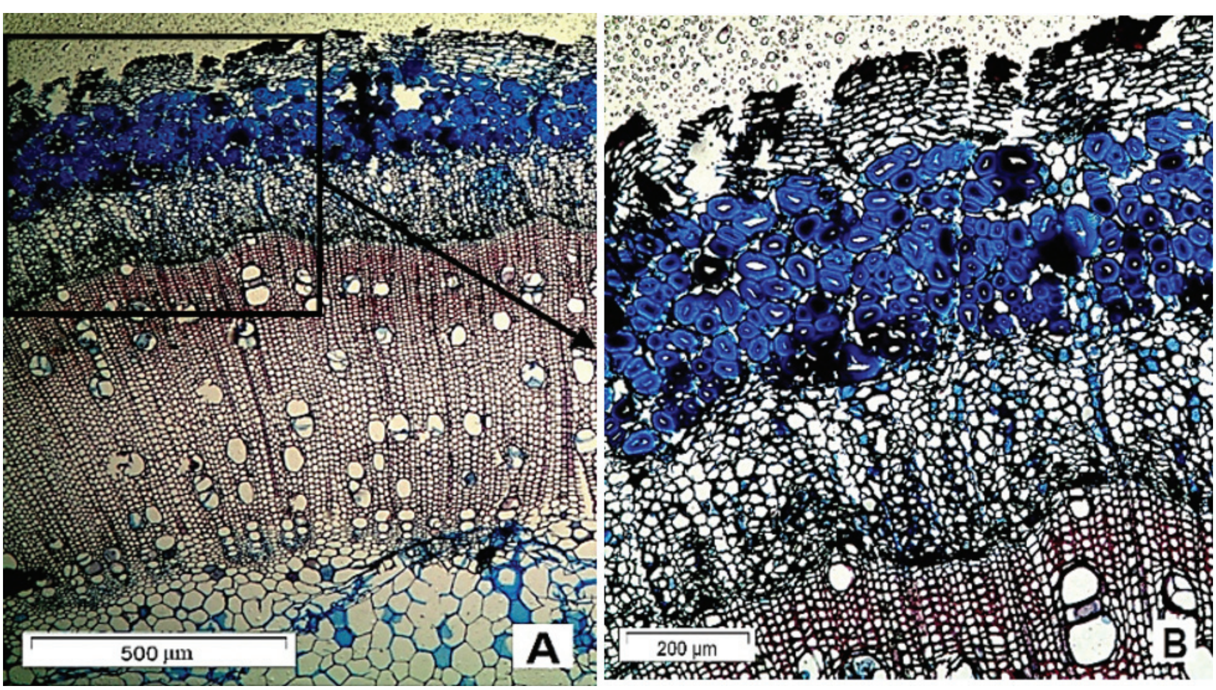

Figure 3. Anatomical views of the cross section of a ramie stem stained with Safranin and Astra blue. A) Overview of the anatomical structure; B) Detail of the cortex region and part of the xylem.

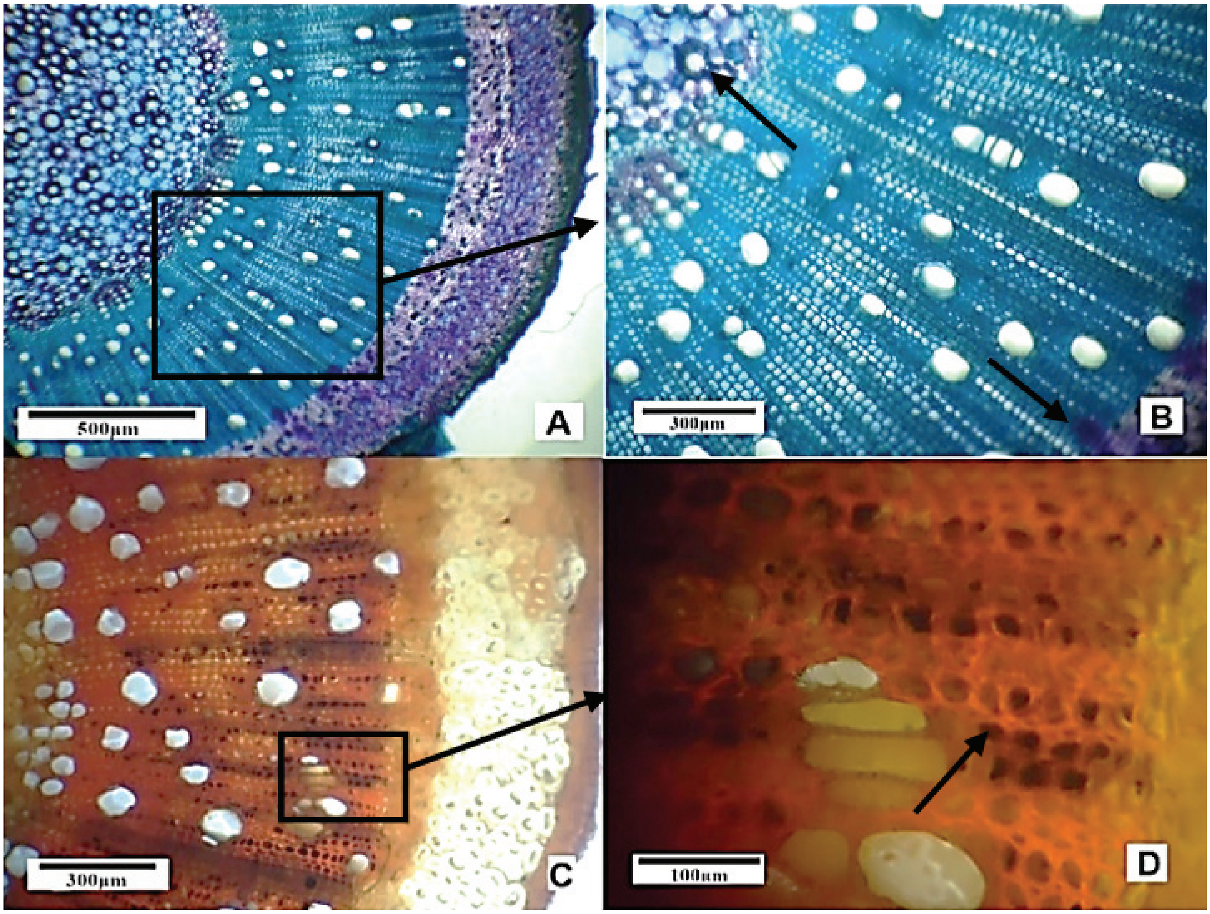

Figure 4. Cross sections of ramie stems. A-B) Methylene Blue; C- D) Lugol.

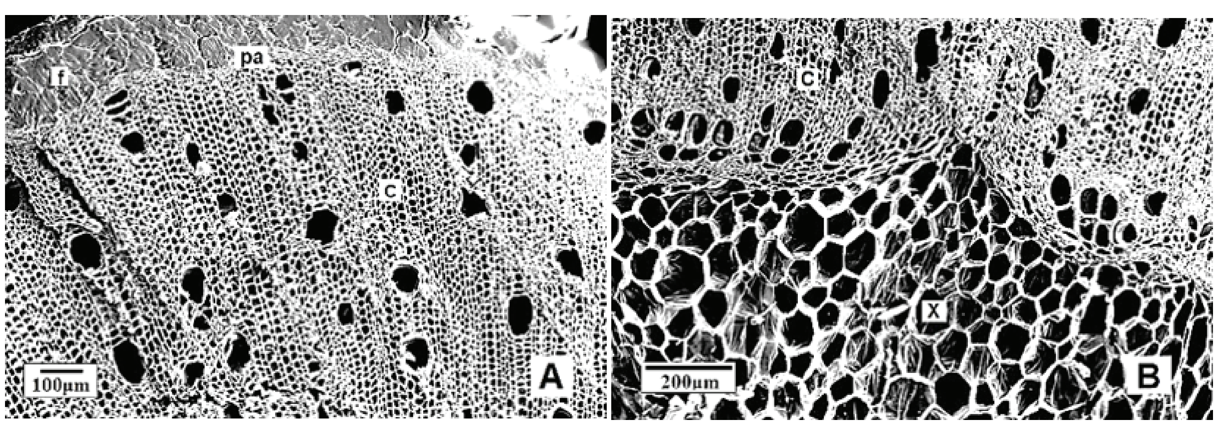

Figure 5. Cross section of the stem region. A) View of cortex with details of the fibrous region (f), parenchyma (pa). B) View of the cortex (C) and xylem (X).

Table 1 presents Ca, K and Si as the elements of higher concentrations in the regions of cortex and xylem (Figure 5B). In the transition region between cortex and xylem (Figure 6), the K as predominant elements were identified (Table 2). Starch deposits in the form of granules have also been identified (Figure 6).

Table 2. Chemical composition of Macro and Micro Mineral Elements between the cortex (C) and xylem (X).

Points	Chemical composition of Macro and Microminerals in different points				
	F (%)	Mg (%)	Al (%)	K (%)	Ca (%)
1	2.72	0.25	0.19	0.61	0.31
2	-	-	-	81.00	-
3	-	-	-	80.59	-
4	-	0.43	0.38	1.08	0.23
5	-	0.36	0.34	0.98	0.26

Note: Region 1 represents general data for the whole region.

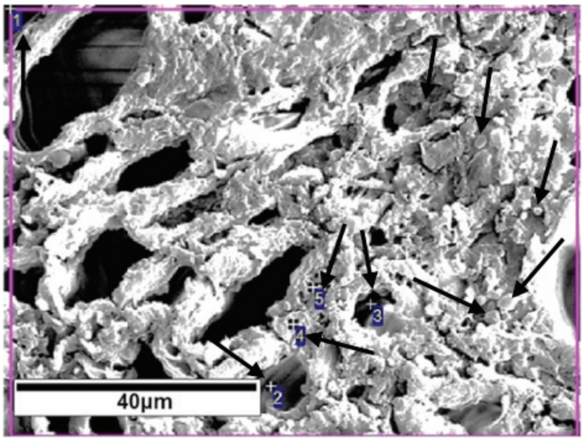

Figure 6. The transition region between cortex and xylem.

Fibers characterization

The surface of ramie fiber after primary processing (debark, drying), shows the layers of parenchyma adhered to their surface by pectic substances (Figure 7A). The ramie fiber surface after chemical treatment present fissures, grooves and striations (Figure 7B).

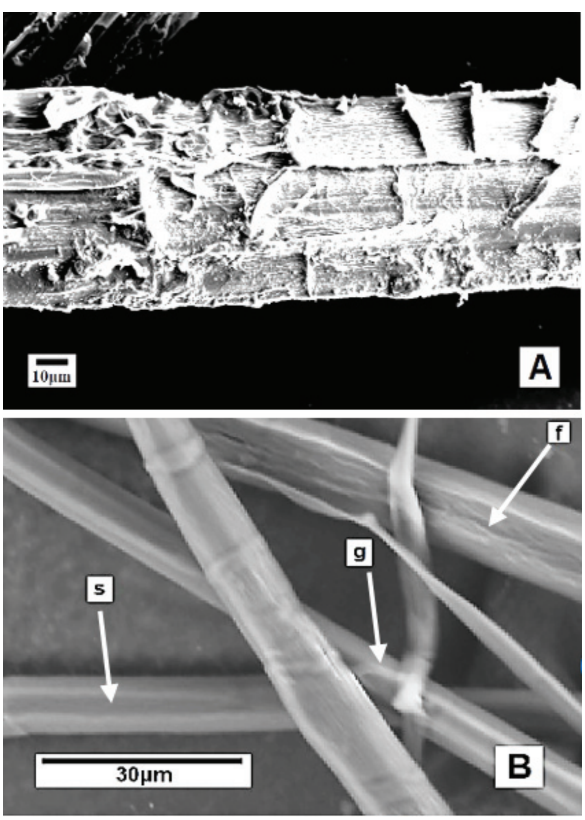

Figure 7. Surface of ramie fiber. A) After primary processing. B) After chemical treatment. (f) fissures, (g) small grooves, (s) striations.

According to Hearle (1963), they are characterized by bright white color and silky appearance. The cells have length between 40-250 mm and width of 16-126 μm, their surfaces are rough and are distinguished by small grooves, striations and deep fissures, easily identified on the coarse and thick cell wall, with lack of twisting on their surface (Figure 7B). More detailed analyses conducted by Batra (2007) revealed the absence of cracks or fissures, instead finding displacement or compression folds along the fibrils in the secondary wall. Another study suggests that these structures (striations) are path by which the nutrients are supplied to the fiber by the adjacent parenchyma cells during the period in which the fiber is a functional member of the plant stem, whose aspect is similar to that shown in Figure 7B.

Table 3 presented the chemical composition of the ramie fibers in the yarn form.

Table 3. Means of chemical composition of ramie fiber and (Standard deviation).

Analyses	Content in Ramie Fibers (%)
Cold water solubility	3.12 (0.10)
Hot water solubility	5.28 (0.11)
Soluble extractives in ethanol-toluene	8.55 (3.16)
Total extractives	9.50 (0.36)
Soluble extractives in NaOH 1%	27.27 (0.27)
Ash	1.70 (0.04)
Lignin	1.06 (0.01)
Cellulose	71.09 (0.78)
Hemicellulose	12.11 (0.8)

Values in parentheses correspond to standard deviation.

The water-soluble extractives (cold and hot) composed 8.40% of the organic elements adhered to the wall of the fibers, consisting of pectic substances (polysaccharides, gums and resins), remains of parenchyma tissue, organic minerals and phenolic substances. The percentage of compounds extractable in organic solvents was 45.32%, consisting of fatty acids, waxes, fats and oils, commonly found in the fiber after the degumming process. The ash content was 1.70%, in the ramie fibers are usually low. Lignin concentration was 1.06%, but varied among the plants, primarily in the different stages (levels) of maturity. The cellulose and hemicelluloses contents were 71.09 and 12.11%, respectively. These values depend on the amount of nutrients available in the soil and the stage of maturity of the plant. In a study of environmental degradation of ramie fiber reinforced biocomposites, Li, Li, Ding, and Yu, (2016) obtained 68-75% (cellulose), 14-16% (hemicelluloses), 0.8-1.5% (lignin), 4-5% (pectin), (5-8) water soluble and 1-2 (wax) in the ramie fibers.

Conclusion

The study of ramie (*Boehmeria nivea*) was of great relevance because it shows the various elements of the stem and its composition, revealing its high potential. The described morphological and chemical characteristics of the plant stem resemble the descriptions of the species performed by other authors. The identification and location of its constituents performed by histochemical tests provides important information for the species. Therefore, this knowledge contributes to a better appreciation of the species and deepening in future studies.

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