



Influence of environment on the leaf morpho-anatomy and histochemical of the ayahuasca leaf: Populations cultivated in extra-Amazonian regions

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ABSTRACT. *Psychotria viridis* Ruiz & Pav. (Rubiaceae) occurs naturally throughout the Amazon and it is traditionally used by indigenous communities, being incorporated into religious use in urban contexts over the last few decades. It is known and cultivated in many regions of South America for possessing valuable bioactive alkaloids. In this paper, we described *P. viridis* leaf morphology, anatomy and histochemistry from three populations cultivated in the southeastern Brazil, in order to identify possible adaptations to local environment and management. All plants presented terminal stipules and basic morpho-anatomical patterns of leaves, consistent with most species of the genus, as heterogeneous dorsiventral mesophyll, uniseriate epidermis, presents large cells with prominent vacuoles and druses. Unicellular non-glandular trichomes and multicellular starry trichomes were present in the primary and secondary veins. Amphi-hypostomatic leaf pattern, not yet described for the species, was common in all studied plants. Variation in the presence of domatia in the same population indicates that this structure cannot be used for taxonomic determination of *P. viridis*, as already described for other species of the genus. Presence of secretory ducts and reduction in stomata density and leaf area represent the main morpho-anatomic adaptations of plants from drier and warmer climates. Histochemical tests were positive for alkaloids, polysaccharides, proteins and phenolic compounds, being negative for starch only in plants subjected to water stress. We concluded that the morpho-anatomical and histochemical alterations found in the plants of this study resulted from seasonal water deficit adaptations and to maintain or attract mutualistic organisms.

Keywords: characterization; alkaloid; trichomes; secretory duct; domatia.

Introduction

Rubiaceae is the fourth-largest family of flowering plants (Robbrecht, 1988; Van Oevelen, Wachter, Robbrecht, & Prinsen, 2003; Jung-Mendaçolli, 2007; Ferreira Junior & Vieira, 2015) standing out among plants of high medicinal, ornamental and economic interests (Gonçalves & Lorenzi, 2007). Among the richest Rubiaceae genus, *Psychotria* is pantropical, with approximately 2000 species, mainly trees (Mathur, Vyas, Kapoor, & Tyagi, 2011). *Psychotria viridis* Ruiz & Pav occurs in Mexico, the Antilles, Bolivia, Argentina (Taylor, 2007) and the North (Amazonas) and Southeast (Minas Gerais and São Paulo) regions of Brazil (Pic-Taylor et al., 2015). *P. viridis* stands out among the genus as an important widespread tropical understory tree providing important bioactive alkaloids (Soares et al., 2017). Leaves of *P. viridis* are used in the composition of Ayahuasca tea, an entheogenic beverage known as Daime, Caapi, Yajé, Hoasca or Vegetal (Reynolds, 1974; Hamill, Hallak, Dursun, & Baker, 2019), remarkably used in religious rituals and indigenous pharmacopoeia (Domínguez-Clavé et al., 2016).

Studies of internal and external *Psychotria* morphology have shown great anatomical and morphological variation (Davis, Bridson, Jarvis, & Govaerts, 2001; Quinteiro, Teixeira, Moraes, & Silva, 2007; Moraes, Rabelo, Alexandrino, Silva Neto, & Cunha, 2011; Nery, Ribas, & Koehler, 2014; Pinheiro, Kolb, & Rossatto, 2018), which makes it difficult to establish determinative morphological structures for the group taxonomy. Domatia was considered determinant for the taxonomy of *P. viridis* in Atlantic Forest (Quinteiro et al., 2007). However, this structure has been identified in several *Psychotria* species of the Atlantic Forest and it

has been considered as an important taxonomic characteristic for genus determination (Moraes et al., 2011). These are key to the leaf system because they are microstructures closely related to the control of fungal pathogens and to several recognition of taxa (Oliveira, Rebouças, Leite, Oliveira, & Funch, 2018; Rowles & O'Dowd, 2009). Thus, studies in other biomes are still necessary to determine important anatomical and morphological characters for correct diagnosis within *Psychotria*.

Variations in leaf anatomy and morphology of *Psychotria* have been related to both environmental adaptation and taxonomic strategies (Quinteiro et al., 2007; Moraes et al., 2011). Leaf and wood anatomical structure adjustments related to light distribution and water availability were found in *Psychotria schlechtendaliana* (Campbell, Mielke, Rabelo, & Cunha, 2018).

Recently, a great number of religious ceremonies and holistic medicine therapies using of Ayahuasca drink containing *P. viridis* leaves in its composition has been growing, which promoted its cultivation in several countries across the Americas, Europe and Oceania. In order to establish which anatomical and morphological characteristics may be influenced by environmental conditions and contribute to more reliable plant identification, which makes its use safer, *P. viridis* leaf morphology and anatomy variations need to be better understood.

In this paper we present the variations of leaf morphology, anatomy and histochemistry across three *P. viridis* populations cultivated in different environmental conditions in the state of São Paulo, Southeast Brazil. Our study intended to generate information for better understanding intraspecific variations and contribute to the elucidation of important characteristics, thus allowing a safer diagnosis. Leaf morphology, anatomy and histochemistry of cultivated plants were described and compared qualitatively and quantitatively.

Material and methods

Study species and plant material

In Brazil, *Psychotria* species occur naturally in tropical and subtropical regions, and nowadays, 236 species were identified, including 137 (58%) endemic (Taylor, Gomes, & Zappi, 2015). From the State of São Paulo, southeastern Brazil, (Jung-Mendaçolli, 2007) 46 species have been found, including *Psychotria viridis* Ruiz & Pav. (Jung-Mendaçolli, 2007). The sampled plants were planted from seedlings brought from the Amazon region and grown under natural shade. Thirty adult individuals of *P. viridis* were selected, obeying a standard of uniformity regarding size, phenology and vegetative aspects. Completely expanded leaves of the mid canopy of each plant were collected randomly for all analyzes. Materials were identified and registered under the numbers ESA 141418, 141419, 141420 and ESA 141421 in the Herbarium of the *Escola Superior de Agricultura "Luiz de Queiroz"* (ESALQ/USP), in the Department of Biological Sciences, Piracicaba, São Paulo, Brazil.

Study area

The populations of *P. viridis* sampled in this study are located in three municipalities in the southeastern region of Brazil, Campinas (22°45'49 "S, 46°58'50" W), Mairiporã, specifically Serra da Cantareira (23°22'44 "S; 46 ° 35'18 "W) and Piracicaba (22 ° 42'0.47" S; 47 ° 17'7.54 "W). They were planted in secondary forests or in abandoned pastures at different times, between 10 and 40 years ago. Vegetation is predominantly Dense Ombrophylous Forest in Campinas and in Cantareira, and Seasonal Semideciduous Forest in Piracicaba. The climate of these regions is classified among the Humid Subtropical Zone, receiving on average 1,200 to 1,400 mm annual rainfall. In Piracicaba the winter is dry and the summer is hot (type Cwa, according to Koppen classification) (Alvares, Stape, Sentelhas, Gonçalves, & Sparovek, 2013), with average temperatures of the hottest months reaching 24.5 °C and precipitation below 50 mm during the six cooler months (Sentelhas, Marin, Ferreira, & Sá, 2003). In Campinas, the climatic subtype without dry season with hot summer (Cfa) is predominant, in which the average of monthly precipitation accumulated is 115 mm, being only two months with rainfall below 50 mm. In Serra da Cantareira region, there is not a dry season either, but it has a temperate summer (type Cwb de Koppen), where the average annual temperature is 23°C in the hottest months and rainfall is concentrated between October and March, reaching 240 mm in January (Sentelhas et al., 2003).

Morphological characterization

Morphological characterization was conducted in the field and in laboratory with the aid of stereomicroscope and super magnifying glass, following descriptions of (Almeida & Almeida, 2018; Gonçalves & Lorenzi, 2007; Metcalfe & Chalk, 1979).

Determination of leaf area and stomata analysis

To measure leaf area, we used a graduated ruler and also a Canon EOS Rebel T6 camera to photograph the leaves of the three populations. The images were used to calculate true leaf area using Image J software. As sampling design and data of distribution pattern of leaf area estimated from *P. viridis* complied with statistical assumptions, we proceeded with analysis of variance, followed by Tukey post-hoc tests.

We used the epidermal impression method to characterize stomata type and density (Segatto et al., 2004), using instant adhesive. Samples were collected from the right side of the main vein until the edge, taking as reference the adaxial surface, in the region of middle third of leaves. Ten leaves (1 leaf plant⁻¹) of each area and three microscopic fields for each leaf of each surface were analyzed (abaxial and adaxial), totalizing 180 observations. Microscopic fields of 1mm² were analyzed using a light microscope, being images randomly captured by the TsView software. As stomata number per mm² adhered better to the Poisson distribution function, we fitted a generalized linear model, followed by Tukey tests for multiple comparisons between the means of different locations.

Scanning Electron Microscopy (SEM)

Tissue samples of approximately 1.5 cm were removed from the middle third of leaves and immediately fixed in Karnovsky's solution (Karnovsky, 1965), modified with phosphate buffer (pH 7.2) for 24 hours. After this period, part of the samples was retained in 30% glycerol for four hours, and then dipped into liquid nitrogen and submitted to cryofracture with blade on metal sheet. All samples were submitted to serial dehydration, followed by drying to the critical point of CO₂ (Horridge & Tamm, 1969). For metallization process, the samples were mounted on aluminum brackets (Stubbs) using double-sided carbon tape, and then, they were covered with a gold layer of 30 to 40 nm. The observations and electron micrographs were made with the scanning electron microscope, model LEO VP 435, operated at 20 kV.

Histological analysis

Cross sections of fresh material were made by hand using a razor blade in the middle third of the central vein and the right margin of the median leaf blade. The samples were stained with Astra blue and basic Fuchsin for analysis of the anatomical structure, and then they were mounted with 50% glycerin between blade and cover slip. Histological sections were photomicrographed using light microscopy, and the images were captured on the same scale using a Samsung camera (SDC-313).

Histochemical analyzes

The chemical nature of ergastic substances was determined by freehand cross sections, stained with Dragendorff for alkaloids and identified by its brown color. Double staining with Schiff Periodic Acid (SPA) and Nafthol Blue Back (Fisher, 1968) was conducted in order to identify polysaccharides by the pink color, starch by dark pink, phenolic compounds by orange and proteins by the blue color. Histological sections were mounted between slide and coverslip with 50% glycerin, analyzed under light microscope. The images captured on the same scale on Samsung camera (SDC-313).

Results

Organographic and anatomical

The plants of the three populations observed in this study presented a shrub-arboreal size of 2-5 m height, cylindrical branches, glabrous aspect, and color ranging from green to greenish brown with well-defined lenticel lines and crossed opposite phyllotaxy. Leaves are simple, lanceolate, with adaxial glabrous surface, pubescent abaxial surface only on the midrib and secondary veins. They present whole margin, acuminate tip, attenuated base, penninerved, venation pattern is brochidodromous, 6-11 pairs of secondary veins, ranging from opposite to alternate in the same leaf (Figure 1A, B, C), and these characteristics were similar between plants of different localities.

There was variation in the presence of domatia on the abaxial leaf surface of the three evaluated populations. When present, they are from the crypt type, located at the intersection of the midrib with secondary veins (Figure 1D), isolated or in pairs (2-4) with wide cavity and aperture restricted to a slit, covered by trichomes (Figure 1E, F). Leaves have short petioles, convex and length varying from 0.5 to 1.5 cm in the three populations (Figure 1G). We recorded the ability of vegetative propagation by induction and development of adventitious roots in distal and proximal regions on the plant's petiole of the three evaluated populations (Figure 1H). In the abaxial surface, we observed trichomes located only in the regions of midrib and secondary veins and such characteristic was found among leaves of the three studied populations (Figure 1I, J, K, L, M). Plants subjected to seasonal stress presented leaf winding towards adaxial face during periods of high luminosity.

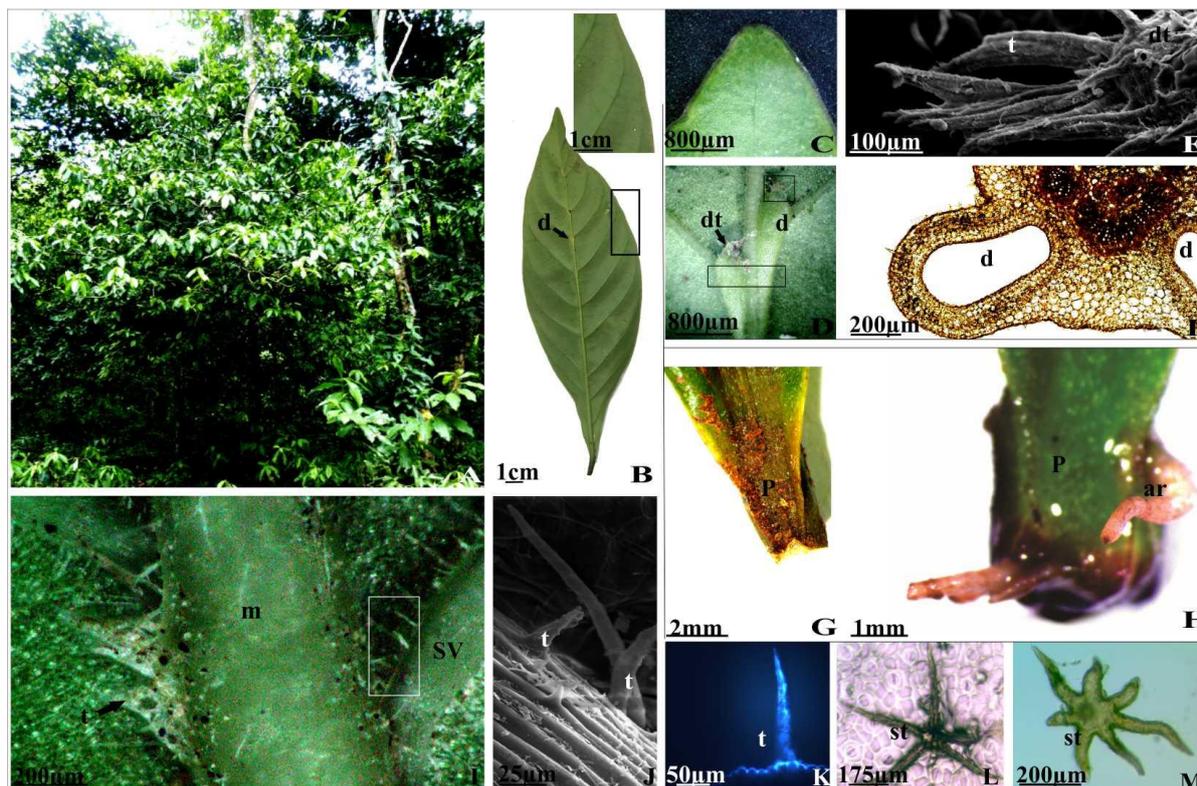


Figure 1. External and internal morphology of three *P. viridis* populations cultivated in the State of São Paulo. (A) *P. viridis* tree. (B) *P. viridis* leaf, evidencing in the rectangle the brochidodromous venation. (C) Leaf apex. (D) Domatia on the abaxial surface. Domatia tip shown in the square was evidenced in Figure E, and the central region shown in the rectangle evidenced in Figure F. (E) Domatia tip and aperture covered with trichomes. (F) Transversal section showing the larger portion of the internal cavity of domatia. (G) Petiole. (H) Adventitious root grown from petiole. (I) Midrib and secondary vein with trichomes. (J) SEM of non-glandular trichomes in the midrib. (K) Unicellular non-glandular trichome captured under polarized light. (L and M) Stellate non-glandular trichome. Abbreviations: ar, adventitious root; d, domatia; m, midrib; sv, secondary vein; p, petiole; st, stellate trichome; t, non-glandular trichome; td, tip of domatia.

Leaf samples of the three populations were significantly different ($F = 24.035$; $df = 2$; $p = 0$), being the leaves from Piracicaba population significantly smaller than those of other locations ($p < 0.01$), (Figure 2A). Petiole length did not differ statistically between the samples ($F = 3.0124$; $df = 2$; $p = 0.0659$). Regarding stomata density, we noted that the population from Piracicaba had significantly lower density than the plants from the other places ($p < 0.01$) (Figure 2B).

Leaf surface analysis in SEM, super-lupus and light microscopy revealed the same basic pattern among the three studied populations (Figure 3A-L). In the frontal view, the epidermal cells are polygonal with a straight anticline wall (Figure 3A). The leaf is amphi-hypostomatic and the paracytic stomata presents a random distribution on both adaxial and abaxial surfaces, (Figure 3B, C, D, E) with reniform guard cell and substomatal chamber right below (Figure 3I). We observed a flat and granular epicuticular wax pattern on the adaxial surface of leaves from the three studied populations (Figure 3E, F), and canalicular and papillary in the abaxial surface (Figure 3 G, H). We recorded the presence of terminal stipules, which in a longitudinal section evidenced the presence of dendroid colleters covered by unicellular and multicellular stellate trichomes (Figure 3J, K, L).

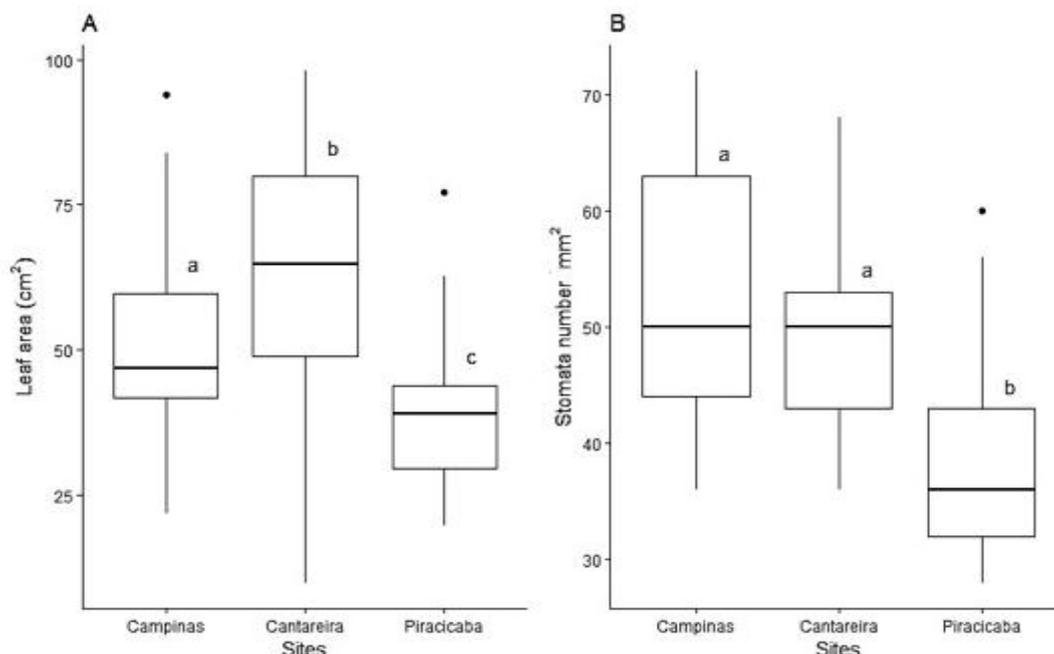


Figure 2. Comparison of leaf area and stomatal density in *P. viridis* leaves from the three study sites. (A) leaf area (cm²); (B) stomatal density (n mm⁻²). Horizontal bars are the medians, boxes represent 50% of the data (50^o quantile), rows are 95^o quantile and points are “outliers”. Different letters indicate a statistically significant difference at the level of 5% by Tukey test.

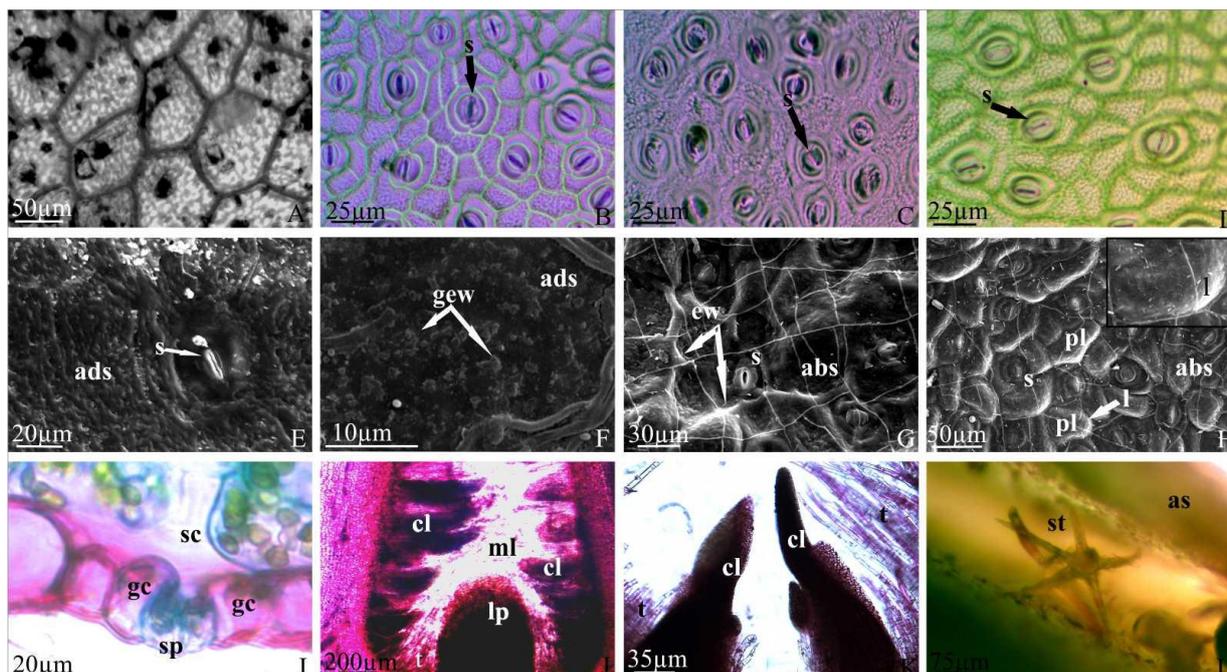


Figure 3. SEM observation, super magnifying glass and light microscopy of leaf surface of *P. viridis* of three populations in the Southeast region of Brazil. (A-D) Superficial view of leaves. (A) Adaxial surface showing cells with polygonal pattern and straight anticline wall. (B-D) Abaxial surface showing stomata. (B, C) Population of Campinas and Serra da Cantareira. (D) Population of Piracicaba. (E-H) SEM images of the leaf surface. (E) Stomata on the adaxial surface and smooth epicuticular wax. (f) Adaxial surface showing granular epicuticular wax. (g) Abaxial surface with canalicular epicuticular wax. (H) Abaxial surface evidencing the lobe of epicuticular papillae. (I) Stomata in cross section. (J-L) Longitudinal section of the apical stipule. (J, K) Colleters. (L) Stellate non-glandular trichome on the adaxial surface of the stipule. Abbreviations: Abs, abaxial surface; ads, adaxial surface; as, apical stipule; cl, colleter; ew, epicuticular wax; gc, guard cell; gew, granulate epicuticular wax; l, lobe; lp, leaf primordium; ml, mucilage; pl, papillae; s, stomata; sc, substomatal chamber; sp, stomatal pore; st, stellate trichome.

Cross sections of *P. viridis* leaves from the three populations, evidenced uniseriate epidermis on both surfaces, with well-developed vacuole and frequent presence of druses (Figure 4A-C), dorsiventral heterogeneous mesophyll, with uniseriate palisade chlorophyll parenchyma, composed of a single layer of elongated and juxtaposed cells on the adaxial surface (Figure 4A) and spongy tissue present on the abaxial surface with 4-6 layers of varying size cells, with irregular and reduced intercellular spaces (Figure 4A, B, C).

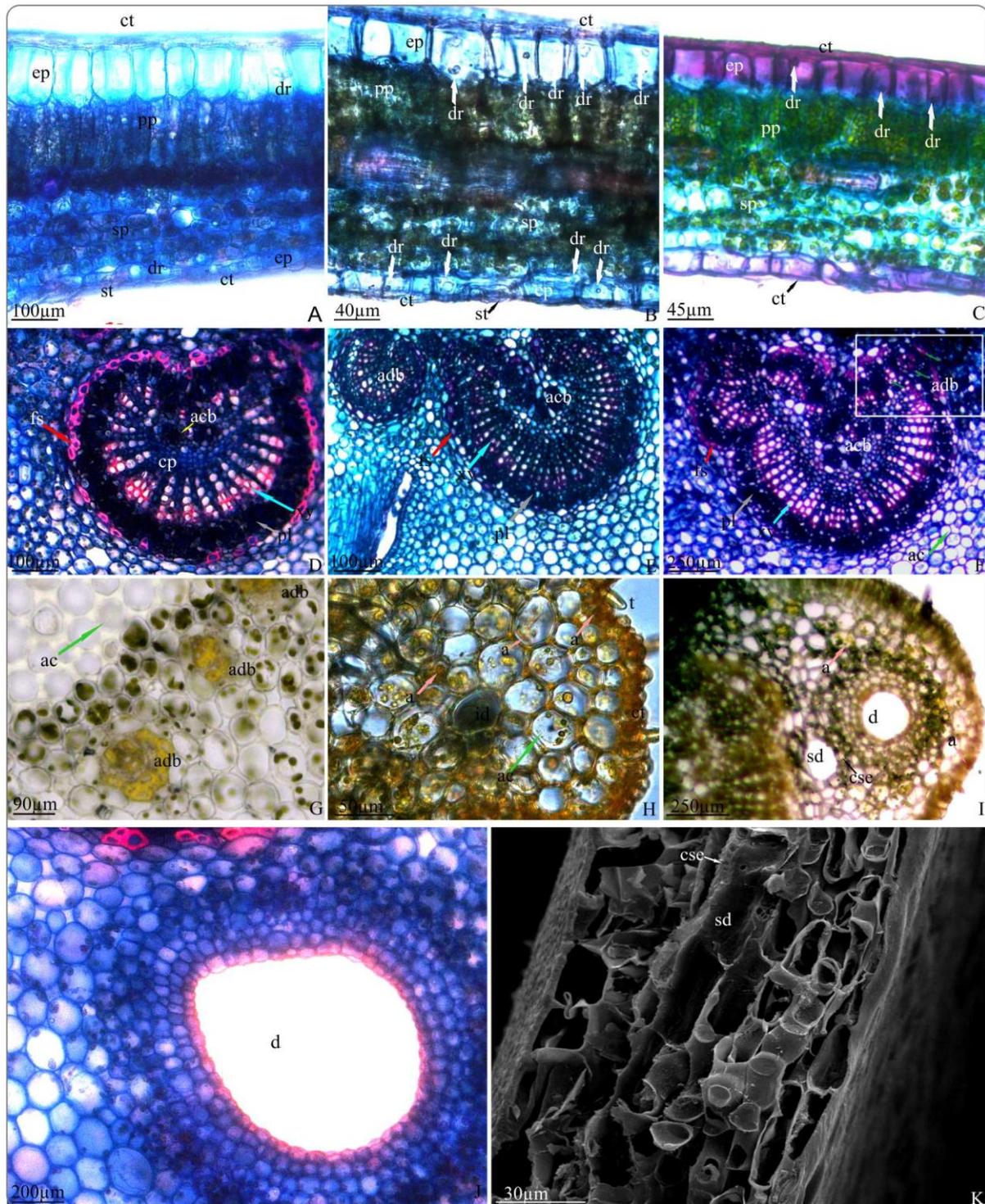


Figure 4. Transversal section of the mid third of leaves of *P. viridis* from the three populations cultivated in the State of São Paulo. (A-C) Dorsiventral mesophyll, druses in epidermis cells, one-layered palisade and multi-layered spongy parenchyma. (B) Several druses observed in epidermal cells of plants from Piracicaba. (C) Thick cuticle and bigger intercellular space in leaves of plants from Serra da Cantareira. (D) Midrib region showing fiber sheath and internal accessory vascular bundles. (E, F) Additional bundle. (F, G) Collenchyma region above and below midrib showing positive staining for alkaloids by the Dragendorff reagent, highlighting collenchyma cells and pattern of additional bundles (adb), which was similar in the three populations. (H) Presence of idioblast and tector trichome. (I) Presence of domatia and secretory duct surrounded by smaller cells in the population of Piracicaba. (J) Domatia internal shape in all populations. (K) SEM image showing secretory duct in longitudinal section. Abbreviations: a, alkaloid (orange arrow); ac, angular collenchyma (green arrow); adb, accessory bundles; acb, accessory bundles; cse, cell of the secretory epithelium; cp, cell of parenchyma; ct, cuticle; d, domatia; dr, druse (white arrow); ep, epidermis; fs, fiber sheath (red arrow); id, idioblasts; pl, phloem (gray arrow); sd, secretory duct; t, trichome; xy, xylem (blue arrow).

The vascular system has the shape of a convex arc, and it faces the adaxial side of the leaf, with convex terminal projections towards the abaxial surface (Figure 4D, E) and medullar parenchyma (Figure 4D). Terminal

projections of the vascular arc present fragmentation of the borders with formation of xylem and phloem agglomerates, with no clear arrangement of how the internal accessory bundles are formed (Figure 4D-F). Xylem cells present radial arrangement, separated by parenchyma rays (Figure 4D, E, F). Sclerenchyma fiber sheath surrounding the main bundle and additional bundles (1-4 pairs) are located laterally on the midrib (Figure 4E, F). Immediately below the adaxial epidermis and in the vein's mid region, there are several layers of angular collenchyma with presence of idioblasts (Figure 4G, H). In the mid region of veins, it was observed the presence of domatia (Figure 4I, J). We also noted sporadic presence of secretory ducts arranged adjacent and accompanying the midrib (Figure 4I and K).

Histochemicals

Histochemical analyzes performed with Dragendorff revealed the presence of alkaloids in the cuticle, epidermis, collenchyma cells, palisade and spongy parenchyma. Trichomes and starch were found in cells of the collenchyma, palisade and spongy parenchyma (Figure 5A, B). The Schiff reactivity (SPA) revealed the presence of phenolic compounds in collenchyma cells and spongy parenchyma (Figure 5C, D). Polysaccharides were observed on the cell wall of the epidermis and collenchyma, and proteins were observed in palisade and spongy parenchyma cells (Figure 5D). Transversal section of control (Figure 5 E, F).

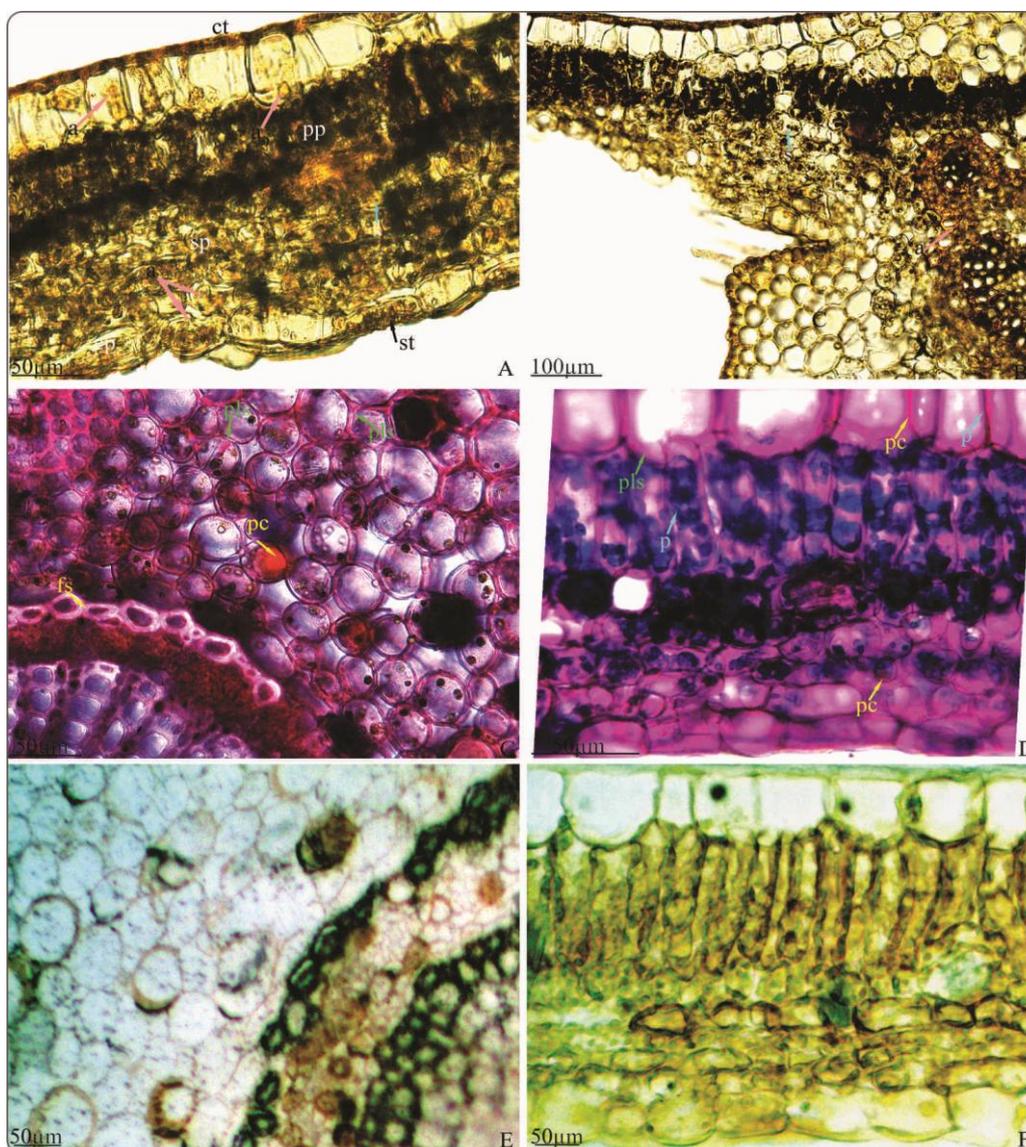


Figure 5. Transversal section of the mid third of *P. viridis* leaves of three populations cultivated in the State of São Paulo. (A, B) Dragendorff reagent revealed the presence of alkaloids in the cuticle, vacuole, cell wall and trichomes of the midrib for the three populations and starch in collenchyma cells, palisade and spongy parenchyma in leaves of the populations from Campinas and Serra da Cantareira. (C, D) Positive reaction for phenolic compounds, polysaccharides, protein by SPA staining. (E, F) Control. Abbreviations: a, alkaloid; ct, cuticle; ep, epidermis; fs, fiber sheath; p, protein; pc, phenolic compounds; pls, polysaccharides; pp, palisade parenchyma; s, starch; st, stomata t, tector trichome.

Discussion

Leaves of *P. viridis* sampled share most of its morphological characteristics with leaves of other species of *Psychotria* (Metcalf & Chalk, 1979; Quinteiro et al., 2007; Gonçalves & Lorenzi, 2007). However, some anatomical characteristics for the species presented here are being described and discussed for the first time, such as the amphi-hypoestomatic pattern and intraspecific variation of the domatia presence, leaf area and stomata density, as well as other anatomical adaptations that favor the water economy of plants under water stress.

Formation of adventitious roots on the petiole, with the consequent development of seedlings, allows to infer a connection with the main vascular system of the petiole, as suggested for *Camellia sinensis* (Koyuncu & Balta, 2004). Adventitious roots in *P. viridis* leaf tissues were also recorded by Salgado, Furlan, Aoyama, Rodrigues, and Cruz (2012). This characteristic points to an alternative reproductive strategy of this species, providing vegetative propagation from the leaves when they detach and reach the ground, which could be very important when pollination and dispersion vectors are absent or reduced.

Domatia is covered with trichomes and it is clearly visible, even with bare eyes, and less prominent or even absent in adult plants of the same population. This change indicates that the presence or absence of these structures is not a feature of taxonomic determination of *P. viridis*, although it is an important structure to the taxonomy of the Rubiaceae family (Metcalf & Chalk, 1979; Judd, Campbell, Kellogg, Stevens, & Donoghue, 2009) and the genus *Psychotria* (Agrawal & Karban, 1997; Mathur et al., 2011). Domatia presence in this species could be more related to the presence of predatory mites, which have been observed using these structures as a shelter in most plants of the three studied populations. The presence of mites, since the beginning of leaf development, could lead to domatia formation in the leaf, which is then benefited by the indirect defense provided by these animals. At the same time, the presence of these leaf structures could increase the recruitment of this defense agent (Agrawal & Karban, 1997; O'Connell, Lee, Monks, & Dickinson, 2010). This type of relation, possibly mutualistic, between arthropods and plants was already suggested previously (Carvalho, Boeger, Brito, & Goldenberg, 2011) (Carvalho, Boeger, Brito, & Goldenberg, 2011; Matos, Pallini, Bellini, & Freitas, 2006; Whiffin, 1972). Still, a more detailed study is required in order to determine the nature of this relationship to *P. viridis* and how environmental factors affect it.

Unicellular and multicellular tector trichomes located in the apical stipule and in the midrib and secondary veins in leaves of plants of the three studied sites indicate a strategy of hydric economy. Lipids present in the walls of the basal cells of the trichomes contribute to reduce the apoplastic loss of water (Barros & Soares, 2013). This way, the strategic location of trichomes in *P. viridis* could contribute to prevent water loss by cells of the vascular bundle and prevent desiccation of apical stipule and, consequently, protect the apical gem from dehydration, mechanical shock, and attack of predators and pathogens.

Dendroid colleters arranged longitudinally on the adaxial surface of the stipule with high density of trichomes and mucilaginous substances were found in the three studied populations. These are arranged as a structure of protection that prevents fungi contact with leaf primordia. As the mucilage has the function of lubrication of the apical gem (Fahn, 1979), its presence may facilitate the sliding of mites between the surfaces of leaf primordia, reinforcing the importance of mites to *P. viridis* leaf adaptation.

Leaves of all populations studied were amphi-hypoestomatic, which have not been reported for *P. viridis*, so far. Stomata density showed a positive relation with leaf area and negative with petiole length for all evaluated plants. In addition, significantly lower stomata density was recorded in plants of the drier area. Once stomata density is determined by the initiation of stomata during ontogenesis (Salisbury, 1928), and it is regulated since its development by hormonal signals (Bergmann, 2004) associated to environmental information (Pompelli, Martins, Celin, Ventrella, & DaMatta, 2010). The reduced number of stomata per mm² in the population of the drier site could be understood as an adaptive anatomical adjustment in response to seasonal hydric stress in which these plants are submitted.

Cuticle is thicker in the adaxial surface than in the abaxial, and it does not seem to be influenced by the environment. Similarly, other species also show thicker cuticle in the adaxial surface (Dickison, 2000; Esau, 1976), which could be attributed to the function of preventing non-stomatic water loss (Kosma & Rowland, 2016), independent of cuticle thickness. Epicuticular wax on the abaxial surface presented stretch marks and papillae patterns to the plants of the three evaluated populations, being smooth or granular on the adaxial

face, as described by Vieira and Gomes (1995) to *P. nuda*, *P. leiocarpa*, *P. stenocalyx*, and *P. ternuinervis*. Regarding the granular pattern, granules form aggregates of irregular distribution, and they were observed only on the adaxial face of plants under seasonal water stress. Granular epicuticular pattern was associated with the wax dissolution by enzymes secreted by mycological flora (Juniper & Jefree, 1983). However, the highest incidence of fungus on the abaxial surface was evident in all plants examined in this study, where it was not observed any granular pattern of ornamentation. Based on these observations, we can infer that the deposition pattern of epicuticular wax to *P. viridis* could also be associated with the specie's strategy for water maintenance.

Uniseriate epidermis with cells presenting larger anticlinal walls than periclinal in the adaxial surface, where it shows varied sizes, is considered as a common characteristic in *Psychotria* (Metcalfe & Chalk, 1979; Quinteiro et al., 2007; Zini, Martins, Toderke, & Temponi, 2016). Idioblasts containing alkaloids, dispersed druses throughout leaf lamina, mainly in epidermis cells, were also found in species of *Borreria* (Zini et al., 2016). Druses have important defense role against herbivory, and according to (Franceschi & Nakata, 2005) they contribute to increase the use of light during photosynthesis in plants that grow under shade.

Vascular system in form of an arch, with the convexity towards the adaxial surface and convex terminal projections facing abaxial were observed for all studied plants, similar to that observed for various species of *Psychotria* (Moraes et al., 2011). Vascular bundles are included in a fiber cylinder, constituting a fibrous sheath, already described by Quinteiro et al. (2007), which confers greater resistance to leaf movements (Cutler, Botha, & Stevenson, 2011). In the leaves of the studied plants, the presence of accessory bundles was observed, which represent agglomerates of xylem and phloem without a defined organization, formed from the fragmentation of the main bundle borders (Mantovani, Gomes, Gomes, & Vieira, 1995). These are characteristics of some members of the genus, such as in *P. carthagenensis* and *P. suturella* (Zini et al., 2016), *Irlbachia nemorosa*, (Corrêa, Melo, Krahl, & Araújo, 2014). In the plants of this study, accessory bundles of concentric and anficrival conformation were always observed in pairs of 1-3, rarely presenting 4 pairs that follow the midrib longitudinally, throughout the extension of leaf lamina.

The environmental differences in the three studied sites, mainly in terms of precipitation, also led to anatomical variations. Secretory ducts following the midrib longitudinally along the entire length of the leaf were recorded in samples from the population located in the drier site (Piracicaba), which is seasonally submitted to water stress, similar to that found by Polesi, Rodrigues, and Almeida (2011) for *Eugenia glazioviana*. It may represent an adaptive response as an irreversible anatomical change that, according to Esau (1976), could occur from the primordium of the leaf gem. The combination of field observation and anatomical traits found in this study, suggests that secretory ducts in *P. viridis* are possibly involved in the process of winding towards the adaxial surface in plants under water stress and its immediate expansion after irrigation, which would occur by the rapid water input and output along these ducts (Polesi et al., 2011). Therefore, this represents a strategy in water economy that was not yet described for the species. Considering mechanisms of preventing water deficit (Begg & Turner, 1976) and the importance of leaf winding for water maintenance (Kramer & Boyer, 1995), ducts and leaf winding in *P. viridis* may be involved in a probable process of water deficit adjustment, also to avoid leaf abscission. However, we recommend further studies that includes more plants of other environments to clearly define this relationship.

Ergastic substances identified in this paper, mucilage in apical stipules, phenolic compounds, polysaccharides, proteins and alkaloids in epidermal cells and mesophyll parenchyma were common to the three studied populations, which had already been cited by Quinteiro et al. (2007). The only exception found was the starch, which was not detected during the analysis on the population subjected to water stress. Reduction in the rate of starch accumulation had already been found in plants under water stress (Pan, Zhu, & Cao, 2007), and evidence that premature starch depletion results in lower growth rates have also been shown (Stitt & Zeeman, 2012). Starch is a storage carbohydrate involved in the development, growth and maintenance of the plant, thus, the exhaustion of this carbohydrate may also have played an important role in reducing the leaf area, as a negative effect of water stress of plants.

Conclusion

The results obtained in this study about the morpho-anatomical and histochemical characteristics of *P. viridis* plants cultivated in the three environmental are consistent with those already described for the species. The differences found in this study, such as secretory ducts, possibly containing osmoregulating

substances, leaf winding, leaf area reduction and depletion of starch might be associated to possible morphological and physiological responses to seasonal water stress.

Such variations reveal the ability of this species to develop tolerance strategies to low water availability, and to attract and maintain mutualistic organisms. However, more extensive studies must be considered, including populations from other localities, mainly from their original dispersal region, since they may provide subsidies for a better comprehension of the anatomical responses of this species to environmental parameters.

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