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Original Article

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

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*
[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*
[d] *Programa de Pós-graduação em Engenharia Florestal, Universidade Federal do Paraná, Curitiba, Paraná, Brazil*

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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.

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. 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

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

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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 $\left[\mathrm{SI} = \frac{S}{E+S} \times 100\right]$, 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 CO_2. 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.