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Original Paper

Leaf anatomy of Rubiaceae species in a semiarid area of Brazil

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Abstract

Considering the importance of anatomical characters for delimiting Rubiaceae subgroups, the difficulties involved in identifying certain taxa and the lack of studies on that group in the semiarid region, this work aimed to characterize the anatomy of the leaves of 15 species belonging to the genera: *Borreria* (2), *Cordiera* (1), *Eumachia* (1), *Hexasepalum* (4), *Mitracarpus* (4), *Richardia* (1), *Staelia* (1), and *Tocoyena* (1) collected in the Serra Branca/Raso da Catarina Environmental Protection Area (Jeremoabo-BA, Brazil), and describe useful elements supporting the group's taxonomy. Variations were found in the shapes and contours of epidermal cells, the presence/absence and types of trichomes, mesophyll type, stomatal type and position, subsidiary cell shapes, vascular system organization, and the occurrence of collector cells and twinned stomata. The results also demonstrated that the anatomical characteristics, when considered together, represent good taxonomic tools for separating the genera and species of Rubiaceae.

Key words: caatinga vegetation, morphoanatomy, Raso da Catarina, taxonomy.

Resumo

Dada a importância dos caracteres anatômicos para delimitação de subgrupos de Rubiaceae, a dificuldade na identificação de determinados táxons, bem como aa escassez de trabalhos sobre o grupo no semiárido, este trabalho objetivou caracterizar a anatomia das folhas de 15 espécies pertencentes aos gêneros *Borreria* (2), *Cordiera* (1), *Eumachia* (1), *Hexasepalum* (4), *Mitracarpus* (4), *Richardia* (1), *Staelia* (1) e *Tocoyena* (1) coletados na APA Serra Branca/Raso da Catarina (Jeremoabo-BA, Brasil), e destacar os elementos úteis para subsidiar a taxonomia do grupo. Os resultados mostraram que houve variação quanto ao formato e contorno das células epidérmicas, presença, ausência e tipos de tricomas, tipo de mesofilo, posição e tipo de estômato, formato das células subsidiárias, organização do sistema vascular, ocorrência de células coletoras e estômatos geminados. Foi observado que as características anatômicas, quando utilizadas em conjunto, servem como uma boa ferramenta taxonômica na separação de gêneros e espécies de Rubiaceae.

Palavras-chave: vegetação de caatinga, morfoanatomia, Raso da Catarina, taxonomia.

Introduction

Rubiaceae is a monophyletic group of easy circumscription (Verdcourt 1958; Bremekamp 1966; Bremer & Jansen 1991) although there are still problems related to intrafamilial classifications, especially delimitations at the subfamily level (Robbrecht & Manen 2006), tribe (Bremekamp 1966; Robbrecht 1988; Bremer & Jansen 1991; Robbrecht & Manen 2006) and genus (Bacigalupo & Cabral 1996). Those problems are related to the requirement for fertile samples to ensure the taxonomic identities of plants collected in the field.

According to Metcalfe & Chalk (1979), traditional study methods that use herborized plants and their external morphologies as the primary tools for defining the taxa often create persistent taxonomic problems. Although plant anatomy, cytogenetics, and molecular genetics are not as accessible as external morphologies,

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new tools have been developed in these areas that can define new characters and parameters useful for solving numerous taxonomic problems (Mayr 1998). Anatomical analyses can provide important additional data (in addition to traditional external morphological characteristics) and can be useful for solving taxonomic problems.

Angiosperm leaves demonstrate wide structural variations among different species having high complexity and either simple or highly subdivided structures (Sinha 1999), which can be used to differentiate between individuals from the same population (Mccauley & Evert 1988).

Although the leaf is the plant organ most exposed to the environment, and therefore the most variable, numerous anatomical characters have systematic value, including the leaf epidermis (Metcalfe & Chalk 1979; Dickinson 2000), which can be used in phylogenetic studies of the Rubiaceae (Andrade *et al.* 2015), as taxonomists have continually sought additional anatomical characters that can help identify its species (Solereder 1908; Metcalf & Chalk 1950, 1979).

The importance of leaf anatomical characters for Rubiaceae was first proposed by Verdcourt (1958) and Bremekamp (1966); Barroso *et al.* (1978) later separated Rubiaceae subfamilies by their leaf raphides and septate trichomes, with the presence of raphides placing *Borreria* into Rubioideae (Spermacoceae tribe) and their absence in *Chiococca*, placing it among the Cinchonoideae (Chiococceae tribe).

Anatomical and micromorphological characters of the leaves of the Rubiaceae with taxonomic significance have been studied in *Coussarea* (Tavares & Vieira 1994), *Psychotria* (Da Cunha & Vieira 1997; Gomes *et al.* 1995; Moraes *et al.* 2011), *Bathysa* (Nascimento *et al.* 1996), *Rudgea* (Mantovani & Vieira 1997; Leo *et al.* 1997), *Rondeletia* (Kocsis *et al.* 2004), *Simira* (Moraes *et al.* 2009), and representatives of the Hamelieae tribe (Martínez-Cabrera *et al.* 2009).

The present study aimed to characterize the leaf anatomy of 15 species of Rubiaceae species occurring in the Brazilian semiarid region to identify useful characters that could support the group's taxonomy.

Materials and Methods

Plant material

Botanical material was collected in the Serra Branca/Raso da Catarina Environmental Protection Area (APASB) (09°53'15.5" to 09°44'34.6"S and 38°49'36,1" to 38°52'20.4"W), located in the municipality of Jeremoabo, in northeastern Bahia state, Brazil. The landscape there is flat, with sandstone formations; the regional climate is very hot, the mean annual rainfall near 500 mm, and the mean annual temperature approximately 23 ºC (Szabo *et al.* 2007).

The adult leaves of three individuals (each), located between the third and fourth node (from the apex to the base) of 15 Rubiaceae species were collected: *Borreria spinosa* (L.) Cham. & Schltdl., *Borreria verticillata* (L.) G. Mey., *Cordiera rigida* (K. Schum.) Kuntze, *Eumachia depauperata* (Müll. Arg.) M.R. Barbosa & M.S. Pereira., *Hexasepalum apiculatum* (Willd.) Delprete & J.H. Kirkbr., *Hexasepalum gardineri* (K.Schum.) J.H. Kirkbr. & Delprete, *Hexasepalum radulum* (Willd.) Delprete & J.H. Kirkbr., *Hexasepalum teres* (Walter) J.H. Kirkbr., *Mitracarpus baturitensis* Sucre, *Mitracarpus longicalyx* E.B. Souza & M.F. Sales, *Mitracarpus robustus* E.B. Souza & E.L. Cabral, *Mitracarpus salzmannianus* DC., *Richardia grandiflora* (Cham. & Schltdl.) Steud., *Staelia galioides* DC., and *Tocoyena formosa* (Cham. & Schltdl.) K. Schum.

Botanical collections were undertaken on different days from May/2014 to June/2015, covering both the rainy and hot/dry seasons, totaling six collections. Samples harvested in the field were herborized following the methodology of Fosberg & Sachet (1965) and Mori *et al.* (1989), and subsequently deposited in the herbarium of the state University of Bahia (HUNEB - Paulo Afonso Collection). Fully expanded sun leaves of the third and fourth nodes were selected for study. The fresh materials were fixed in 70% FAA for 72 hours, according to the methodology described by Johansen (1940). The leaves were then transferred to 70% ethyl alcohol (v/v) for anatomical procedures.

Light microscopy

Cross- and paradermic sections of the leaves were prepared following the methodology described by Kraus & Arduim (1997). The samples were sectioned manually using a razor blade, and stained with astra blue, safranin, and toluidine blue. Semi-permanent slides were prepared using 50% glycerin (v/v) and photographed using a digital camera (AxioCam ERc5s) coupled to a light microscope (Zeiss Primo Star). The anatomical classifications followed Solereder (1908), Metcalfe & Chalk (1950), and Appezzato-da-Glória B & Carmello-Guerreiro SM (2012).

Similarity analysis

Cluster analysis was performed to evaluate the degrees of similarity among the species studied based on the absence (0) or presence (1) of certain characters, using Euclidean distances and the Average method. Analyses with co-phenotype indices greater than 0.7 were considered significant. Cluster analyses were performed using SYSTAT version 13.0 software (SYSTAT Inc., USA).

Results

Leaf anatomy

The leaves of Rubiaceae plants have a thick cuticular layer covering the uniseriate epidermis. The adaxial epidermal surfaces have papillae and anticlinal walls.

The anticlinal adaxial walls appeared straight and polygonal in frontal view in *Hexasepalum* (except *Hexasepalum apiculatum*), *Richardia* (Fig. 1a), and *Tocoyena*, curved in *Cordiera*, *Borreria verticillata,* and *Hexasepalum apiculatum* (Fig. 1b), and sinuous in *Borreria spinosa* (Fig. 1c), *Mitracarpus,* and *Staelia*. Most species show sinuous and anticlinal abaxial walls. *Hexasepalum apiculatum*, *Hexasepalum radulum*, *Cordiera,* and *Tocoyena*, however, show curved anticlinal abaxial walls, while those in *Hexasepalum teres* and *Eumachia* they are polygonal and straight.

Epidermal cells of different sizes were found on both leaf faces in most species, although they were isodiametric on both faces in *Hexasepalum teres* and *Eumachia; Borreria*, *Cordiera*, *Tocoyena,* and *Mitracarpus baturitensis*

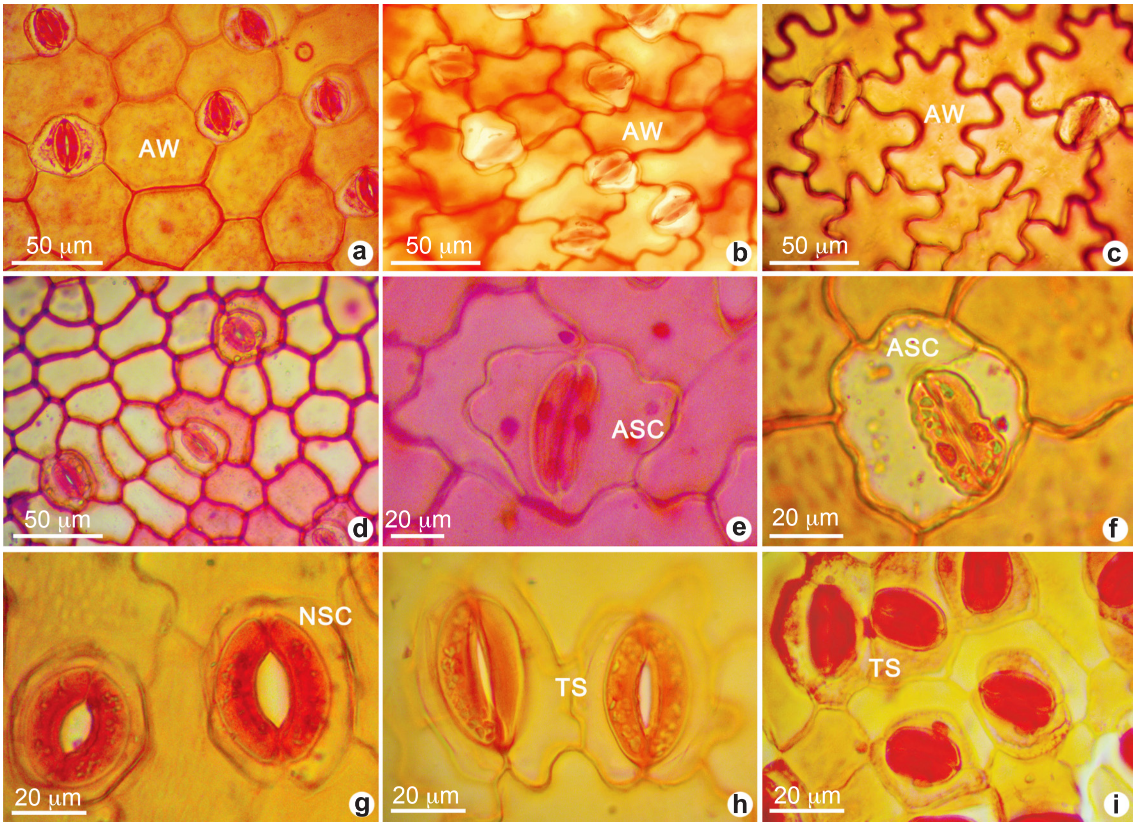

Figure 1 – a. *Richardia grandiflora* with anticlinal adaxial walls appeared straight and polygonal; b. *Hexasepalum apiculatum* with anticlinal adaxial walls appeared curved; c. *Borreria spinosa* with anticlinal adaxial walls appeared sinuous; d. *Eumachia depauperata* with paracytic and anisocytic stomata; e. *Mitracarpus robustus* with paracytic stomata with abnormal subsidiaries cells; f. *Mitracarpus baturitensis* showing stomata with abnormal subsidiaries cells; g. *Staelia galioides* presenting stomata with normal subsidiaries cells; h. *Mitracarpus* with twinned stomata; i. *Tocoyena formosa* with grouped twinned stomats. AW=anticlinal wall, ASC=abnormal subsidiarires cells, NSC= normal subsidiarires cells, TS=twinned stomata.

demonstrated variations, with isodiametric adaxial cells and different sized abaxial cells.

The leaves of *Cordiera*, *Eumachia*, *Richardia*, *Tocoyena,* and *Hexasepalum radulum* are hypostomatic, while the other species presented amphistomatic leaves. Paracytic stomata were observed in all of the species, although *Hexasepalum* (except *Hexasepalum radulum*), *Eumachia,* and *Mitracarpus baturitensis* also had anisocytic stomata (Fig. 1d).

Malformed subsidiary stomatal cells or abnormal cells were observed in most species (Fig. 1e,f); only *Cordiera*, *Staelia* (Fig. 1g), and *Hexasepalum teres* demonstrated all normal cells. Twinned stomata (Fig. 1h,i) were often seen in *Cordiera*, *Hexasepalum*, *Mitracarpus* (except *Mitracarpus robustus*), and *Tocoyena*.

A dorsiventral mesophyll was observed in most species; only *Hexasepalum apiculatum* and *Staelia* had isobilateral mesophylls. Collector cells (Fig. 2a) were quite common in the mesophyll, but absent in *Eumachia*, *Richardia*, *Tocoyena*, *Mitracarpus baturitensis* and *Mitracarpus longicalyx*.

The numbers of palisade parenchyma layers varied greatly, with *Borreria verticillata*, *Hexasepalum gardineri*, *Hexasepalum radulum,* and *Eumachia* being unistratified (Fig. 2b), *Cordiera* and *Tocoyena* being multistratified (Fig. 2c), while the other species were bistratified.

A vascular midrib system of the collateral type in an open arc (Fig. 2d) was seen in all of the species studied. *Tocoyena* differed from the others, however, by having a continuous principal vascular bundle in a closed loop (Fig. 1e), with two subjacent accessory bundles; the vascular systems of *Cordiera* and *Eumachia* had half-moon outlines (Fig. 1f).

Most species showed angular collenchyma on the abaxial surface of the midrib (Fig. 1g); only *Mitracarpus salzmannianus*, *Richardia,* and *Staelia* did not exhibit that characteristic. Additionally, angular collenchyma were observed on the adaxial faces of *Hexasepalum* (except *Hexasepalum gardineri*), *Tocoyena*, *Mitracarpus baturitensis,* and *Mitracarpus robustus*. The taxa *Borreria verticillata*, *Hexasepalum gardineri*, *Eumachia,* and *Staelia* differed by having collenchyma on the adaxial face of the central cylinder; *Cordiera rigida* demonstrated both of those characters on the adaxial face.

The vascular bundles of all of those species were composed of sclerenchyma fibers as well as primary phloem and xylem that partially or completely surrounded the main vascular bundles. *Cordiera* also showed abundant fibers in its secondary bundles.

Tocoyena formosa, which was the only petiolate species in this study, presented a non-fistulous cross section and planar petiole with semi-depressed margins, angular collenchyma with 4–5 cell layers, oval cortical parenchyma cells, and sclerenchyma fibers surrounding the vascular system (which consists of a primary vascular bundle in a closed, continuous arc with accessory strands (Fig. 2h) in the medulla and along the petiole edges), and medulla with irregular cells.

Most species showed various types of raphide-like (Fig. 2i,j) and druse (Fig. 2k) calcium oxaloacetate crystals. *Hexasepalum radulum* and *Tocoyena* also contained single crystals (Fig. 2l).

Secretory idioblasts were observed in the mesophyll of *Mitracarpus longicalyx* (Fig. 2m); *Hexasepalum radulum* and *Tocoyena formosa* also demonstrated idioblasts in their leaf midribs, and *T. formosa* in the petiole (Fig. 2n).

Unicellular non-glandular trichomes were observed in all species (except *Eumachia depauperata*). Multicellular non-glandular trichomes were present in *Borreria spinosa*, *Cordiera*, *Hexasepalum apiculatum*, *Hexasepalum radulum*, *Mitracarpus baturitensis*, *Mitracarpus robustus*, *Richardia,* and *Tocoyena*.

Long, non-glandular trichomes were observed in all species, being mostly erect or curved. *Hexasepalum gardineri* differed by having hook-shaped trichomes, and *Tocoyena* had abundant and tangled trichomes throughout the leaf. *Borreria, Staelia, Mitracarpus baturitensis,* and *Mitracarpus salzmannianus* demonstrated short, thick trichomes, and *Hexasepalum teres* also bore unicellular glandular trichomes on the blade borders.

Similarity analyses

Similarity analyses performed based on presence/absence matrices (Tab. 1) distinguished three different groups among the Rubiaceae (Fig. 3a) species studied here. The first group comprised the Psychotrieae tribe (Fig. 3b) (represented by *Eumachia depauperata*), and the second group the Spermacoceae tribe (Fig. 3c) (represented by *Borreria*, *Hexasepalum*, *Mitracarpus*, *Richardia,* and *Staelia*) - both belonging to the Rubioideae subfamily. A third group was formed by the Gardeniae tribe (Fig. 3d) (represented by *Cordiera* and *Tocoyena*), belonging to the Ixoroideae subfamily.

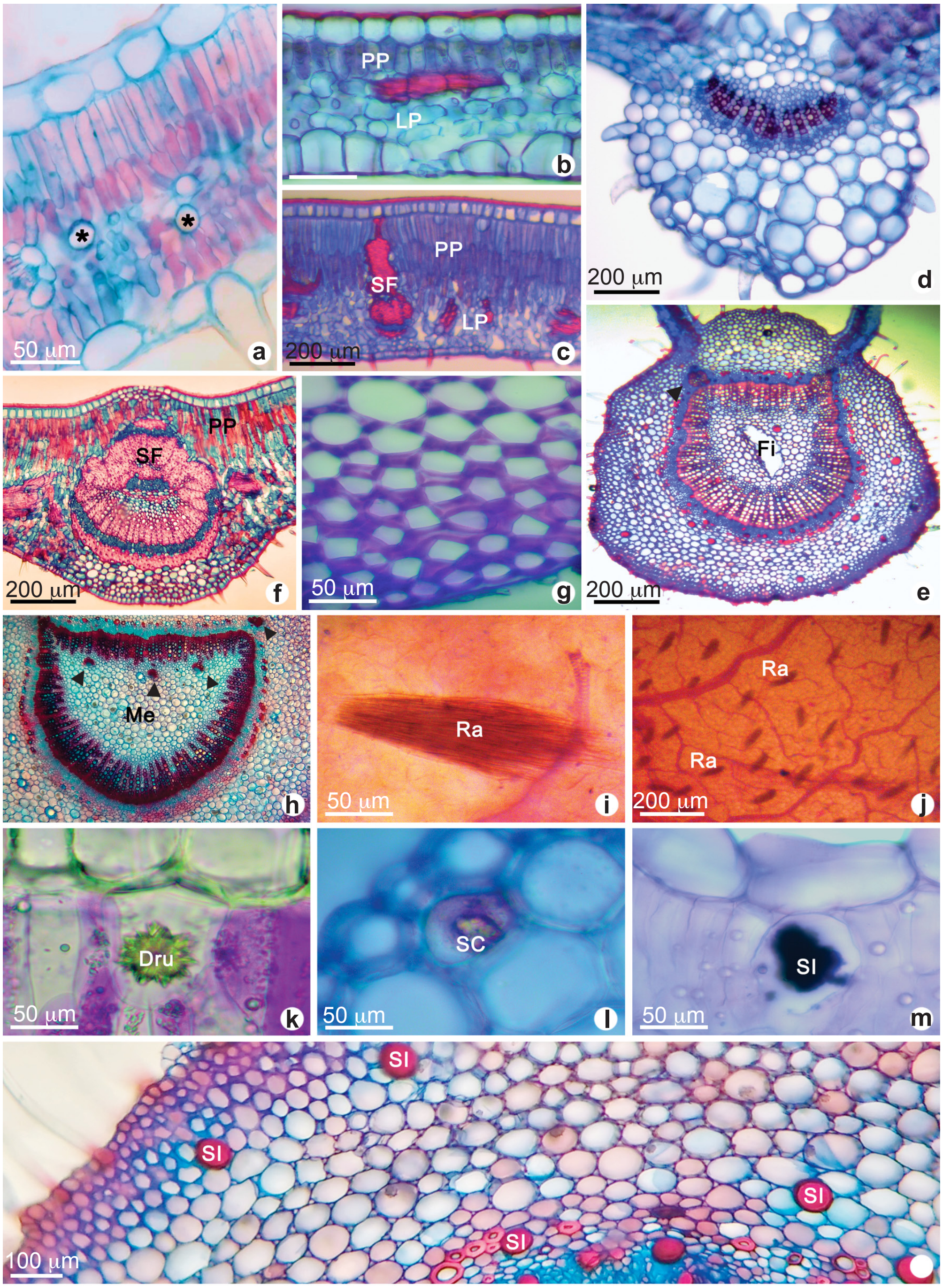

Figure 2 – a. Mesophyll of *Hexasepalum apiculatum* with biostratified palisadic parenchyma with collector cells; b. *Eumachia depauperata* with unstratified palisadic parenchyma; c. *Cordiera rigida* with multistrata palisade parenchyma and sclerenchyma fibers in the lower bundle; d. *Mitracarpus salzmannianus* with open arch form vascular system; e. *Tocoyena formosa* with a continuous principal vascular bundle in closed loop form and subjacent accessory bundles, fistula in the medulla; f. *C. rigida* with abundant sclerenchyma fiber and vascular system in the form of half moon; g-h. *T. formosa* with angular collenchyma on the abaxial surface of the midrib and accessory bundles in the petiole; i-k. *Hexasepalum teres* showing raphides and drusen; l. *Hexasepalum radulum* showing single crystal; m. *Mitracarpus longicalx* with scattered idioblast in the mesophyll; n. *T. formosa* petiole detail with druse and midrib with scattered idioblasts. PP=palisade parenchyma, LP=lacunar parenchyma, SF=sclerenchyma fibers, Fi=fistula, SI=secretory idioblasts, *= collector cell ▼= accessory bundles, Dru=drusen, Ra=raphides, Me=medulla.

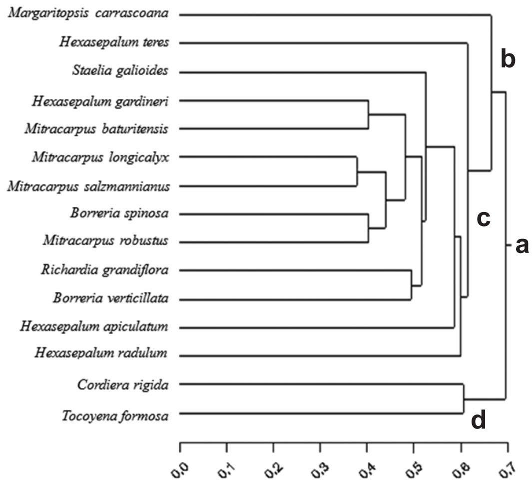

Figure 3 – Similarity analysis – a. Rubiaceae; b. Psychotrieae tribe; c. Spermacoceae tribe; b-c. Rubioideae subfamily; d. Gardenieae tribe, Ixoroideae subfamily.

The characteristics that distinguished Psychotrieae from Spermacoceae were the absence of trichomes, the presence of normal and abnormal subsidiaries cells in the same individual, half-moon shaped vascular bundles, and isodiametric and bulky epidermal cells on both leaf faces.

The most shared characteristics in the Spermacoceae tribe were: amphistomatic leaves, stomata level with the adaxial face, anomalous subsidiary cells, mesophyll with collector cells, arc-shaped vascular bundles, angular collenchyma in the midrib, abaxial face, epidermal cells and adaxial papillose, and the absence of druses.

The characters that grouped the Gardenieae tribe were: cell walls with anticlinal curves on their abaxial faces, the absence of papillae on the adaxial epidermis, hypostomatic leaves, only paracytic stomata, multistrata palisade parenchyma, the presence of angular collenchyma in the midrib and adaxial face, and the presence of druses.

Discussion

The leaf epidermis proved to be useful for the systematics of the Rubiaceae, providing important distinguishing features such as presence of papillae, different epidermal cell sizes, wall shapes and thicknesses, stomatal diversity, and trichome morphologies and distributions (Metcalf & Chalk 1979; Dickinson 2000; Araújo 2008).

All of the species have leaf blades with uniseriate epidermises, usually with epidermal cells of different sizes on both faces; isodiametric epidermal cells were also commonly found on the adaxial face. Similar results were reported by Mantovani *et al*. (1995) for *Rudgea* species. The adaxial faces of the leaves were bulky in most species. Vitarelli (2008) reported the same trait when studying *Psychotria carthagenensis*. Light and water availability can modulate cell growth and influence cell division processes, epidermal development, and cell volumes (Taiz & Zeiger 2004; Rizzini 1997; Lambers *et al*. 1998; Larcher 2000; Zini *et al*. 2016). The greater volumes of the epidermal cells on the adaxial faces of xeromorphic individuals likely reflects increases in vacuolization to ensure greater water reserves when water restrictions exist due to the high solar irradiation exposure, as in the APASB.

Most of the species studied showed adaxial epidermal papillary cells, with conservative characteristics in *Borreria*, *Hexasepalum*, *Mitracarpus* (except *M. robustus*), and *Staelia* that could be used to solve delimitation problems in Spermacoceae, as noted by Mattos (2011) for *Borreria*. Kay *et al*. (1981) and Zini *et al.* (2016) suggested that a papillose epidermis could help reflect sunlight, thus reducing water losses and stabilizing the physiological balances of the plant.

The contours of the leaf epidermal cells corresponded to the patterns described by Metcalfe & Chalk (1950), with most species demonstrating sinuous cells on the abaxial face, but straight and polygonal cells on the adaxial surface. Isanogle (1944), Combes (1946), Hughes (1959), Gusmão *et al*. (1992), Dickinson (2000), and Alquini *et al*. (2012) reported that the sinuous anticlinal walls of epidermal cells are directly related to light effects, and the greater shading the greater the sinuosity. In this study, however, *Mitracarpus*, *Staelia,* and *B. spinosa* demonstrated twisted adaxial epidermal cells even in non-shaded sites; although growing in shaded sites, *Hexasepalum teres* and *Eumachia depauperata* had straight, polygonal abaxial epidermal cells. Light, therefore, is not influencing that character in those Rubiaceae species, which enhances its taxonomic usefulness – corroborating Mantovani *et al*. (1995) and Mattos (2011). According to Medri & Lleras (1980), the low sinuosity of the cell walls reflects an adaptive strategy against water losses, while Haberlandt (1928) observed that wall sinuosity increased cell stiffness, thus preventing cell collapse under conditions of water stress.

Table 1 - Matrix of presence (1) and absence (0) of the anatomical characters of 15 species of Rubiaceae from the Serra Branca Environmental Protection Area

Characteristics/Species	**Bsp**	**Bve**	**Cri**	**Hap**	**Hga**	**Hra**	**Hte**	**Mca**	**Mba**	**Mlo**	**Mro**	**Msa**	**Rgr**	**Sga**	**Tfo**
Epidermis: adaxial cells and isodiametric	1	1	1	0	0	0	1	1	1	0	0	0	0	0	1
Epidermis: abaxial cells and isodiametric	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
Epidermis: adaxial cells of different size	0	0	0	1	1	1	0	0	0	1	1	1	1	1	0
Epidermis: abaxial cells of different size	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
Epidermis: adaxial cells polygonal, walls straight and anticlinal	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1
Epidermis: abaxial cells polygonal, walls straight and anticlinal	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
Anticlinal wall pattern of adaxial surface curved	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Anticlinal wall pattern of abaxial surface curved	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1
Adaxial surface with sinuous anticlinal walls	1	0	0	0	0	0	0	0	1	1	1	1	0	1	0
Abaxial surface with sinuous anticlinal walls	1	1	0	0	1	0	0	0	1	1	1	1	1	1	0
Papillose epidermis cells	1	1	0	0	1	1	1	0	1	1	0	1	0	1	0
Unicellular trichomes	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Multicellular trichomes	1	0	1	1	0	1	0	0	0	1	1	0	0	1	1
Glandular trichomes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Amphistomatic mesophyll	1	1	0	1	1	0	1	0	1	1	1	1	0	1	0
Hypostomatic mesophyll	0	0	1	0	0	1	0	1	0	0	0	0	1	0	1
Paracytic and anisocytic stomata	0	0	0	1	1	0	1	1	1	0	0	0	0	0	0
Stomata leveled with the adaxial face	1	1	0	1	1	0	1	0	1	1	1	1	1	1	0
Stomata leveled with the abaxial face	1	0	0	1	0	0	0	1	0	0	1	0	0	0	1
Slightly protruding stomata on the abaxial face	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0
Protruding stomata on the face abaxial	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0
Twinned stomata	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1
Subsidiary anomalous cells	1	1	0	1	1	1	0	1	1	1	1	1	1	0	1
Dorsiventral mesophyll	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1
Isobilateral mesophyll	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Mesophyll with guard cells	1	1	1	1	1	1	1	0	0	0	1	1	0	1	0
Palisade unstratified parenchyma	0	1	0	0	1	1	0	1	1	0	0	0	0	0	0
Bistratified Palisade parenchyma	1	0	0	1	0	0	1	0	0	1	1	1	1	1	0
Palisade multistrata parenchyma	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Vascular bundle in the form of open arcs	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0
Vascular bundle in the form of half moon	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
Vascular bundle in the form of closed arcs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Angular collenchyma in the adaxial midrib	0	0	1	1	0	1	1	0	1	0	1	0	0	0	1
Angular collenchyma in the abaxial midrib	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1
Palisade parenchyma in the adaxial midrib	1	0	1	0	1	0	0	1	0	0	0	0	0	1	0
Raphides type crystals	0	1	0	1	0	1	1	1	0	1	0	0	0	0	0
Druses type crystals	0	0	1	1	1	1	1	0	0	0	1	0	0	0	1
Single crystals	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1

Abbreviations: Bsp- *Borreria spinosa*; Bve- *Borreria verticillata*; Cri- *Cordiera rigida*; Hap- *Hexasepalum apiculatum*; Hga- *Hexasepalum gardineri*; Hra- *Hexasepalum radulum*; Hte- *Hexasepalum teres*; Mca- *Margaritopsis carrascoana*; Mba- *Mitracarpus baturitensis*; Mlo- Mitracarpus longicalyx; Mro- *Mitracarpus robustus*; Msa- *Mitracarpus salzmannianus*; Rgr- *Richardia grandiflora*; Sga- *Staelia galioides*; Tfo- *Tocoyena formosa*.

The amphistomatic stomatal positions observed in the present study differed from the hypostomatic pattern described for most Rubiaceae species by Solereder (1908), Metcalfe & Chalk (1950), Robbrecht (1988), and Mattos (2011). Only *Cordiera*, *Eumachia*, *Tocoyena,* and *Hexasepalum radulum* demonstrated hypostomatic leaves. That character therefore demonstrated taxonomic value that could be used in genera circumscriptions, as well as differentiating *H. radulum* from other species in its genus. That stomatal type also differed from the traditional paracytic stomatal pattern associated with Rubiaceae (the 'Rubiaceous type') (Solereder 1908; Accorsi 1947; Metcalf & Chalk 1950; Bahadur *et al*. 1971). Here, we identified anisocytic stomata in *Hexasepalum* (except *H. radulum*), *Eumachia,* and *Mitracarpus baturitensis* in addition to the paracitic stomata type. Those characteristics are essential for differentiating *H. radulum* and *M. baturitensis* from the other species of the genus. Variations of stomatal type in Rubiaceae were also observed by Mantovani *et al.* (1995), Da Cunha & Vieira (1997), Vitarelli (2008), and Mattos (2011), who all reported paralelocytic stomata.

Accorsi (1947) and Pant & Mehra (1965) observed that the stomatal subsidiaries cells in Rubiaceae species showed anomalous morphological defects due to incomplete and uneven development. Although the ontogeny of those peculiar stomata in the species studied here was not examined, they were considered anomalous because of their similarities to those described by the aforementioned authors.

Some species demonstrated grouped stomata (described as twinned stomata by Accorsi [1947]) or malformed subsidiary cells; those finding agree with the studies of Mantovani *et al*. (1995) and Gavilanes *et al*. 2016. Accorsi (1947) reported the presence of twinned stomata in 39.45% of a total of 601 Rubiaceae species. Most of the species studied here showed anomalous stomatal cells, and only the genera *Cordiera* and *Staelia,* and *H. teres* genera showed normal cells. The genus *Eumachia* demonstrated both normal and abnormal stomata cells; twinned stomata were recorded in the genera *Cordiera*, *Hexasepalum*, *Mitracarpus* (except *M. robustus*), and *Tocoyena.*

The most common type of vascular midrib seen here was in the form of an arc, corroborating the studies of Holm (1907), Solereder (1908), and Metcalfe & Chalk (1950); a half-moon type variation was seen in the genera *Cordiera* and *Eumachia*. The genus *Tocoyena* could be distinguished by the arrangement of its vascular system, with a continuous main bundle in a closed arc, with two subjacent accessory bundles on the adaxial face; Coelho *et al*. (2006) reported that same morphology.

Sclerenchyma fibers were associated with the phloem in most species, completely or partially surrounding the vascular bundles. Sclerenchyma fibers were abundant in the principal and secondary veins in the genus *Cordiera* genus, but those fibers were scarce and ungrouped in *Tocoyena* and collateral (except in *T. formosa*), and were usually surrounded by an endoderm layer; a vascular sheath was seen in the species *B. verticillata*, *H. apiculatum*, and *H. teres*, as well as in *Richardia* and *Staelia*. The abundances of those fibers are related to greater tissue stability in xerophytic plants, and they help avoid cell collapse during dry periods as well as mechanical stress due to strong winds (Esau 1977; Krahl *et al*. 2013; Krahl & Krahl 2017).

A dorsiventral mesophyll was observed in the majority of the species studied here, corroborating with the family descriptions of Solereder (1908) and Metcalfe & Chalk (1950). *Hexasepalum apiculatum* and *Staelia galioides*, however, showed an isobilateral mesophyll, which is an important character for their differentiation. Collector cells were observed in *Cordiera*, *Borreria*, *Hexasepalum*, *Staelia*, *Mitracarpus baturitensis,* and *M. longicalyx*. Collector cells are formed by the connection of spongy parenchyma cells to palisade parenchyma cells (thus differing from other foam cells). Those cells consistently occur in Rubiaceae species, and have taxonomical value (Lersten 1974; Scatena & Scremin-Dias 2012).

According to Mattos (2011) and Teixeira *et al*. (2016), trichomes have taxonomic value in the Rubiaceae. Their importance may vary according to the hierarchical level analyzed, with Kocsis *et al*. (2004) demonstrating that trichome types, especially abaxial leaf trichomes, can be used to distinguish *Rodeletia* species. Martínez-Cabrera *et al*. (2009) observed that the types, sizes, and distributions of trichomes are important characteristics for separating genera in the Hamelieae tribe. Trichomes have an indirect influence on water conservation in plants by reflecting back solar radiation that strikes the leaves (Salatino *et al*. 1986; Larcher 2000) and, at high densities, they are responsible for maintaining a saturated water vapor atmosphere (Fahn & Cutler 1990; Larcher 2000). Trichomes were found here to be important in distinguishing species such as *Hexasepalum apiculatum,* which has dense, elongated multicellular trichomes, but *H. teres* does not; *H. teres*, on the other hand, has glandular marginal trichomes but *H. apiculatum* does not. The presence of glandular trichomes in *H. teres* was also reported by Mussury *et al.* (2012). Those species are commonly confused in taxonomic studies, resulting in misidentifications. The anatomical characters observed here point to identifiable differences between the species.

Hexasepalum apiculatum is described here for the first time or the APASB.

Similarity analyses showed that the presence of papillae on the adaxial epidermis, the presence or absence of trichomes, stomatal type, variations of palisade layer morphologies, stomatal classification and position, subsidiary cell shape changes during vascular system formation, epidermal cell sizes, the morphologies of the anticlinal walls, the presence of collector cells, the types of crystals, and the angular dispositions of the midrib collenchyma are useful characteristics for the taxonomy of Rubiaceae species. According to Vasconcelos *et al.* (2017), crystal types represent important diagnostic features for the genera and species of Rubiaceae.

Some of these characteristics have been used in anatomical studies applied to the taxonomy of the Rubiaceae (Tavares &Vieira 1994; Nascimento *et al.* 1996; Kocsis *et al.* 2004; Moraes *et al.* 2009; Martínez-Cabrera *et al.* 2009; Moraes *et al.* 2011; Mattos 2011), so that the results reported here can contribute to a better understanding of the phylogenetic relationships among the taxa of that family.

The similarity analyses corroborated the current circumscription of Rubiaceae proposed by Bremer & Eriksson (2009).

These results confirm the importance of leaf anatomical characters as additional tools that can support taxonomic studies in Rubiaceae and increase our anatomical, ecological, and physiological knowledge of the subfamily, tribe, genera, and species, and indicate promising characters for future taxonomic and phylogenetic approaches.

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