

Brazilian Journal of Pharmacognosy revista brasileira de farmacognosia





Original Article

Comparative leaf morpho-anatomy of six species of *Eucalyptus* cultivated in Brazil

Check for updates

Izabel Pietczak Migacz^a, Paola Aparecida Raeski^b, Valter Paes de Almeida^a, Vijayasankar Raman^c, Silvana Nisgoski^d, Graciela Inês Bolzón de Muniz^d, Paulo Vitor Farago^a, Ikhlas Ahmed Khan^c, Jane Manfron Budel^{a,*}

^a Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil

^a Programa de Pós-graduação em Engenharia Florestal, Universidade Federal do Paraná, Curitiba, Paraná, Brazil

rograma de ros-gradadção em Engennaria rorestal, Oniversidade reaerar do rarana, Carnoa, Farana, Brazi

ARTICLE INFO

Article history: Received 21 February 2018 Accepted 25 April 2018 Available online 14 May 2018

Keywords: Plant anatomy Calcium oxalate crystals Energy-dispersive X-ray spectroscopy Light and scanning electron microscopy Morphology

Introduction

The genus *Eucalyptus* L'Hér., Myrtaceae, is represented by more than 800 species, and a majority of them are native to Australia (Flores et al., 2016; The Plant List, 2017). Species of *Eucalyptus* are economically important and are being used in the production of essential oils, wood, paper and cellulose (Balbino et al., 2011). *Eucalyptus* flowers contribute to the production of honey (Birtchnell and Gibson, 2006). Essential oils of *Eucalyptus* are rich in monoterpenes and are extensively used in pharmaceuticals, perfumes, food flavorants and agrochemicals (Brooker and Kleinig, 2006; Flores et al., 2016; Barbosa et al., 2016).

Species of *Eucalyptus* contain phenolic compounds and essential oils as main groups of secondary metabolites (Metcalfe and Chalk, 1950; Santos et al., 2008). Several biological activities, such as acaricidal, antioxidant, antibacterial, insecticidal, fungicide and herbicidal, have been reported for different species of *Eucalyptus*. These activities are attributed mainly to the chemicals present in the essential oils (Barbosa et al., 2016).

Six species of Eucalyptus, namely E. badjensis Beuzev. & Welch, E. benthamii Maiden & Cambage, E. dunnii Maiden, E. grandis W.Hill,

E-mail: jane@uepg.br (J.M. Budel).

ABSTRACT

The present work provides a comparative account of the morpho-anatomy of six species of *Eucalyptus*, namely *E. badjensis* Beuzev. & Welch, *E. benthamii* Maiden & Cambage, *E. dunnii* Maiden, *E. grandis* W.Hill, *E. globulus* Labill. and *E. saligna* Sm., Myrtaceae. Leaf samples of these six species were investigated by light and scanning electron microscopy. The observed microscopic features that can be useful in the identification and quality control of the studied species include the morphology of epicuticular waxes, presence of prismatic crystals on the leaf surface, leaf midrib shape and arrangement of its vascular system, and the presence or absence of the sclerenchymatous fiber caps in the vascular bundle.

© 2018 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

E. globulus Labill. and *E. saligna* Sm., Myrtaceae, are analyzed in the present study. Previous studies have shown that all six species have insecticidal activities. In addition, *E. grandis* has antifungal and *E. globulus* has acaricidal and antifungal properties (Barbosa et al., 2016).

Previous studies have reported that many *Eucalyptus* species have similar morphologies, making their morphological identification difficult (Santos et al., 2008; Flores et al., 2016). For instance, *E. grandis* can be confused with *E. dunni*, *E. deanei* Maiden, *E. saligna* and *E. botryoides* Sm. (Flores et al., 2016). In this situation, comparative morpho-anatomical studies, like the present work, can help in the distinction and the identification of the species. Therefore, the aim of the present work was to examine and compare the leaf morphological and anatomical characteristics of the six *Eucalyptus* species by light and scanning electron microscopy.

Material and methods

Plant material

Seedlings of six species of *Eucalyptus*, namely *E. badjensis* Beuzev. & Welch, *E. benthamii* Maiden & Cambage, *E. dunnii* Maiden, *E. grandis* W.Hill, *E. globulus* Labill. and *E. saligna* Sm., Myrtaceae, were obtained from "Registro Nacional de Sementes e Mudas" in 2015 (collection numbers 00605/1-6) and were

https://doi.org/10.1016/j.bjp.2018.04.006

0102-695X/© 2018 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http:// creativecommons.org/licenses/by-nc-nd/4.0/).

^b Programa de Pós-graduação em Ciências da Saúde, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil ^c National Center for Natural Products Research, School of Pharmacy, University of Mississippi, Oxford, MS, USA

^{*} Corresponding author.

housed in the garden located in São João do Triunfo, Paraná. These seedlings were grown from certified seeds authenticated by Ministério da Agricultura, Agropecuária e Abastecimento, Brazil.

The seedlings of the six species, with four replicates for each species, were acclimatized in the same environment, in São Mateus do Sul, Paraná (latitude 25°41′00″ S; longitude 50°17′50″ W; altitude: 840 m) in October 2015, in an entirely random design. For the anatomical studies, leaf samples were collected from 12-month old plants. At least six samples of mature and young leaves were collected from each species and used for microscopic analyses.

Preparation of samples for light microscopy

The leaf materials were fixed in formalin-acetic acid-alcohol (FAA) solution (Johansen, 1940) for 7 days and washed in distilled water and then stored in 70% ethanol (v/v) (Berlyn and Miksche, 1976). Transverse sections of the leaf blade were prepared by free-hand using razor blades. The sections were hydrated and stained with toluidine blue (O'Brien et al., 1964) or double-stained with basic fuchsin and Astra blue (Roeser, 1972). The sections were then mounted on glass slides in a drop of glycerin solution (50% in water).

For the analysis of leaf epidermal characters, the leaf specimens were cleared by dipping them in commercial bleach (2.5% sodium hypochlorite) solution until translucent. Then, the samples were immersed briefly in a diluted acetic acid solution, washed with water and stained in safranin (Fuchs, 1963). The prepared specimens were observed and photomicrographs were prepared using an Olympus CX31 microscope equipped with Olympus C-7070 digital camera.

The terminology of Barthlott et al. (1998) was used to describe the epicuticular waxes.

Micromeasurements

Quantitative studies of stomata were performed by taking twenty measurements from multiple leaf specimens. The stomatal index (SI) was calculated using the following formula $\left[SI = \frac{S}{E+S} \times 100\right]$, wherein *S* = number of stomata per unit area, and *E* = number of epidermal cells in the same unit area (including overlying cells). The length and width of stomata were measured from 20 stomata at different locations on the leaf blade for each species to determine the average stomatal size.

Histochemical analyses

Standard solutions of ferric chloride (Johansen, 1940) and potassium dichromate (Gabe, 1968) were used to detect the presence of phenolics; phloroglucinol/HCl to identify lignified tissues (Sass, 1951); iodine solution to stain starch (Berlyn and Miksche, 1976) and sudan III was used to detect lipophilic compounds (Foster, 1949).

Preparation of samples for scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) analyses

The leaf samples fixed in FAA were washed in water and passed through a series of ethanol solutions (80, 90 and 100%). The samples were then dried in a Balzers CPD-030 critical point dryer supplied with liquid CO₂. The fully dried samples were mounted on aluminum stubs with double-sided adhesive tapes and then coated with gold using a Quorum SC7620 sputter coater in order to make the samples conductive. The samples were analyzed and imaged using a Mira 3 Tescan Field-Emission SEM (Oxford Instruments, Oxford, UK) in high vacuum mode at 15 kV accelerating voltage. Qualitative and quantitative X-ray microanalyses were performed for selected crystals using an EDS detector attached to the SEM. The

SEM and EDS analyses were carried out at the multi-user laboratory in the State University of Ponta Grossa.

Results and discussion

In the present work, morpho-anatomical characters of the leaves of six species of *Eucalyptus* were examined and compared (Table 1). The leaves (Fig. 1A–F) of all the six species have similar morphologies; they are simple, petiolate and alternately arranged, and the leaf blades are acuminate at apex, acute to attenuate at base, entire along margins, reticulately veined and are glabrous and smooth on both surfaces. These features are in agreement with previous reports (Nisgoski et al., 1998; Flores et al., 2016).

The morpho-anatomical features of the leaves of the six species of *Eucalyptus* are compared in Table 1. *Eucalyptus badjensis* has the narrowest and smallest leaves, while *E. saligna* has the largest and *E. grandis* has the longest leaves. In the case of leaf shape, *E. badjensis* has linear to narrowly lanceolate leaves; *E. badjensis, E. dunnii, E. grandis* and *E. globulus* are falciform; and *E. saligna* has lanceolate leaves. The leaves are green on both sides in all species. However, the leaf of *E. globulus* is light green and presents white points more evident on the adaxial side. *Eucalyptus badjensis, E. benthamii* and *E. grandis* present as papyrus consistency, whereas *E. dunnii, E. globulus* and *E. saligna* are classified as coriaceous. The leaves of *E. benthamii* can be confused with those of *E. globulus*. As also noted by Flores et al. (2016), the leaves of *E. grandis* can be confused with those of *E. dunnii*.

Previous studies report that the leaf epidermal cells in *Eucalyptus* species usually have straight anticlinal walls (Oliveira et al., 2005; Malinowski et al., 2009). The present study confirms this observation; the anticlinal cell walls are observed to be straight on both leaf epidermises in all the six species examined (Fig. 2A–I).

Anomocytic stomata have been frequently reported for *Eucalyptus* species (Santos et al., 2008; Al-Edany and Al-Saadi, 2012; Saulle et al., 2018). However, anisocytic type is also found in the genus, such as in *E. camaldulensis* Dehnh (Tantawy, 2004). In all species studied, stomata are slightly sunken below the leaf surface (Fig. 3B–G, J–L). This characteristic has been reported for *E. camaldulensis* (Santos et al., 2008), *E. platypus* Hook.f, *E. spathulata* Hook., *E. yalatensis* Boomsma and *E. viridis* F.Muell. ex R.T.Baker (Knight et al., 2004).

Considering the occurrence of stomata in the leaves, amphistomatic leaves are common in *Eucalyptus* (Santos et al., 2008; Döll-Boscardin et al., 2010; Saulle et al., 2018). However, hypostomatic feature has also been found in the genus, such as in young leaves of *E. globulus* subsp. *Bicostata* Maiden, Blakely & Simmonds (Malinowski et al., 2009). In this study, all six species have amphistomatic (Fig. 2A–I) leaves. And the stomata are of the anomocytic type.

The stomatal index is the percentage of the number of stomata made by the total quantity of epidermal cells, including the stomata, each stoma being counted as one cell. The size and the stomata index have greater taxonomic relevance (Cutter, 1986). Micro-measurements of stomata show that the smallest stomata are present in *E. benthamii* on both adaxial ($28.57 \times 21.43 \mu$ m) and abaxial ($28.70 \times 22.88 \mu$ m) epidermises among the studied six species. The largest stomata are observed in *E. grandis* ($65.61 \times 51.06 \mu$ m) on the adaxial side and in *E. globulus* on the abaxial side ($56.48 \times 46.16 \mu$ m).

Stomatal index of the adaxial side is lower than that of the abaxial side in all the species studied. *Eucalyptus benthamii* has the highest stomatal index for both epidermises (around 8%), whereas *E. dunnii* shows the lowest indices on both abaxial (4.84%) and adaxial (1.86%) sides.

Table 1

Comparative morpho-anatomy of Eucalyptus species.

Leaf morpho-anatomical features		E. badjensis	E. benthamii	E. dunnii	E. grandis	E. globulus	E. saligna
Leaf size (length × width in Leaf shape	n cm)	0.35–1.1 × 14.5–15.5 Linear to narrowly lanceolate	1.1–2.1 × 15.5–22 Narrowly lanceolate	1.3-3.6 × 16-25 Falcate	2.3–3.8 × 14.5–20 Falciform to lanceolate	1.5–3 × 15.35–17.5 Falcate	1.8–2.8 × 14.5–19 Lanceolate
Texture		Papery	Papery	Coriaceus	Papery	Coriaceus	Coriaceus
Stomatal index %	ad	4.66	8.19	1.86	3.53	3.77	3.92
	ab	5.05	8.26	4.84	5.53	7.48	6.55
Stomatal size (average length \times width in μ m)	ad	$\begin{array}{c} 56.62 \pm 7.70 \\ 42.03 \pm 4.10 \end{array}$	$\begin{array}{c} 28.57 \pm 2.77 \\ 21.43 \pm 1.98 \end{array}$	$48.55 \pm 5.82 42.20 \pm 4.65$	$58.34 \pm 5.95 \\ 49.87 \pm 3.70$	$\begin{array}{c} 65.61 \pm 4.87 \\ 51.06 \pm 4.10 \end{array}$	$\begin{array}{c} 39.29 \pm 3.24 \\ 31.35 \pm 1.78 \end{array}$
	ab	$\begin{array}{c} 44.78 \pm 3.73 \times \\ 33.28 \pm 3.59 \end{array}$	$\begin{array}{c} 28.70 \pm 2.77 \\ 22.88 \pm 2.79 \end{array}$	$47.75 \pm 4.89 \qquad 38.36 \pm 4.65$	$\begin{array}{c} 56.48 \pm 5.29 \\ 46.16 \pm 4.23 \end{array}$	$\begin{array}{c} 45.90 \pm 4.89 \\ 36.11 \pm 2.97 \end{array}$	$\begin{array}{c} 38.36 \pm 3.96 \\ 30.36 \pm 2.51 \end{array}$
Cuticle		Slightly striated	Smooth	Slightly striated	Slightly striated	Slightly striated	Slightly striated
Epicuticular wax		Granules (on both sides)	Densely aggregated platelets inside epistomatal cavities	Granules (on both sides)	Parallel platelets on the adaxial side	Tubules on the abaxial side	Crystalloid form (rosettes) and crust-like type on abaxial side
Prismatic crystals on epidermal surface		Absent	Absent	Absent	Absent	Present	Absent
Mesophyll (with number of pal- isade/spongy/palisade layers)		Isobilateral (2/2/2)	Isobilateral (2/2/2)	Isobilateral (4/2/3)	Isobilateral (2/2/4)	Isobilateral (2/2/2)	Isobilateral (3/2/3)
Midrib shape		Slightly biconvex	Slightly biconvex	Flat-slightly convex	Flat-slightly convex	Flat-convex	Flat-convex
Vascular system pattern		Circular	Open arc with two dorsal traces	Open arc with two dorsal traces	Open arc with two dorsal traces	Open arc with one dorsal plate	Open arc with invaginated ends
Sclerenchymatous fiber caps		Present	Present	Present	Absent	Absent	Absent

ad, adaxial; ab, abaxial.



Fig. 1. Morphology of leaves. Eucalyptus badjensis (A), E. benthamii (B), E. dunnii (C), E. globulus (D), E. grandis (E), E. saligna (F) [ad, adaxial side; ab, abaxial side]. Scale bar: A-F=2 cm.



Fig. 2. Leaf epidermis in *Eucalyptus* [light microscopy; stained in safranin]. *E. badjensis* (A, B), *E. benthamii* (C, D), *E. dunnii* (E, F), *E. globulus* (G, H), *E. grandis* (I, J), *E. saligna* (K, L). Adaxial side (A, C, E, G, I, K), Abaxial side (B, D, F, H, J, L) [oc, overlying cell; pa, papillae; st, stomata; wa, aggregated waxes]. Scale bar: A-L=50 µm.



Fig. 3. Anatomy of *Eucalyptus* – epidermis in surface view [scanning electron microscopy]. *E. badjensis* (A–B), *E. benthamii* (C, D, E), *E. dunnii* (F, G), *E. globulus* (H–J), *E. grandis* (K, L), and *E. saligna* (M, N). Adaxial side (A, C, F, H, I, K, M), abaxial side (B, D, E, G, J, L, N). [ct, cuticle; le, lenticel; pa, papillae; pr, prismatic crystal; st, stomata; wa, aggregated waxes; wc, crusts waxes; wg, granules waxes; wp, parallel platelets waxes; wr, rosette waxes; wt, wax tubules. Scale bars: A, C, E, I, L=50 µm; B, D, G, M, N=20 µm; F, K=10 µm; I=5 µm; H=2 µm.

Santos and co-workers (2008) have calculated stomatal index values for *E. grandis* as 1.03 for adaxial side and 16.05 for abaxial, and for *E. saligna* as 0.35 for adaxial and 15.55 for abaxial side. However, these authors used leaves of young plants (120 days-old) for their analysis.

Overlying cells are associated with secretory cavities and are distinguished from ordinary cells in terms of shape, size and/or coloration and have taxonomic value (Gomes et al., 2009). On both sides of the leaves of the studied six species of *Eucalyptus*, a pair of cells overlying the secretory cavities are observed at the same level as the stomata (Fig. 2B, D–F, I, K, L). The overlying cells are also observed in several other species of *Eucalyptus* (Santos et al., 2006; Santos et al., 2008). However, variations in the number of overlying cells can be found, for example four

overlying cells are found in *E. pyrocarpa* F.Muell. (Santos et al., 2008).

In species of *Eucalyptus*, the cuticle on the leaf epidermal surfaces is usually smooth to slightly striated, and sometimes papillae are also present (Santos et al., 2008; Iftikhar et al., 2009; Döll-Boscardin et al., 2010). However, papillae are not observed in *E. platypus*, *E. spathulata* and *E. viridis* (Knight et al., 2004). All the species included in this study show slightly striated cuticle, especially around the stomata (Fig. 3A, B, F–H, L, M), except *E. benthamii* in which smooth cuticle if found (Fig. 3C, D). Papillae are also observed in all the six species (Figs. 2A–I, L and 3A–C, D, F, L–M).

Pinkard et al. (2006) have studied intumescences on leaves of *E. globulus* and *E. nitens* H.Deane & Maiden and called them lenticels or lenticel-like structures. They affirmed that these structures are



Fig. 4. Spectra of X-ray energy dispersive elemental analysis of isolate crystals. (A) Prismatic crystals found on the leaf epidermal surface in *Eucalyptus globulus*; (B) prismatic crystal of midrib of *E. dunnii*; druse of mesophyll of *E. globulus*.

formed in response to environmental factors. Döll-Boscardin et al. (2010) mentioned that the presence of this structure helps in the identification of *E. benthamii*. In the present study, all six *Eucalyptus* showed lenticel-like structures, as evidenced in Fig. 3C.

The morphology of epicuticular wax is especially valuable for the classification of taxonomically complex genera, such as Eucalyptus (Wilkinson, 1979). Different types of epicuticular waxes have been described for several species of Eucalyptus, such as digitate-edged platelets in E. platypus, entire-edged platelets in E. viridis, parallel platelets in E. platypus, parallel-stacked platelets in E. yalatensis (Knight et al., 2004; Malinowski et al., 2009; Döll-Boscardin et al., 2010). In the present study, epicuticular waxes were found in different shapes, as granules in *E. badjensis* (Fig. 3B) and E. dunnii (Fig. 3F) on both sides, densely aggregated platelets inside epistomatal cavities in E. benthamii (Fig. 3E), tubules shape in E. globulus on the abaxial side (Fig. 3]), parallel platelets on both sides of E. grandis (Figs. 2] and 3K) and two types in E. saligna, namely crystalloid form (rosettes) and crust-like on the abaxial leaf surface (Fig. 3N). The presence and the type of the epicuticular waxes can help in species identification.

During the present study, pyramidal and sand crystals are observed externally on the adaxial leaf surface (Fig. 3H, I). This feature has not been previously reported for *Eucalyptus* species. Elemental composition of the prismatic crystals was confirmed by EDS. The spectrum presented prominent peaks for carbon (56%), oxygen (35%) and calcium (7%) (Fig. 4), indicating that the crystals are indeed made up of calcium oxalate. The presence, type and

distribution of the crystals are useful in species identification (Franceschi and Nakata, 2005; Meric, 2009; Santos et al., 2018; Saulle et al., 2018).

In cross-section, the leaf epidermis is unilayered on both sides (Fig. 5A–F), presenting cells varying from polyhedral to rounded in shape. The cuticle is thick and reacted positively with sudan III.

All the six species of *Eucalyptus* show isobilateral mesophyll formed by 2–3 layers of palisade and two layers of spongy parenchyma (Fig. 5A–F). Isobilateral mesophyll has been reported for several species of *Eucalyptus* (Metcalfe and Chalk, 1950; Tantawy, 2004; Santos et al., 2008; Saulle et al., 2018). However, dorsiventral mesophyll has also been reported in the genus (Santos et al., 2008). According to Malinowski et al. (2009), dorsiventral mesophyll is common in young leaves and isobilateral mesophyll in mature leaves in *E. globulus*. In this study, only mature leaves from the plants growing in the same environment were analyzed.

Phenolic compounds are detected in the mesophyll, especially in the palisade parenchyma region in all *Eucalyptus* species, as shown in *E. grandis* (Figs. 5E, F and 6I). Minor bicollateral vascular bundles traverse the spongy parenchyma and are encircled by a parenchymatous sheath containing phenolic compounds in all analyzed species (Fig. 6I). The adaxial phloem was not always clearly evident. Secondary veins presented sclerenchymatous layers adjoining the phloem on both sides (Fig. 6C).

Secretory cavities (Fig. 5A–F) containing essential oils (Figs. 5A, C and 6B, G) that reacted with sudan III are present and distributed throughout the mesophyll, especially below the epidermis on both sides (Figs. 5A–F and 6G), as also observed in numerous other species of *Eucalyptus* (Santos et al., 2008; Malinowski et al., 2009). However, *E. pilularis* Sm. showed cavities only on the adaxial side of the epidermis (Santos et al., 2008).

The midrib in cross-section show different shapes in the present study. Slightly biconvex shape is observed in *E. badjensis* and *E. benthamii*, flat-slightly convex in *E. dunnii* and *E. globulus*, and flat-convex in *E. grandis* and *E. saligna*. The uniseriate epidermis is coated with a thick cuticle that reacted with sudan III (Fig. 6B). Papillae are observed in the epidermis of leaf midrib.

Chlorenchyma is interrupted and is substituted by few layers of angular collenchyma in all the species of *Eucalyptus* on both sides (Fig. 5A–F). Sclerenchymatous fiber layers (Fig. 6A) are adjoined the phloem on both sides of *E. badjensis* (Fig. 5A), *E. benthamii* (Fig. 5B) and *E. dunnii* (Fig. 5C). This characteristic is not found in *E. grandis* (Fig. 5D), *E. globulus* (Fig. 5E) and *E. saligna* (Fig. 5F). The lignification is well evidenced with phloroglucinol/HCl as seen in Fig. 6F.

In all the six studied species, bicollateral vascular bundle is embedded in the ground parenchyma, however the organization is different in some species. A single vascular bundle, circular in shape, is present in *E. badjensis* (Fig. 5A). *Eucalyptus benthamii* (Fig. 5B), *E. dunnii* (Fig. 5C) and *E. globulus* (Fig. 5D) present a vascular bundle in open arc and two dorsal traces. *Eucalyptus grandis* (Fig. 5E) shows a bicollateral vascular bundle in open arc with one dorsal plate. *Eucalyptus saligna* (Fig. 5F) presents a bicollateral vascular bundle in open arc with invaginated ends (Fig. 6L).

Phenolic compounds are observed in the phloem in all studied species, as shown in *E. badjensis* (Figs. 5A and 6A) and *E. benthamii* (Figs. 5B and 6C–D), in higher amounts in *E. grandis* (Fig. 5E) and *E. saligna* (Fig. 6K, L). This feature was observed in *E. saligna* by Saulle et al. (2018). Starch grains are found in the xylem parenchyma. They are small and rounded and found solitary or in pairs (Fig. 6J).

Crystalliferous idioblasts are frequent in several genera of Myrtaceae (Cardoso et al., 2009). In the present study, prismatic crystals (Fig. 6E) and druses (Fig. 6H) are observed in the mesophyll and in the midrib of all species. These crystals were analyzed by EDS and the results confirm that the chemical composition of these crystals is calcium oxalate (Fig. 4C) for all species analyzed. The spectrum of isolated prismatic crystal (Fig. 4B) presents prominent



Fig. 5. Anatomy of *Eucalyptus* – leaf midrib in cross-section. *E. badjensis* (A), *E. benthamii* (B), *E. dunnii* (C), *E. globulus* (D), *E. grandis* (E), and *E. saligna* (F). [co, collenchyma; eo, essential oil; ep, epidermis; fi, fibers; ph, phloem; pp, palisade parenchyma; sc, secretory cavity; sp, spongy parenchyma; sv, secondary vein; vb, vascular bundle; xy, xylem]. Scale bars: A–F = 200 µm.

peaks for carbon (12.24%), oxygen (29.39%) and calcium (58.37%). The spectrum of druse (Fig. 4C) shows prominent peaks for carbon (7.47%), oxygen (9.34%) and calcium (83.19%). Both spectra indicating that the crystals are composed of calcium oxalate. The major unlabeled peaks characterize gold element used to spraying the samples.

Conclusions

In the present work, the leaf anatomy of six species of *Eucalyptus* is investigated and compared. Although the basic anatomical features in these species are relatively similar, they showed distinctive differences in some characteristics that can be used as anatomical markers for species identification and differentiation. The key diagnostic features observed in the studied species include the morphology of epicuticular waxes, presence of prismatic crystals on the leaf surface, leaf midrib shape and arrangement of its vascular system, and the presence or absence of the sclerenchymatous fiber caps in the vascular bundle.

Authors' contributions

IPM collected the plants and carried out the laboratory work. PAR and VPA carried out the laboratory work. VR and IAK provided critical reading and insightful recommendations of the manuscript. JMB, PVF, SN, GIBM created the project. JMB supervised the laboratory work and wrote the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors are grateful to CAPES/Brazil (008.640/2016), Universidade Estadual de Ponta Grossa for financial and technical support and Paraiso Verde Nursery for the plant material.



Fig. 6. Anatomy of Eucalyptus - [scanning electron microscopy (E, H) and normal light (all others) microscopy]. E. badjensis (A, B), E. benthamii (C, D), E. dunnii (E, F, G), E. globulus (H), E. grandis (I, J), and E. saligna (K, L). [co, collenchyma; ct, cuticle; dr, druses; eo, essential oil; ep, epidermis; fi, fibers; gp, ground parenchyma; pc, phenolic compounds; ph, phloem; pp, palisade parenchyma; pr, prismatic crystal; sc, secretory cavity; sg, starch grains; sp, spongy parenchyma; sr, sclerenchymatous fiber caps; sv, secondary vein; vb, vascular bundle; xy, xylem]. Scale bars: C, F, L=100 μm; A, B, D, G, I, J, K=50 μm; H=5 μm; E=2 μm.

References

- Al-Edany, T., Al-Saadi, S., 2012. Taxonomic significance of anatomical characters in some species of the family (Myrtaceae). Am. J. Plant Sci. 3, 572-581.
- Balbino, L.C., Cordeiro, L.A.M., Silva, P.V., Moraes, A.M., Gladys, B., Alvarenga, R.C., Kichel, A.N., Fontaneli, R.S., Santos, P.H., Franchini, J.C., Galerani, P.R., 2011. Evolução tecnológica e arranjos produtivos de sistemas de integração lavourapecularia-floresta no Brasil. Pesq. Agropecu. Bras. 46, 1–10. Barbosa, L., Filomeno, C., Teixeira, R., 2016. Chemical variability and biological activ-
- ities of Eucalyptus spp. essential oils. Molecules 21, 1671–1676
- Barthlott W Neinhuis C Cutler D Ditsch F Meusel I Theisen I Wilhelmi H 1998. Classification and terminology of plant epicuticular waxes. Bot. J. Linn. Soc. 126, 227-236
- Berlyn, G.P., Miksche, J.P., 1976. Botanical Microtechnique and Cytochemistry. Iowa State University Press, Ames, IA.
- Birtchnell, M.J., Gibson, M., 2006. Long-term flowering patterns of melliferous Eucalyptus (Myrtaceae) species. Aust. J. Bot. 54, 745-754.
- Brooker, M.I.H., Kleinig, D.A., 2006. Field Guide to Eucalyptus, Austrália.
- Cardoso, C.M.V., Proenca, S.L., Sajo, M.G., 2009. Foliar anatomy of the subfamily Myrtoideae (Myrtaceae). Aust. J. Bot. 57, 148–161. Cutter, E.G., 1986. Anatomia vegetal Parte I – Células e tecidos, Roca, São Paulo.
- Döll-Boscardin, P.M., Farago, P.V., Nakashima, T., Santos, P.E.T., Paula, J.F.P., 2010. Estudo anatômico e prospecção fitoquímica de folhas de Eucalyptus benthamii Maiden et Cambage. Lat. Am. J. Pharm. 29, 4–101.
- Flores, T.B., Alvares, C.A., Souza, V.C., Stape, J.L., 2016. Eucalyptus no Brasil: Zoneamento climático e guia para identificação. IPEF, Piracicaba, São Paulo.
- Foster, A.S., 1949. Practical Plant Anatomy, 2nd ed. D. Van Nostrand, Princeton.

- Franceschi, V.R., Nakata, P.A., 2005. Calcium oxalate in plants: formation and function, Annu, Rev. Plant Biol, 56, 41-71.
- Fuchs, C.H., 1963. Fuchsin staining with NaOH clearing for lignified elements of whole plants or plants organs. Stain Technol. 38, 141–144.
- Gabe, M., 1968. Techniques histologiques. Masson & Cie, Paris.
- Gomes, S.M., Somavilla, N.S.D., Gomes-Bezerra, K.M., Miranda, C.S.C., Plauto, S., Graciano-Ribeiro, D., 2009. Anatomia foliar de espécies de Myrtaceae: contribuições à taxonomia e filogenia. Acta Bot. Bras. 23, 224-238
- Iftikhar, A., Qaiser, A., Syed, H., Mansoor, N., Nargis, Z., Sara, K., 2009. Leaf anatomical adaptations in some exotic species of Eucalyptus L. (Myrtaceae). Pak. J. Bot. 41, 2717-2727
- Johansen, D.A., 1940. Plant Microtechnique. McGraw Hill Book, New York.
- Knight, T.G., Wallwork, K., Meredith, A.B., Sedgley, M., 2004. Leaf epicuticular wax and cuticle ultrastructure of four Eucalyptus species and their hybrids. Int. J. Plant Sci. 165. 27-36.
- Malinowski, L.R.L., Nakasshima, T., Alquini, Y., 2009. Caracterização morfoanatômica de folhas jovens de Eucalyptus globulus Labill ssp. bicostata (Maiden et al.) J.B. Kirkpat (Myrtaceae). Lat. Am. J. Pharm. 28, 756-761.
- Meric, C., 2009. Calcium oxalate crystals in some species of the tribe Inuleae (Asteraceae). Acta Biol. Cracov. Bot. 51, 105-110.
- Metcalfe, C.R., Chalk, L., 1950. Anatomy of the Dicotyledons. Clarendon Press, Oxford. Nisgoski, S., Muniz, G.I.B., Klock, U., 1998. Caracterização anatômica da madeira de Eucalyptus benthamii Maiden et Cambage. Cienc. Florest. 8, 67-76.
- O'Brien, T.P., Feder, N., McCully, M.E., 1964. Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59, 368-373.
- Oliveira, F., Akisue, G., Akisue, M.K., 2005. Farmacognosia. Atheneu, São Paulo.

- Pinkard, E., Gill, W., Mohammed, C., 2006. Physiology and anatomy of lenticel-like structures on leavez of *Eucalyptus nitens* and *Eucalyptus globulus* seedlings. Tree Physiol. 26, 989–999.
- Roeser, K.R., 1972. Die Nadel der Schwarzkiefer-Massenprodukt und Kunstwerk der Natur. Mikrokosmos 61, 33–36.
- Santos, L.D.T., Thadeo, M., Iarema, L., Meira, R.M.S.A., Ferreira, F.A., 2008. Foliar anatomy and histochemistry in seven species of *Eucalyptus*. Rev. Arvore 32, 769–779.
- Santos, T.L.D., Ferreira, L.R., Ferreira, F.A., Duarte, W.M., Tiburcio, R.A.S., Machado, A.F.L., 2006. Intoxicação de eucalipto submetido à deriva simulada de diferentes herbicidas. Planta Daninha 24, 521–526.
- Santos, V.L.P., Raman, V., Bobek, V.B., Migacz, I.P., Franco, C.R.C., Khan, I.A., Budel, J.M., 2018. Anatomy and microscopy of *Piper caldense*, a folk medicinal plant from Brazil. Rev. Bras. Farmacogn. 28, 9–15.

Sass, J.E., 1951. Botanical Microtechnique, 2nd ed. Iowa State College, Ames.

- Saulle, C.C., Raman, V., Oliveira, A.V.G., Maia, B.H.L.N.S., Meneghetti, E.K., Flores, T.B., Farago, P.V., Khan, I.A., Budel, J.M., 2018. Anatomy and volatile oil chemistry of *Eucalyptus saligna* cultivated in South Brazil. Rev. Bras. Farmacogn., http://dx.doi.org/10.1016/j.bjp.2018.03.001.
- Tantawy, M.E., 2004. Morpho-anatomical study on certain taxa of Myrtaceae. Asian J. Plant Sci. 3, 274–285.
- The Plant List, 2017. A working list of all plant species. *Eucalyptus*. http://www. theplantlist.org/1.1/browse/A/Myrtaceae/Eucalyptus (accessed 15.10.17).
- Wilkinson, H.P., 1979. The plant surface Mainly Leaf Part VII: epicuticular wax and its morphology. In: Anatomy of the Dicotyledons: Leaves, Stem and Woods in Relation to Taxonomy with Notes on Economic Uses Metcalfe. C.R. & L. Chalk, Claredon Press, Oxford, pp. 158–161.