Original Article

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

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A B S T R A C T

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 used 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.

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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 (Birchnell and Gibson, 2006). Essential oils of Eucalyptus are rich in monoterpenes and are extensively used in pharmaceuticals, perfumes, food flavo-
rants and agrochemicals (Brooker and Kleing, 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. dunnii, 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...
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 (Roesser, 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.

**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: 

\[
SI = \frac{S \times 100}{E}
\]

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 aluminium stubs with double-sided adhesive tapes and then coated with gold using a Quorum SCI7620 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 (Nigoski 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 anticinal walls (Oliveira et al., 2005; Malinowski et al., 2009). The present study confirms this observation; the anticinal cell walls are observed to be straight on both leaf epidermis 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 (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, amphistomotic 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 stomatal 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 ± 21.43 μm) and abaxial (28.70 ± 22.88 μm) epidermis among the studied six species. The largest stomata are observed in E. grandis (65.61 ± 51.06 μm) on the adaxial side and in E. globulus on the abaxial side (56.48 ± 46.16 μm).

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 epidermis (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.

<table>
<thead>
<tr>
<th>Leaf morpho-anatomical features</th>
<th>E. badjensis</th>
<th>E. benthamii</th>
<th>E. dunnii</th>
<th>E. grandis</th>
<th>E. globulus</th>
<th>E. saligna</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf size (length × width in cm)</td>
<td>0.35–1.1 × 14.5–15.5</td>
<td>1.1–2.1 × 15.5–22</td>
<td>1.3–3.6 × 16–25</td>
<td>2.3–3.8 × 14.5–20</td>
<td>1.5–3 × 15.35–17.5</td>
<td>1.8–2.8 × 14.5–19</td>
</tr>
<tr>
<td>Texture</td>
<td>Papery</td>
<td>Papery</td>
<td>Coriaceus</td>
<td>Papery</td>
<td>Coriaceus</td>
<td>Coriaceus</td>
</tr>
<tr>
<td>Leaf shape</td>
<td>Linear to narrowly lanceolate</td>
<td>Narrowly lanceolate</td>
<td>Falcate</td>
<td>Falciform to lanceolate</td>
<td>Falcate</td>
<td>Lanceolate</td>
</tr>
<tr>
<td>Stomatal index % ad</td>
<td>4.66</td>
<td>8.19</td>
<td>1.86</td>
<td>3.53</td>
<td>3.77</td>
<td>3.92</td>
</tr>
<tr>
<td>Stomatal index % ab</td>
<td>5.05</td>
<td>8.26</td>
<td>4.84</td>
<td>5.53</td>
<td>7.48</td>
<td>6.55</td>
</tr>
<tr>
<td>Stomatal size (average length × width in μm)</td>
<td>56.62 ± 7.70</td>
<td>28.57 ± 2.77</td>
<td>48.55 ± 5.82</td>
<td>58.34 ± 5.95</td>
<td>65.61 ± 4.87</td>
<td>39.29 ± 3.24</td>
</tr>
<tr>
<td>Cuticle</td>
<td>Slightly striated</td>
<td>Smooth</td>
<td>Slightly striated</td>
<td>Slightly striated</td>
<td>Slightly striated</td>
<td>Slightly striated</td>
</tr>
<tr>
<td>Epicuticular wax</td>
<td>Granules (on both sides)</td>
<td>Densely aggregated platelets inside epistomatal cavities</td>
<td>Granules (on both sides)</td>
<td>Parallel platelets on the adaxial side</td>
<td>Tubules on the abaxial side</td>
<td>Crystallloid form (rosettes) and crust-like type on abaxial side</td>
</tr>
<tr>
<td>Prismatic crystals on epidermal surface</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Mesophyll (with number of palisade/spongy/palisade layers)</td>
<td>Isobilateral (2/2/2)</td>
<td>Isobilateral (2/2/2)</td>
<td>Isobilateral (4/2/3)</td>
<td>Isobilateral (2/2/4)</td>
<td>Isobilateral (2/2/2)</td>
<td>Isobilateral (3/2/3)</td>
</tr>
<tr>
<td>Midrib shape</td>
<td>Slightly biconvex</td>
<td>Slightly biconvex</td>
<td>Flat-slightly convex</td>
<td>Flat-slightly convex</td>
<td>Flat-convex</td>
<td>Flat-convex</td>
</tr>
<tr>
<td>Vascular system pattern</td>
<td>Circular</td>
<td>Open arc with two dorsal traces</td>
<td>Open arc with two dorsal traces</td>
<td>Open arc with one dorsal plate</td>
<td>Open arc with one dorsal plate</td>
<td>Open arc with invaginated ends</td>
</tr>
<tr>
<td>Sclerenchymatous fiber caps</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

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, 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.
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, I, M), except *E. benthamii* in which smooth cuticle is found (Fig. 3C, D). Papillae are also observed in all the six species (Figs. 2A–I, I, and 3A–C, D, F, I–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
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 lentil-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. ualatensis* (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. 3J), 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 unlayered 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 bicellular 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 6C), 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). Papilae are observed in the epidermis of leaf midrib.

Chloroenceyma 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, bicellular 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 bicellular vascular bundle in open arc with one dorsal plate. *Eucalyptus saligna* (Fig. 5F) presents a bicellular vascular bundle in open arc with invaginated ends (Fig. 6I).

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. budjensis* (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

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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; sv, 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


