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ARTICLE

Domatia and leaf blade structure of *Rudgea eugenioides* (Rubiaceae)

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ABSTRACT: (Domatia and leaf blade structure of *Rudgea eugenioides* (Rubiaceae)). *Rudgea eugenioides* is a rare woody species that grows in the understory of well-preserved Atlantic Forest of Serra dos Órgãos, in the state of Rio de Janeiro, Brazil. Leaves were collected in *Reserva Ecológica de Macaé de Cima*, Rio de Janeiro. The anatomical characteristics of the leaf blade were examined, especially the epidermis, using light and electron microscopy. The abaxial surface was smooth, with gap domatia between the midrib and the secondary veins and papillae. Micromorphological analyses showed epicuticular wax without any ornamentation. Leaf cross sections of *R. eugenioides* showed that the epidermis consisted of one-layer of cells covered with a fine cuticle and parallelocytic stomata. Light microscopy revealed that the periclinal cell walls were flat and that the anticlinal walls were predominantly straight and thicker. The leaf blade contained dorsiventral mesophyll with crystal idioblasts and collateral vascular bundles. Transmission electron microscopy allowed the arrangement of the outer periclinal cell wall to be observed. The results identified anatomical and ultrastructural characteristics that may contribute to understanding the taxonomy of this species and the survival strategies it uses in this forest environment.
Key words: Leaf anatomy, cuticle, domatia, outer epidermal cell wall, papillae, plant ultrastructure.

RESUMO: (Estrutura da lâmina foliar e da domácia de *Rudgea eugenioides* (Rubiaceae)). *Rudgea eugenioides* é uma espécie arbórea rara no sub-bosque de florestas bem preservadas da Serra dos Órgãos, na Floresta Atlântica do Estado do Rio de Janeiro, Brasil. Folhas foram coletadas na Reserva Ecológica de Macaé de Cima, Rio de Janeiro e observadas características anatômicas da lâmina foliar, com especial atenção à epiderme, usando microscopia óptica e eletrônica. A superfície abaxial é lisa, apresentando domácias do tipo em fenda entre a nervura principal e as secundárias e papilas. Análises micromorfológicas mostraram filme contínuo de cera sem qualquer ornamentação. Cortes transversais da lâmina foliar de *R. eugenioides* mostrou epiderme adaxial e abaxial com uma camada de células cobertas com uma cutícula delgada e estômatos paralelocíticos apenas na superfície abaxial. Em microscopia óptica, as paredes periclinais mostraram-se planas e as paredes anticlinais predominantemente retas e espessas. A lâmina foliar apresenta mesofilo dorsiventral com idioblastos cristalíferos e feixes vasculares colaterais. Em microscopia eletrônica de transmissão, foi observado o arranjo da parede periclinal externa. Os resultados identificaram características anatômicas e ultraestruturais que podem ser relevantes para subsidiar a taxonomia e estratégias de sobrevivência no ambiente de floresta.
Palavras-chave: anatomia foliar, cutícula, domácia, parede periclinal externa, papila, ultraestrutura vegetal.

INTRODUCTION

The Rubiaceae are comprised of approximately 650 genera and 12,000 species, which have a wide geographical distribution and occur mostly in tropical and subtropical regions. Fifty-seven species of Rubiaceae have been identified at the *Reserva Ecológica de Macaé de Cima*, in the Nova Friburgo municipality, within the state of Rio de Janeiro, Brazil. *Rudgea eugenioides* is a rare species found in the understory of well-preserved Atlantic Forest, of Nova Friburgo and Teresópolis, in this state (Gomes 1996). The Atlantic Forest constitutes the second most threatened ecosystem in the world, and has been reduced over the years (Myers *et al.* 2000). The drastic reduction of this forest may be attributed mainly to timber exploitation, urban sprawl and an increase in the amount of land used for cultivation and livestock. The high diversity and complex ecological organization of these forests are still poorly known and, unfortunately, insufficiently protected.

Anatomical and ultrastructural studies have indicated important characteristics capable of distinguishing taxa, and have also related structural characteristics to the environment (Bredenkamp & Van Wyk 2000; Kong 2001). The comparative leaf anatomy and micromorphology of *Psychotria* (Da Cunha & Vieira 1997, Gomes *et al.* 1995, Vieira & Gomes 1995), *Rondeletia* (Kocsis *et al.* 2004), *Rudgea* (Mantovani & Vieira 1997, Leo *et al.* 1997, Alves *et al.* 2004, Silva *et al.* 2004), *Rustia* (Vieira *et al.* 2001), *Coussarea* (Tavares & Vieira 1994), *Bathysa* (Nascimento *et al.* 1996, Barros *et al.* 1997, Moraes 2005), and *Simira* (Moraes *et al.* 2009) have been studied, as well as the wood anatomy of several timber species of Rubiaceae from the Atlantic Forest (Callado *et al.* 1997).

The plant epidermis plays a number of key roles, including interruption of apoplastic movement, pre-

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vention of transpiration, helping to focus light in shady environments and protecting the plant against high amounts of radiation (Juniper & Jeffree 1983, Bone *et al.* 1985, Tenberge 1992). Taxonomic features of epidermal micromorphology, ultrastructure and composition have been studied (Bussotti *et al.* 1995, Schreiber & Riederer 1996, Barros & Miguens 1998), and it is known that cuticle arrangement and composition may vary depending on the plant species and environmental conditions (Koteyeva 2005).

The epidermis also presents different structures like papillae and domatia, which help the plant interact with the environment. Previous leaf anatomical studies of *Rudgea* identified papillae on the lower surface and a striate cuticle on its epidermal cells (Mantovani *et al.* 1995, Leo *et al.* 1997).

Leaf domatia are small structures that often harbor predaceous arthropods that are potentially beneficial to the plant (Agrawal *et al.* 2000). All known types of domatia (tufts of hairs, pocket-, pit-, cave- and dome-excavation; glabrous, ciliate or hairy excavations) and a number of transitional types occur in the Rubiaceae (Robbrecht 1988, Brouwer & Clifford 1990).

In an effort to increase our knowledge of the anatomy of Rubiaceae species from the Atlantic Forest, this work aimed to investigate the leaf blade anatomy and ultrastructure of *Rudgea eugenioides* in order to gain detailed information about the epidermis and arrangement of domatia in this species. In particular, this study focused on identifying anatomical and ultrastructural characteristics that could be relevant to the taxonomy of this species, and the group it belongs to, or that could be a survival strategy used in this forest environment.

MATERIALS AND METHODS

Plant

Young and adults leaves of *Rudgea eugenioides* Standl. (Rubiaceae, Psychotrieae) were obtained from plants collected at the *Reserva Ecológica de Macaé de Cima* (22° 21' to 22° 28' S 42° 27' to 42° 35' W), in the Nova Friburgo municipality, within the sate of Rio de Janeiro (Brazil). The specimens were deposited in the Rio de Janeiro Botanical Garden Herbarium.

Light microscopy

Mature leaves were selected and the samples were fixed in formalin-acetic-alcohol, dehydrated in ethanol and embedded in paraffin (58–60° C) (Johansen, 1940). Sections (10–12 μm) were stained with fucsin-astrablau (Roeser 1962). Sudan IV was used to localize lipids, chlor-zinc-iodine to recognize cellulose, and 0.2% Ruthenium red was used to identify pectin (Johansen 1940). The specimens were observed with a ZEISS Axioplan photomicroscope. Digital data were obtained using ZEISS Analysis® software.

Electron microscopy

Leaf fragments (2.0 mm²) were fixed in a 2.5% glutaraldehyde and 2.0% formaldehyde, in a 0.05 M cacodylate buffer, at 7.4 pH, for two hours at room temperature. The fragments were post-fixed with 1.0% OsO_4 in the same buffer. Subsequently, samples were rinsed in distilled water, stained with 0.5% aqueous uranyl acetate at room temperature for two hours, dehydrated in acetone and embedded in epoxi resin (Polybed). The contrast of the sections was enhanced with uranyl acetate followed by lead citrate and observed using a ZEISS 900 transmission electron microscope (TEM). The following cytochemical procedures for TEM were performed: periodic acid--thiocarbohydrazide-silver proteinate (PATAg) for polysaccharides containing 1, 2-glycol groups. The controls performed were (i) periodic acid was left out and (ii) TCH was left out (37); 1% Ruthenium red (Luft 1971) was used to detect pectin. Imidazole-buffered osmium tetroxide was used to detect lipid compounds (Angermüller & Fahimi 1982). For scanning electron microscopy, after fixation, the leaves were dehydrated in acetone, critical point dried in CO_2, sputter coated with 20 nm gold and observed with a ZEISS 962 scanning electron microscope. In addition, to verify the presence of epicuticular waxes, samples were boiled in chloroform for 2 minutes before critical point drying.

RESULTS

Adult leaves of *Rudgea eugenioides* have an epicuticular wax without any ornamentation on the adaxial surface (Fig. 1A), which was confirmed by the use of chloroform as a control. Scanning electron microscopy revealed that the outer periclinal cell wall of the adaxial epidermis is nearly flat and that the anticlinal cell walls could not be clearly observed in frontal view (Fig. 1A). Ordinary epidermal cells of the abaxial surface were convex, smooth and their limits could be seen in frontal view. The abaxial surface had a smooth cuticle (Fig. 1B). As expected for plants collected in the field, both leaf surfaces had fungal hyphae and precipitated granules (Figs. 1A and 1B).

On the abaxial surface, domatia were observed in young and adult leaves on the acute angle between the midrib and the secondary veins (Figs. 1C to 1F). The lengths of the domatia varied relative to leaf maturity. In this species, the domatia were characterized as a cavity covered with trichomes. The development of these structures begins in young leaves that are less than 3.0 cm long (Fig. 1D), with the differentiation of trichomes. Afterwards, invaginations form on the leaf surfaces that become the cavities, which extend to the mesophyll or midrib (Fig. 1E). Epidermal cells of the domatia were smaller when compared to ordinary epidermal cells (Figs. 1E and 1F).

The transverse section of the *R. eugenioides* leaf blade showed an adaxial and abaxial epidermis with a layer of cells that was compactly arranged and covered with a fine cuticle (Fig. 2A). Light microscopy showed that the periclinal cell walls were flat and that the anticlinal walls were predominantly straight and thicker. The abaxial epidermis had papillae with a remarkably thicker cuticle (Fig 2B). Trabeculae (resembling crossbars) were observed in some ordinary epidermal cells (Fig. 2B). The leaf of *R. eugenioides* was hypostomatic, with parallelocytic stomata (Fig. 1B). The mesophyll was dorsiventral, with one palisade layer and 5 to 6 layers of spongy parenchyma. Crystalliferous idioblasts with raphids were observed among the mesophyll cells (Fig. 2C). The midrib showed

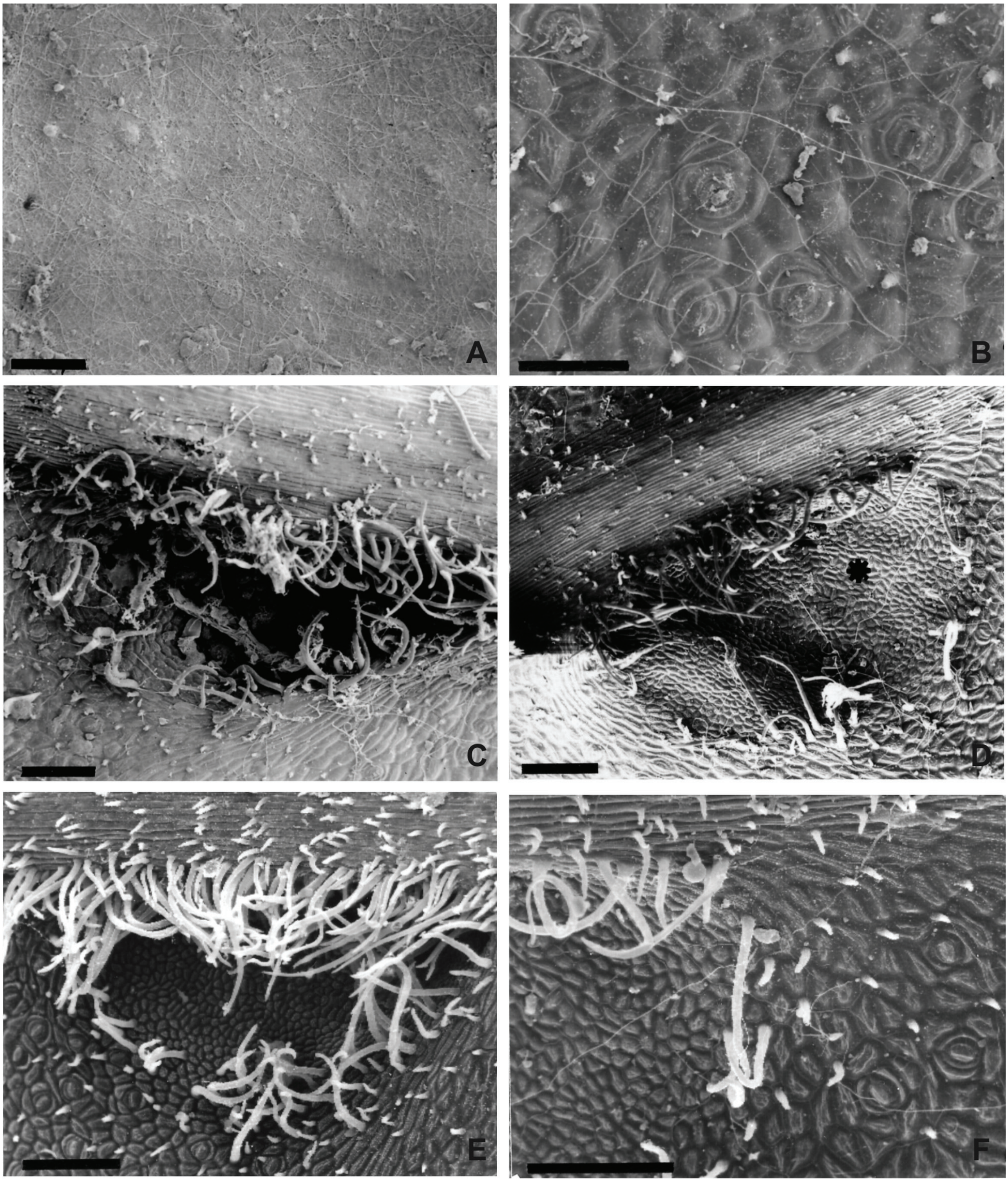

Figure 1. Surface of *Rudgea eugenioides* Standl. Leaf blade. A. Adaxial surface showing epicuticular wax without ornamentation; B. Abaxial surface with smooth cuticular relief; C. Domatia (of a mature leaf) between the midrib and secondary vein, covered by trichomes; D. Domatia of immature leaf (3.0 cm) showing trichomes and epidermal cell differentiation (asterisk); E. Domatia of immature leaf (4.0 cm) showing trichome differentiation and cavity development; F. Domatia of immature leaf (4.0 cm). Note the difference between the ordinary epidermal cells and the epidermal cells of the domatia. Scanning electron microscopy. Scale bars = 100 µm (A and B); 200 µm (C–F).

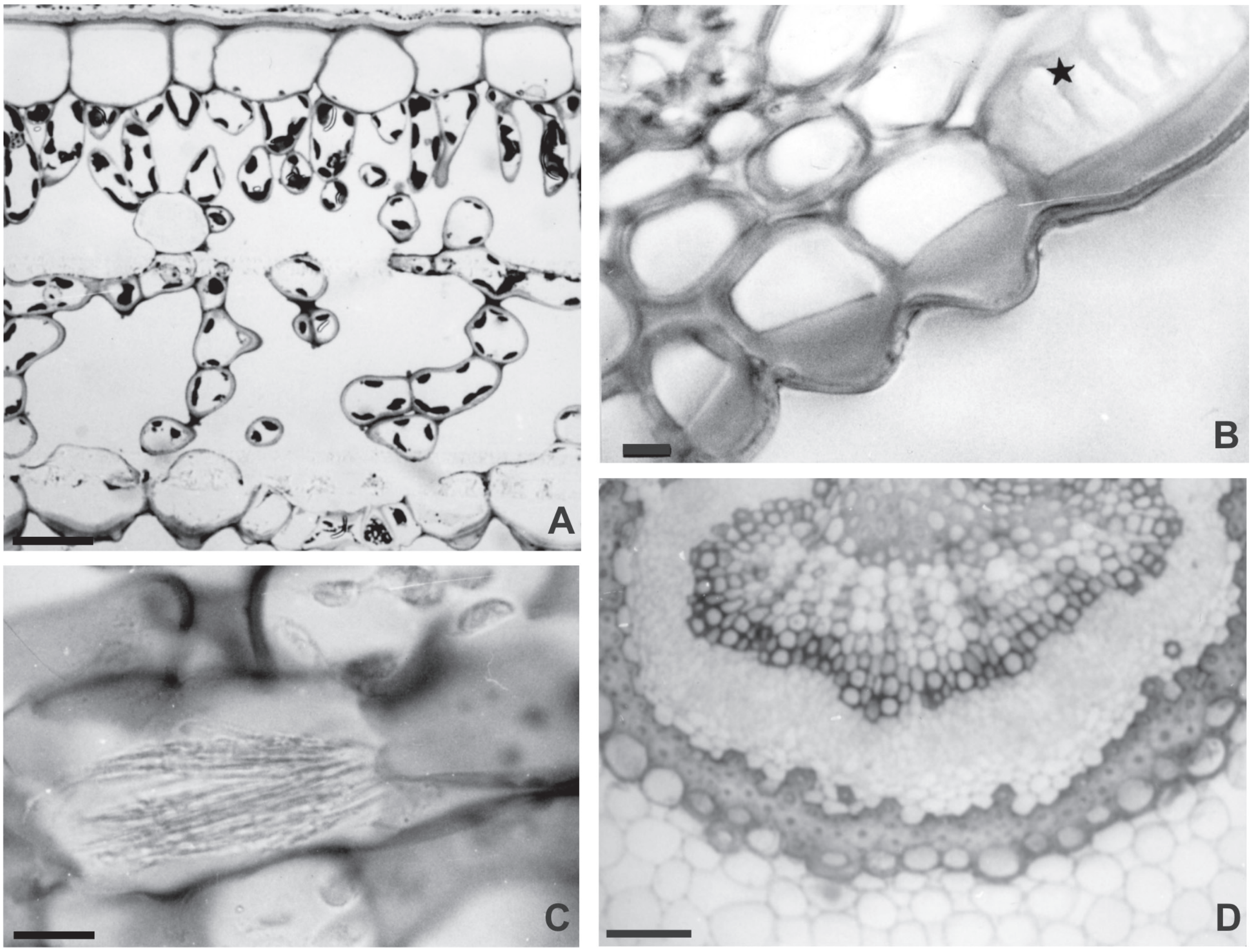

Figure 2. Transverse section of *R. eugenioides* Standl. A. Leaf blade showing unisseriate adaxial and abaxial epidermis surface and dorsiventral mesophyll; B. Papillae in abaxial epidermis. Note thickness of crossbar (star); C. Raphids in mesophyll cells; D. Circular collateral vascular bundle in principal vein. Light microscopy. Scale bars = 20 µm (A); 5 µm (B); 3 µm (C); 2 µm (D).

a circular collateral vascular bundle from the petiole to the leaf apex, which was surrounded by perivascular fibers (Fig. 2D). Angular collenchyma occurred close to epidermis in the midrib.

Transmission electron microscopy showed that the cuticular membrane had three morphological layers in the outer periclinal cell wall of the epidermis (Fig. 3A). According to Tenberge (1992), these layers are the lamellar layer (L1), cuticular layer (L2), and the cuticle proper (L3). The inner lamellar layer was mainly composed of polysaccharides; in the papillae, an increase in the rich polysaccharide layer could be observed (Figs. 3B and 3C). The cuticular layer was subdivided into a tree-like polysaccharide network and reticulated layer, immersed in a matrix of lipid compounds. The outer layer was a cuticle proper that appeared homogenous. The cuticle proper and reticulated layer reacted strongly when treated with the Imidazol/Osmium method (Fig. 4A), which revealed their lipid compositions. The tree-like polysaccharide network and the polysaccharide layer reacted with both PATAg (specific for polysaccharides) (Fig. 4B) and Ruthenium red (specific for pectins) (Fig. 4C).

DISCUSSION

The anatomical study of the leaf blade of *Rudgea eugenioides* disclosed that its structure reflects the general characteristics found in the Rubiaceae, i.e., dorsiventral mesophyll and crystals as cited for Metcalfe & Chalk (1950) and Robbrecht (1988). In this species, no differences in the vascular system were observed, which is similar to other species of *Rudgea*, i.e., *R. ovalis* and *R. tinguana* (Leo *et al.* 1997) and *R. jasminoides* (Silva *et al*. 2004).

Rudgea eugenioides has domatia located at the acute angle between the midrib and secondary veins on the abaxial surface. Brouwer & Clifford (1990) found that 122 genera and 780 species of Rubiaceae have domatia. Leaf domatia have also been observed in other Rubiaceae genera, i.e., *Bathysa* and *Simira* (Moraes 2005, Moraes *et al.* 2009), *Rudgea* (Leo *et al.* 1997) and *Psychotria* (Da Cunha & Vieira 1997). Domatia are small morphogenetic structures that commonly occur in the vein axils and margins on the leaf undersurface of many woody dicotyledons (Brouwer & Clifford 1990, O´Dowd & Pemberton 1994). Leaf domatia are found predominantly in woody plants of humid tropical or subtropical regions

(Nakamura *et al.* 1992, Agrawal & Karban 1997), such as the *Reserva Ecológica de Macaé de Cima*, where *R. eugenioides* grows. These structures frequently shelter small predators or fungivorous arthropods. It has been suggested that these organisms are indirectly beneficial for the plant because they reduce the herbivorous arthropods and pathogenic fungi that commonly inhabit the surfaces of plant leaves (Agrawal 1997, Agrawal *et al* 2000, O' Dowd & Wilson 1989).

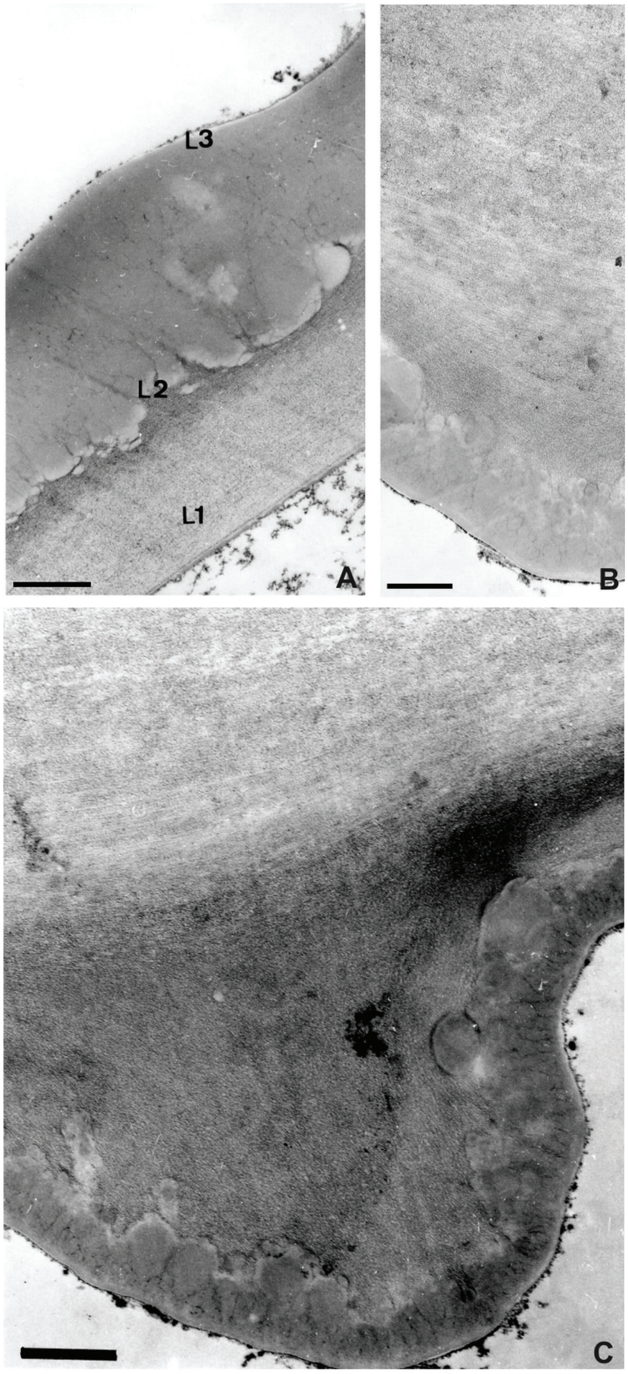

Figure 3. Outer periclinal cell wall of *R. eugenioides* leaf blade. A. Adaxial epidermal surface. (L1) polysaccharide layer, (L2) cuticular layer, (L3) the proper cuticle; B. Papillae in abaxial surface; C. Papillae in abaxial epidermal surface. Transmission electron microscopy. Scale bars = 0.9 µm (A and B); 1.02 µm (C).

Micromorphological studies have been an important contribution to the characterization of surface structures, to relating structural characteristics to the environment where a plant grows, and to indicating the specific functions of vegetative and reproductive organs (Klein *et al.* 2007). Some aspects of leaf micromorphology might reflect the adaptation of plants to their habitats (Juniper & Jeffree 1983); however, several characters can also provide conclusive data for the separation of taxa (Barthlott 1981). Bergen (1904) reported smooth cuticles in leaves that grow in shady, mesophytic and hygrophytic environments, and ornamented cuticles in species that grow in xeric environments. *Rudgea eugenioides,* which grows in the understory, showed a fine structured epicuticular wax without ornamentation. This was verified by washing the leaves in chloroform and comparing them to unwashed specimens using an SEM. The abaxial leaf surface of *R. eugenioides* had a smooth cuticular relief, except for the stomata, and reflected the contours of the cells bellow. The adaxial leaf surface had a tenuous wavy cuticular relief. These features contrasted with other species of Rubiaceae were both surfaces are smooth, such as in species of *Psychotria* (Vieira *et al.* 1992, Gomes *et al.* 1995, Vieira & Gomes 1995), or striated, such as in species of *Rudgea* that grow in sandy coastal vegetation (Mantovani *et al.* 1995). Smooth cuticles have been reported for the leaves of plants that grow in shady environments and ornamented cuticles have been reported for plants with leaves that are exposed to direct sunlight, such as in olive trees (Bergen 1904) and dicotyledons in general (Dunn *et al.* 1965). These data suggest that the occurrence of smooth and ornamented cuticles in Rubiaceae species, including *R. eugenioides*, may be related to the amount of exposure to light.

The periclinal cell walls of *R. eugenioides* were convex and the anticlinal walls were predominantly straight and thicker. Papillae were observed in the abaxial epidermis. The cells that had papillae on the surface were more resistant than the cells that did not possess them. Chemically, papillae are composed of callose, lignin or other phenolic derivatives, cellulose, or silicon. It is possible that papillae function as a mechanical barrier to fungi by inhibiting the penetration of fungal hyphae (Dickinson 2000). In *R. eugenioides* and other species of the Atlantic Forest it is common to find many microorganisms on leaf blades. The results suggest that the papillae help protect the leaf surface. In addition, previous leaf anatomical studies about *Rudgea* identified papillae on the lower surface and a striate cuticle in epidermal cells and suggested that these characters are of possible taxonomic importance (Mantovani *et al.* 1995, Leo *et al.* 1997).

Haberlandt (1914), following a study of plants in tropical rain forests, thought that the function of papillose epidermal cells was to concentrate limited light by acting as lenses. Bredenkamp and Van Wyk (1999) speculated that, in *Passerina*, the convex outer periclinal epidermal cell wall may well focus light onto the mesophyll, and that large vacuoles filled with phenols and the mucilage

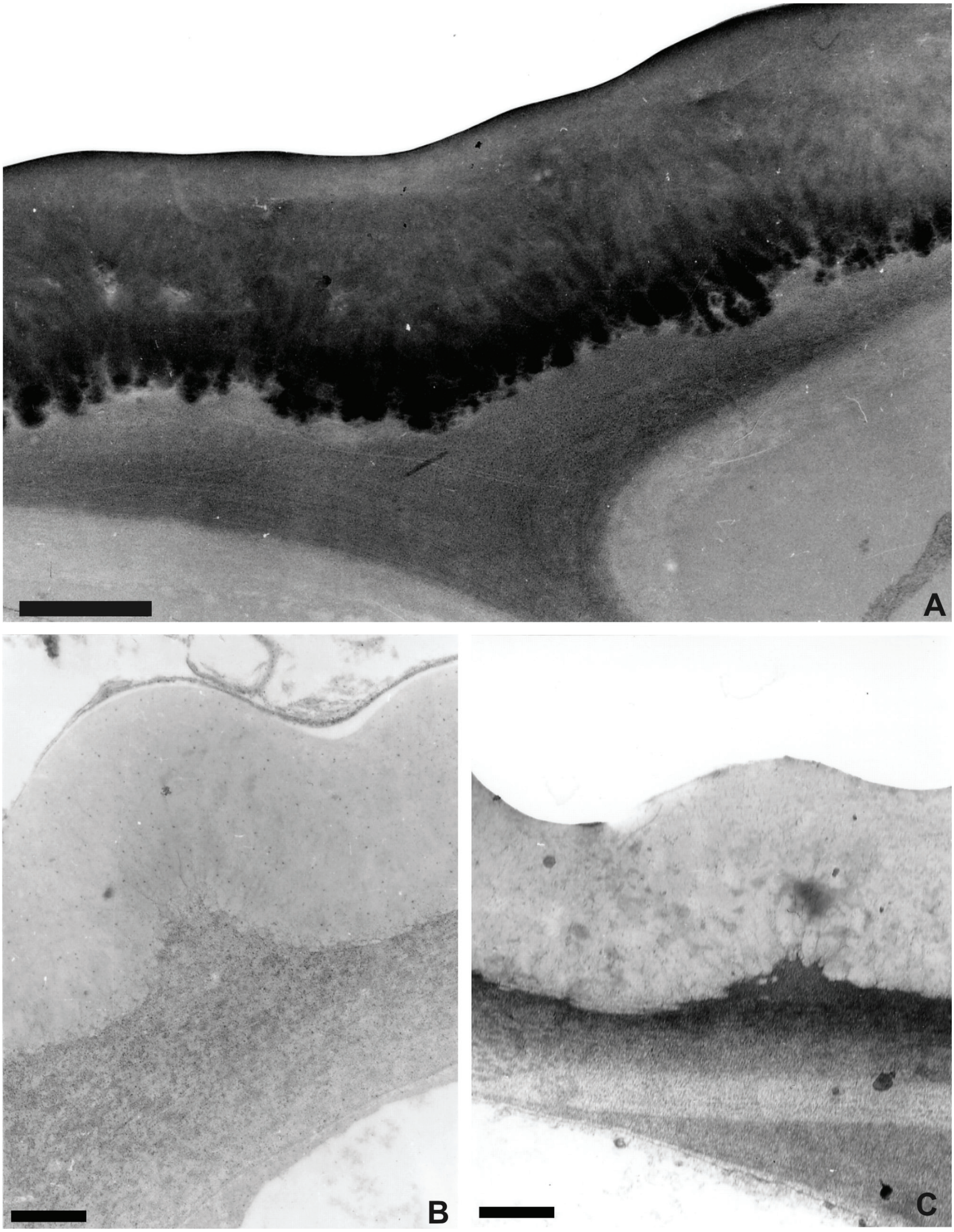

Figure 4. Cytochemical tests in outer periclinal cell wall. A. Adaxial epidermis with osmium-imidazole treatment; B. Adaxial epidermal surface with Thiery treatment; C. Adaxial epidermal surface with Ruthenium red treatment and Thiery Treatment. Transmission electron microscopy. Scale bars = 2.04 µm (A); 0.6 µm (B and C).

formed by the cellulose slimes (inner periclinal walls) protect the mesophyll from potentially dangerous UV-B radiation. According Wilkinson (1979) the presence and prominence of papillae are diagnostically unreliable because they vary with the climate or distribution of the species; only morphologically distinct types can be used for diagnostic purposes, such as in *Passarine* (Bredenkamp & Van Wyk 2000).

Concave epidermal cells with unicellular, unisseriate trichomes that are relatively small, and stomata, with guard cells at same level of the epidermis, are characteristic of shade leaves, as found in *R. eugenioides*. The indumentum likely plays an important role in water relations of the plant, and leaf arrangement is of vital importance to the physiology of the plant. The epidermis serves as an envelope, physically protecting the mesophyll, and the largest part of the abaxial epidermis forms a multifunctional barrier to the outside environment.

Several types of crystalline inclusions occur in Rubiaceae species and may be good taxonomic characters. In the present investigation, crystal idioblasts with raphids were observed in the mesophyll, corroborating the occurrence of crystalline inclusions in other species of the genus, i.e., *R. ovalis* (Leo *et al.* 1997) and *R. viburnoides* (Alves *et al.* 2004), as well as other species in the family (Metcalfe & Chalk 1950). The major functions of calcium oxalate (CaOx) crystals in plants include calcium (Ca) regulation and protection against herbivory (Franceschi & Nakata 2005).

In *R. eugenioides*, the arrangement of the outer periclinal cell wall, when observed using transmission electron microscopy, was 3-layered: the inner layer was polysaccharide-rich and mainly composed of cellulose (L1); the intermediate cuticular layer (L2), which was subdivided into a tree-like polysaccharide-rich network (TL) and reticulated layer immersed in a matrix of cutin; and the outer the cuticle proper (L3). Although the interpretation proposed by Martin and Juniper (1970) for the cuticle of plants has been widely followed by many researchers, Holloway (1982) reviewed historical perspectives of the plant cuticle and attempted to adopt the most workable interpretation of the cuticular membrane (CM) in practice. In response to this, we followed Jeffree (1986), whose uncomplicated and pragmatic interpretation distinguishes three main zones, namely the cuticle proper, the cuticular layer and the cell wall. The cuticular membrane comprises the cuticle proper plus the cuticular layer and is bonded to the outer periclinal walls of the epidermal cells by a pectin-rich layer, which is equivalent to the continuous middle lamella. This species presents a cuticle structure corresponding to cuticular structural type 3, described by Holloway (1982). Cuticular thickness and composition may be affected by light, temperature, soil, atmospheric moisture and altitude (Wilkinson 1979); however, it is difficult to speculate about the functional significance of the relatively thin cuticle in *R. eugenioides* and how this relates to cellular permeability and structure.

This work provides a detailed description of the leaf blade of *R. eugenioides*, and increases what is known about this species. In addition, the characteristics found in this study contribute to understanding Rubiaceae taxonomy, the genus *Rudgea*, and the flora of the Atlantic Forest.

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