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Seed anatomy and histochemistry of *Myrciaria dubia* (Kunth) McVaugh, an Amazonian Myrtaceace

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ABSTRACT

Myrciaria dubia, or camu-camu, is a perennial species that produces fruits of great nutritional interest because they have high levels of antioxidant compounds. The species occurs spontaneously on Amazonian river margins and requires a low amount of nutritional resources. Further, *Myrciaria dubia* fruits are collected via extractivism and managing this process is still at an incipient stage. The present study investigated the anatomical and histochemical aspects of the seeds of five clones of *Myrciaria dubia*. The external seed coat has an epidermis comprising a single layer of tabular cells that are rich in phenolic content, pectins, alkaloids, and terpenes. The cotyledons have a uniseriate epidermis, polyhedral parenchyma cells with a high concentration of starch grains, dispersed vascular bundles, alkaloids, and idioblasts with phenolic content, lipids, and proteins. The embryonic axis has a protoderm with cuboid, juxtaposed cells with thin walls and a conspicuous nucleus. Secondary metabolites in the seeds are mainly in the seed coat. This work provides new information about the anatomical and histochemical aspects of *Myrciaria dubia* seeds. No differences in the structural characteristics of the seeds and ovules were recorded among the clones since these are the most conserved reproductive organs in seed plants.

1. Introduction

The flora of Brazil is extremely diverse in edible fruits of which many are produced by native species of Myrtaceae. This family is of great botanical importance and comprises around 142 genera and 3500 to 5800 species distributed throughout the tropics and subtropics (Frauches et al., 2016; Musthafa et al., 2017). The main centers of diversity of the Myrtaceae family are in America and Australia. (Heywood et al., 2007; Wilson, 2011).

In Brazil, Myrtaceae are represented by 23 genera and around 1025 species, of which 244 have been recorded in the northern Brazil (Sobral et al., 2015). Myrciaria O.Berg is a genus of shrubs and small trees. It occurs from Mexico to the Caribbean to the north of Argentina. It is estimated that there are up to 30 species in Brazil, mainly in the southeast (Landrum and Kawasaki 1997). Further, the family has numerous representatives that are of economic and commercial interest, such as *Myrciaria dubia* (Kunth) McVaugh or camu-camu (Maeda and Andrade, 2003). *Myrciaria dubia* grows spontaneously on the margins of lakes and rivers in Amazonian Brazil and Peru (Zapata and Dufour, 1993; Silva and Andrade, 1997; Šmíd et al., 2017). Its fruits are of interest to the agro-industrial, medicinal, pharmaceutical, and cosmetic industries due to their high level of antioxidants, such as ascorbic acid and phenolic compounds (Chirinos et al., 2010; Neves et al., 2017). The level of bioactive components in the fruits of *Myrciaria dubia*, such as ascorbic acid, is above that found in fruits of *Malpighia glabra* Linn. (acerola), Prunus avium L. (cereja) and *Rubus* spp. (amora) (Yuyama, 2002; Kuskoski et al., 2005; Ferreira et al., 2010; Neves et al., 2017).

The fruit of *Myrciaria dubia* is poorly known in most of Brazil and commercialized on a small scale as frozen pulp. However, in Europe, Japan, Canada, and the United Sates it is of great interest; the pulp is imported and made into a sparkling drink, vinegar, ice cream, and sweets (Yuyama, 2011; Akter et al., 2011). Despite the nutritional and economic importance, obtaining *Myrciaria dubia* fruits for commercialization is still exclusively done by extractivism and managing this

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process is at an incipient stage.

Seeds are of great importance to humans as food and for propagation. In addition, variation in seed structures of angiosperms and the consistency of specific characters in related groups makes them useful in the classification of plants. Therefore, anatomical, and morphological studies about seeds are important, as well as works about germination, maturation, dormancy, and dispersal, which contribute to better understanding and conserving plant species (Beltrati, 1994).

Knowledge about the chemical composition and mobilization of seed reserves is crucial for seed production technology. The compounds present in seeds influence germination, vigor, and storage potential (Bewley et al., 2013). Reserves in seeds are commonly found in the form of lipids, proteins, and starch (Abud et al., 2017). During germination and seedling development, carbohydrates and lipids are mobilized and used as an energy and carbon source (Graham, 2008; Bewley et al., 2013). Proteins mainly store nitrogen and sulfur that are essential for the synthesis of other proteins, nucleic acids, and secondary compounds used during seedling growth (Bewley et al., 2013). There are some studies about *Myrciaria dubia* seeds, but the chemical composition and type of predominant reserve in the seeds are unknown (Ferreira and Gentil, 2003; Gentil et al., 2004; de Andrade et al., 2006; Yuyama, 2011).

The family Myrtaceae is a complex, highly diverse group in Brazilian ecosystems and its taxonomy is still poorly studied (Landrum and Kawasaki, 1997; Gomes et al., 2009). In this context, anatomical studies can contribute to a better understanding of how physiological and structural processes interact with environmental growth conditions and the distribution of plants in a habitat, as well as provide other important information about the natural history of species (Mourão et al., 2002; Corrêa et al., 2019).

In general, anatomical, and histochemical studies of tropical seeds are uncommon. Although the fruits of Myrtaceae are ecologically and economically important and there is an anatomical and histochemical description of seeds of some species of this family (Ciccarelli et al., 2005; Luqman et al., 2018; Mendes and Mendonça, 2020), nothing is known about the seed anatomy and histochemistry of *Myrciaria dubia*. Thus, the seeds of five *Myrciaria dubia* clones were studied with the objective of comparing histochemical and anatomical characteristics that have not been described in the literature.

2. Materials and methods

2.1. Plant material and preliminary procedures

This study was carried out with fruits of *Myrciaria dubia* (Kunth) McVaugh acquired from 45 plants of five clones present in the Active Germplasm Bank (BAG) of Embrapa Amazônia Oriental (48°26'45"W, 1°26'31" S) (Fig. 1). Anatomical and histochemical analyses were performed at the Botanical Laboratory of the Museu Paraense Emílio Goeldi. The epicarp and mesocarp of the fruits were removed manually with a domestic knife. After separation, the seeds were soaked in distilled water for 24 h and the embryos were cut with a steel razor blade.

2.2. Anatomical evaluation

The samples were fixed in 50% FAA (Johansen, 1940) for 24 h and stored in 70% ethyl alcohol. Subsequently, they were dehydrated in an ethylic series and butyl acetate, embedded in histological paraffin (Johansen, 1940), sectioned with a rotary microtome (12 μm thick), stained with 0.5% toluidine blue, at pH 4.7 (O'Brien et al., 1964), and safranine (Kraus and Arduin, 1997), and mounted in Entellan®.

2.3. Histochemical evaluations

For the histochemical tests, fresh transversal and longitudinal sections were used. Some histological sections were mounted and photographed without submitting them to reagents to observe the natural aspect of the substances. The reagents used were the following; aqueous solution of 10% ferric chloride for phenolic compounds (Johansen, 1940); Wagner's reagent for general alkaloids (Furr and Mahlberg, 1981); Sudan III for detecting total lipids (Johansen, 1940); aqueous solution of 0.02% ruthenium red to identify diverse polysaccharides and pectins (Jensen, 1962); lugol solution (iodine + potassium iodide) for starch (Johansen, 1940); xylidine ponceau for proteins (O'Brien and McCully, 1981); and NADI reagent (David and Carde, 1964) for terpenes. The controls for each test followed those cited by the above authors. Temporary slides were mounted in glycerin. For the anatomical analyses and histochemical tests, evaluations were made in triplicate for each clone.

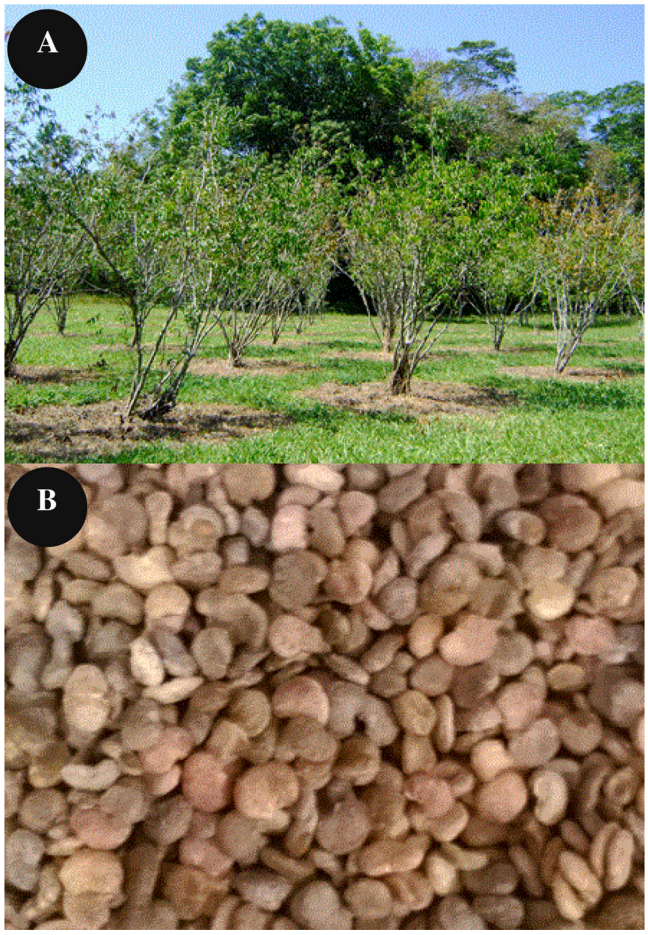

Fig. 1. Plants (Fig. 1A) and seeds (Fig. 1B) of *Myrciaria dubia* (Kunth) McVaugh (camu-camu) clones belonging to the Active Germplasm Bank at Embrapa Amazônia Oriental.

Photographs of the anatomical aspects of the seeds were made with a digital camera (Canon Power Shot A6 40) coupled to a light microscope (Zeiss Axiolab). For scanning electron microscopy (SEM), sections of the seed coat, cotyledons and protuberance of the seed were fixed in a 2.5% glutaraldehyde solution in a 0.1 M, pH 7.3, phosphate buffer, known as Karnovsky solution (Karnovsky, 1965). They were then post-fixed in 1% osmium tetroxide, washed three times in the same buffer, dehydrated in an alcohol series (Gahan, 1984), critically point dried using CO_2 as the transition liquid (Bozzola and Russel, 1991), fixed with graphite on aluminum stubs, and coated with carbon and gold. The images were obtained in the Scanning Electron Microscopy Institutional Laboratory, at Museu Paraense Emílio Goeldi, using an electron microscope (LEO model 1450 VP).

3. Results

3.1. Seed anatomy

The external periclinal wall of the seed coat cells has an irregular surface and, probably, residues of the mesocarp, and the cells are tabular and very irregular (Fig. 2A). The internal periclinal walls are faviform (Fig. 2B). In transversal section, the seed coat has approximately 15 cell layers (Fig. 2C) divided into two strata. The first stratum has juxtaposed, lignified, elongated cells forming a palisade layer (Fig. 2D). The second stratum comprises large intercellular spaces (Fig. 2E) and more rounded cells that have thick walls (Fig. 2F-I).

In the meristematic region, located at one of the seed apices where the protrusion of the root of the epicotyl occurs, the external periclinal wall of the epidermis has a layer of reticulated cells with globose, elevated, irregular punctations that are presumably secretory structures (Fig. 3A and B). The cotyledons have an epidermis of juxtaposed, rectangular cells, forming a uniseriate layer, with thin and thick walls, irregular parts, and no ornamentation (Fig. 3D). The secretory cavities are side by side near the epidermis, solitary in the subepidermis and have an isodiametric lumen and uniseriate epithelium formed by tabular cells (Fig. 3E). The mesophyll of the cotyledons comprises parenchymatous tissue, with a reserve characteristic, dispersed vascular bundles (Fig. 3G) and secretory ducts irregularly distributed throughout the tissue. The tissue is homogeneous with various layers of isodiametric, polyandric cells, with a conspicuous nucleus (Fig. 3H and I).

The isodiametric cells have anticlinal walls that are thin with punctations and intercellular spaces (Fig. 4C). The embryo is homogeneous, without apparent delimitations of its main structures, and comprises a dense cotyledonary mass. The embryonic axis is indistinct, the cotyledons are thick, with undifferentiated cells (Fig. 4H), and the protoderm is composed of cuboid, juxtaposed cells, with a conspicuous nucleus and thin walls. It was not possible to describe the ground meristem of the cortex, procambium and ground meristem of the pith.

3.2. Seed histochemistry

In transversal section, the seed coat has approximately 15 cell layers.

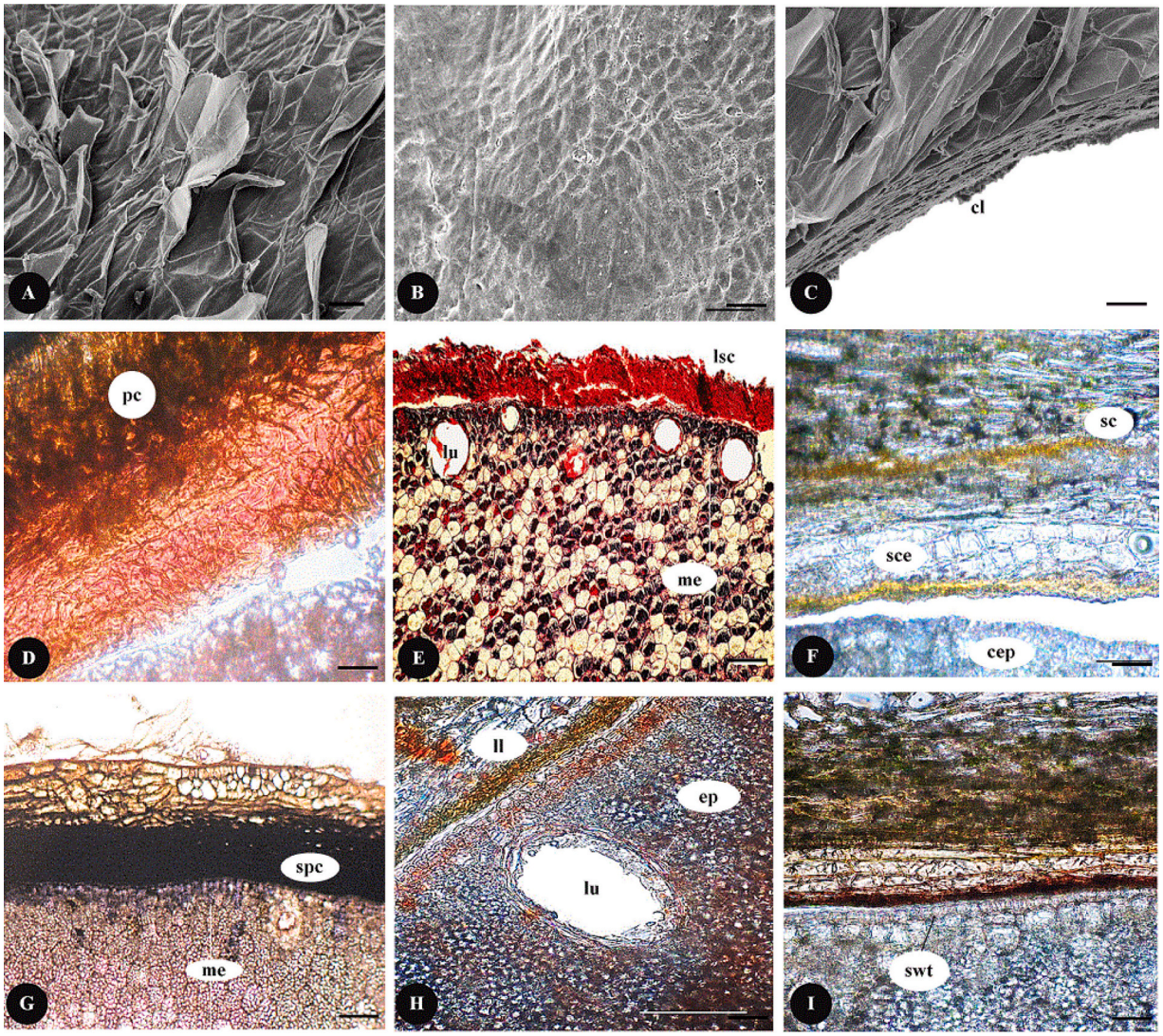

Fig. 2. Seed coat of *Myrciaria dubia* (Kunth) McVaugh. SEM images of longitudinal (Fig. 2A and 2B) and transversal (Fig. 2C) sections, and light electron microscopy images of transversal sections (Fig. 2D-I): 1A- external surface of seed coat, 2B- internal seed coat, 2C- subepidermal parenchyma in the seed coat, 2D- positive reaction to pectins, 2E- lignified seed coat, 2F- Unstained seed coat, 2G- positive reaction to phenolic compounds, 2H- positive reaction to total lipids, 2I- positive reaction to terpenes. Legend: cc - layers of cells; ce- spread out cells; cl- lipid layer; cp- palisade cells; ct- tabular cells; ecf- stratum with phenolic compounds; ep- epithelium; et- stratum with terpenes; lu- lumen; ma- adhered mesocarp; tg- seed coat; tl- lignified seed coat; me- mesophyll. Scale bars: 2A-C, E- 50 μm; 2D, 2F, 2I- 100 μm; 2G- 20 μm; and 2H- 30 μm.

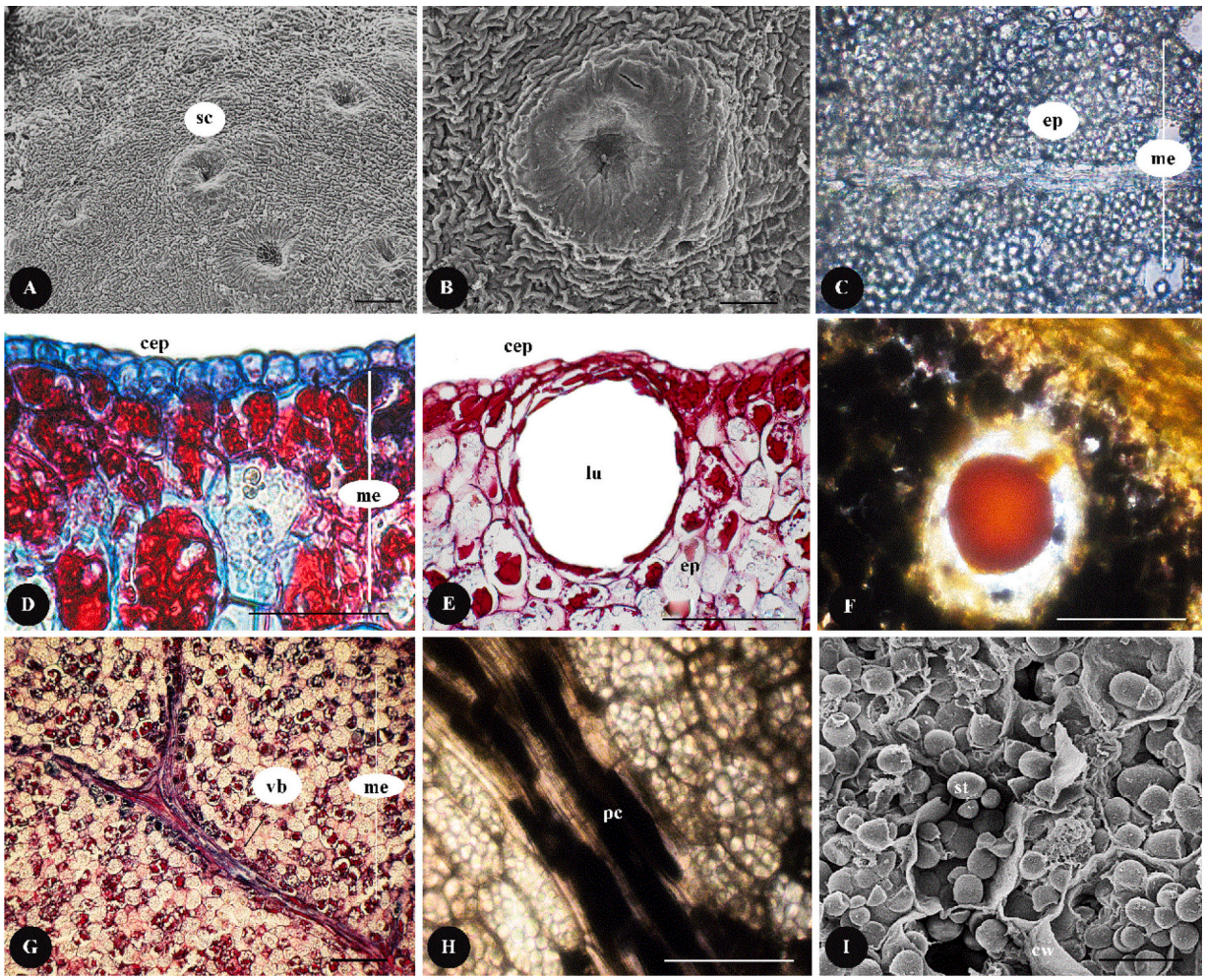

Fig. 3. Cotyledons of *Myrciaria dubia* (Kunth) McVaugh seeds. SEM images of longitudinal sections (Fig. 3A, 3B and 3I) and light electron microscopy images of longitudinal (Fig. 3D, 3 G) and transversal (remaining images) sections: 3A- region of the protuberance, 3B- detail of the secretory cavity of the protuberance, 3C- cotyledon mesophyll without reagent, 3D- uniseriate epidermis, 3E- detail of lumen, 3F- detail of terpenes in the lumen, 3G- vascular bundles in the cotyledon mesophyll, 3H- secretory duct with phenolic compounds in the cotyledon mesophyll, 2I- starch grains in the amyloplasts. Legend: am- starch; cf- phenolic compounds; cs- secretory cavity; ep- epithelium; epc- cotyledon epidermis; fv- vascular bundle; lu- lumen; mc- cotyledon mesophyll; pc- cell wall. Scale Bars: 3B- 20 μm; 3I- 30 μm; 3A, 3E, 2F, 3H −50 μm; and 3D, 3C, 3 G −100 μm.

The second layer are rich in phenolic compounds, pectins, lipids and terpenes (Fig. 2F-I). The secretory cavities present positive reaction (reddish brown) to reagent of NADI was evidence of terpenes in the lumen (Fig. 3F). The mesophyll of cotyledons present secretory ducts with phenolic content irregularly distributed throughout the tissue. The tissue is homogeneous with numerous simple, spherical starch grains that have a conspicuous nucleus (Fig. 3H and I), affirmed by the positive reaction to the lugol reagent (Fig. 4A). The starch grains are present throughout the cellular cytoplasm. Globose idioblasts with terpenes, evidenced by the positive reaction to the NADI reagent, and phenolic compounds, evidenced by the positive reaction (black color) to 10% ferric chloride (Fig. 4C), were observed in the mesophyll of the cotyledons. The parenchymatous cell walls are rich in polysaccharides, mainly pectins, which was shown by the positive reaction to ruthenium red (Fig. 4D). Lipid bodies (positive reaction to Sudan III) (Fig. 4E) and abundant proteins and alkaloids were observed in the cytosol (Fig. 4F and G).

4. Discussion

The seeds of *Myrciaria dubia* described in this study have a voluminous embryo, short hypocotyl-radical axis, and indistinct, partially fused cotyledons. In seeds, there is a close relationship between the large size of embryos and recalcitrance, as is the case with species of the genus Eugenia (Von Teichman and Van Wyk, 1991). *Myrciaria dubia* seeds do not tolerate desiccation and humidity below 20% (Yuyama, 2011). For this reason, they are considered as recalcitrant, according to the Roberts (1973) classification. Seeds of *Myrciaria dubia* show differences and similarities in comparison to seeds of other plant species. For example, Moreira-Coneglian (2007) described similar characteristics for the seeds of Eugenia punicifolia (Kunth) DC. However, in study with mature seed of *M. rufa* (Colla) Skottsb. ex Kausel seeds, Retamales et al. (2014) describe the absence of endosperm and an embryo occupying most of the seed, as found in the present study, as well as a conspicuous hypocotyl. Unlike the embryonic axis of seeds of *Myrciaria dubia* described in the present study, (Justo et al., 2007) observed a differentiated embryonic axis in a seed of *E. pyriformes* Camb.

Regarding seed coat, we did not classify the seed testa strata of *Myciaria. dubia* because we did not conduct an ontogenetic analysis of the seeds to understand the histological constitution throughout the development of *Myrciaria dubia* seeds. However, several studies have found different descriptions for the testa in seeds of species belonging to the family Myrtaceae. For example, Corner (1976) observed multiple tests, with 15 to 26 layers of sclerified cells. Moreira-Coneglian (2011) describes the presence of an exotesta with macrosclereids and palisade cells with thick, lignified walls, a mesotesta, and an endotesta formed by

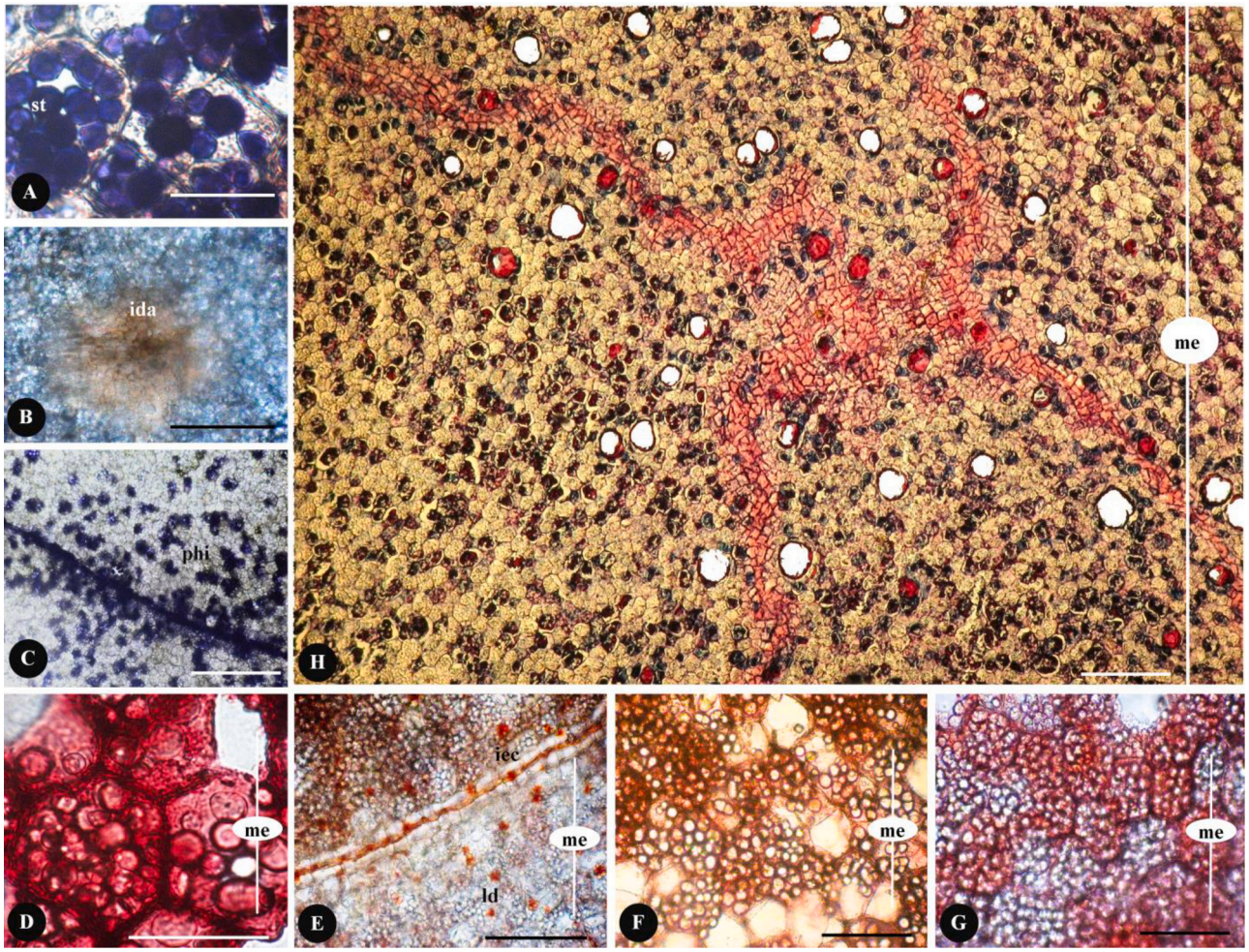

Fig. 4. Cotyledons of *Myrciaria dubia* (Kunth) McVaugh seeds. Light electron microscopy images of longitudinal (Fig. 4 G) and transversal (remaining images) sections: 4A- positive reaction to the lugol test, 4B- idioblasts with terpene in the cotyledon mesophyll, 4C- cotyledon mesophyll with phenolic idioblasts, 4D- positive reaction to ruthenium red, 4E- lipid droplets in the cotyledon mesophyll and internal epidermis of the cotyledons, 4F- positive reaction to alkaloids in the cotyledon mesophyll, 4G- positive reaction to proteins in the cotyledon mesophyll, 4H- general aspects of the embryonic axis. Legend: am- starch; epi- internal epidermis of the cotyledons; gl- lipid droplets; ida- idioblasts with alkaloids; idf- phenolic idioblasts; mc- cotyledon mesophyll. Scale Bars: 4A, 4D, 4F- 50 μm; and 4B, 4C, 4E, 4 G, 4H- 100 μm.

brachysclereids with phenolic content, lignified walls and punctations in *E. bimarginata* DC. Retamales et al. (2014) in *M. rufa* observed a testa with two sclerified layers.

In *Myrciaria dubia* phenolic compounds mainly accumulate in the seed coat. However, they were also found in the ducts and idioblasts in the cotyledons (Table 1). According to Rocha et al. (2011) phenolic substances can have different functions in plants, including protection against herbivory and seed dormancy due to these compounds in the seed coat (Villavicensio et al., 2007). Myoda et al. (2010) analyzed the oxidative potential of residues of *Myrciaria dubia* juice and reported elevated levels of phenolic compounds in *Myrciaria dubia* seed extracts, suggesting that the quantity of these substances in the seeds is greater than that in the fruit epicarp. Myoda et al. (2010) indicated that the phenolic compounds present in *Myrciaria dubia* seeds could be responsible for the antioxidant activity, which would make the seeds an efficient and applicable source of great relevance to the food industry compared to acerola, pineapple, passion fruit and other tropical fruit residues. In addition, Cunha (2018) observed specific phenolic compounds, such as rutin, quercetin, ρ-coumarin, epicatechin and catechin, in extracts of *Myrciaria dubia* seeds. The color of the seed coat in several species is due to the presence of phenolic compounds. In the seeds, the presence of compounds is correlated with the restriction to germination due to the permeability to water, oxygen or mechanical resistance to the protrusion of the radicle (Debeaujon et al., 2007). Phenolic compounds or polyphenols constitute a broad group of phytochemicals that have at least one aromatic ring that binds to one or more hydroxyl groups. The chemical structure of these compounds varies from a simple phenolic molecule to complex polymers of high molecular weight (Balange and

Table 1
Presence (+) or absence (-) of organic compounds in *Myrciaria dubia* (Kunth) McVaugh seed structures.

Seed structure	Starch	Terpene	Phenol	Lipid	Protein	Alkaloid	Pectin
Cotyledon mesophyll	+	+	–	–	–	–	–
Seed coat	–	+	–	+	+	–	+
Ducts in the cotyledon	–	–	+	–	–	–	–
Idioblasts in the cotyledon	–	–	+	–	–	–	–
Secretory cavities	–	+	–	–	–	+	–

Benjakul, 2009; Cartea et al., 2011; Oliveira et al., 2014). Phenolic and terpenoid compounds such as abscisic acid, a terpenoid derived from the metabolism of carotenoids, can act as inhibitors of germination (Bewley et al., 2013).

Moreira-Coneglian (2011) describing *E. bimarginata* observed that the cotyledons have a protoderm with juxtaposed cells and a ground meristem with thin walls and starch grains, subepidermal secretory canal, dispersed procambium strands and some cells with phenolic content. Similar characteristics were encountered in the seeds of the species investigated in the present work. According to Souza Filho et al. (2011) alkaloids in plants play an important role against herbivory and have medicinal properties. Yazawa et al. (2011) observed betulinic acid (triterpene) in an extract of *Myrciaria dubia* seeds. These authors suggest that this substance is responsible for the anti-inflammatory activity in the seeds. Inflammation is the immune system's response to infections and tissue damage. It has been implicated in the pathogenesis of arthritis, cancer, and stroke, as well as in neurodegenerative and cardiovascular diseases. The inflammatory process is mediated by the synthesis of prostaglandins catalyzed by the enzyme cyclooxygenase, which can be inhibited by anti-inflammatory substances (Ricciotti and FitzGerald, 2011). The terpene found in the seeds of *Myrciaria dubia* is in the most internal layer of the seed coat (Table 1). However, the main substances in the reserve of mature *Myrciaria dubia* seeds are protein and lipid bodies, as well as carbohydrates in the form of starch (Werker, 1997).

There were no differences in the histochemistry and anatomy of the seeds of the *Myrciaria dubia* clones evaluated. The consistency of seed characters between species of the same genus is common. For example, in three species of *Banisteriopsis* (*B. campestris, B. oxyclada* and *B. stellaris*) Souto and Oliveira (2008) found no differences in the structures and development of the seeds. For seeds and ovules, the consistency of structural characteristics is expected and reported in the literature, since these are the most conserved reproductive organs in seed plants (Von Teichman and Van Wyk, 1991). Additionally, in the present study, the same soil, climatic and management factors among the cultivated clones could have contributed to the absence of anatomical and histochemical differences in the seeds, since morphological and micromorphological characteristics are strongly influenced by the environment.

This is the first study of *Myrciaria dubia* that reports the location of phenolic compounds in the seed coat, as well as terpenes in the most internal layer of the seed coat. These results, based on histochemical tools, increase what is known about the chemical composition of the seeds of this species.

5. Conclusion

The seed anatomy of *Myrciaria dubia* (Kunth) McVaugh has the same anatomical distribution pattern described for most Myrtaceae. The seeds have a homogeneous embryo comprising a large cotyledonary mass and indistinct embryonic axis. Determining the location of the secondary metabolites in the seed coat could contribute to subsequent studies that isolate these substances. Protein and lipid bodies, as well as carbohydrates in the form of starch, were the main nutrient reserves found in the seeds. We recommend conducting ontogenetic studies of *Myrciaria dubia* to better understand the origin of each seed coat layer. The anatomical and histochemical aspects described in this study increase what is known about the seeds of Myrtaceae and clarify uncertainties related to seed characteristics of *Myrciaria dubia*.

CRediT authorship contribution statement

Olívia Domingues Ribeiro: Investigation, Conceptualization, Methodology, Writing – review & editing. **Walnice Maria Oliveira do Nascimento:** Investigation, Conceptualization, Methodology, Writing – review & editing. **Flávio José Rodrigues Cruz:** Writing – review & editing. **Ely Simone Cajueiro Gurgel:** Investigation, Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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