

## Leaf blade anatomy and ultrastructure of six *Simira* species (Rubiaceae) from the Atlantic Rain Forest, Brazil

TARSILA MARIA DA SILVA MORAES<sup>1</sup>, CLAUDIA FRANCA BARROS<sup>2</sup>, SEBASTIÃO JOSÉ DA SILVA NETO<sup>3</sup>, VALDIRENE MOREIRA GOMES<sup>4</sup> AND MAURA DA CUNHA<sup>1\*</sup>

1. Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil.
2. Laboratório de Botânica Estrutural, Programa Mata Atlântica, Diretoria de Pesquisas, Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, RJ, Brazil.
3. Programa Mata Atlântica – Diretoria de Pesquisas, Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, RJ, Brazil.
4. Laboratório de Fisiologia e Bioquímica de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil.

**Key words:** leaf anatomy, structure, morph-anatomy

**ABSTRACT:** *Simira* is a predominantly woody Neotropical genus comprising 41 taxa, 16 of which occur in Brazil and eight of them in the southeastern region of Brazil. Leaf blades of *Simira eliezeriana* Peixoto, *S. glaziovii* (K. Schum.) Steyer., *S. grazielae* Peixoto, *S. pikia* (K. Schum.) Steyer., *S. rubra* (Mart.) Steyer., *S. sampaioana* (Standl.) Steyer. were collected in the southeastern region of Brazil and fixed according to usual methods for light and electron microscopy. The leaf blades show typical characteristics of the Rubiaceae family as dorsiventral mesophyll and paracytic stomata. The presence of two bundle sheaths that extend to the upper epidermal layer, prismatic crystal and crystal-sand, alkaloids in the mesophyll and the organization micromorphological of the outer periclinal wall are considered characteristics representative for the genus. This study also demonstrates some leaf blade characteristics that can be used to *Simira* species identification (leaf surface, domatia types, epicuticular wax types and patterns of epidermis anticlinal cell walls).

### Introduction

The Atlantic Rain Forest constitutes the second most threatened ecosystem in the world, and has been reduced over the years (Myers *et al.*, 2000). This biome presents a high diversity of plant families and Rubiaceae is one of the most important families in several remnant forests in Rio de Janeiro state/Brazil (Guedes-Bruni, 1998). The Rubiaceae family has ca. 650 genera and 13,000 species with a wide geographical distribution in the tropical and subtropical areas (Struwe, 2002). Among

these, *Simira* Aubl. is a predominantly woody Neotropical genus consisted of 41 species, 16 of which occur in Brazil, and eight from southeastern Brazil (Silva Neto, 2000). This genus is important due to its economic value, e.g. production of handmade pieces, urban landscaping and dyestuff.

The Rubiaceae family is a monophyletic group (Judd *et al.*, 1999) and belongs to the Gentianales order (Struwe, 2002). Robbrecht (1988) divided this family into four subfamilies: Antirheoideae, Cinchonoideae, Ixoroideae and Rubioideae based on morphological characters. Phylogenetic studies based on molecular data alone or in combination with morphological data have drastically changed the Rubiaceae classification at subfamily (e.g. Bremer *et al.*, 1995; Bremer, 1996), tribal (e.g. Bremer

\*Address correspondence to: Maura Da Cunha.

E-mail: [maura@uenf.br](mailto:maura@uenf.br)

Received: September 10, 2008. Revised version received: July 13, 2009. Accepted: July 19, 2009.

and Thulin, 1998; Rova *et al.*, 2002), generic (e.g. Nepokroeff *et al.*, 1999; Andersson, 2001), and species level (e.g. McDowell and Bremer, 1998; Persson, 2000). The molecular studies proposed a new classification in three subfamilies - Rubioideae, Cinchonoideae and Ixoroideae (Bremer *et al.*, 1995; Rova *et al.*, 2002). Together, macromolecular data, morphological and anatomical characters have contributed to characterize several subgroups of this family (Piesschaert *et al.*, 2000; Dessein *et al.*, 2001; Jansen *et al.*, 2001, 2002). The genus *Simira* has been classified in the Cinchonoideae subfamily and Rondeletieae tribe (Robbrecht, 1988) based on morphological data. A recent phylogenetic study based on molecular data, however, placed *Simira* in the tribe Simireae within the Ixoroideae subfamily (Rova *et al.*, 2002). Because of this change in the *Simira* classification, it is necessary to reunite data of several study areas.

Anatomical and micromorphological characters of the leaf blade with possible taxonomic significance have been studied as a source for solving taxonomic problems. The comparative leaf anatomy and micromorphology in *Psychotria* (Da Cunha and Vieira, 1993/97; Gomes *et al.*, 1995; Vieira and Gomes, 1995), *Rondeletia* (Kocsis *et al.*, 2004), *Rudgea* (Mantovani and Vieira, 1993/97; Leo *et al.*, 1997), *Rustia* (Vieira *et al.*, 2001), *Coussarea* (Tavares and Vieira, 1994) and *Bathysa* (Nascimento *et al.*, 1996; Barros *et al.*, 1997) have been studied as well as the wood anatomy of several species of Rubiaceae of the Atlantic Forest timber (Callado *et al.*, 1997). In *Simira* genus, the comparative structure of colleters (Klein *et al.*, 2004) and wood anatomy (Callado and Silva Neto, 2003) in several species has been studied.

The present study aims to characterize the leaf blade anatomy and micromorphology of six *Simira* species of Atlantic Rain Forest from Southeastern Brazil, and to contributing to the knowledge the implications in survival strategies and taxonomic importance below the genus level. We particularly want to identify anatomical and morphological variations, which may be useful in species identification as well as to test the validity of the sections currently recognized in *Simira*.

## Material and Methods

### Plants

Mature leaves of *Simira eliezeriana* Peixoto, *S. glaziovii* (K. Schum.) Steyererm., *S. grazielae* Peixoto, *S. pikia* (K. Schum.) Steyererm., *S. rubra* (Mart.) Steyererm., *S. sampaioana* (Standl.) Steyererm. were collected at

Reserva Florestal da Companhia do Vale do Rio Doce (19° 06' - 19° 18' S and 39° 45' - 40° 19' W), in the Municipal District of Linhares, Espírito Santo state, Brazil, at Barragem de Saracuruna (22° 12' - 22° 17' S and 43° 30' - 43° 35' W), in the Municipal District of Duque de Caxias, and at Reserva Biológica de Tinguá (22°28' - 22°39' S and 22° 35 - 43° 34' W), in the Municipal District of Duque de Caxias and Nova Iguaçu, in Rio de Janeiro state, in the southeastern region of Brazil.

### Light microscopy

Leaf fragments were fixed in a solution of 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.05 M cacodylate buffer at pH 7.2, for two hours. Subsequently, the samples were rinsed three times with the buffer and post-fixed for two hours at room temperature with 1.0% osmium tetroxide in 0.05 M cacodylate buffer at pH 7.2. The post-fixed samples were dehydrated in a graded series of acetone solutions. Subsequently, the material was infiltrated and embedded in epoxy resin (Polybed). Microtome sections (1.0 µm) were cut and stained with toluidine blue (0.05% aqueous solution). The slides were sealed with Entellan® (Merck) and examined with an Axioplan ZEISS microscope. For epidermis, dissociation was achieved using a solution 1:1 of 10% nitric acid and 10% chromium oxide (Jensen, 1962), and stained with 10% safranin. Epidermis outer periclinal cell walls visualization, images were obtained using an ANALYSIS SIS LINK/OXFORD - ZEISS.

### Histochemistry

Histochemical tests were carried out on freehand sections of the collected material alkaloids were localized by the Dragendorff test described by Costa (1982); and crystals were tested by the insolubility in acetic acid and solubility in hydrochloric acid (McLean and Cook, 1958), and examined with an Axioplan ZEISS microscope.

### Scanning electron microscopy

The fragments of leaf blades were fixed, post-fixed and dehydrated as for light microscopy. The samples were subsequently critically point dried in CO<sub>2</sub>, sputter coated with 20 nm gold, and observed with a digital scanning electron microscope (ZEISS DSEM 962). The authenticity of epicuticular wax was verified by washing leaves in chloroform for one minute and comparing to the unwashed specimens under SEM.

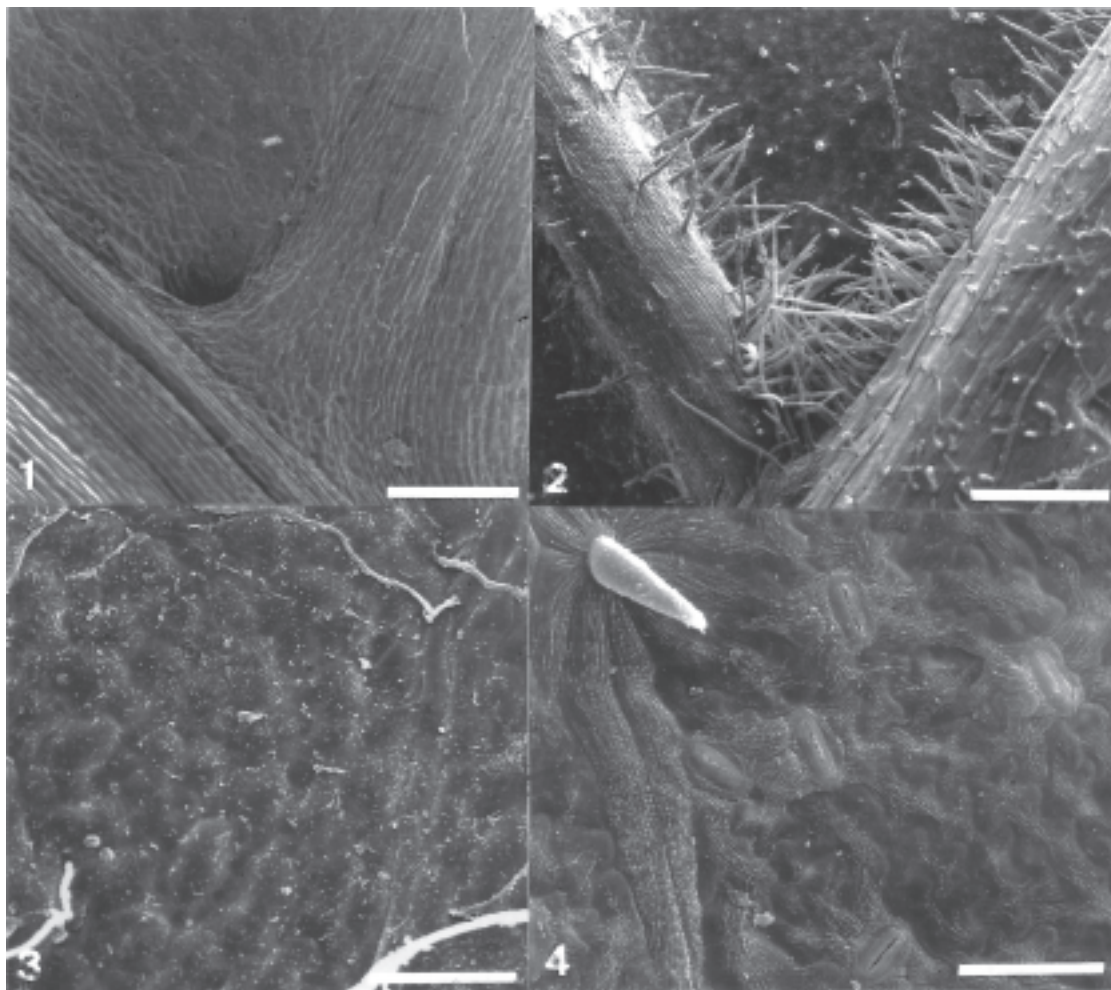
### Transmission electron microscopy

The samples were fixed, post-fixed, dehydrated and embedded as described previously. The ultrathin sections (80 nm) were collected in copper grids (300 mesh), stained with acetate with 1.0% uranyl followed by 5.0% lead citrate for routine observation. Three other stains were used to elucidate the cytochemical of the outer periclinal cell walls. (1) The preservation and contrast of lipids were enhanced and observed using imidazole-buffered osmium tetroxide (Angermüller and Fahimi, 1982). (2) 0.5 mg.ml<sup>-1</sup> Ruthenium red was used to detect negatively charged components of the outer periclinal cell walls (Luft, 1971). (3) Periodic acid-thiocarbohydrazide (THC)-silver proteinate (PATAg) was used to detect polysaccharides containing 1,2-glycol groups. For the latter technique, sections were stained with periodic acid and solutions not containing peri-

odic acid and THC as control (Thiéry, 1967). Sections were observed at 80 kV using a transmission electron microscope (ZEISS EM 900).

### Extraction and determination of leaf proteins

The protein extraction was performed as described by Granier (1988) with some modifications. About 5 g of leaf blades were dry crushed in a mortar cooled with liquid nitrogen. The dry powder was resuspended in 5 mL of extraction buffer containing 100 mM Tris-HCl, pH 8.7, 1 mM dithiothreitol (DTT), 1 mM ascorbic acid, 5 mM Mg Cl<sub>2</sub> and 1% polyvinylpyrrolidone (PVP). The extract was centrifuged at 20,000 g for 1 hour at 4°C. The supernatant was then mixed with 10% Trichloroacetic acid, 0.07% 2-mercaptoethanol in cold acetone and kept at -18°C for 1 h. After a 30 min centrifugation at 30,000 g, the supernatant was removed and the pellet was recov-



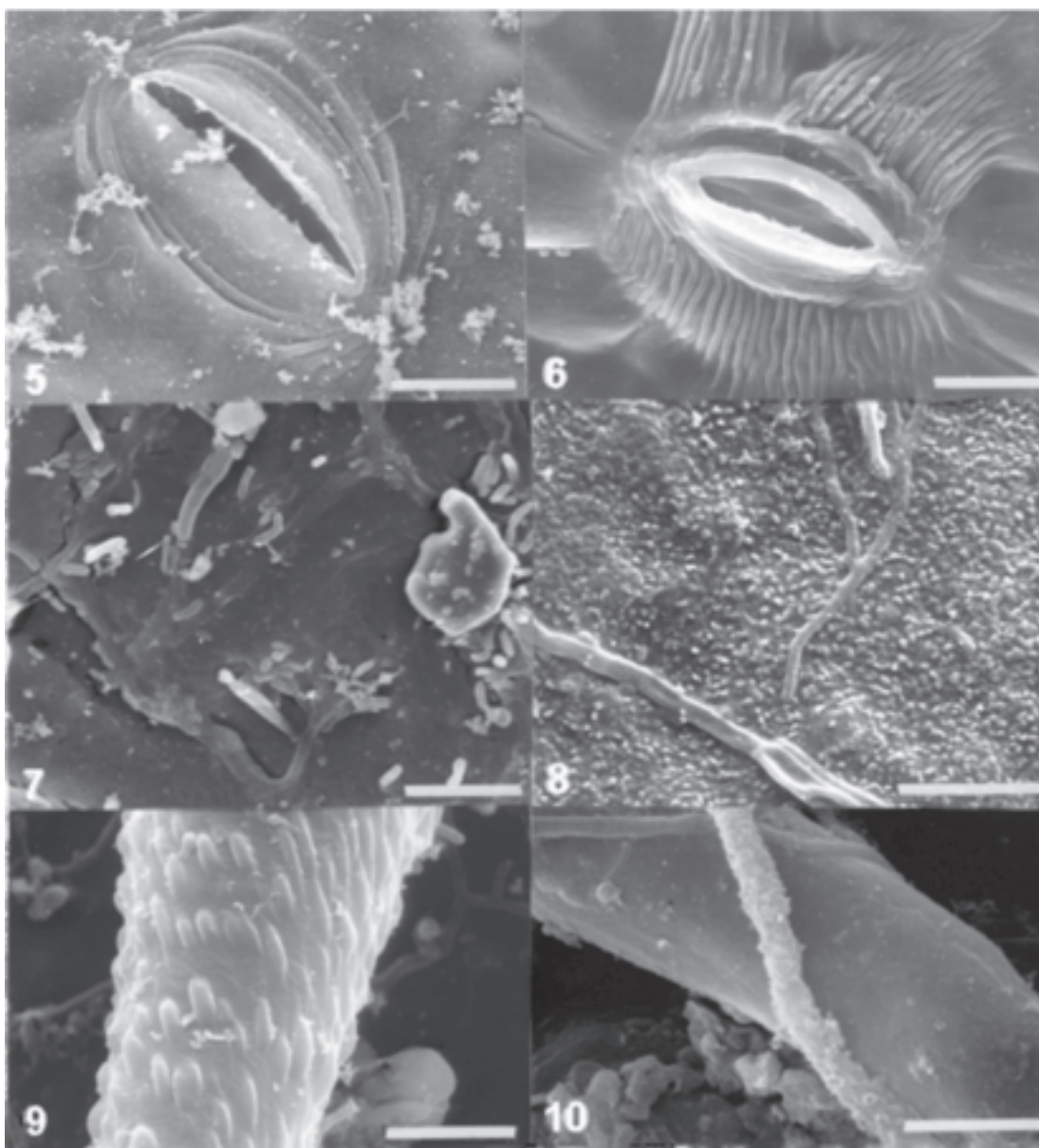
**FIGURES 1-4.** Scanning electron microscopy of *Simira* Aubl. species. **1.** Pocket domatia in *S. grazielae* Peixoto. **2.** Hair-tuft domatia in *S. pikia* (K. Schum.) Steyerl. **3.** *S. glaziovii* (K. Schum.) Steyerl. adaxial surface. **4.** *S. pikia* (K. Schum.) Steyerl. abaxial surface. Scale bars= (1) 200  $\mu$ m; (2) 500  $\mu$ m; (3, 4) 50  $\mu$ m.



ered and submitted to further electrophoresis. The protein determination was performed by the method of Bradford (1976), using bovine serum albumin as standard and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Proteins utilized as molecular mass standards for PAGE-SDS were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and  $\beta$ -lactoglobulin (14 kDa).

## Results

The presence of domatia is observed in the abaxial surface between the midrib and secondary veins. *Simira grazielae* (Fig. 1) presented domatia in pockets, while the other species presented domatia in tufts of hairs (Fig. 2). Cuticular ornamentation is observed on both surfaces as in *S. glaziovii* (Fig. 3), except in *S. pikia* (Fig. 4), and is restricted near the stomata of the other species studied. The cuticle of epidermic cells is non-striated (Fig. 3) on

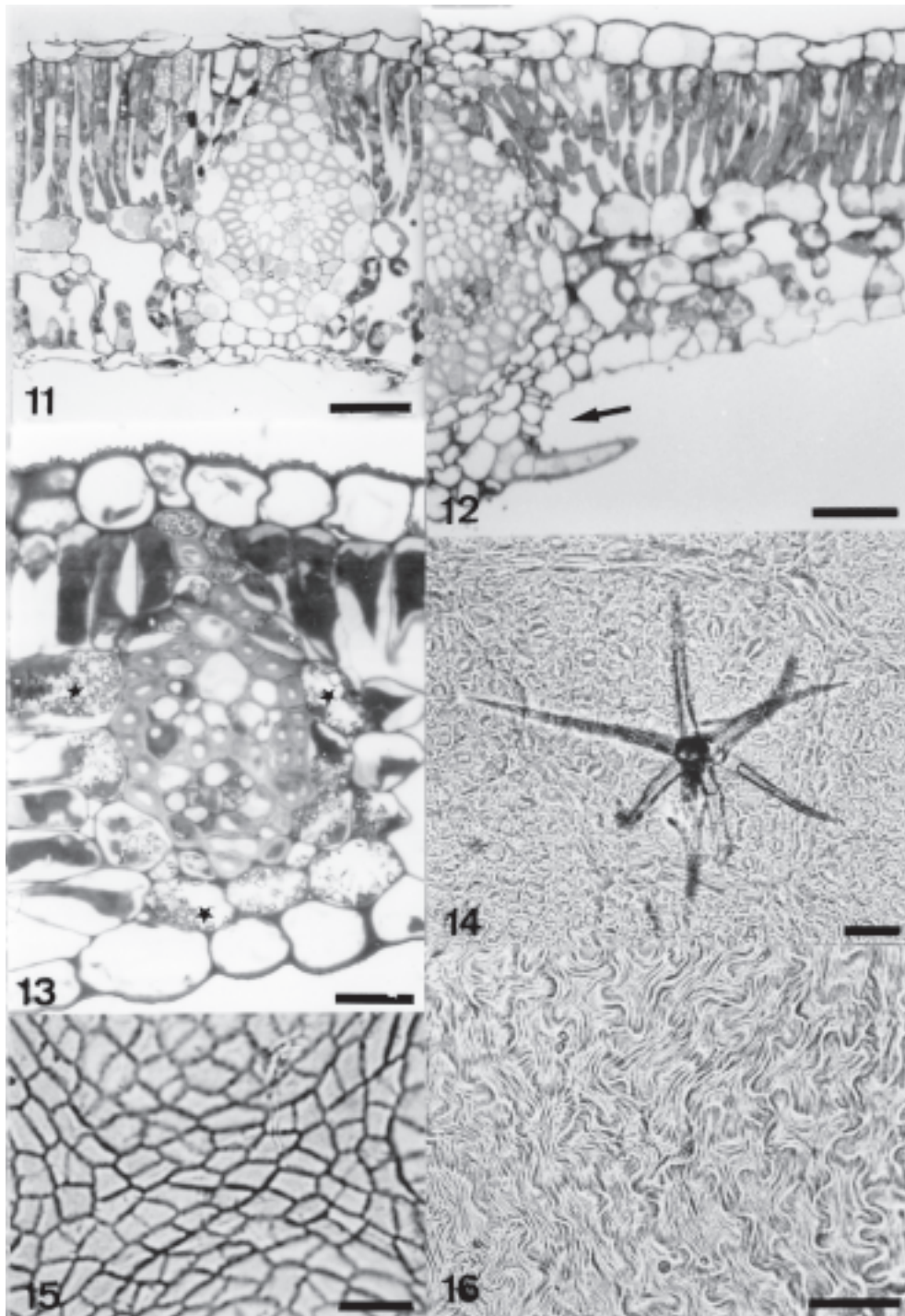


**FIGURES 5-10.** Scanning electron microscopy of *Simira* Aubl. species. **5.** *S. grazielae* Peixoto abaxial surface showing stomata with parallel cuticular striations. **6.** *S. rubra* (Mart.) Steyerl. abaxial surface showing stomata with perpendicular cuticular striations. **7.** *S. elizeriana* Peixoto adaxial surface showing smooth layer of epicuticular wax. **8.** *S. grazielae* Peixoto adaxial surface showing epicuticular wax in granules. **9.** *S. sampaioana* (Standl.) Steyerl. trichome with cuticular striations. **10.** *S. elizeriana* Peixoto trichome without cuticular striations. Scale bars= (5, 6, 7, 8, 10) 10  $\mu$ m; (9) 5  $\mu$ m.

both surfaces, except in *S. pikia* (Fig. 4). The cuticle ornamentations is observed only near the stomata, except in *S. elizeriana*. In *S. grazielae* (Fig. 5), these cuticular striations are parallel to the stomata axis, while in other species they are perpendicular (Fig. 6). Epicuticular wax is observed in all species studied. *Simira glaziovii*, *S. pikia*, *S. rubra*, and *S. sampaioana* present a fine film; however, a smooth layer is seen *S. elizeriana* (Fig. 7) and granules in *S. grazielae* (Fig. 8).

The species studied exhibit unicellular, pluricellular (Fig. 12) and stellate (Fig. 14) trichomes on both surfaces, except in *S. grazielae* that has a glabrous surface. Trichomes of *S. pikia*, *S. rubra*, and *S. sampaioana* (Fig. 9) show surface ornamentations, not observed in *S. elizeriana* (Fig. 10) and *S. glaziovii*.

In a transverse section of the leaf blade, the adaxial and abaxial epidermis present a single layer covered with a fine cuticle in all species as can be observed in *S. rubra*



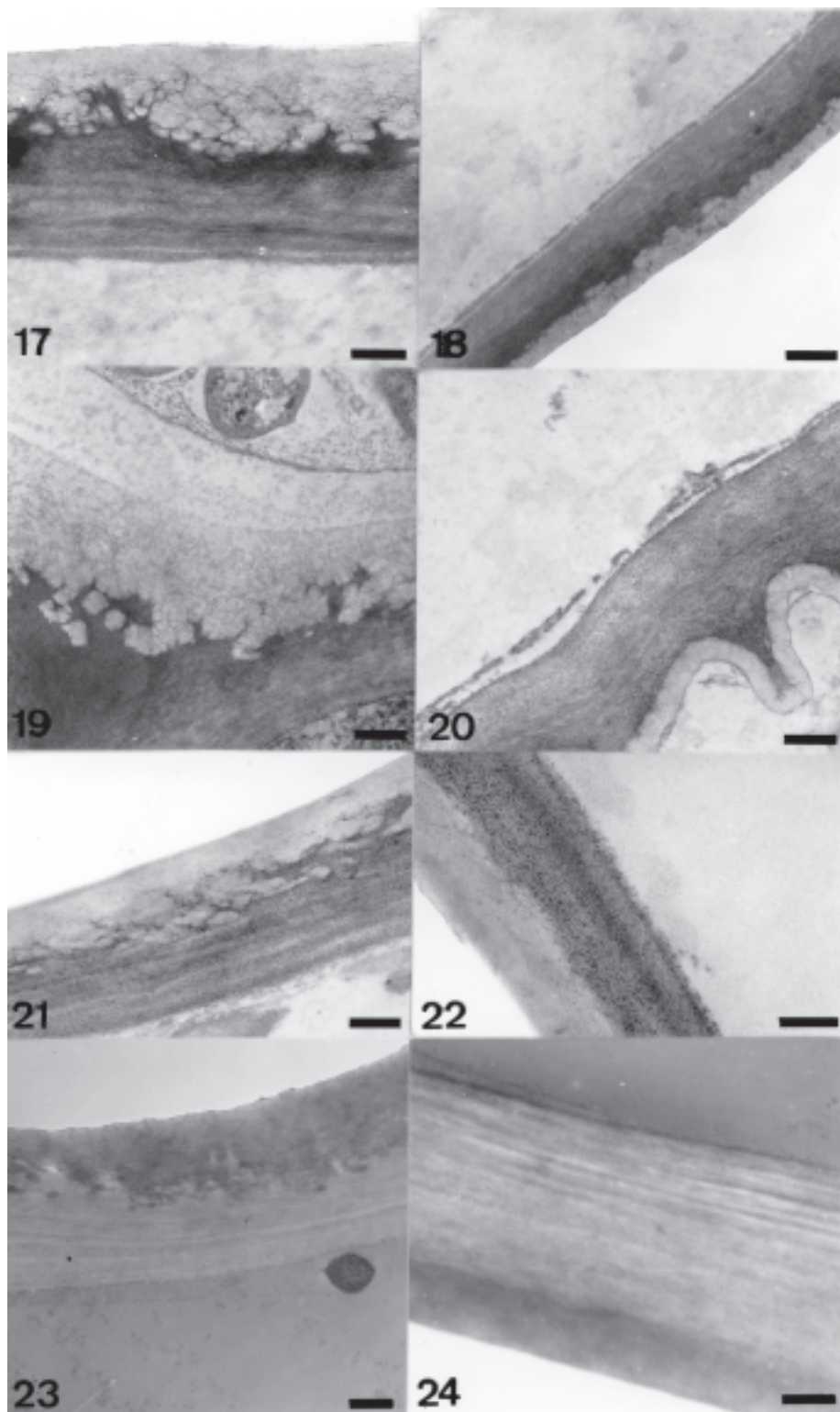
**FIGURES 11-16.** Light microscopy of *Simira* Aubl. species. **11.** Transverse section of *S. rubra* (Mart.) Steyer. leaf blade showing dorsiventral mesophyll. **12.** Transverse section of *S. rubra* (Mart.) Steyer. leaf blade showing lateral projections along main vein forming domatia (arrow). **13.** Transverse section of *S. pikia* (K. Schum.) Steyer. Note two layers of the bundle sheath and parenchymatic layer forming crystalliferous series (stars). **14.** Frontal view of stellate trichome in abaxial surface of *S. rubra* (Mart.) Steyer. **15.** Straight anticlinal wall in *S. sampaioana* (Standl.) Steyer. leaf blade. **16.** Strong sinuous anticlinal wall in *S. pikia* (K. Schum.) Steyer. leaf blade. Scale bars= (11) 30  $\mu\text{m}$  (12, 14) 50  $\mu\text{m}$  (13) 20  $\mu\text{m}$ ; (15, 16) 40  $\mu\text{m}$ .



(Fig. 11, 12), and in *S. pikia* (Fig. 13). The *Simira* species studied have dorsiventral mesophyll. Palisade tissue comprises two layers in *S. elizeriana* and one or two layers in the other species; and the number of spongy tissue layers varies from three layers in *S. sampaioana* to six in *S. glaziovii*. The species present prismatic crys-

tals and crystal-sand, identified by the insolubility in acetic acid and solubility in hydrochloric acid, as well as alkaloids, identified by Dragendorff reagent, in the vacuole of mesophyll cells.

Paracytic stomata slightly protrude above the level of the other epidermal cells. Stomata are not usually



**FIGURES 17-24.** Transmission electron microscopy of *Simira* Aubl. outer periclinal cell wall. **17.** *S. glaziovii* (K. Schum.) Steyer. adaxial epidermis. **18.** *S. elizeriana* Peixoto abaxial epidermis. **19.** *S. sampaioana* (Standl.) Steyer. adaxial epidermis with Ruthenium red treatment. **20.** *S. pikia* (K. Schum.) Steyer. abaxial epidermis with Ruthenium red treatment. **21.** *S. glaziovii* (K. Schum.) Steyer. adaxial epidermis with Thiéry treatment. **22.** Abaxial epidermis of *S. rubra* (Mart.) Steyer. with Thiéry treatment. **23.** *S. elizeriana* Peixoto adaxial epidermis with imidazole treatment. **24.** *S. grazielae* Peixoto abaxial epidermis with imidazole treatment. Scale Bars= (17, 18, 20, 24) 0.25  $\mu\text{m}$ ; (21) 0.15  $\mu\text{m}$  (22) 0.9  $\mu\text{m}$  (19, 23) 0.4  $\mu\text{m}$ .

seen over primary and secondary veins. In transverse sections, the guard cell presents a parietal thickening that is uniform.

In epidermis, at both leaf surfaces, the pattern of anticlinal cell walls varies from straight to sinuous: straight anticlinal walls in *S. sampaioana* (Fig. 15); slightly undulating walls forming an “S” in the *S. elizeriana*, *S. glaziovii* and *S. rubra*; and *S. grazielae* and *S. pikia* (Fig. 16) exhibit a strong sinuous. Anticlinal walls of epidermal cells are thin or moderately thick.

The vascular system shows a collateral arrangement and two layers of the bundle sheath (Fig. 11). The inner sheath is constituted by bundle perivascular fibers presenting an interruption in the phloematic tissue and parenchymatic cells in the outer bundle. The parenchymatic cells of the *S. pikia* bundle sheath contain a large amount of prismatic crystals, forming a crystalliferous series (Fig. 13). The bundle sheaths are extended towards adaxial epidermis in all species (Fig. 11, 13).

In the TEM cross sections, the outer periclinal cell walls in both epidermis show a fine epicuticular wax and three distinct layers: the inner polysaccharide-rich layer, mainly composed of cellulose; the intermediate cuticular layer, with a tree-like polysaccharide-rich network, immersed in a matrix of cutine; and a cuticle proper layer (Fig. 17-24). The cuticle proper is homogenous and relatively electron lucent. The cuticular layer is formed by a reticulated network layer and composed of a cutine matrix and a tree-like layer composed of polysaccharides and pectins, as observed with cytochemistry stains. The polysaccharide portion of the cuticular layer reacts with PATAg for polysaccharides and with ruthenium red, for pectin (Fig. 19-21). In contrast, the lipid portion of the cuticular membrane, the cuticle proper and the matrix of the cuticular layer react with imidazole (Fig. 23, 24). The species studied present differences in cuticular layer organization showing the tree-like layers more prominent in the adaxial surface (Fig. 17, 19, 21, 23). In *S. pikia* that presents a cuticular striated relief, the cuticular layer keeps up with a striated organization, mainly the tree-like layer (Fig. 20).

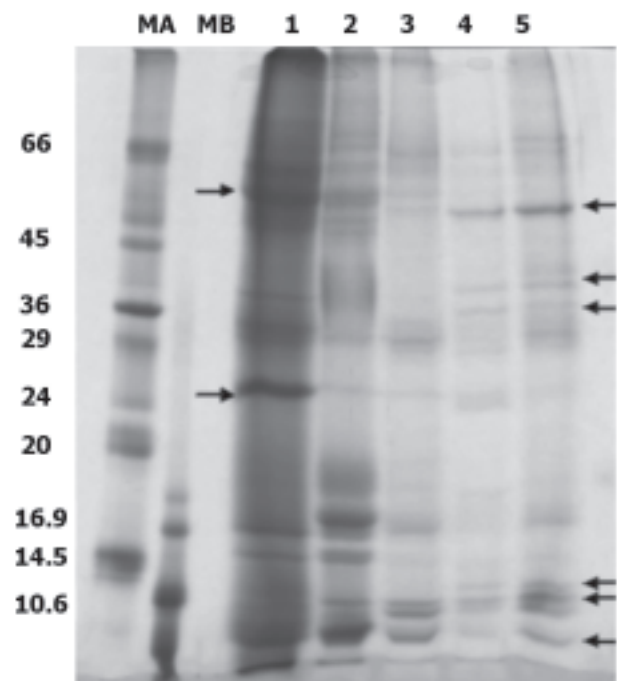
The preliminary analysis of the soluble proteins of *Simira* and *Bathysa* species shows the presence of several specific proteins between 66 and 14.5 kDa. Two bands of the same molecular weight of specific proteins between 66 and 45 kDa are observed for the *Simira* and *Bathysa* species (Fig. 25, arrows). Two other proteins close to 36 kDa are also detected in the *Bathysa* species (Fig. 25, arrows). A band of approximately 24 kDa is observed just in the lanes of the *Simira* species. Our study also demonstrated proteins with low molecu-

lar masses, more specifically below 14.5 kDa, present in all species of the *Bathysa* and *Simira* species analysed.

## Discussion

Anatomical and ultrastructural studies have indicated important characteristics capable of distinguishing taxa, as well as relating structural characteristics with the environment (Bredenkamp and Van Wyk, 2000; Kong, 2001). The characteristics observed may be useful for identifying characters of genera, and to distinguish species and possible implications in survival strategies in the Atlantic Rain Forest.

The presence of domatia in the abaxial surface of the leaf blades between the midrib and secondary veins occur in the six species studied, and Robbrecht (1988) relate that it is a common character in the Rubiaceae family. Domatia are structures that frequently shelter small predators or fungivore arthropods. These structures appear scattered among 277 families of angiosperms, occurring mostly in tropical and subtropical areas (Agrawal and Karban, 1997). The presence



**FIGURE 25.** SDS-polyacrylamide gel electrophoresis of the proteins extracted from the leaf blades of the *Simira* and *Bathysa* species. (1) *Simira pikia*; (2) *S. glaziovii*; (3) *S. rubra*; (4) *Bathysa gymnocarpa*; (5) *B. stipulata*. (M) Markers (kDa).

and type of domatia constitute a morphological character with useful systematic value to separate genera and species (Adâmolli Barros, 1959). The domatia in pockets are only found in *Simira grazielae* and is a good character to separate this species from the others. Lateral projections in the midrib vein were also observed forming wakes covered with hairs in *S. rubra*, described as a domatia type in *Psychotria velloziana* (Da Cunha and Vieira, 1993/97). The organisms that living in domatia, are indirectly beneficial for the plant because they reduce the herbivore arthropods or the pathogenic fungi that commonly inhabit the surface of the plant leaves (O'Dowd and Wilson, 1989; Agrawal *et al.*, 2000). These structures are possibly associated with defense mechanisms against microorganisms in Atlantic Rain Forest. Further studies are required to determine the possible role of these structures in plant protection.

The leaf micromorphology aspects can sometimes reflect the adaptation of plants to their habitat (Juniper and Jeffree, 1983); however, several characters can also provide conclusive data for taxa separation (Barthlott, 1981). Only *S. pikia* exhibit cuticular ornamentation in both surfaces, distinguishing the others species studied. In the species studied, the adaxial surfaces are smooth, except in *Simira pikia* that exhibit cuticular ornamentation that distinguish this species from the others. The cuticle of the plants is covered with epicuticular waxes that possess considerable ultrastructural and chemical diversity. These waxes show variable shapes that can be used in taxonomy (Barthlott *et al.*, 1998). In the species studied, three types of epicuticular waxes were observed according to Barthlott *et al.* (1998): a smooth layer in *Simira elizeriana*; a granular type in *S. grazielae*, and a fine film in the other species. These different types were a complementary characteristic for separating *S. elizeriana* and *S. grazielae*.

Structural aspects of the epidermis, such as the form of the anticlinal cell walls, seem to be related to the light intensity. According to Combes (1946), shaded leaves exhibit epidermis with sinuous anticlinal cell walls. The anticlinal cell wall of *Simira sampaiiana* is straight in the adaxial epidermis, as in *Psychotria nuda* and *P. leiocarpa* (Vieira *et al.*, 1992), and *Coussarea meridionalis* and *C. graciliflora* (Tavares and Vieira, 1994). Other species of *Simira* show variation in the sinuosity, such as *S. grazielae* and *S. pikia*, suggesting that others factors, besides luminosity, may be related to the expression of this characteristic. According to Barthlott (1981), the form of the anticlinal cell wall can be useful as a taxonomic value. The

straight anticlinal cell wall of *S. sampaiiana* was used to separate it from the other species.

The outer periclinal cell walls are organized forming layers of varied compositions that are observed by TEM. Few studies have utilized technical cytochemistry to identify these components (Lyshede, 1978; Tenberge, 1992; Barros and Miguens, 1998). In all species studied, three distinct layers were observed: the inner polysaccharide-rich layer mainly composed of cellulose, the intermediate cuticular layer, with a tree-like polysaccharic-rich network immersed in a matrix of cutine, and the cuticle proper in all species studied. Comparative analyses in *Simira* leaf blades have shown evidence of biochemical similarities that can be useful for taxonomy.

Rubiaceae trichomes have a relatively simple structure and present only minor diversity (Robbrecht, 1988). According to Metcalfe and Chalk (1950), they can be unicellular, uniseriate, in tuff or rarely stellate. In the species studied, trichomes were unicellular, pluricellular and stellate at both surfaces, except in *Simira grazielae*. Cuticular ornamentations were observed on the surface of the trichomes of *S. pikia*, *S. rubra* and *S. sampaiiana* as described for others Rubiaceae, i.e. *Bathysa australis* and *Psychotria suterella* (Barros *et al.*, 1997).

The vascular system in the *Simira* species shows a collateral arrangement described before in other rubiaceous species (Robbrecht, 1988). The presence of two layers of the bundle sheath extending until the adaxial epidermis observed in *Simira* species can constitute a diagnostic characteristic of the genus. The bundle sheath prevents the vascular terminations from being exposed to the air contained in the intercellular spaces; involved in the short distance transportation between the bundle and the mesophyll; the sheath extensions have facilitated this function, taking the product of the bundle to the epidermis cells (Dickison, 2000). The same author believes that these extensions provide an additional mechanical support for the leaf blade.

Several types of crystalline inclusions are found in Rubiaceae species and may be a taxonomic character. In the present investigation, prismatic crystals and crystal-sand were observed in the mesophyll cells corroborating with the occurrence of crystalline inclusions for this genus as described by Metcalfe and Chalk (1950). These crystal types have been already observed in wood of many genera of Cinchonoideae and Ixoroideae (Jansen *et al.*, 2002). In *S. pikia*, prismatic crystals were also observed in the parenchymatic bundle sheath. The presence of crystals in plants has



been attributed to a possible defense function against herbivory, ion balance role or tissue support (Franceschi and Horner Jr., 1980).

Robbrecht (1988) reported the presence of indolic, quinolinic and isoquinolinic alkaloids in tribes of Rubiaceae. Some benzilquinolinic alkaloids identified have been mentioned as an important taxonomic character (Dahlgren, 1975; Cronquist, 1981). Chromatographic analyses have isolated alkaloids from the wood and bark extract of the *S. glaziovii* (Alves *et al.*, 2001; Bastos *et al.*, 2001), *S. salvadorensis* and *S. rubra* (Arnason *et al.*, 1983). In the species studied, alkaloids were identified in mesophyll cells through the positive reaction to the Dragendorff reagent. Phytochemistry studies are necessary to determine their chemical composition.

The accumulation of specific proteins has a taxonomic value and can be used to additionally characterize plant taxa (Chen *et al.* 1997). Several studies have shown the importance of protein markers identification present in some families as: Poaceae (Chen *et al.*, 1997), Brassicaceae (El Naggar, 2001; Marques *et al.*, 1998), Cucurbitaceae (Pasha and Sen, 1991; Castro *et al.*, 1999), Fabaceae (Misset and Fontenelle, 1992) and Leguminosae (=Fabaceae) (Niknam *et al.*, 2004). These techniques have also been used in phylogenetic studies in the Rubiaceae family. Through the preliminary analysis of the present soluble proteins in the Rubiaceae species, the presence of a great diversity of molecular masses of several proteins can be observed. Some bands seem to be characteristic of each species or each genus and may come to represent specific markers to be used for identification of *Bathysa* and *Simira* genus.

The results reported here as the presence of domatia, "rubiaceous" stomata and dorsiventral mesophyll in *Simira* leaf blades confirms the leaf characteristics for the Rubiaceae family (Metcalf and Chalk, 1950; Robbrecht, 1988). The presence of two layers of the bundle sheath extending until the adaxial epidermis, as well as the presence of alkaloids may be taxonomic value in *Simira* genus, helping to segregate this genus. Micromorphological and anatomical aspects such as cuticle ornamentation, domatia type, epicuticular wax type, the pattern of anticlinal cell walls, and the presence or absence of trichomes, can be used to segregate the *Simira* species studied. The combination of anatomical and micromorphological aspects of the leaf blade may be helpful in the systematic of these species. However, it is necessary more information about others *Simira* species for the genus comparative study.

## Acknowledgements

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Amparo a Pesquisa do Rio de Janeiro (FAPERJ), Coordenação de Aperfeiçoando de Pessoal de Nível Superior (CAPES), the Fundação Margareth Mee, and the PETROBRÁS for their financial support. We thank the technicians M.A.S.C. Dutra, B.F. Ribeiro, and G.A. de Moraes of LBCT/CBB/UENF. Mr. Walter da Silva for the help in plant collects. This study forms part of the MSc degree thesis of T.M.S.M., carried out at the Universidade Estadual do Norte Fluminense.

## References

- Adâmolí de Barros MA (1959). Ocorrência das domácias na família Rubiácea. *Anais da Escola Superior de Agricultura "Luiz de Queiroz"* **16**: 311-337.
- Agrawal AA, Karban R, Colfer RG (2000). How leaf domatia and induced plant resistance affect herbivores, natural enemies and plant performance. *Oikos* **89**: 70-80.
- Agrawal AA, Karban R (1997). Domatia mediate plant arthropods mutualism. *Nature* **387**: 562-563.
- Alves CCF, Cranchi DC, Carvalho MG, Silva SJ (2001). Triterpenos, esteróide glicosilado e alcalóides isolados de *Simira glaziovii*. *Floresta e Ambiente* **8**: 174-179.
- Andersson L (2001). *Margaritopsis* (Rubiaceae, Psychotriaceae) is a pantropical genus. *Systematics and Geography of Plants* **71**: 73-85.
- Angermüller S, Fahimi DH (1982). Imidazole-buffered osmium tetroxide: an excellent stain for visualization of lipids in transmission electron microscopy. *Histochemical Journal* **14**: 823-825.
- Arnason T, Morand P, Salvador J, Reyes I, Lambert J, Towers N (1983). Phototoxic substances from *Flaveria trinervis* and *Simira salvadorensis*. *Phytochemistry* **22**: 594-595.
- Barros CF, Callado CH, Da Cunha M, Costa CG, Pugialli HRL, Marquete O, Machado RD (1997). Anatomia ecológica e micromorfologia foliar de espécies de floresta montana na Reserva Ecológica de Macaé de Cima. In: *Serra de Macaé de Cima: florística e conservação em mata atlântica* (HC Lima, RR Guedes-Bruni, eds.), p. 275-296. Instituto de Pesquisa do Jardim Botânico do Rio de Janeiro Rio de Janeiro, Rio de Janeiro.
- Barros CF, Miguens FC (1998). Ultrastructure of the epidermal cell of *Beilshmeidia rigida* (Mez) Kosterm (Lauraceae). *Acta Microscopica* **7**: 1-11.
- Barthlott W (1981). Epidermal and seed surface characteristics of plants: systematic applicability and some evolutionary aspects. *Nordic Journal of Botany*. **1**: 345-354.
- Barthlott W, Neinhuis C, Cutler D, Ditsch F, Meuse I, Theisen I, Wilhelmi H (1998). Classification and terminology of plant epicuticular waxes. *Botanical Journal of the Linnean Society* **126**: 237-260.
- Bastos ABFDO, Carvalho MG, Velandia JR, Braz-Filho R (2001). Constituintes químicos isolados de *Simira glaziovii* (K. Schum) Steyerl. e a distribuição dos deslocamentos

- químicos dos átomos de carbono e hidrogênio do alcalóide ofiorina e seus derivados. *Química Nova* **25**: 241-245.
- Bradford MM (1976). A rapid sensitive method for the quantification of microgram quantities of protein utilising the principle of dye binding. *Biochemistry* **72**: 248-254.
- Bredenkamp CL, Van Wyk AE (2000). The epidermis in *Passarina* (Thymelaeaceae): structure, function and taxonomic significance. *Bothalia* **30**: 69-86.
- Bremer B (1996). Phylogenetic studies within Rubiaceae and relationships to other families based on molecular data. *Opera Botanica Belgica* **7**: 33-50.
- Bremer B, Andreasen K, Olsson D (1995). Subfamilial and tribal relationships in the Rubiaceae based on *rbcL* sequence data. *Annals of the Missouri Botanical Garden* **82**: 383-397.
- Bremer B, Thulin M (1998). Collapse of Isertieae, re-establishment of Mussaendae, and a new genus of Sabiceae (Rubiaceae); phylogenetic relationships based on *rbcL* data. *Plant Systematics and Evolution* **211**: 71-92.
- Callado CH, Pugialli HRL, Costa CG, Da Cunha M, Marquete O, Barros CF (1997). Anatomia do lenho de espécies da mata atlântica: interpretação ecológica e indicações para aproveitamento. In: *Serra de Macaé de Cima: florística e conservação em Mata Atlântica Lima* (HC Lima, RR Guedes-Bruni eds.), p. 251-274. Instituto de Pesquisa do Jardim Botânico do Rio de Janeiro, Rio de Janeiro.
- Callado CH, Silva Neto SJ (2003). Anatomia do lenho de três espécies do gênero *Simira* Aubl. (Rubiaceae) da Floresta Atlântica do Estado do Rio de Janeiro. *Rodriguésia* **54**: 23-33.
- Castro HÁ, Gonzalez SR, Prat MI, Villmil CB (1999). Use of the western blot in botanical taxonomy. *Phyton* **65**: 185-196.
- Chen LX, Fischer H, Jensen U (1997). Accumulation of seed storage proteins and the taxonomy of Poaceae. *Plant Systematics and Evolution* **206**: 243-257.
- Combes R (1946). *La forme des végétaux et le milieu*. Librairie Armand Colin, Paris.
- Costa AF (1982). *Farmacognosia*, Vol. 3. Fundação Calouste Gulbekian, Lisboa.
- Cronquist A (1981). *An integrated system of classification of flowering plants*. Columbia University Press, New York.
- Da Cunha M, Vieira RC (1993/97). Anatomia foliar de *Psychotria velloziana* Benth. (Rubiaceae). *Rodriguésia* **45/49**: 39-50.
- Dahlgren RT (1975). A system of classification of the angiosperms to be used to demonstrate the distribution of characters. *Botaniska Notiser* **128**: 119-147.
- Dessein S, Jansen S, Huysmans S, Robbrecht E, Smets E (2001). A morphological and anatomical survey of *Virectaria* (African Rubiaceae), with a discussion of its taxonomic position. *Botanical Journal of the Linnean Society* **137**: 1-29.
- Dickison WC (2000). *Integrative plant anatomy*. Harcourt Academic Press, San Diego.
- El Naggar SM (2001). Implications of seed proteins in Brassicaceae systematics. *Biologia Plantarum* **44**: 547-553.
- Franceschi VR, Horner Jr HT (1980). Calcium oxalate crystals in plants. *Botanical Review* **46**: 361-427.
- Gomes DMS, Mantovani A, Vieira RC (1995). Anatomia foliar de *Psychotria ternuinerives* Müll. Arg. e *Psychotria stenocalix* Müll. Arg. (Rubiaceae). *Arquivos de Biologia e Tecnologia* **38**: 15-33.
- Granier F (1988). Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis* **9**: 712-718.
- Guedes-Bruni RR (1998). Composição, estrutura e similaridade florística de dossel em seis unidades de Mata Atlântica no Rio de Janeiro. Tese de doutorado, Universidade de São Paulo, São Paulo.
- Jansen S, Lens F, Ntore S, Piesschaert F, Robbrecht E, Smets E (2001). Contributions to the wood anatomy of the Rubioideae (Rubiaceae). *Journal of Plant Research*. **14**: 269-289.
- Jansen S, Robbrecht E, Beeckman H, Smets E (2002). A survey of the systematic wood anatomy of the Rubiaceae. *IAWA Journal* **23**: 1-67.
- Jensen WA (1962). *Botanical Histochemistry (Principles and Practice)*. W. H. Freeman and Company, São Francisco.
- Judd WS, Campbell CS, Kellogg EA, Stevens PF (1999). *Plant systematics: A phylogenetic approach*. Sinauer Associates Inc. Publishers Sunderland, Massachusetts.
- Juniper BE, Jeffree D (1983). *Plant Surfaces*. Edward Arnold Publishers Ltd., London.
- Klein DE, Gomes VM, Silva-Neto SJ, Da Cunha M (2004). The structure of colleters in several species of *Simira* (Rubiaceae). *Annals of Botany* **94**: 733-740.
- Kocsis M, Darók J, Borhidi A (2004). Comparative leaf anatomy and morphology of some neotropical *Rondeletia* (Rubiaceae) species. *Plant Systematics and Evolution* **218**: 205-218.
- Kong H (2001). Comparative morphology of leaf epidermis in the Chloranthaceae. *Botanical Journal of the Linnean Society* **136**: 279-294.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage  $\phi_4$ . *Nature* **227**: 680-685.
- Leo RRT, Mantovani A, Vieira RC (1997). Anatomia foliar de *Rudgea ovalis* Müll. Arg. e *R. tinguana* Müll. Arg. (Rubiaceae). *Leandra* **12**: 33-44.
- Luft JH (1971). Ruthenium red and violet. ii. fine structural localization in animal tissues. *Anatomical Record* **171**: 369-376.
- Lyshede OB (1978). Studies on outer epidermal cell walls with microchannels in a xerophytic species. *New Phytology* **80**: 421-426.
- Mantovani A, Vieira RC (1993/97). Leaf surface of two understory shrubs *Rudgea decipiens* Müll. Arg. and *Rudgea macrophylla* Benth. (Rubiaceae). *Rodriguésia* **45/49**: 7-13.
- Marques MR (1998). *Interação entre uma endopoligalacturonase de Mucor ramosissimus (mucorales) e a pectina da parede celular de Palicourea marcgravii (Rubiaceae) na indução de respostas de defesa de plantas*. 166 p.. Escola Paulista de Medicina – Universidade Federal de São Paulo, São Paulo.
- McDowell T, Bremer B (1998). Phylogeny, diversity, and distribution in *Exostema* (Rubiaceae): implications of morphological and molecular analyses. *Plant Systematics and Evolution* **212**: 215-246.
- McLean RC, Cook WRL (1958). *Plant Science Formulae*. Macmillan & Company Ltd., London.
- Metcalf CR, Chalk L (1950). *Anatomy of dicotyledons*. Clarendon Press, Oxford.
- Misset MT, Fontenelle C (1992). Protein relationships between natural populations of *Ulex europaeus* and *U. galli* (Faboideae, Genisteae) and their hybrids. *Plant Systematics and Evolution* **179**: 19-25.
- Myers N, Mittermeier RA, Mittermeier CG, Fonseca GAB, Kent J (2000). Biodiversity hotspots for conservation priorities. *Nature* **403**: 853-858.
- Nascimento MVO, Gomes DMS, Vieira RC (1996). Anatomia foliar de *Bathysa stipulata* (Vell.) Presl. (Rubiaceae). *Revista Unimar* **18**: 387-401.

- Nepokroeff M, Bremer B, Systma K (1999). Reorganization of the genus *Psychotria* and tribe Psychotrieae (Rubiaceae) inferred from ITS and *rbcL* sequence data. *Systematic Botany* **24**: 5-27.
- Niknam V, Sharifizadeh B, Ebrahimzadeh H, Zarre Sh, Izadpanah M (2004). Comparative study of proteins in seed of some species of *Trigonella* from Iran. *Iranian International Journal of Science* **5**: 1-11.
- O'Dowd DJ, Willson MF (1989). Leaf domatia and mites on Australasian plants: ecological and evolutionary implications. *Botanical Journal of Linnean Society* **37**: 191-238.
- Pasha MK, Sen SP (1991). Seed protein patterns of Cucurbitaceae and their taxonomic implications. *Biochemical Systematics and Ecology* **19**: 569-576.
- Persson C (2000). Phylogeny of the Neotropical *Alibertia* group (Rubiaceae), with emphasis on the genus *Alibertia*, inferred from ITS and 5S ribosomal DNA sequences. *American Journal of Botany* **87**: 1018-1028.
- Piesschaert F, Andersson L, Jansen S, Dessein S, Robbrecht E, Smets E (2000). Searching for the taxonomic position of the African genus *Colletocema* (Rubiaceae): morphology and anatomy compared to an rps16- intron analysis of Rubioideae. *Canadian Journal of Botany* **78**: 288-304.
- Robbrecht E (1988). Tropical woody Rubiaceae. Characteristic features and progressions. Contributions to a new subfamilial classification. *Opera Botanica Belgica* **1**: 1-271.
- Rova JHE, Delprete PG, Anderson L, Albert VA (2002). A trnL-F CPDNa sequence study of Condamineae-Rondeletieae-Sipaneeae complex with implications on the phylogeny of Rubiaceae. *American Journal of Botany* **89**: 145-159.
- Silva Neto SJ (2000). *O gênero Simira Aubl. (Rubiaceae, Rondeletieae) no Brasil extra-amazônico*. Magister thesis, Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro.
- Struwe L (2002). *Gentianales (Coffeees, Dogbanes, Gentians and Milkweeds)*. In: Macmillan Publishers Ltd, Nature Publishing Group/ www.els.net Encyclopedia of life.
- Tavares ES, Vieira RC (1994). Anatomia foliar de *Coussarea meridionalis* (Vell.) Muel. Arg. e *Coussarea graciliflora* Benth. & Hook (Rubiaceae). *Bradea* **39**: 320-330.
- Tenberge KB (1992). Ultrastructure and development of the outer epidermal wall of spruce (*Picea abies*) needles. *Canadian Journal of Botany* **70**: 1467-1487.
- Thiéry JP (1967). Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *Journal de Microscopie* **6**: 987-1016.
- Vieira RC, Delprete PG, Leitão GG, Leitão SG (2001). Anatomical and chemical analyses of leaf secretory cavities of *Rustia formosa* (Rubiaceae). *American Journal of Botany* **88**: 2151-2156.
- Vieira RC, Gomes DMS (1995). Superfície da lâmina foliar de *Psychotria nuda* (Cham. & Schltdl.) Wawra, *P. leiocarpa* Cham. & Schltdl., *P. stenocalix* Müll. Arg. e *P. tenuinervis* Müll. Arg. (Rubiaceae). *Acta Botanica Brasilica* **9**: 263-270.
- Vieira RC, Gomes DMS, Ferraz CL (1992). Anatomia foliar de *Psychotria nuda* Wawra e *Psychotria leiocarpa* Mart. (Rubiaceae). *Hoehnea* **19**: 185-195.



