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Bioactive compounds and leaf anatomy of yerba mate morphotypes

Abstract – The objective of this work was to evaluate the bioactive compounds and foliar anatomy of yerba mate (*Ilex paraguariensis*) morphotypes. The antioxidant capacity, total phenolic compounds, methylxanthines, and caffeoylquinic acids were determined from the aqueous extract of leaves of the following yerba mate morphotypes: "sassafras", dark green, dull green, gray, and "peludinha". Light microscopy and scanning electron microscopy were used to observe the anatomical differentiations. The dark-green morphotype showed the highest contents of caffeine (20.4 mg g^{-1}), differing significantly only from the "peludinha" morphotype (8.8 mg g^{-1}). All morphotypes showed similar and high antioxidant activity (886.0 and 588.1 µmol Trolox equivalent antioxidant activity per gram of sample for ABTS and DPPH, respectively), as well as high total phenolic compounds content (73.9 mg gallic acid equivalent per gram of sample). Although the anatomy of the leaf mesophyll is similar, the wax deposition and cuticle striation configuration on the adaxial surface differ between all five morphotypes.

Index terms: *Ilex paraguariensis*, antioxidant capacity, genotype selection, methylxanthines.

Manoela Mendes Duarte[1] ✉, Mônica Moreno Gabira[1], Jéssica de Cássia Tomasi[1], Erika Amano[2], Antonio Carlos Nogueira[1] and Ivar Wendling[3]

[1] Universidade Federal do Paraná, Departamento de Ciências Florestais, Campus Jardim Botânico, Avenida Prefeito Lotário Meissner, nº 900, Jardim Botânico, CEP 80210-170 Curitiba, PR, Brazil. E-mail: manu.florestal@gmail.com, monica.gabira@gmail.com, jehtomasi@gmail.com, acnogueira.ufpr@gmail.com

[2] Universidade Federal do Paraná, Departamento de Botânica, Centro Politécnico, Avenida Coronel Francisco H. dos Santos, nº 100, Jardim das Américas, CEP 81530-000 Curitiba, PR, Brazil. E-mail: erika.amano@gmail.com

[3] Embrapa Florestas, Estrada da Ribeira, Km 111, Parque Monte Castelo, CEP 83411-000 Colombo, PR, Brazil. E-mail: ivar.wendling@embrapa.br

✉ Corresponding author

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Compostos bioativos e anatomia foliar de morfotipos de erva-mate

Resumo – O objetivo deste trabalho foi avaliar os compostos bioativos e a anatomia foliar de morfotipos de erva-mate (*Ilex paraguariensis*). A capacidade antioxidante, os compostos fenólicos totais, as metilxantinas e os ácidos cafeoilquínicos foram determinados a partir do extrato aquoso das folhas dos seguintes morfotipos de erva-mate: sassafrás, verde-escuro, verde fosco, cinza e peludinha. A microscopia de luz e a microscopia eletrônica de varredura foram utilizadas para observar a diferenciação anatômica. O morfotipo verde-escuro apresenta os maiores teores de cafeína (20,4 mg g^{-1}) e difere significativamente apenas do morfotipo peludinha (8,8 mg g^{-1}). Todos os morfotipos apresentaram atividade antioxidante semelhante e alta (886,0 e 588,1 µmol de atividade antioxidante equivalente a Trolox por grama de amostra, para ABTS e DPPH, respectivamente) e elevado conteúdo de compostos fenólicos totais (73,9 mg de equivalente a ácido gálico por grama de amostra). Embora a anatomia do mesofilo da folha seja semelhante, a deposição de cera e a configuração do estriamento da cutícula na superfície adaxial diferem entre os cinco morfotipos.

Termos para indexação: *Ilex paraguariensis*, capacidade antioxidante, seleção de genótipos, metilxantinas.

Introduction

Widely consumed in southern Brazil and Mercosur countries, yerba mate (*Ilex paraguariensis* A.St.-Hil.) is responsible for one of the most important extractive activities in Brazil. Several southern Brazil municipalities have their economy based on yerba mate plantations or extraction from native forests. In addition to the traditional uses as *chimarrão*, *tereré*, and *mate* tea, the current scenario evidences an increasing potential of yerba mate, which has been successfully applied for cosmetic, aesthetic, and culinary purposes (Godoy et al., 2013; Alkhatib & Atcheson, 2017; Barboza & Cazal, 2018).

The versatility of yerba mate due to the range of its bioactive compounds has aroused interest in the national and international markets. The presence of methylxanthines, saponins, minerals, phenolic acids, and their derivatives gives yerba mate a high antioxidant capacity and other beneficial actions to human health (Valduga et al., 2019). These attributes launch opportunities for research, new technologies, and product development. Also, it contributes to increase importance of this economic sector due to the higher added value of final products.

There is a high phenotypic variation in yerba mate plants whether in natural populations or commercial plantations, which are mainly related to size, hairiness, and leaves and stem color (Resende et al., 1995; Wendling et al., 2016). Morphologically similar plants are commonly called morphotypes. This polymorphism may be associated with ecological variations or species peculiarity. With large-scale commercial production, another assumption would be the anthropic migration of genotypes from different origins and subsequent crosses.

The knowledge about morphotype peculiarities is essential for prospecting future products and markets, as well as leaf anatomical characteristics, an important feature for the ecology of species. However, species morphological diversity is still little known and explored, and literature on yerba mate morphotypes is quite restricted (Resende et al., 1995; Wendling et al., 2016). For the next years, the increasing interest of industries and the potential of this species for developing food, energetic drinks, cosmetics, and pharmaceutical products from yerba mate leaves.

Due to yerba mate's economic, social, and ecological importance, it is imperative to research it and observe if, as besides its morphotype visual differences, there are other essential variations.

The objective of this work was to evaluate the bioactive compounds and leaf anatomy of yerba mate morphotypes.

Materials and Methods

The morphotypes used in the present study were "sassafras", dark green, dull green, gray, and "peludinha" (Wendling et al., 2016). All plant materials were collected in a progeny test, in the municipality of Ivaí (25º01'S, 50º47'W, at 650-750 m altitude), in the state of Paraná, Brazil. The morphotypes classification was based in Wendling et al. (2016), who described the characteristics of the morphotypes as: "sassafras" - leaves with a dark green tone and extreme brightness on the limbus adaxial surface; dull green - leaves with a lighter and opaque green color, absence of glare on the adaxial surface; dark green - dark green tone, absence of glare on the leaf adaxial surface; gray morphotype - adaxial surface with greenish-gray color; and "peludinha" - presence of trichomes, especially on the abaxial surface (Figure 1).

In June 2019, mature and injury-free leaves were collected across the canopy circumference of 10 individuals of each morphotype. The leaves were dried in a microwave (Electrolux MEP37, 1,000 W, 2,450 MHz, Manaus, AM, Brazil), and crushed until a thin and homogeneous powder was attained. To prepare the aqueous extract, 10 mg of the ground sample was added to 2 mL of ultrapure water and mixed for 30 s by pulse-vortex. The extractions were performed in Thermomixer Eppendorf equipment (Hamburg, Germany) at 60°C and 300 rpm, for 1 hour, and later filtered through a 0.22 mm nylon filter.

Methylxanthines and caffeoylquinic acids analyses were performed in a liquid chromatograph (Shimadzu, Kyoto, Honshu, Japan) controlled by a LC solution software (Shimadzu). The chromatograph was equipped with an automatic injector and UV detector (SPD-20A), with a Shim-Pack CLC-ODS (M) C18 column (250 x 4.6 mm i.d., 5 µm particle size), protected by a Shim-Pack CLC G-ODS pre-column (100 x 4.0 mm i.d.), both Shimadzu (Kyoto, Japan).

The conditions for compound separation (20 µL injection) were: 30°C with a 0.5 mL per min flow, and the mobile phases consisted of a gradient elution

of water with acetic acid 99.9:0.1 v/v (solvent A – Alphatec, Brussels, Belgium) and acetonitrile 100% (solvent B – Merck, Darmstadt, Hesse, Germany). The gradient elution program was: 0−15 min (3% B); 15−20 min (3-20% B); 20−40 min (20% B); 40−45 min (20-30% B); 45−55 min (30-100% B); 55−75 min (100% B); 75−80 min (100−3% B); and 80−95 min (3% B). The wavelength used to detect the compounds was 280 nm (fixed).

The identification and quantification of methylxanthines caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) was performed using analytical curves (Sigma Standard, Darmstadt, Hesse, Germany), with concentrations from 0 to 1.0 mg mL^{-1} (R^2 = 0.9933) and 0 to 0.5 mg mL^{-1} (R^2 = 0.9827), respectively. Caffeoylquinic acids (3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA) were identified and semiquantified from an analytical curve (Sigma) from 0 to 10 mg mL^{-1} 3-CQA (R^2 = 0.9914). The results were expressed in milligrams of compound per gram of sample (mg g^{-1}) on a dry basis.

To determine the total phenolic compounds, the Folin-Ciocalteu spectrophotometric methodology (Singleton & Rossi, 1965) was followed, with minor modifications. In a volumetric flask, 0.1 mL extract, 6 mL distilled water, and 0.5 mL Folin-Ciocalteu reagent (Sigma) were added and stirred for 1 min in a vortex mixer (Vortex-Genie 2, Scientific Industries, Bohemia, New York, United States). Then, 2 mL of 15% aqueous Na_2CO_3 solution was added and stirred for 30 s. The final volume was adjusted to 10 mL with distilled water. The reaction remained in the dark, at room temperature (23±2ºC), for 2 hours; afterward, absorbance was measured at 760 nm in the UV-1800/ visible scanning spectrophotometer (Shimadzu, Kyoto, Japan). The analytical curve was obtained with gallic acid (3,4,5-trihydroxy benzoic acid) (Dinâmica,

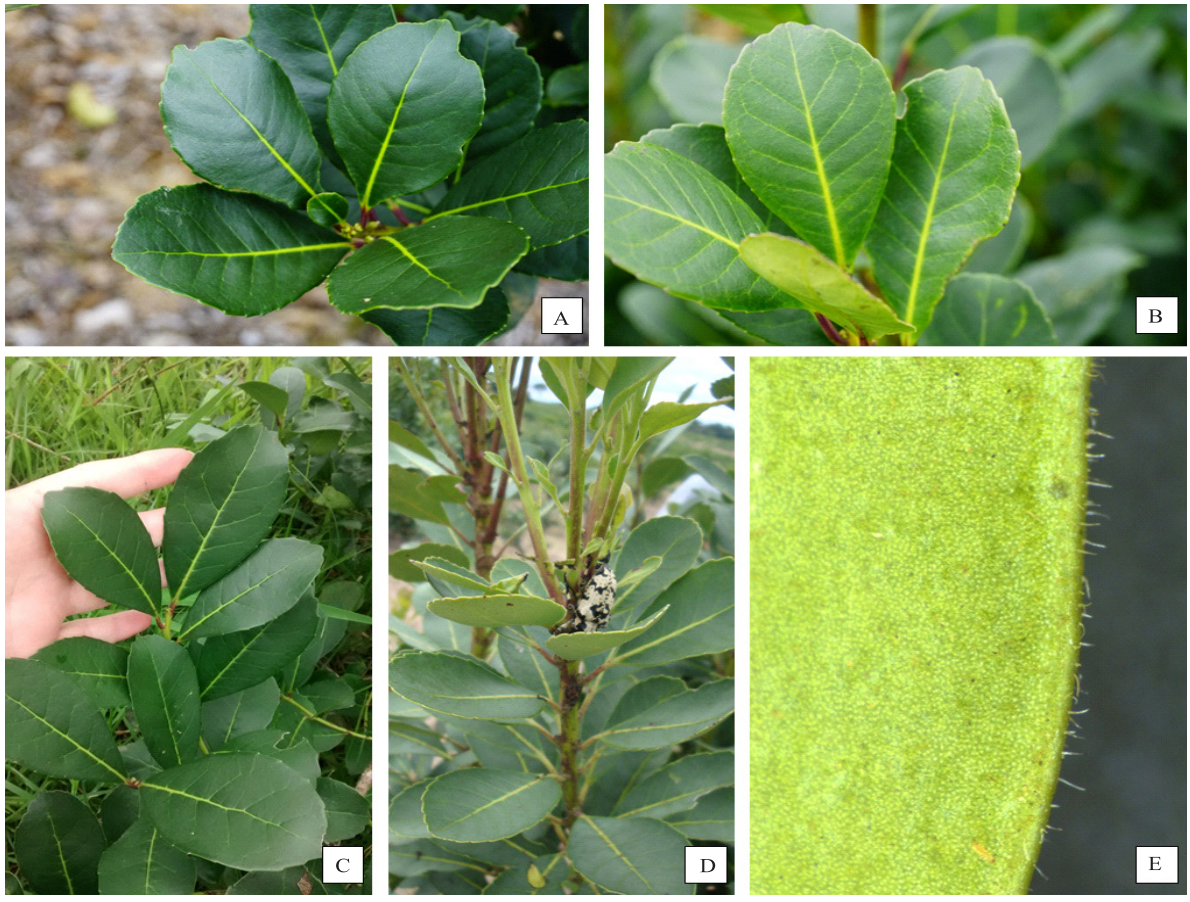

Figure 1. *Ilex paraguariensis* morphotypes: A, "sassafras"; B, dull green; C, dark green; D, gray; E, "peludinha". A, B, C, D: photo courtesy of Manoela Mendes Duarte. E: photo courtesy of Letícia Siqueira Walter.

Indaiatuba, São Paulo, SP, Brazil) with 0.25 to 13 mg L^{-1} (R^2 = 0.9988), and the results expressed in milligrams of gallic acid equivalent per gram of sample (mg EAG g^{-1} sample) on a dry basis.

The antioxidant capacity of the extract by the radical ABTS − 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) − was determined by the reaction of 10 mL ABTS (7 mmol L^{-1}, Sigma Aldrich) with 176 µL potassium persulfate (140 mmol L^{-1}), for 16 hours, in the dark. Subsequently, 1 mL of this solution was added to sodium acetate buffer (80 mmol L^{-1}; pH 4.5), and the solution absorbance was adjusted to 0.70±0.05. An aliquot of 3 mL of the adjusted solution was added to 30 µL of extract. The samples were kept in the dark for 2 hours, at room temperature (23±2ºC), and absorbance was measured at 734 nm in the UV-1800/visible scanning spectrophotometer (Shimadzu, Kyoto, Japan), following Re et al. (1999) and Yim et al. (2013), with minor modifications.

The antioxidant capacity of the extract via free radical DPPH (2,2-diphenyl-1-picryl-hydrazine) was determined by using 0.1 mL extract diluted in 3.9 mL DPPH methanolic solution (0.06 mmol L^{-1}, Sigma Aldrich) (absorbance 0.50±0.05) and maintained for 30 min in the dark, at room temperature (23±2ºC). Absorbance was measured in the UV-1800 UV/visible scanning spectrophotometer (Shimadzu, Kyoto, Japan), at 515 nm, following Brand-Williams et al. (1995), with minor modifications.

The analytical curves were obtained with Trolox (6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid, Sigma Aldrich), with concentrations from 0 to 2,500 µmol L^{-1} (R^2 = 0.9896), for the conversion of ABTS radical scavenging absorbance, and concentrations from 0 to 1000 µmol L^{-1} (R^2 = 0.9868) for the conversion of DPPH radical scavenging absorbance. Results were expressed in micromoles of Trolox equivalent antioxidant capacity per gram of sample (µmol TEAC g^{-1} sample) on a dry basis.

All chemical analyses were performed in triplicate. Data were tested for the homogeneity of variances (Bartlett's test) and normality of residues (Shapiro-Wilk's test). When there was a significant difference in the analysis of variance for the parametric variables, the comparison of means was performed by the Tukey's test, at 5% probability, and the Kruskal-Wallis' test for the nonparametric variables.

Mature leaves, from the fourth to the eighth knot, from three plants of each morphotype, were collected and fixed in FAA_{50} (formalin-acetic acid-alcohol). The median region of the leaf blade was fixed, dehydrated in an alcoholic series, embedded in Leica historesin (Wetzlar, Hesse, Germany), and sectioned in a rotation microtome. The slides were stained with toluidine blue, and observed under light microscope (Olympus BX41, Feasterville-Trevose, PA, USA). The same fixation and dehydration process was used to analyze epidermis in a scanning electron microscopy (SEM). Subsequently, the samples were dried by the critical point method, adhered to a metallic support with carbon adhesive tape, metalized with gold and, then, analyzed on an electron microscope (JEOL JSM 6360-LV, Akishima, Tokyo, Japan).

Results and Discussion

Among the bioactive compounds, only the caffeine content showed a significant difference between morphotypes (Figure 2). The dark green morphotype showed the highest caffeine content (20.4 mg g^{-1}), differing significantly only from the "peludinha" morphotype (8.8 mg g^{-1}). Theobromine and the sum of methylxanthines showed no significant difference between all morphotypes.

Differences for caffeine and theobromine content between morphotypes have already been reported in the literature; however, the results are controversial. Reginatto et al. (1999) concluded that *I. paraguariensis* var. *vestita* ("peludinha" morphotype) shows much

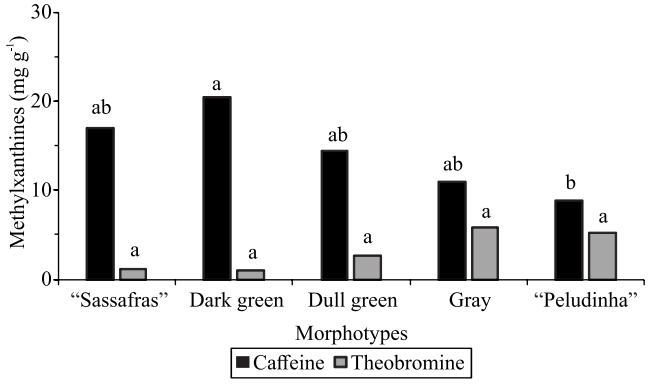

Figure 2. Total contents of methylxanthines (caffeine and theobromine) in *Ilex paraguariensis* morphotypes. Bars followed by equal letters do not differ by the Kruskal-Wallis' test.

lower methylxanthine content than that observed for typical *I. paraguariensis*; the same trend was observed by Coelho et al. (2001). In contrast, Borille et al. (2005) did not identify significant differences for caffeine and theobromine, when studying three yerba mate morphotypes (yellow, gray, and "sassafras"), which is corroborated by the findings of the present study for the morphotypes gray and "sassafras".

There was an inverse relation among caffeine and theobromine content in all morphotypes (Figure 2). This result is due to the gene regulation of caffeine biosynthesis route for *I. paraguariensis* (Yin et al., 2015), for which theobromine is the direct precursor. This inverse relationship was also reported by Cardozo Junior et al. (2010) and Vieira et al. (2021) in yerba mate genotypes. In a study developed with *Camellia*, the genotypes with high theobromine and low caffeine contents showed a higher gene expression related to the caffeine degradation pathway (Zhu et al., 2019).

From a commercial point of view, different caffeine contents are attractive because they provide an expanded range of products and potential applications. In the same way that high levels of caffeine are desired in energetic and stimulating groceries/beverages, those with low caffeine levels or which are decaffeinated are also the target of several consumers. Alternative products for problems associated with insomnia, hyperactivity, heartburn, and tachycardia represent a market to be exploited (Valduga et al., 2019).

There are still few studies reporting caffeine content as a target for yerba mate breeding programs, but results observed in the literature for this and other compounds indicate the possibility of genetic gains with genotypes selection, especially for caffeine high heritability (Cardozo Junior et al., 2010; Duarte et al., 2020; Tomasi et al., 2021; Vieira et al., 2021). Morphotypes differentiation concerning bioactive compounds can become the target of yerba mate silviculture, with the possibility of introducing genotypes with differentiated raw material.

The antioxidant capacity for both radicals, ABTS and DPPH, and the content of total phenolic compounds did not differ between morphotypes (Table 1). There were similar results for total methylxanthines and caffeoylquinic acids (3-CQA, 4-CQA, 5-CQA, and total CQA) contents. Phenolic compounds act in different ways on human health, showing antioxidant, anti-inflammatory, and anti-allergenic activities, also inhibiting tumors (Chaicouski et al., 2014). *I. paraguariensis* is a natural source of phenolic compounds, especially chlorogenic acids and their derivatives that are the main responsible for the species' high antioxidant activity (Riachi & Maria, 2017). In the present study, all morphotypes showed high antioxidant activity and contents of total phenolic compounds, resulting in excellent raw material for human consumption.

Regarding the epidermal anatomic characteristics, the deposition of epicuticular wax and striation pattern on the leaf adaxial surface differ between all morphotypes, which was the main anatomical difference observed in the present work (Figure 3). "Sassafras" morphotype (Figure 3 A) shows a deposition of epicuticular wax in poorly defined filaments, with a tendency toward a smoother cuticle and some dispersed wax granules. The dark green morphotype shows a very striated cuticle, with well-defined and prominent filaments and few wax granules (Figure 3 B). The dull green morphotype shows a smooth cuticle, with discrete unidirectional filaments and numerous wax granules that are similar to those of "sassafras" (Figure 3 C). The gray morphotype shows

Table 1. Antioxidant capacity ABTS [2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH (2,2-diphenyl-1-picryl-hydrazine) radicals, total phenolic compounds (PC), total methylxanthines (MX), and caffeoylquinic acids (CQA), in *Ilex paraguariensis* morphotypes, in 2019, in the municipality of Ivaí, in the state of Paraná, Brazil[(1)].

Morphotype	ABTS (μmol TEAC g^{-1})	DPPH (μmol TEAC g^{-1})	Total PC (mg GAE g^{-1})	Total MX	3-CQA	4-CQA	5-CQA	Total CQA
				(mg g^{-1})				
"Sassafras"	830.6±64.2a	569.9±60.7a	75.4±5.7a	18.1±5.9a	19.3±5.1a	15.6±3.1a	42.8±6.9a	77.7±13.8a
Dark green	902.1±90.1a	594.3±52.2a	75.2±7.3a	21.4±3.4a	18.4±3.0a	16.7±2.2a	48.0±7.6a	83.1±9.9a
Dull green	882.5±106.8a	568.4±75.2a	72.5±7.3a	17.1±4.6a	20.4±5.9a	16.8±3.7a	44.9±9.3a	82.1±17.2a
Gray	889.0±107.9a	611.8±81.7a	71.6±10.9a	16.7±8.0a	20.0±4.4a	15.3±4.7a	40.0±9.9a	75.2±16.5a
"Peludinha"	925.8±99.5a	596.0±82.7a	74.9±8.5a	14.0±8.2a	20.9±5.4a	18.6±3.7a	47.9±6.8a	87.3±11.5a

[(1)]Averages followed by equal letters in the columns do not differ, by Tukey's test, at 5 % probability.

a slight striation, and filaments are less prominent than those observed for the dark green morphotype, with wax granules (Figure 3 D). "Peludinha" morphotype (Figures 3 E and G) shows a very pronounced striation, with wax granules and the presence of trichomes randomly scattered along the adaxial surface, at low density.

The abaxial surfaces of all morphotypes were very similar, with high stomatal density, stomatic pores without obstruction, and few dispersed wax granules (Figure 3 F). Stomata were not observed on the leaf adaxial surfaces. "Peludinha" morphotype is differentiated on the abaxial surface by the presence of trichomes in higher numbers, especially in the petiole and veins (Figures 3 E and G). In addition to their taxonomic importance, trichomes play a fundamental role for plant protection as a natural barrier against herbivores, radiation, pathogen attack, and excessive transpiration; the distribution and morphology of this anatomical apparatus are related to several factors, including the geographical ones (Xiao et al., 2017).

For yerba mate, trichomes are described and attributed to the botanical variety *Ilex paraguariensis* var. *vestita* ("peludinha" morphotype). Its main characteristic is the presence of trichomes on the leaves, inflorescences, and branches surface (Coelho et al., 2001); according to these authors, the *vestita* variety occurs naturally in the northeast of Paraná and in the southwest of São Paulo, in a *cerrado* and

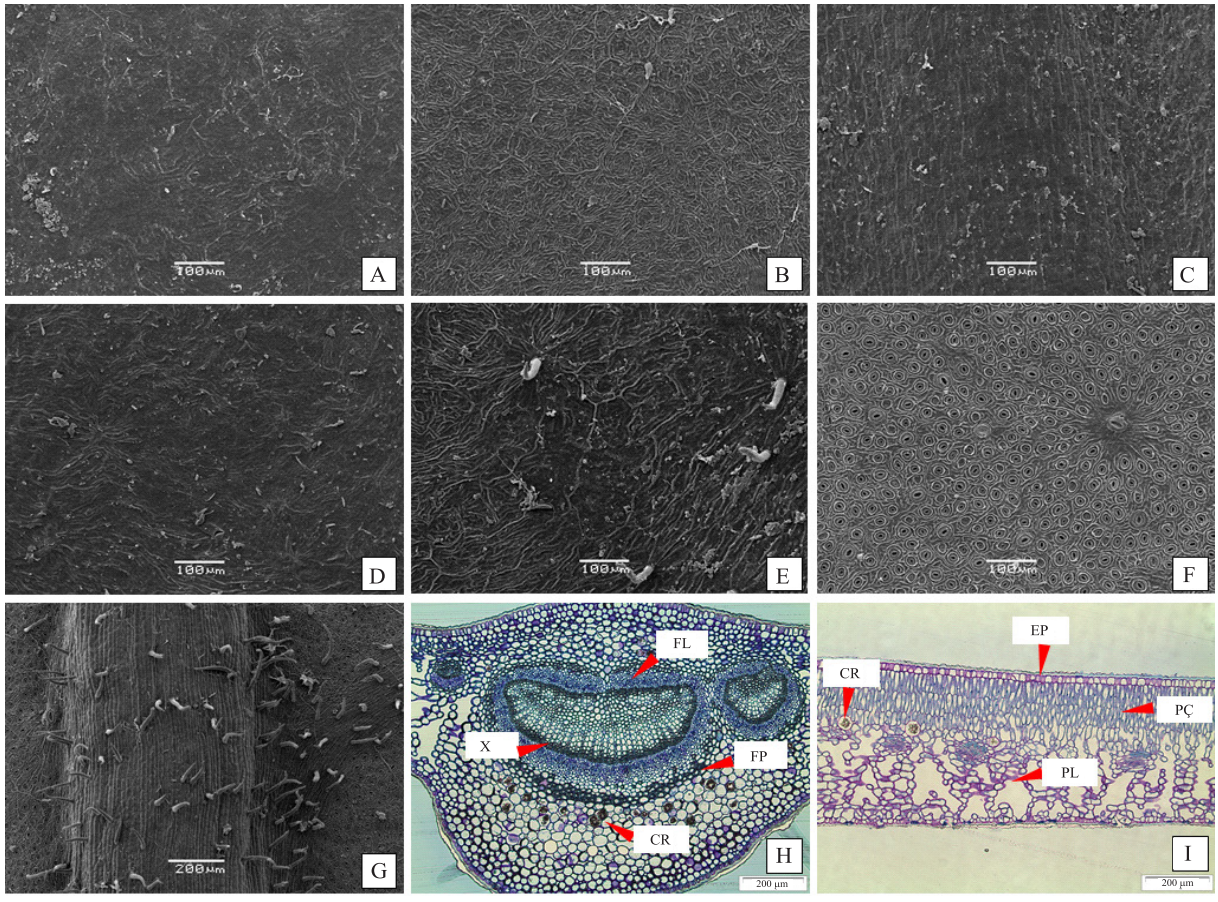

Figure 3. Leaf anatomy of *Ilex paraguariensis* morphotypes, with the general characterization of the adaxial face in scanning electron microscopy of the morphotypes: A, "sassafras"; B, dull green; C, dark green; D, gray; and E, "peludinha". The presence of trichomes is illustraded in: F, abaxial face, image of "sassafras" morphotype; and G, central vein detail of "peludinha" morphotype. General anatomical characterization under light microscopy and cross-section showing the central rib (H, "sassafras" morphotype), and dorsiventral mesophyll (I, dull green morphotype). FL, phloem; X, xylem; CR, calcium oxalate crystals; PC, palisade parenchyma; PL, lacunous parenchyma; EP, epidermis; FP, perivascular fibers.

transition region. It is assumed that the presence of trichomes is associated with the plant's origin.

The leaf epidermis is uniseriate, with juxtaposed cells and a larger cell diameter in cross-section on the adaxial surface. The mesophyll is dorsiventral, with palisade parenchyma composed of two to three strata of elongated and juxtaposed cells (Figures 3 H and I). Lacunous parenchyma is formed by cells of different shapes, with irregular distribution loosely arranged. Central vein is biconvex, with a closed arc vascular system, with bundle surrounded by a fiber sheath. Below the palisade parenchyma, large isodiametric parenchymal cells were observed, with druses along the leaf blade (Figures 3 H and 3 I). Same crystals are also observed in the central vein of leaves, close to the vascular bundle (Figure 3 H). All morphotypes showed the same pattern for these anatomical characteristics.

The few anatomical studies for yerba mate focus on differences between leaves subjected to distinct environments (Fermino Junior & Fockink, 2017; Bastias et al., 2018), and there are no reports on morphotypes. The results obtained in the present study are of great relevance for the yerba mate ecology and silviculture, and it shows the potential for improving its production system to obtain differentiated products. These results suggest further studies focusing on the sensory evaluation and potential of new products for each morphotype.

Conclusions

1. The caffeine content differs only between the dark green (highest content) and "peludinha" (less content) *Ilex paraguariensis* morphotypes.

2. All five yerba mate morphotypes – "sassafras", dark green, dull green, gray, and "peludinha" – show similar and high antioxidant capacity, and high content of phenolic compounds.

3. Although the anatomy of the leaf mesophyll is similar, the deposition of wax and cuticle striation on the adaxial surface differs between all five morphotypes.

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