Laguncularia Racemosa Leaves from a Mangrove of the Southeast Atlantic Coast, Brazil: Epicuticular Wax, Morphoanatomical Traits and Minerals

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Research Article

Keywords: Sepetiba Bay, epicuticular wax, leaf cuticle, mangroves, heavy metals, micromorphology

Posted Date: July 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-540578/v1

Abstract

Leaves of *Laguncularia racemosa* (L.) Gaertn. f. were collected from the following mangroves along Brazil's southeastern Atlantic coast: Coroa Grande (CG), Pedra de Guaratiba (PG) and Marambaia (M). This work aimed to evaluate the presence of minerals by Energy Dispersive X-Ray Spectroscopy (EDS) and Inductively coupled plasma - optical emission spectrometry (ICP-OES); the chemical composition of epicuticular waxes by gas chromatography–mass spectrometry (GC-MS) and the leaf morphoanatomical features. Results revealed variation in metal contents among mangroves in the following ranges: Al (0.307–0.73), Cd (0.004–0.016) and Pb (0.095–0.325) mg/mL by ICP-OES. Leaf epicuticular wax contained more than 50% of triterpenes, in particular the pentacyclic triterpenes lupeol (41.61–55.63%) and β-amyrin (8.81–16.35%), as well as n-alkanes, such as hentriacontane and tetratetracontane. In particular, we observed differences in the micromorphology of the epicuticular wax in the leaves of plants from each of the three evaluated sites, especially around stomatal entrances. Histochemical reaction indicated the presence of zinc in fiber cell walls and druse crystals of leaves.

1. Introduction

Mangroves are affected by domestic and industrial pollutants. For example, domestic waste increases phosphates and plasticizers in the environment, while industrial waste varies by industry. These residues are dumped in rivers and flow into the mangroves, tending to accumulate as a result of the large amount of organic matter in sediments and/or filtered particulate matter with the help of tree roots (Souza et al. 2018). Such residues may also enter and accumulate into living organisms or come into contact with microbes inhabiting the soil and, as a consequence, affect different trophic levels (Fernandes 2012).

Leaves correspond to most of the primary production in the mangrove ecosystem and are the main constituents of the litter (Clough et al. 2000), comprising, in turn, the food resource of insects and arboreal crabs. Leaf evaluation allows researchers to detect pollutants in different ecosystems by various morphoanatomical changes or changes in plant metabolism (Bargagli et al. 1998; Flores et al. 2018; Victório et al. 2020). Different studies of mangroves have shown the presence of heavy metals and plasticizers in leaves (Alzahrani et al. 2018; Almahasheer et al. 2018; Victório et al. 2020; Victório et al. 2021; Flores et al. 2021). These substances are taken up by plants through their leaves, but roots are the first and most common organs in contact with pollutants (Cheng et al., 2017). In the roots, pollutants are freely diffused via apoplast or symplast or carried across cells to xylem from which contaminants are transported throughout the plant (Kvesitadze et al. 2015).

Sepetiba Bay is an aquatic saline environment surrounded by a large restinga and a mangrove, ecosystems that have been severely impacted by anthropic activities since the 1970s. This Bay has experienced an increase in industrialization with the construction of the Sepetiba Industrial Complex, the Itaguaí Harbour area and the Industrial District of Santa Cruz. These areas were and are the sites of several metallurgical, petrochemical, and pyrometallurgical smelters, as well as chemical, textile, beverage, and paper plants (SEMA 1998; Wasserman et al. 2013; Victório et al. 2020; Silva and Victório

2021). The entire region is dubbed the “sacrifice zone” by the extraordinary damage caused to such coastal systems as mangroves, not to mention societal issues arising from the ever-expanding industrial complex and port that receives industrial waste (Viégas 2006). Metal smelting, including Zn, Cd, Al, Fe, and alloy steel are the major economic activities located in Sepetiba Bay's basin, followed by the chemical and paper industries (Lacerda et al. 2004; Wasserman et al. 2001, 2013; Tonhá et al. 2020). Previous studies reported improper disposal of solid wastes, such as Zn and Cd, which come from an industrial site on Madeira Island, one of the main sources of heavy metal to Sepetiba Bay (Fonseca et al. 2012). Most heavy metal inputs to the bay arrive from rivers, mainly as drainage from the most industrialized and urbanized areas of Sepetiba Bay (Guandu River, Guarda River and Sao Francisco Channel) (Marins et al. 1999; Fonseca et al. 2012). Cyclical periods and the action of marine tidal currents create a dynamism that alters the chemical and physical condition of mangroves; and bring pollutants directly to mangroves (Silvan and Madureira 2012). Heavy metals also reach Sepetiba Bay through the atmosphere. Atmospheric deposition of pollutants emitted outside the Bay area may further contribute to the total heavy metal load (Marins et al. 1999). Heavy metals are not degraded; instead, they become concentrated in water, sediments and plants themselves posing a threat to the entire food chain (Almahasheer et al. 2018).

Laguncularia racemosa (L.) Gaertn. f. (Combretaceae) is an arboreal plant which occurs in mangrove swamps on the Atlantic coasts of the Americas and West Africa (Sugiyama 1995, Nyananyo et at. 2009). A typical pioneer found in the interior of the mangroves and in the transition to the restinga forest, *L. racemosa* is popularly known as mangue-branco. The leaves are slightly succulent with red or purplish petiole. *L. racemosa* exhibits a complex root system with five types of roots: anchoring, cable, pneumatophores that form pneumathodes at the tip with hypertrophied lenticels, feeding, and lateral aerial roots arising from the pneumatophores (Angeles et al. 2002) that contain lenticels favoring oxygenation in a flooded environment (Tomlinson 1986). This plant does not have barriers in roots to protect against the entry of salt that comes from seawater; instead, it is excreted through specialized glands in the leaves, which, in addition, produce solutes in their tissues that contribute to the maintenance of osmotic balance (Kathiresan and Bingham 2001). It is well known that mangrove plants can absorb, accumulate, degrade/transform and volatilize metals by green remediation processes (Fernandes 2012).

In this study, we investigated the presence of minerals and heavy metals in leaves of *L. racemosa* collected in different mangroves of Rio de Janeiro situated around Sepetiba Bay - Marambaia, Pedra de Guaratiba and Coroa Grande. In addition, we analyzed the leaf epicuticular waxes for chemical composition and micromorphology, including histochemical tests to evidence the presence of zinc in internal tissues.

2. Materials And Methods

2.1. Plant materials and mangrove areas

Leaves of *Laguncularia racemosa* (L.) Gaertn. f. used in the present study were collected from five plants during the reproductive stage at mangroves around Sepetiba Bay between June and July 2012: Coroa Grande-CG (22°54'42.23"S and 43°52'48.88" W); Pedra de Guaratiba-PG (23° 0'27.98"S and 43°37'22.41"W); and Marambaia -M (23° 2'32.46"S and 43°35'43.99"W) (Figs. 1, 2).

Approximately 30 expanded and mature leaves (Fig. 2) were collected from the third to fifth node, from the apex of the branches, at a height of approximately 1.75 cm above substrate level, from five individuals for each mangrove site. Vouchers are deposited at the Herbarium RBR at UFRRJ, Rio de Janeiro, Brazil.

2.2. Analysis of Sepetiba Bay: physicochemistry and heavy metals

Sepetiba Bay has brackish water according to resolution n. 357/2005 of CONAMA, Brazil (Conselho Nacional do Meio Ambiente) and considering OD, salinity and pH (see Table 1).

Table 1. Total concentration of heavy metals in sediments from Sepetiba Bay, as analyzed by ICP-OES and physicochemical measurements of water at different sampling stations in mangroves around Sepetiba Bay.

Heavy metals	Mangrove sediments* (mg/kg)	
Cadmium (Cd)	3.112[a]	0.3 – 23[b]
Copper (Cu)	7.706 [a]	4 - 1.160 [b]
Lead (Pb)	23.918 [a]	3 - 78 [b]
Nickel (Ni)	19.641 [a]	1 - 51[b]
Aluminum (Al)	-	52.64-85.55[c]
Iron (Fe)	-	35.25- 48.27[c]
Manganese (Mn)	-	227 – 872[d]
Zinc (Zn)	317.862 [a]	17 - 3.440[b]
Selenium (Se)	-	-
Physical parameters[e]		
pH	6.5-8.1	
Salinity (%)	20-33	
Conductivity (mS/cm)	17.7-21.9	
OD (mL/L)	7.0-8.3	
Turbidity (UNT)	9.1-11.7	

*Sediment of Sepetiba Bay. (-) not determined. Letters indicate references: [a]Waserman 2005, [b]Rocha et al. 2010, [c]Herms and Lanzillotta 2012, [d]Gomes et al. 2009, and [e] (Ferreira et al. 2010; Silva 2017 and Monte 2014).

2.3. Leaf traits

For each species and sampling location, ten fully expanded and undamaged leaves were removed from the collected branches. The leaves were photographed individually on a known area for determination of leaf area using ImageJ, v. 1.42q (Rasband 2010) (Fig. 2 D) for subsequent calculation of extract yields. The experiment was conducted in a randomized design with ten replicates for each species and locality. The means of ten replicates and their standard deviations were calculated.

The weight measures were done with a digital analytical balance with four decimal places, applying the parameter in grams. For fresh weight, ten leaves of each individual were used. They were conditioned in paper at room temperature for drying and maintained in an oven at 50ºC. After seven days, the leaves were unwrapped and weighed to calculate the dry weight. Later, they were weighed for confirmation of the values. Specific leaf area (SLA) was calculated using the ratio of the leaf area to the corresponding leaf dry weight. These procedures were repeated in the three areas, and the average dry and fresh weight for each species was calculated.

2.4. Analysis of heavy metals from ashes of leaves by Energy Dispersive X-Ray Spectroscopy (EDS)

The ashes for metal presence analysis were obtained by burning the leaves in a muffle furnace, as described by the Association of Official Analytical Chemists (AOAC, 1995). Six leaves from each study area were deposited in porcelain crucibles and previously dried at 60ºC in an air circulation oven for one hour, followed by storage at room temperature. Afterwards, porcelain crucibles that contained leaves were put in an EDG 3P-S muffle furnace for 60 min at 800ºC.

Ashes from the burning leaves were analyzed by Energy Dispersive X-Ray Spectroscopy (EDS or EDX, Thermo®, Noran System Six model, coupled to SEM, Jeol JSM-6380L), following the protocol of Resende (2013) with adaptations. The setup works such that the main electron beam of the Scanning Electron Microscope (SEM) focuses on the sample. Its atoms emit X-rays. Each element of the periodic table has specific and well-defined energy peaks; consequently, it is possible to identify elements present in the sample. The detector will capture the X-ray photons emitted by the sample and measure its energy in electron volts (Ev), counting how many photons are detected with certain energy content. From these results, a spectrum is produced indicating the number of photons for each X-ray-emitted value. An Ev is the amount of energy gained by a single electron when accelerated by a power of one volt in vacuum. Qualitative data were obtained by means of replicates.

2.5. Analysis of minerals by spectrometry

Nitric acid digestion of leaves was based on protocols found in the literature (Raposo Junior et al. 2007). In order to determine the minerals, mainly heavy metals (metals with high atomic weight/number/density), leaf samples were dried in an oven at 60°C with air circulation, crushed and submitted to digestion with nitric acid 65% ultrapure (Merck) in a sealed Erlenmeyer. To an Erlenmeyer were transferred 0.2 grams of leaves to which 10 mL of HNO_3 were added under an exhaust hood. Then, the samples were placed in an ultrasound apparatus for 30 minutes. Following this, samples were transferred to an oven to dry at 60°C for 60 minutes. After cooling, an additional 5 mL of HNO_3 were added and evaporated off at 95±5°C for 2 more hours. Digested samples were filtered through a quantitative filter (n^0 40), diluted to 50 ml with distilled-deionized water plus 5 drops of HNO_3, and stored in Falcon tubes at low temperature until analysis.

Samples were analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) 2100 using a PerkinElmer Optima 7300 V ICP/OES apparatus. To prepare the standard curves, a multi-element standard of concentration of 100 mg/L with the same elements (Spex CertiPrep or Fluka®) was used. The calibration curves were adjusted according to the analysis of elements. For Al, Ba, Fe, Zn, Mg, Mn, and Sr, the curves ranged from 0.01 to 1.0 mg/L; for Co, Cr, Cu, Ti, V, Li, Mo, and Ni, they ranged from

0.005 to 0.2 mg/L; for B, Ca, Na, and K, they ranged from 0.100 to 1.000 mg/L; and for Se, As, Pb, Be, Cd, and Sb, they ranged from 0.002 to 0.025 mg/L. For K, Ca, Na, and Mg, the curves ranged from 1 to 50 mg/L, for Si from 1 to 50 mg/L, and for Sn, the curves ranged from 0.01 to 0.1 mg/L. Highly efficient ion extraction and transport through the mass spectrometer, as well as detection, afford ICP-OES its ultra-trace elemental detection features. Data were obtained by means of three replicates from three to five individuals of each mangrove.

2.6. Extraction of epicuticular waxes and chemical analysis by gas chromatography (GC) coupled with mass spectrometry (MS)

After leaf area determination, each leaf was placed in a separate pre-weighed flask containing 50 mL of chloroform (spectroscopy grade; Tedia®) and maintained for 30 seconds under gentle manual agitation. This procedure extracts only surface chloroform-soluble compounds without disturbing the leaf interior. Chloroform extracts were maintained at room temperature for solvent evaporation to obtain the solid residue. The amount of wax was expressed per unit leaf area ($\mu g/mm^2$). The experiment was conducted in a randomized design with ten replicates for each individual and locality. The means of three replicates and their standard deviations were calculated. Two mg of dried extract were dissolved in 200 mL chloroform. Subsequently, 1 µL was injected into a gas chromatograph (GC-2010-Shimadzu) equipped with a flame ionization detector (FID). The analyses were performed on a DB-1MS capillary column (30 m x 0.25 mm x 0.2 mm), using He as carrier gas at 1 mL/min and a split ratio (50:1). Temperature was increased by 10^oC/min from 140 to 300^oC and then maintained at 300^oC for 15 min. The injector was maintained at 290^oC and the detector at 300^oC. Quantification was performed from GC/FID profiles, using relative area (%). Identification of n-alkanes was based on injection of commercial standards (Sigma Fluka® Alkane standard solution C21-C40). Analysis of a subsample was performed in a GC/MS QP 2010 Plus Shimadzu mass detector, using the same operating conditions as above (except the column; ZB-5MS column 30 m x 0.25 mm x 0.2 mm), and the MS scanned for 50–650 amu at 2 sec/decade with an electron impact ionization potential of 70 eV. Triterpenoid compounds were identified by comparison of the corresponding mass spectra with library data (Spectrum Libraries: NIST05.LIB) complemented with proton nuclear magnetic resonance (1H NMR) spectrometry (Bruker DRX 400 MHz), using deuterated chloroform as solvent.

2.7. Microscopy analyses

For light microscopic observations and measurements, three leaves from each site were fixed in FAA, dehydrated in ethanol series, and stored in ethanol 70% (Johansen, 1940). Middle portions of leaves were cut in appropriate pieces and embedded in glycolmethacrylate (Historesin®), cross sectioned (8 µm thickness) in a rotary microtome (Leica RM 2155), and stained with Toluidine Blue 1% (O'Brien et al. 1965). Photomicrographs of leaf sections were made using light microscopy (Leica) fitted with a digital camera. The measurements were taken from images obtained from leaf cross sections using a Nikon Eclipse CI microscope equipped with a digital camera (Moticam Pro 252b). To identify the presence of zinc in plant tissues, free-hand cuts were performed on collected leaves, dehydrated at room temperature and exposed to Zincon® reagent, following the method of Seregin et al. (2015) and Seregin and Kozhevnikova (2011). For scanning electron microscopy observations (SEM), segments of dry leaves were mounted on stubs and coated with a thin layer of gold (Denton vacuum Desk IV, LLC). The abaxial and adaxial surfaces of leaves were analyzed with a JEOL-JSM 6390 LV scanning electron microscopy (JEOL, Tokyo, Japan). To analyze the epidermis in frontal view and count the secretory glands/mm^2, leaf epidermises were obtained after treatment with a dissociation solution of hydrogen peroxide and glacial acetic acid (1: 1) and heating in an oven at 60ºC for 24h (Franklin 1945). Afterwards, the epidermises were washed in distilled water many times to remove the dissociation solution. Then, they were stained with 0.05% basic fuchsine aqueous and mounted in 50% glycerin on semi-permanent slides (Kraus and Arduin 1997).

2.8. Statistical analyses

Morphoanatomical data from leaves and mineral content obtained by ICP were subjected to a two-way analysis of variance (ANOVA), multiple comparisons, considering data for each mangrove (CG, PG and M), followed by Tukey`s test. A difference was considered to be statistically significant when $P< 0.05$. All statistical analyses were performed with GraphPad Prism software, version 8.0 for Windows.

3. Results

3.1. Morphological leaf traits

Leaf weight of *L. racemosa* from Marambaia showed high values in comparison with leaves from CG and PG. Meanwhile, the leaves from PG presented low values for weight and area (Fig. 3). Data from leaf area, together with leaf weight, confirm the low average development of *L. racemosa* in PG compared to CG and M. Significant difference attributable to leaf area was observed when the data from three locations were analyzed, showing that leaves from PG had smaller values. Leaves from M presented lower specific leaf area (SLA) than that from CG and PG, suggesting the influence of pollutants on plant development since CG and PG are surrounded by widespread industrialization.

3.2. Epicuticular wax chemical composition

The amount of triterpene content in epicuticular wax per leaf follows the descending order of CG> PG. (Table 2). Epicuticular wax composition presented the major pentacyclic triterpene fagarasterol (lupeol) and -amyrin found in plants of *L. racemosa* from CG and PG mangroves. Considering lupeol and -amyrin, the chemical profiles of L. racemosa leaf waxes were consistent. The *n*-alkanes hentriacontane and 8-octadecanone were identified in leaves from CG, while tetratetracontane was only found in leaf samples from PG. This is the first time the epicuticular wax of *L. racemosa* from mangroves of the southeast Atlantic coast has been chemically profiled.

Table 2. Composition of triterpenes identified in epicuticular wax of *Laguncularia racemosa* collected in Coroa Grande (CG), Pedra de Guaratiba (PG) and Marambaia (M) mangroves, Rio de Janeiro (Brazil), as

obtained by gas chromatography.

Constituent	RT (min)	Relative area (%)*		
		CG	PG	M
Hentriacontane	61.50	28.01	nd	nd
Tetratetracontane	66.17	nd	10.93	nd
8-octadecanone	70.16	6.31	nd	nd
β-amyrin	74.63	8.81	16.35	nd
Fagarasterol (lupeol)	75.57	53.63	41.61	nd
Total triterpenes		62.44	57.96	nd

*Mean amount of wax extracted with chloroform. RT – Retention time obtained by GC. nd- not detected.

3.3. Mineral content analyzed by EDS

The analysis of leaf ash made it possible to quantify the ATOM (%), which is the percentage in number of atoms of the elements present in the sample. Using EDS, the major essential mineral levels found in leaves of *L. racemosa* collected in M, CG and PG were Na>Cl>K>Ca>K. These minerals are common in saline ecosystems that have input of seawater (Fig. 4a).

The presence of heavy metals was found mainly in leaves of *L. racemosa* from CG and PG. For leaf samples from M, only Al, Si and Fe were detected. Zn was detected only in leaves from CG, the site nearest the industrial center (CG) (Fig. 4b). Cd was not detected.

3.4. Mineral content analyzed by ICP- OES

As shown in Table 3, it was possible to observe higher levels of calcium, magnesium and potassium, both in CG and PG, when compared to the levels of the same minerals in M.

Among the heavy metals in leaf samples, Cd and Zn were identified. The levels of the heavy metals As, Al, Cr, Cu, Pb and Se were similar for *L. racemosa* samples from all three mangroves evaluated. PG and CG samples showed equal results in terms of the order that they bioaccumulated in the leaves: Fe>Al>Zn>Mn>Pb>Cu>Cd (Table 3), with Cd varying by having a higher level in PG (0.016 mg/L) compared to CG (0.0012 mg/L). The order of accumulation of metals in leaves of *L. racemosa* from Marambaia was similar to that found in the other mangroves; however, Al (0.37 mg/L) was detected at a higher concentration than Fe (0.35 mg/L). For comparison, the total concentration of heavy metals within a normal range for plants is presented in Table 3. Specifically, the normal range in plants is 0.03 to 0.9 mg/kg for Cd and 50.8 mg/kg for Zn (Table 3). When compared to chemical fingerprinting of reference plants, *L. racemosa* leaves contain a lower concentration of heavy metals (Table 3). For analysis of minerals by ICP, collections were made at two points in CG. Differences between the two CG points

(CG^1 and CG^2) were observed; specifically, Al and Na presented higher content in CG^2, while Sr and Ca presented lower content in CG^2 when compared to CG^1 (Table 3).

Table 3. Content of minerals, heavy metals and other chemical elements in leaves (mg/kg) of *Laguncularia racemosa* by ICP-OES compared to reference plants. Micro- and macronutrients are separated based on physiological use by plants. Leaves were collected in mangroves around Sepetiba Bay, Rio de Janeiro (Brazil): Coroa Grande (CG), Pedra de Guaratiba (PG) and Marambaia (M).

Mineral (mg/kg)	CG[1]	CG[2]	PG	M	Reference plants†
Metals					
Al	0.31±0.31	0.74±0.45	0.38±0.1	0.37±0.061	80
As	0.002±0	0.002±0	0.002±0	0.002±0	
Ba	0.025±0.007	0.027±0.004	0.039±0.0005	0.025±0.004	40
Be	0.002±0	0.002±0	0.002±0	0.002±0	
Cd	0.004±0.003	0.032±0.01	0.016±0.01	0.009±0.02	0.03-0.9
Pb	0.095±0.16	0.32±0.13	0.16±0.12	0.1±0.17	1.54
Li**	0.005±0	0.005±0	0.005±0	0.005±0	
Sr	0.46±0.07	0.29±0.036	0.45±0.147	0.24±0.07	
Cr	0.005±0	0.005±0	0.005±0	0.005±0	
Sn	0.01±0	0.01±0	0.01±0	0.01±0	
Ti**	0.008±0.002	0.006±0.003	0.005±0	0.005±0	5.0
V	0.005±0	0.005±0.0	0.005±0	0.005±0	
Micronutrients					
Co	0.005±0	0.005±0.0	0.005±0	0.005±0	0.2
B	0.126±0.45	0.1±0.0	0.21±0.06	0.35±0.79	
Mn*	0.088±0.0026	0.091±0.007	0.20±0.02	0.13±0.033	15-100
Mo	0.01±0	0.01±0.0	0.1±1.69	0.01±0	
Ni*	0.007±0.0081	0.005±0.0	0.087±0.141	0.005±0.004	3.10
Zn*	0.30±0.0587	0.26±0.11	0.33±0.025	0.25±0.16	50.80
Cu*	0.024±0.0051	0.028±0.005	0026±0.002	0.018±0.003	10.0
Fe*	0.27±0.0982	0.33±0.08	0.51±0.006	0.36±0.09	89.22
Macronutrients					
Na	31.988±6.84	68.71±5.512	38.33±4.73	92.0±11.56	
Mg	15.778±3.03	12.718±1.13	14.034±3.83	9.71±3.006	
Ca	62.94±11.10	37.25±3.10	69.23±19.85	42.18±8.50	
K	27.09±5.43	29.126±1.78	26.025±2.63	29.063±7.23	

Other elements					
Se	0.005±0.0	0.007±0.001	0.006±0.001	0.006±0.001	
Si	0.602±0.16	0.5±0.07	0.55±0.04	0.76±0.24	0.1
Sb***	0.002±0.0	0.002±0.001	0.002±0.0	0.002±0.0	(<0.5) 1.5[††]

* Also heavy metals, but considered micronutrients used by plant. **Light metal. ***Antimony (or stibium) metalloid. [†]References: Markert 1992, values in mg/Kg dry weight. [††]Levels in leaves of different plants: Pérez-Sirvent et al. 2011. [1,2]For analysis of minerals by ICP, collections of leaves were made at two points in CG.

3.5. Microscopic diagnosis

The qualitative and quantitative parameters evaluated in *L. racemosa* leaves from the three sites are presented in Fig. 6.

In investigated plants, the epicuticular wax layer covering the leaf surface presented distinct patterns, those being plates, granules, rodlets and crusts, according to the site of collection (Fig. 5). Leaves from Marambaia presented epicuticular wax in crusts and granules covering most of the surface area, but in scales near stomata (Fig. 5a, b). In leaves from Pedra de Guaratiba, the wax layer consisted of crusts and plates throughout the surface, including stomatal cells (Fig. 5c, d). In leaves from Coroa Grande, the epicuticular wax consisted of granules over common epidermal cells, but a rod pattern near stomata (Fig. 5e, f). Pores of the cavity where salt secretory glands are located were seen in both surfaces (Fig. 5g, h).

In cross section, the epicuticular wax and cuticular layer are not easily distinguished under light microscopy, so they were measured together. The thickness of wax+cuticular layer was smaller in M compared to that for CG for both sides with few differences on the adaxial side between CG and PG (Fig. 6).

Laguncularia racemosa has amphistomatic leaves; the ordinary epidermal cells present straight walls (Fig. 6a, b). Unicellular trichomes were detected only in plants collected in CG (Fig. 6a). In cross section, the epidermis consisted of a single layer of epidermal cells (Fig. 6d-f). Common epidermal cells stored phenolic compounds in both surfaces more intensely as detected in plants from CG (Fig. 6e). The adaxial epidermal cells in individuals from M were higher than those of individuals from PG (Fig. 6).

Salt-secreting glands are located in epidermal cavities that invade the mesophyll (Fig. 6 b, c-e). The number of glandular trichomes was constant between the two faces of the leaf and also between the three sites evaluated (2-3 glands/mm^2). The secretory structure is supported by short peduncle cells with 3-4 (basal cells) sustaining the secretory cells that form a nearly globose structure (Fig. 6b-c, e). The epidermal cells that surround the opening of the cavity (pore) are palisade-like in cross section, with tabular shape, containing phenolics. The salts, mucilage and other substances excreted by this secretory structure first accumulate in the cavity and thereafter are likely released to the external environment through the epidermal pore (Fig. 6e).

The mesophyll is heterogeneous and isolateral in leaves from the three sites investigated and was comprised of two layers of adaxial and abaxial palisade parenchyma with shorter cells when compared with adaxial layers and 4-5 layers of spongy, or water storage, parenchyma (Fig. 6d-f). The thickness of the mesophyll was greater in leaves from PG, but less so in plants from M (Fig. 7). The length of palisade parenchyma cells (adaxial and abaxial) was similar in the leaves evaluated for individuals of the same site; however, when different sites were compared, longer cells were observed in PG and shorter cells in CG. In the first layer of palisade parenchyma under both sides of the epidermis, a strong reaction to phenolic compounds was observed (Fig. 6d-f). The median part of mesophyll is occupied by a spongy parenchyma, similar to water storage parenchyma, with voluminous cells with few, or no, chloroplasts (Fig. 6c). This tissue is thicker in leaves from CG than that from PG. In all samples evaluated, the cell wall of water storage parenchyma presented intense reaction to the mucilage test. The mucilage was observed spread between cells in the mesophyll (Fig. 6f). Idioblasts with druse crystals were observed in the cells of the spongy parenchyma, proportionally more abundant in PG leaves (Fig. 6). Druse crystals presented positive reaction to the Zincon® histochemical test with more intensity in leaves from CG and PG, indicating the presence of this metal (Fig. 6i-j).

The vascular bundles are the collateral type with fibers in the most developed units, forming a cap protecting the phloem (Fig. 6c). Positive reaction to the presence of zinc was observed in vascular fibers in CG and PG leaves (Fig. 6i-j). A parenchymal sheath (endodermis) is present and is better observed in the larger bundles. It does not touch the epidermis. Phenolic compounds and idioblasts with druse crystals are associated with vascular tissue. Regarding the vascular system, we did not observe any difference among leaf samples from different collection sites.

Midvein - The epidermal cells on the adaxial face had a rectangular shape (Fig. 6g), and those on the abaxial face were square to papillose. The height of the epidermal cells and the cuticle layer was higher in PG and lower in M. Under adaxial and abaxial epidermal sides, we observed 6-8 layers and 1-2 layers, respectively, of regular parenchyma storing phenolic compounds and crystals. In the region of the main vein, the parenchyma cells are isodiametric, separated by intercellular spaces filled with mucilage (Fig. 6g). The main vascular bundle is the collateral type, with a reniform shape, surrounded by fibers and collenchyma (Fig. 6h). The width of the main rib is proportional to the length in height of the main vascular bundle, longer in PG and shorter in CG. The width of the main rib was longer in M and shorter in CG.

Discussion

Among the studied mangroves of Sepetiba Bay, Marambaia is thought to be the most preserved since it lies east of the Coroa Grande and Santa Cruz industrial complex and, hence, the most distant mangrove from anthropic pressure. It is also located in an area under military protection. In 2010 Ferreira et al. found that the concentration of heavy metals in Sepetiba Bay did not exceed the limits recommended in CONAMA Resolution No. 344/2004. However, Cd, Zn and Cu in the water did show unsatisfactory values in some parts of Sepetiba Bay when assessed according to resolution Nº357/2005. One of the many factors that alter the concentration of minerals in Sepetiba Bay is the frequent movement of tidewater and sediment from water entering the bay. In addition, industrial activities have production rates that can alternately reduce and increase pollution. Sepetiba Bay is a coastal area sensitive to regional environmental changes that can create an ecotone where interactions between land and water take place. According to Ribeiro et al. (2013), metal mobility may also change in the short term. The average of dry weight showed a decreasing pattern following the order M> CG> PG (Fig. 4).

Severoglu et al. (2015) suggest that heavy metals constitute one of the main abiotic agents related to growth reduction and alteration in physiological processes. The high SLA value of *L. racemosa* collected in CG and PG mangroves is consistent with the pollution data based on proximity to the industrial complex. SLA is an important indicator of the impact of pollution in the environment, and it can may be a parameter involved in protection and adaptation of plants (Wuytack et al. 2011).

The amount of Na is three times greater in leaves of Marambaia compared to leaves examined from CG and PG mangroves. This result could be attributed to the degree of salinity in the region where a high hydrodynamism is known to significantly change salinity. Soil salinity can interfere with the uptake and exchange of ions in the soil-plant system, which, in turn, is reflected in the morphology and structure of plants (Bartz et al. 2015).

Leaf structure of *L. racemosa* was analyzed by Francisco et al. (2009) who reported the presence of leaf glands and trichomes, as well as salt secretory glands. They considered these features to be adaptations to the saline ecosystem. Here we added information about the quantity of salt secretory glands in the leaves of *L. racemosa* plants growing in different mangroves around Sepetiba Bay and data about the micromorphology of epicuticular wax. Some microscopic views showed the high salt content on leaves and around salt glands (Fig. 5). Evidence suggests that mature leaves of *L. racemosa* can secrete salt according to its concentration in the soil. That is, if salt increases in the substrate, then salt secretion will be higher in leaves (Sobrado 2004). Through salt glands, salt-tolerant plants also can expel heavy metals (Arrivabene et al. 2016). Leaf structures are particularly relevant to the ecological success of this species, which occupies environments where salinity is high. No leaf samples from mangroves evaluated showed morphological or structural damage.

The plant cuticle consists of nonpolar substances such as cutin, which is the matrix associated with waxes. It is secreted by cells of the epidermis and deposited on an organ of the plant's surface, such as the leaf. It also seals flowers, stems, and fruits, protecting them from biotic and abiotic stresses (Kunst and Samuels 2009). Intracuticular wax is embedded into the cuticle, and epicuticular wax is found on the cuticle's surface (Koch and Ensikat, 2005). Cuticular waxes are involved in guarding against excessive leaf water loss (residual transpiration) (Hasanuzzaman et al. 2017). The presence of n-alkanes and triterpenes, as the main constituents of epicuticular wax, is recurrent among restinga and mangrove plants that use residual transpiration as a mechanism of tolerance under salinity stress (Oku et al. 2003; Zorat et al. 2011; Hasanuzzaman et al. 2017; Victório et al. 2020). Pentacyclic triterpenoids, such as β-amyrin and lupeol, which are representative of the oleanane and lupane groups, are widely distributed in mangrove plants associated with features tolerant to salt (Basyuni et al. 2012). Pentacyclic triterpenes were identified as the main components of *A. shaueriana* epicuticular wax (Victório et al. 2020), as well as the main components of *L. racemosa* leaves in this study. However, our results are different from those of Rafii et al. (1996) who verified only trace amounts of triterpenes in leaf wax of *L. racemosa* from Guyana (western Atlantic coast) and 8.5% in wax extract in a population from Gabon (eastern Atlantic coast) that did not present lupeol.

The presence of hydrocarbons in leaf epicuticular wax has proven valuable in chemotaxonomic studies of different botanic families. Alkanes were the most abundant compounds among the hydrocarbons, highlighting hentriacontane (28.01-45.36%) in leaf wax collected in CG. The hydrocarbons hentriacontane and octadecanone are present in leaves of *Rhizophora mangle* from Africa (Dodd et al. 1995), and hentriacontane is present in leaves of A. shaueriana (Victório et al. 2020). The presence of the hydrocarbon hentriacontane is recorded for the epicuticular waxes of several plant species (Wang et al. 1999), but this was the first evidence of its presence in the *Laguncularia* genus. As constituents of epicuticular wax, n-alkanes, but not triterpenes, seem to be related to a reduction in cuticular water loss (Buschhaus and Jetter 2012).

In studies with conifer (*Pinus sylvestris* L.) seedlings, Burkhardt and Pariyar (2014) verified that air pollution degraded (´erosion`) epicuticular waxes that revealed an amorphous appearance resulting in low drought tolerance. These symptoms, which are easily visible through an analysis of the leaf surface by scanning electron microscopy (SEM), are provoked by very diverse chemical environments, such as acid rain or fog, simultaneous exposure to SO_2 and NH_3 or car exhaust, as pointed out by Viskari et al. (2000). Studies of Arrivabene et al. (2015) detected a higher amount of particulate material, including iron, on the leaf surface of *A. schaueriana* and *L. racemosa*, the leaves of which contain salt glands in comparison to *Rhizophora mangle*. The presence of hydrophobic epicuticular wax covering the leaf blades may be associated with the absorption of chemicals of equal polarity, such as phthalates, pesticides and others. Plasticizers were detected in the leaf epicuticular wax of *A. shaueriana* and *R. mangle* from the same mangroves (Victório et al. 2021). Cuticular waxes also act in controlling loss and uptake of polar solutes (Hasanuzzaman et al. 2017).

Cutin is integrated into superimposed waxes; it is an extracellular layer that lines the epidermal cells externally. When plant cells are subjected to stress, the results show up later as alterations in the synthesis of wax, in turn affecting gene expression. Apart from internal processes, biotic and abiotic stresses are involved in the regulation of plant cuticle biosynthesis (Fich et al. 2016; Tafolla-Arellano et al. 2018). Some reports show consistency between the morphology of wax and its composition (Koch and Ensikat 2008). However, variations in wax types caused by changing environmental conditions have been suggested during crystallization, and genes associated with cuticle formation have shown responsiveness to environmental conditions (Koch and Ensikat 2008).

It has been reported that uptake of metals from both soil and leaves influences cuticular wax layer and permeability. A positive correlation between transpiration rate and cadmium (Cd) concentration was reported in Beta vulgaris plants, potentially affecting cuticle biosynthesis and composition. Consequently, changes in chemical composition are reflected in permeability which can then result in high water losses through the cuticle (Greger and Johansson 1992). Evidence suggests that heavy metals alter the cuticle, e.g., the expression of genes involved in Cd tolerance, while Fe deficiency was shown to reduce the amount of cuticular lipids that influence water retention, solute permeability, pathogen infection and disease resistance (Fernández et al. 2008; Tafolla-Arellano et al. 2018).

Mangroves are also exposed to pollution of particulate material through the atmosphere owing to their coastal distribution and proximity to urban centers (Bayen 2012). Particulate pollutants deposited on the leaf surface may be absorbed by leaf tissues and alter the chemical structure of epicuticular waxes, thereby causing morphoanatomical damage, such as chlorosis, necrosis, reduction in photosynthesis and gas exchange (Prasad and Hagemeyer 1999; Naidoo and Chirkoot 2004; Arrivabene et al. 2015). Such absorption may also induce physiological responses of plants and alter production of metabolites. In studies reporting on the epicuticular waxes of *Coffea arabica*, Lichston and Godoy (2006) verified a decrease in wax content and morphological alteration of wax when leaves were exposed to a copper-based fungicide.

Plants from mangroves, mainly in polluted areas, incorporate some metals in organ tissues. However, results show that metal concentration attained in biota is only high if the plants have accumulated features and are hyperaccumulators (Saenger and McConchie, 2004). *Laguncularia racemosa* plants accumulate high concentrations of Cr in roots, but low mobility of this element results in correspondingly low content in leaves (Rocha et al. 2009). Saenger and McConchie (2004) also indicate that young leaves accumulate more metals than mature leaves. The metal contents in leaves of mangroves sampled in this study are lower than those observed in normal reference plants grown in uncontaminated soils. Machado et al. (2002) conducted a study with *L. racemosa* in Guanabara Bay (RJ) and found that heavy metals tend to accumulate in chemical forms that reduce their mobility and, hence, absorption by biota. In addition, a low translocation of heavy metals was observed from the leaves of the litter to other trophic levels, suggesting the low bioavailability of these heavy metals. Leaves may accumulate heavy metals or metabolize them. When leaves fall in the mangrove substrate, a low amount of metals present in the leaf litter is returned to the soil by decomposition and mineralization processes, becoming bioavailable in the environment in a way that culminates in biomagnification processes along the food chain. However, the release of metals from fallen leaves is low (Saenger and McConchie 2004). This ability to keep heavy metals in unavailable forms, together with resistance to these elements in the sediment, may explain the low levels of some heavy metals found in the aerial part of L. racemosa. Still, leaves are important organs in the analysis of heavy metals, essentially because many processes of primary and secondary metabolism occur in leaves. In addition, because of transpiration, the water rises from the root carrying the pollutants dissolved in the water when they are not deposited on the leaf owing to their presence in the atmosphere (Liang et al. 2017).

EDS analysis evidenced high concentrations of calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) (Fig. 4), indicating the high salinity of the mangrove environment surrounding Sepetiba Bay where the marine influence is greater (Lacerda et al. 1985). The ability to tolerate high concentrations of salt may be associated with tolerance to heavy metals since both depend, in part, on common physiological mechanisms (Manousaki and Kalogerakis 2011). Ca, K, and Mg will work by inhibiting the absorption of heavy metals, and their levels will increase whenever stress is caused by heavy metals. Potassium will act to restore osmotic pressure, and the plant will protect itself by increasing the levels of Ca and Mg to a threshold, after which they start to decrease (Severeglu et al. 2015). Using EDS, Zn was detected only in leaves from CG (0.39 ATOM%). Its absence in other mangroves may be associated with experimental conditions or optimization of the analysis by EDS since the presence of Zn for all mangroves was verified in analyses by ICP-OES. Comparing these methods, a large number of minerals were identified through ICP, revealing that this type of analysis is more accurate than that performed with EDS. However, the use of standards is required in EDS analysis to better indicate the concentration of minerals.

Considering the analysis by ICP-OES, it follows that CG, as the mangrove area nearest the industrial complex, would have high values of heavy metals, as verified by both spectroscopy and spectrometry. The high concentrations of Zn (0.335 mg/L) and Cd (0.016 m/L) for PG samples in relation to other mangroves also suggest the influence of industrial pollutants in this area, in contrast to Marambaia, which would be the most distant from industrial pollution. Through the analysis by ICP-OES, only high concentrations of Zn were confirmed in CG. Zn is a metal present in the lithosphere, but it also indicates anthropogenic pollution. Zn is a naturally occurring metal, but it is often associated with anthropogenic sources, and it can be considered a key indicator of polluted areas, such as contaminated urban areas (Alharbi et al. 2019). Also, Zn is probably associated with the waste from the metallurgical plants in the West Zone of Rio de Janeiro and Coroa Grande (Itaguai City). The higher concentration of Zn (<50 ppm) was also observed in leaves of *L. racemosa* in experiments carried out by Bernini et al. (2010) from leaves collected in an estuarine mangrove of the São Mateus River, Espírito Santo, Brazil.

According to Ramos and Silva (2006), mangrove forests are important biochemical filters of heavy metals to coastal areas since these elements would otherwise be accumulated in the organs of perennials, such as branches and leaves, with high renewal rate. Heavy metals like Cd, Cr, Pb and Zn present high toxicity to environment, and their significant bioaccumulation causes many problems in ecosystems because they are not biologically degraded, but rather accumulate in biota and in abiotic environments as trapped particulate in mangrove sediments (Mathivanan and Rajaram 2013).

Mineral resources correspond to an important material base for socioeconomic progress. Besides Zn and Cd, other metals, such as Ba, Si, Sb, Ti and V, are employed in different industries, in particular, those in the Sepetiba Bay area, and thus also found in leaves of *L. racemosa*. For example, Ti, Sb and V are used in the production of metal alloys. Because of the lower values from some minerals, it is possible that they do not originate from industrial sources, but, instead, are the result of biogeochemical cycling.

Barium (Ba) is a toxic chemical used in various industries, such as oil well drilling, production of rubber and paper, fireworks, manufacture of glass, paints and pigments, composition of batteries, and the composition of fluorescent lamps, and it causes many disturbances in plant development (Sleimi et al. 2021). Silicon (Si) has important applications in computers and the production of silicone polymers. Antimony (Sb) is employed mainly in metal alloys, and some fire-resistant compounds are also used in paintings, ceramics, enamels, rubber vulcanization and fireworks. However, Sb is potentially toxic and has no role in biological functions. Sb in plants can lead to toxicity, so low to moderate concentrations can result in damage to plant growth and development, including photosynthesis, lipid peroxidation and oxidative stress (Natasha et al. 2019). The maximum value reported in leaves is 1.5 mg/kg, but in most samples, like our data (0.2 mg/kg), the concentrations were below 0.5 mg/kg (Pérez-Sirvent et al. 2011). These chemical elements are indicators of industrial development and were detected in samples of L. racemosa leaves, but not in concentrations considered toxic.

Vanadium (V) is a metal widely present in the environment and distributed in leaf organs of plants in low concentrations. Compared to other species, the leaves of *L. racemosa* accumulated it at very low concentration (0.05 mg/kg). The lowest registered to leaves was found in smilograss - *Piptatherum miliaceum* (0.1 mg/kg of V) (Aihemaiti et al. 2020). Studies indicated that some phosphate fertilizers present high concentrations of V (90-180 mg/kg) as a contaminant (Vachirapatama et al. 2002), suggesting that this element is widespread in soils, water and vegetables through phosphorus fertilization. For plants, depending on concentration, V can be harmful to development in high concentrations by disrupting energy metabolism and matter cycling. It can also inhibit some enzymes, protein synthesis, and ion transport, as well as reduce growth rate, cause root and shoot abnormalities, or even death of plants. In low levels, the results may be positive by elevating plant height, root length, and biomass production associated with increased chlorophyll content, seed germination, essential mineral uptake, and assimilation of nitrogen and its utilization (Aihemaiti et al. 2020).

From an anatomical point of view, the *L. racemosa* leaf presented tissue organization similar to that described for the family and genus mentioned in the classical literature (Metcalfe and Chalk 1950). The anatomical pattern observed in the samples of *L. racemosa* differed slightly from that described by Silva et al. (2010), who evaluated the same species in a mangrove in the state of São Paulo by comparing a highly impacted area with a non-impacted one. In the epidermis, for example, the authors observed only epicuticular wax in granules. Baker (1980) suggests that epicuticular wax in plates are produced by primary alcohols, such as triterpenoids, resulting in amorphous morphology, as we observed here.

Salt-secreting structures in the epidermis seem to be an unusual feature. It has been suggested that plants evolutionarily tend to adapt to the saline condition rather than having salt glands. Only a few orders of Angiosperms such as Poales and Myrtales, including Combretaceae, Caryophyllales, Lamiales, and Solanales have salt glands (Flowers et al. 2010), indicating an independent evolutive origin. The secretory glands actively eliminate salts, keeping them within certain limits (Larcher 1995). In our study, the number of salt glands varied (2-3 glands/mm^2), different from what was observed by Silva et al. (2010) who found less than 1 gland/mm^2. This reduction may be related to the levels of salinity found in each collection site, especially from the proximity of large rivers that flow into the site investigated by Silva et al. (2010) and which would have reduced the salinity of the soil. The very high content of Na in leaves from M, compared to that found in leaf samples from CG and PG mangroves, is simply suggestive of high salinity as a characteristic of the local ecosystem. This study did not verify any changes in the patterns of salt gland distribution for *L. racemosa*. The anatomical characteristics of the salt glands, which are located at the bottom of an epidermal cavity, corroborate the description provided by Francisco et al. (2009) and Dassanayake and Larkin (2017) regarding cell organization and composition.

In the mesophyll of *L. racemosa* herein evaluated, we observed that the thickness varied from 397.94 to 430.13 µm, values similar to those found in leaves collected in northern Brazil (Lucena et al. 2011) in a place not affected by industrial pollution, suggesting that the leaves of the species evaluated here did not present significant alterations in the organization of the palisade and spongy parenchyma. In leaves investigated here, we observed an isolateral structure in the three sites selected, even as we acknowledge the dorsiventral mesophyll cited by Lima et al. (2013) and Lucena et al. (2011). Isolateral leaves are found in plants living in sites where incident light is received from upper and lower orientations, possibly improving the photosynthetic process in an otherwise growth-limiting environment. As part of the mesophyll, the spongy parenchyma, similar to an aquiferous tissue, is a common, but no less remarkable, tissue in halophytes and has a fundamental role in the dilution of salts that are absorbed together with water and that can accumulate in levels toxic to the plant (Silva et al. 2020; Larcher 1996).

In the epidermis and mesophyll, we observed an intense reaction pointing to the presence of phenolic compounds in all collection areas. The occurrence of phenolic compounds, including flavonoids and derived phenolic acids, in the epidermal cells and in the palisade parenchyma close to the adaxial and abaxial surfaces may be related to photoprotection of the underlying tissues, guaranteeing their integrity, even under conditions of intense luminosity, as they relieve the photo-oxidative stress and limit the formation of reactive oxygen species (ROS) in chloroplasts or reduce their formation (Zhang et al. 2018).

Histochemical analyses revealed the presence of Zn associated with druses in the parenchymatic tissue of the leaves. According to Silva et al. (2010), most vascular plants store some type of mineralized material, with druses being the most common form. One of the functions of the deposition of calcium oxalate in the leaves, the main substance of the composition of druses, is to maintain a low concentration of Ca in the vicinity of the cells of the stoma. Ca is engaged during the opening of the stomata, and the greater number of druses may be related to several changes in metabolism (Silva et al. 2010), including those caused by heavy metal stress, which explains the large number of druses in histological analyses.

Conclusion

The *L. racemosa* epicuticular wax studied revealed a high concentration of the pentacyclic triterpenes fagarasterol (lupeol) and β-amirine, as well as n-alkanes, such as hentriacontane (28.01%), in Coroa Grande mangrove. This study showed variability in wax layer micromorphology among leaves from each mangrove investigated, including the morphological patterns mentioned for plants with the presence of terpenoids in the wax composition. The anatomical features correspond to the physiology of plants growing in saline environments under intense luminosity, as indicated by isolateral structure, not described up to now for *L. racemosa*.

In general, the results showed that accumulation of heavy metals in leaves of *L. racemosa*, as revealed by ICP-OES, did not reach phytotoxic concentrations or toxic levels, according to the reference values. The metal content found in *L. racemosa* leaves is instructive insofar as pollutants associated with anthropic activity in the study area. We herein validated an association with industrial plants engaged in metallurgy, electronics, and other industries that deposit toxic materials into the water and soil. Thus, the values of Pb and Cd indicated significant anthropogenic contributions to pollution of Sepetiba Bay.

Through histochemical tests, it was possible to observe the presence of Zn in such leaf tissues as sclerenchyma associated with vascular system and crystals, suggesting influence of heavy metals on leaf metabolism. However, other variations in physical conditions may change leaf metabolism in the same Bay. These data also point to point to this plant as a bioindicator and/or bioaccumulator in accordance with the proposal of Ramos and Geraldo (2007). Further studies are necessary to analyze the concentration limits of heavy metals in the organs of this species. Nonetheless, it can be speculated that the leaves of such plants can serve as monitors of environmental quality.

Declarations

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]

Competing interests

The authors declare that they have no competing interests.

Funding

Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, RJ, Brazil): E-26/111.687/2011 and E-26/110.781/2011.

Authors' contribution

We declare that all work performed in compiling this article was done by the authors.

C.P.V. designed the study, collected plant material, conducted the leaf extractions and analysis of minerals and epicuticular wax, interpreted the results, and drafted the manuscript.

M.S.S. evaluated morphological parameters, extracted the epicuticular wax for analysis, and prepared leaf samples for mineral analysis.

A.C.D. evaluated morphological parameters and prepared leaf samples for mineral analysis.

J.P.S.P.B. prepared anatomical slides and descriptions and performed leaf tissue measurements and light photomicrography.

M.C.S. collected and identified plant material.

N.K.S. analyzed the composition of epicuticular wax and interpreted the results.

R. C. O. A. conducted the anatomical analysis and description, prepared the histochemical tests, interpreted the results, and drafted the manuscript.

Acknowledgements

We are grateful to the Brazilian Army for providing access to the Marambaia restinga to collect raw material used in this study. We also acknowledge the technician Luiz Felipe Plaça from UFMS for his support in the SEM-EDS analyses and Companhia de Pesquisa de Recursos Minerais – CPRM, RJ for the ICP-OES analysis.

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Figures

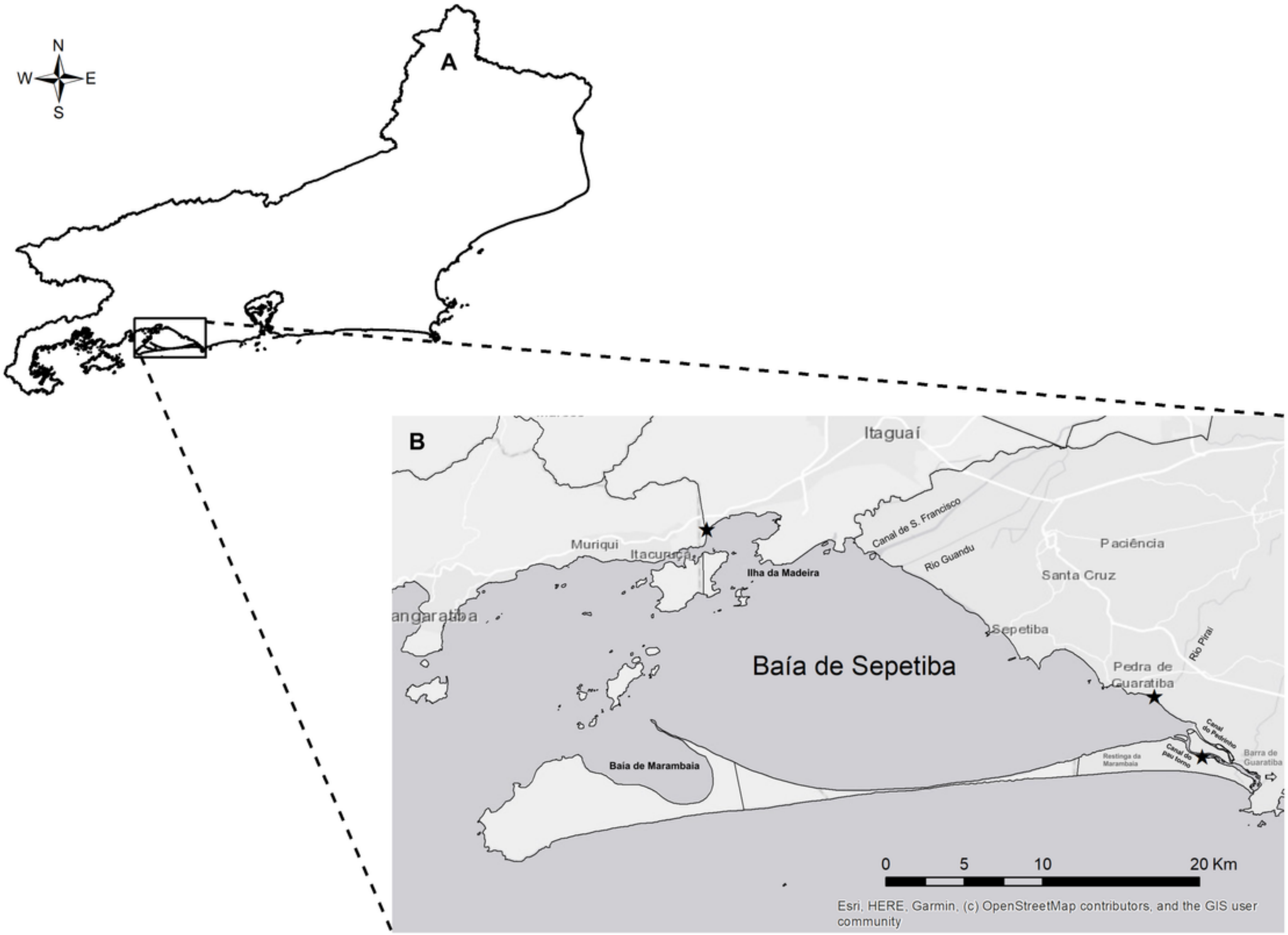

Figure 1

A. Rio de Janeiro City. B. Location of mangroves around Sepetiba Bay (Baía de Sepetiba, Rio de Janeiro) where Laguncularia racemosa leaves were collected (*). The black stars indicate mangroves: Coroa Grande (Itaguaí City), Pedra de Guaratiba (Rio de Janeiro City) and Marambaia (Rio de Janeiro City). In Itaguaí and Santa Cruz, there are industrial centers.

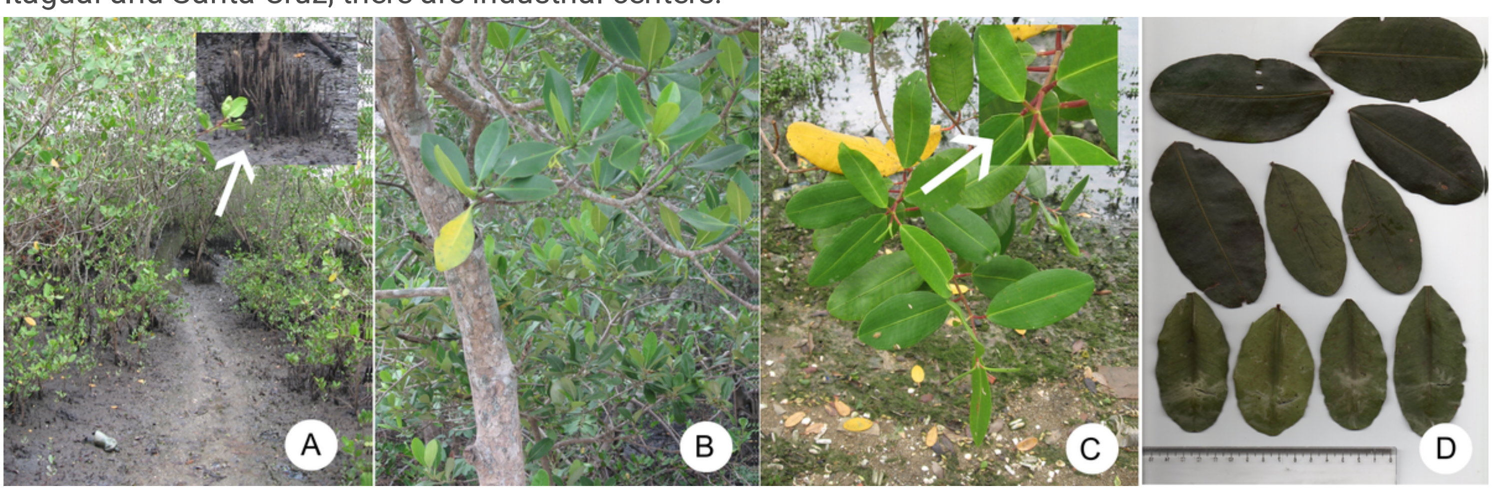

Figure 2

Plant habit, collection sites and leaves of Laguncularia racemosa in Rio de Janeiro State, Brazil, around Sepetiba Bay. A-C. Tree with pneumatophores (in detail) from Coroa Grande Mangrove. C. Red petiole of leaves (in detail). D. Morphological variation in leaves of L. racemosa in mangrove and organized to scan for analysis of leaf area (D).

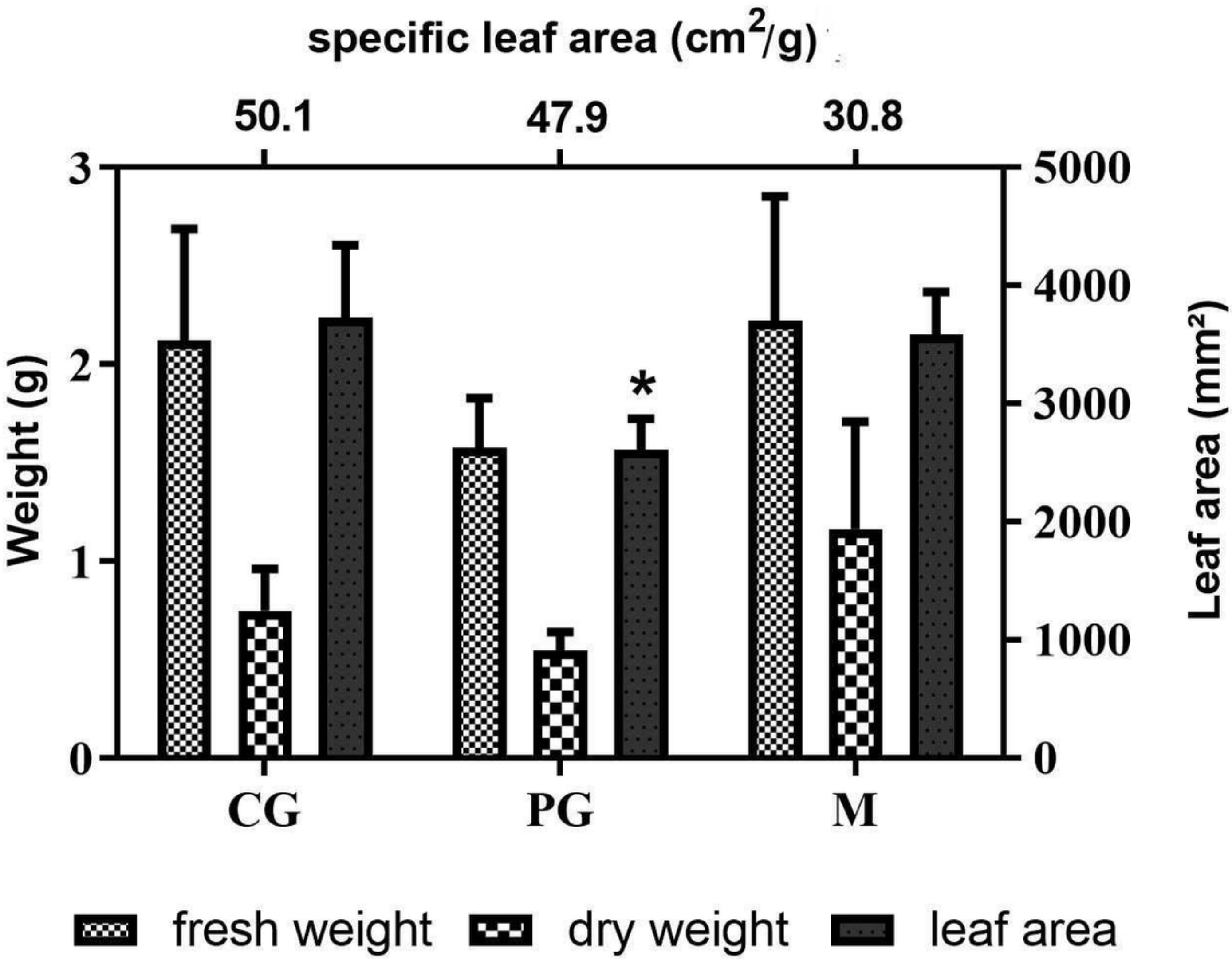

Figure 3

Fresh and dry weight (g), leaf area (mm2) and specific leaf area (SLA) of Laguncularia racemosa collected in mangroves of Marambaia (M), Coroa Grande (CG) and Pedra de Guaratiba PG), around Sepetiba Bay. *Significant difference, p< 0.0002.

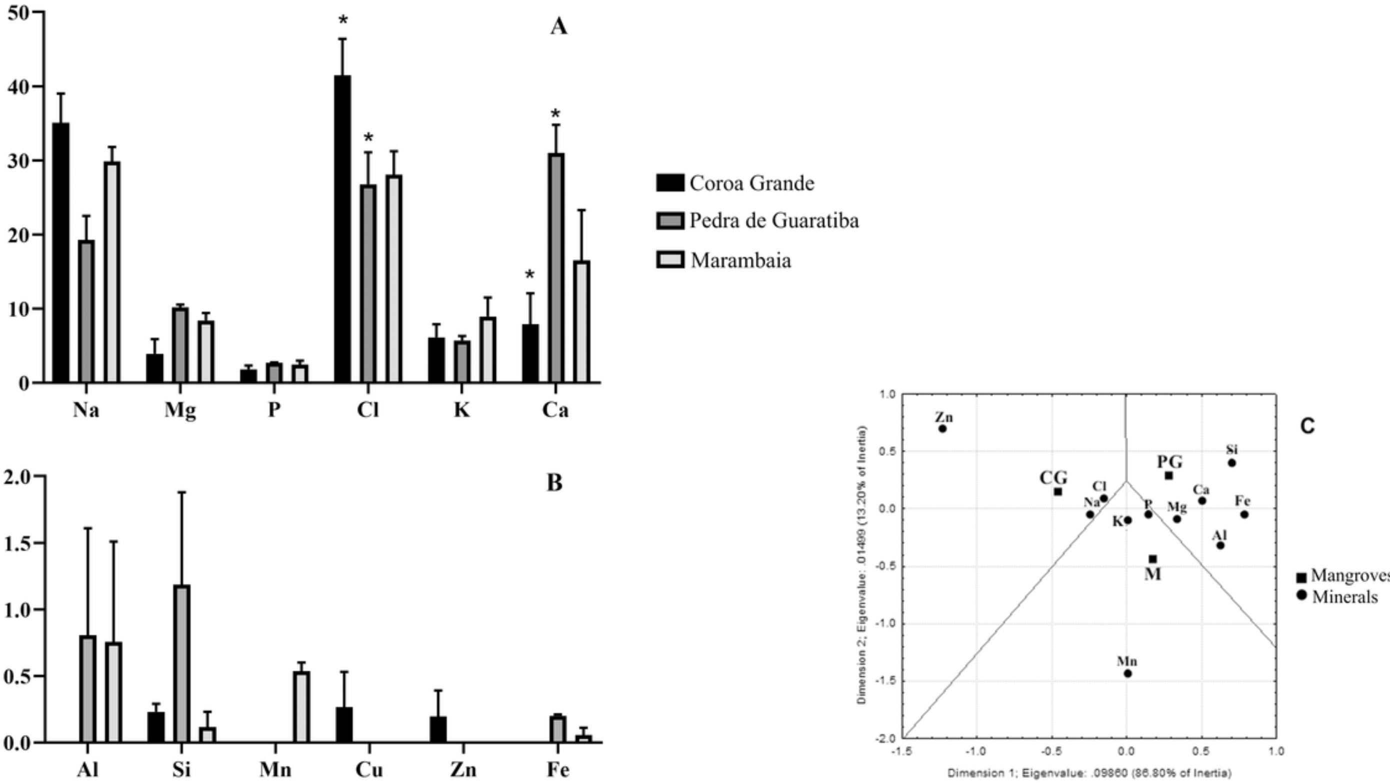

Figure 4

Relative quantity in number of atoms per mineral (chemical element) expressed in ATOM (%) of leaf of Laguncularia racemosa by analysis of ashes of leaves using Energy Dispersive X-Ray Spectroscopy (EDS). Al: aluminum; Si: Silicon, Fe: iron; Mn: manganese; Cu: copper, Zn: zinc; Ca: calcium; K: potassium; Mg: magnesium; Na: sodium, Cl: chlorine; P: phosphorous. Mangroves around Sepetiba Bay, Rio de Janeiro (Brazil): Coroa Grande (CG), Marambaia (M) and Pedra de Guaratiba (PG). A. Essential minerals; B. Heavy metals. *Significant difference Cl (CG and PG, $p < 0.01$) and Ca (CG and PG, $p < 0.0001$). C. Correspondence analysis of the number of atoms per mineral (chemical element) expressed in ATOM (%) of L. racemosa leaves collected in the mangroves.

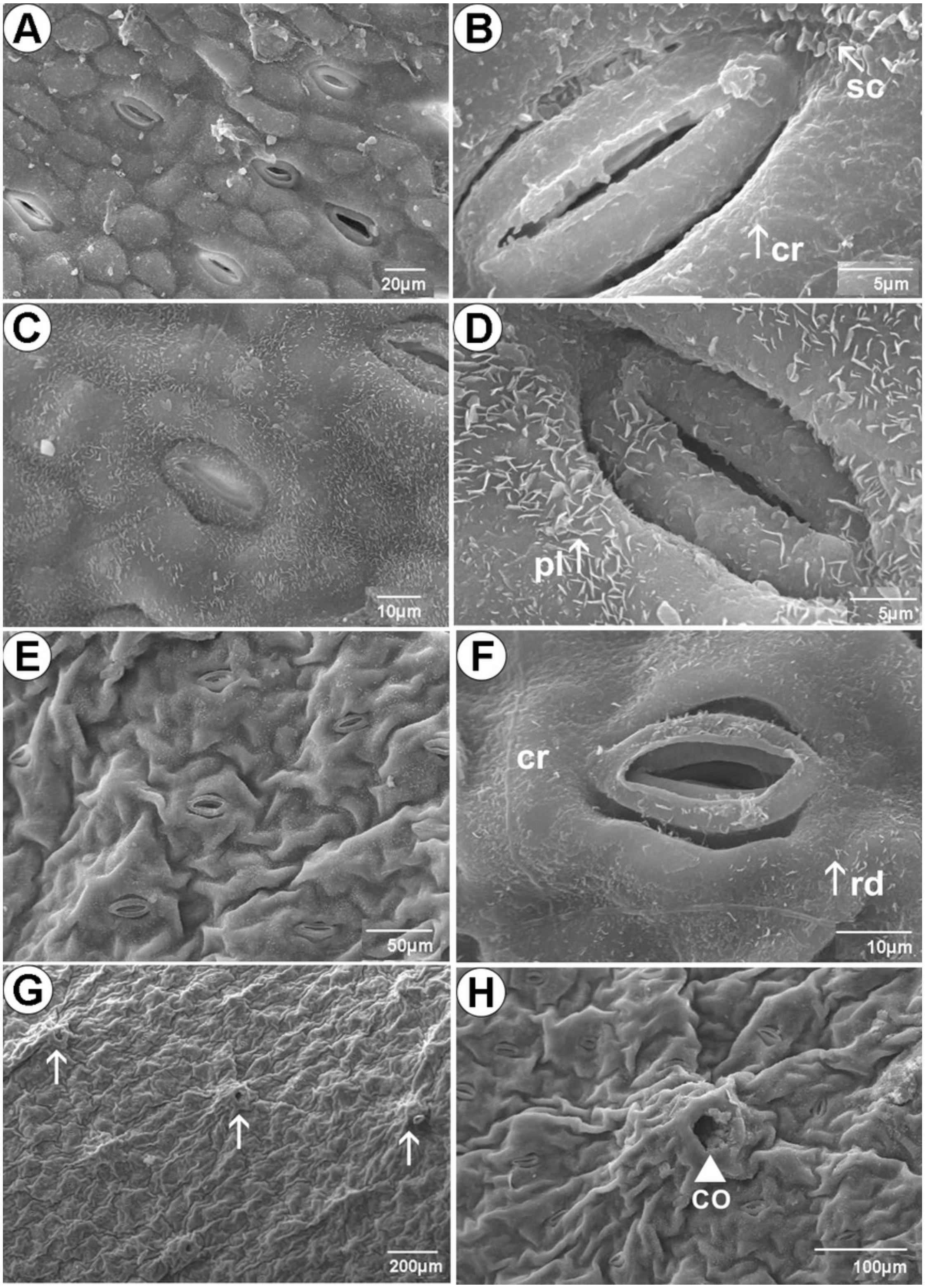

Figure 5

Leaf micromorphology of Laguncularia racemosa from three mangrove areas in Rio de Janeiro State (Brazil), showing different epicuticular wax deposition, stomata and pore of salt secretory structure located in a cavity by electronic microscopy. A-B. Marambaia (M) mangrove: epicuticular wax layer in crusts (cr) and scales near a stoma; C-D. Pedra de Guaratiba (PG) mangrove: epicuticular wax in plates (pl) in all surface, including next stoma. E-H. Coroa Grande (CG) mangrove: epicuticular wax in crusts, some rodlets (rd) over ordinary epidermal cells and stoma. G-H: abaxial epidermis of L. racemosa from CG mangrove showing opening of epidermal cavity (co) where secretory multicellular glandular trichomes are located. A, C-H. Abaxial surface. B. Adaxial surface.

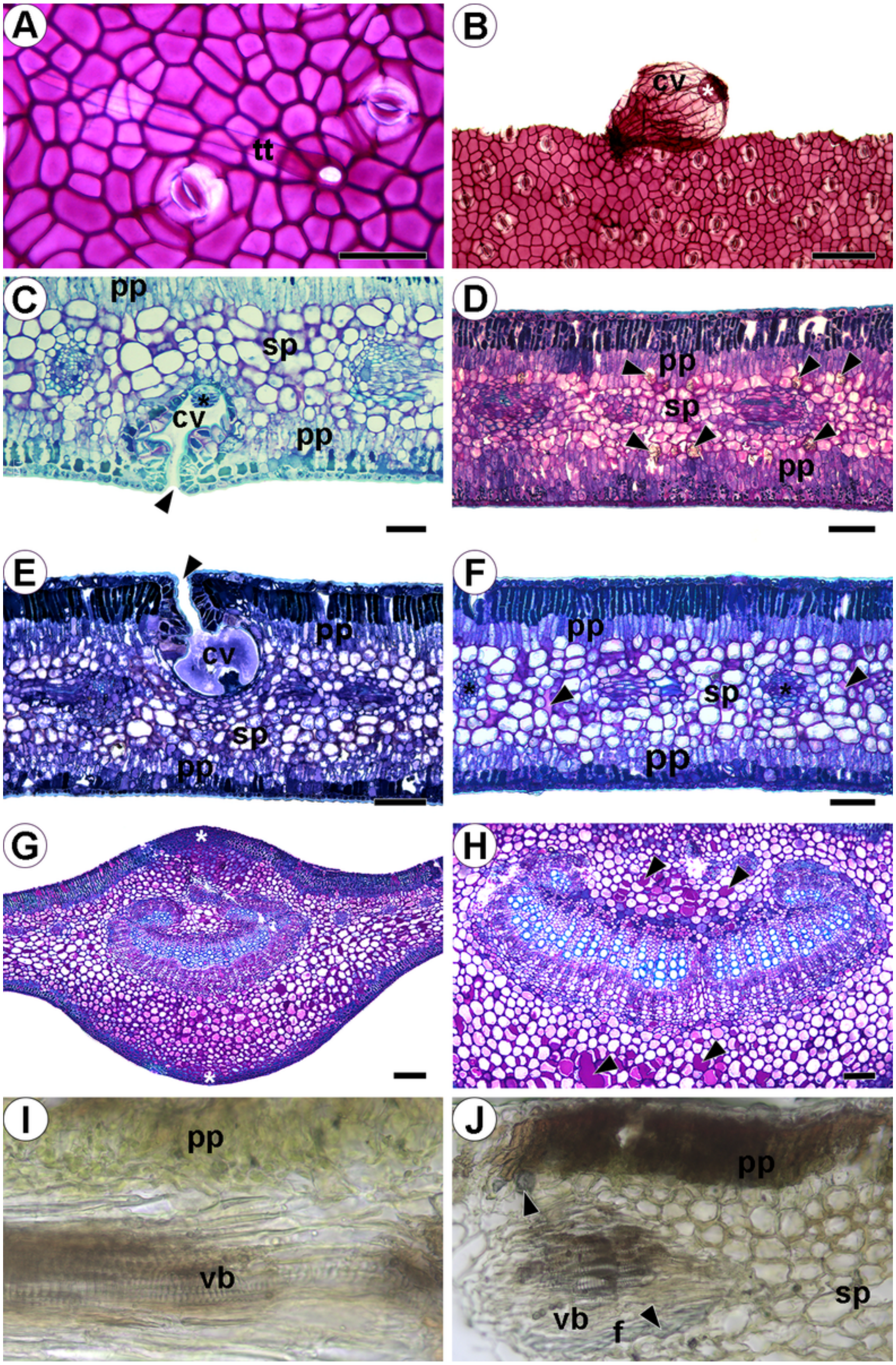

Figure 6

Anatomical analysis of Laguncularia racemosa leaves collected in different mangroves in Rio de Janeiro (Brazil), under light microscopy. A-B. epidermises in frontal view showing ordinary epidermal cells, stomata, unicellular non-glandular trichome (tt), and secretory cavity (cv) after removal of the mesophyll with a salt secretory trichome at the bottom (*); C. Leaf cross section showing cavity (cv) on the abaxial epidermal surface where the salt secretory trichome (*) is located, with opening to external environment (black arrowhead), palisade parenchyma (pp) and spongy parenchyma (sp). D. Leaf from Coroa Grande (CG): palisade parenchyma (pp) and spongy parenchyma (sp), crystal idioblasts with druses (black arrowheads). E. Leaf from Pedra de Guaratiba (PG): adaxial secretory cavity (cv) with pore (black arrowheads). F. Leaf from Marambaia (M): palisade parenchyma (pp), spongy parenchyma, collateral vascular bundles (*), black arrowheads point to mucilage from spongy parenchyma cells. G. Midvein cross section showing cortical and medullary parenchyma storing phenolic compounds (*). H. Main vascular bundle evidencing phenolic idioblasts and mucilage (black arrowheads). I. Cross section of leaf with Zincon® reagent; plant from Marambaia. J. Cross section with leaf with Zincon® reagent, evidencing zinc in fibers and idioblasts with druse crystals; plant from CG. A, E. Coroa Grande mangrove; B, F, H. Marambaia mangrove and D, G. Pedra de Guaratiba mangrove.

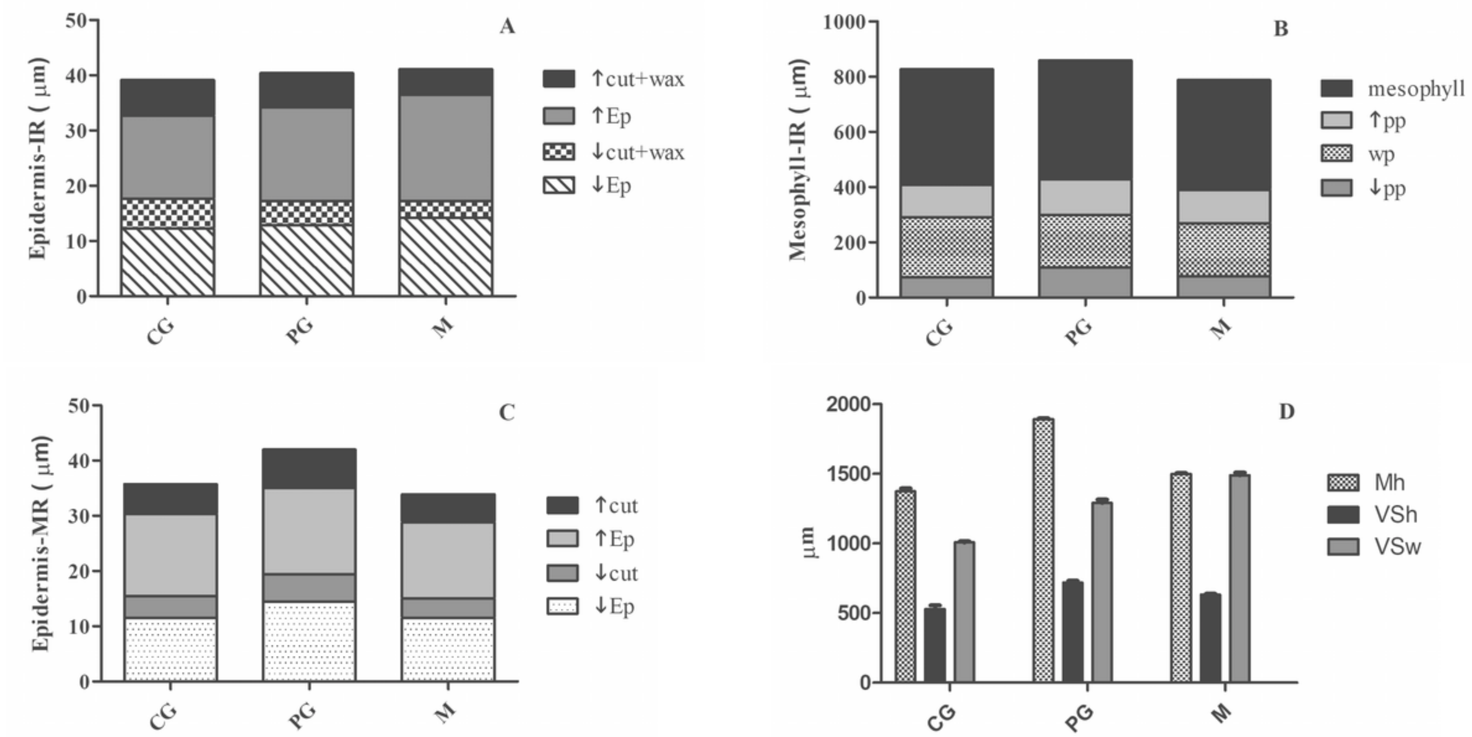

Figure 7

Leaf anatomical parameters from intercostal region (epidermis and mesophyll) and midvein (epidermis and vascular system) from leaf samples of Laguncularia racemosa collected in the Coroa Grande (CG), Pedra de Guaratiba (PG) and Marambaia (M) mangroves of Rio de Janeiro (Brazil). No significant differences were verified (Tukey`s test, $p < 0.05$). A. wax (w) + cuticle (cut) + epidermis (ep) intercostal region (IR). B. mesophyll IR: palisade parenchyma – pp, water parenchyma -wp. C. cuticle + epidermis midvein region (MR). ↑- adaxial ↓- abaxial, D. Mh - Midvein height, VSh - Vascular system height, VSw - Vascular system width.