Original article

Are the anatomical traits of stems and leaves good indicators of habitat specificity in closely related Myrtaceae species from Patagonia?

Javier Puntieri[1*] and Ana María González[2]

ABSTRACT

The anatomical attributes of young stems and leaves of newly formed shoots were studied for five Patagonian species of Myrtaceae: *Amomyrtus luma*, *Ugni molinae*, *Luma apiculata*, *Myrceugenia exsucca* and *M. ovata* var. *nannophylla*. The latter three species are more common in periodically-flooded areas than the former two. The vascular cambium and the first cork cambium of a shoot become active at about the same time, two months after budbreak. The cork cambium derives from cells located towards the inside of the perivascular fibers in *A. luma*, *L. apiculata* and *U. molinae*, and towards the outside in *M. exsucca* and *M. ovata*. Crystals and starch deposits were found in cortex, ray parenchyma and pith of all species. Total vessel area relative to total xylem area was higher in *Myrceugenia* spp. and *L. apiculata* than in *A. luma* and *U. molinae*. Interspecific differences in leaf anatomy concerned midvein structure and the development or not of substomatal chambers delimited by column-like cells (found only in *Myrceugenia* spp.). The anatomical traits of young shoots allow the distinction between species of this family and may contribute to explain each species' ecological affinities.

Keywords: Andean forests, cork cambium, leaf anatomy, Myrtaceae, secondary growth, shoot size, stem anatomy, trichomes, vessel lumen.

Introduction

The anatomical traits of plant stems and leaves are key for the understanding of plant functioning and evolution (Du *et al.* 2020). A better knowledge about anatomical variations among related species may contribute to determining which functional attributes are more closely related to plant diversification (Zanne & Falster 2010).

Many comparative anatomical studies focus on leaves in accordance to their relevance in photosynthesis and hydraulic balance (*e.g.* Ali *et al.* 2009; Ocampo *et al.* 2013; Tian *et al.* 2016; Bertel *et al.* 2017; Rashid *et al.* 2020; Rafique *et al.* 2021), not to mention their significance as key features in plant recognition. Anatomical leaf traits of adaptive relevance, such as mesophyll structure, epidermal thickness and cutinization, and distribution of support tissues, have

1 Instituto de Investigaciones en Recursos Naturales, Agroecología y Desarrollo Rural (IRNAD), Universidad Nacional de Río Negro y Consejo Nacional de Investigaciones Científicas y Técnicas, Anasagasti 1463, CPA: 8400, San Carlos de Bariloche, Argentina.

2 Instituto de Botánica del Nordeste (IBONE)- Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste y Consejo Nacional de Investigaciones Científicas y Técnicas, Sargento Cabral 2131, CPA: W3402BKG, Corrientes, Argentina.

* Corresponding author: jpuntieri@unrn.edu.ar

been evaluated for many species (Ali *et al.* 2009; Zhang *et al.* 2013; Bertel *et al.* 2017; Crang *et al.* 2018). Stems are relevant in various aspects of plant functioning, such as the transport of water, minerals, hormones and assimilates, the provision of physical support to leaves and other stems, and reserve storage, which highlights the importance of comparative studies on stem anatomy (Maguire & Kobe 2015; Furze *et al.* 2019).

Among the most investigated anatomical stem traits are vessel size and density, on which a plant's hydraulic efficiency depends (Pandey 2021). The development of vessels with a wide lumen favors the water transport capacity of a stem, at the cost of a higher vulnerability to cavitation, so that plants belonging to species from dry regions tend to have narrower vessels than those from humid regions (Rodriguez-Zaccaro *et al.* 2019, but see Lens *et al.* 2022). The large majority of studies on anatomical stem traits of woody species have focused on axes (mainly trunks) with a number of years of secondary growth. However, there is no *a priori* reason why the same principles and hypotheses would not apply to axis portions (i.e. shoots) of recent extension; in fact, the length growth and the addition of new stems and leaves of an axis depend upon the capacity of new shoots to achieve positive carbon balance. The scarcity of comparative studies on the anatomy of recently extended shoots have precluded inter-specific comparisons among woody species regarding stem-anatomy features with functional, adaptive and taxonomic significance, such as the location of the first cork cambium and the development of crystals (*e.g.* Nijsse *et al.* 2001; McKinley *et al.* 2018; Campilho *et al.* 2020; Peschiutta *et al.* 2020).

Since the size of a shoot (measured in terms of length, stem diameter or number of nodes) and its physiology are related to one another (Hallé *et al.* 1978; Puntieri *et al.* 2003; Barthélémy & Caraglio 2007; Normand *et al.* 2009), the anatomical traits of a stem may be expected to depend on shoot size. Several studies have shown that, within a species, shoots of different sizes also differ in leaf morphology and anatomy, in the number and types of branches they develop (Puntieri *et al.* 2001; 2003; Gamage 2011; Ostria-Gallardo *et al.* 2015; Edwards *et al.* 2016), and in their capacity to bear reproductive structures (Laurie & Trottier 2004; Heuret *et al.* 2006; Fernández *et al.* 2007). However, the link between shoot size and stem anatomy has been little investigated (Nicolini & Chanson 1999).

Inter-specific anatomical differences and their relationships with stem and leaf morphology may provide evidence for different plant strategies under similar environmental conditions. For example, it has been proposed that there are two survival strategies under anoxic conditions due to immersion: one of them concerns leaf acclimation, aerenchyma formation and the development of longer shoots; the other one involves growth stop and the increment in carbohydrate storage (Nakamura & Noguchi 2020). In related species with similar morphological

attributes, the comparative anatomical study of stems and leaves may contribute significantly to the understanding of the evolutionary steps leading to speciation (*e.g.* Poorter *et al.* 2010; Schweingruber *et al.* 2014).

The forests of Patagonia (in Chile and Argentina) are among the most important wet-temperate forests worldwide (Alaback 1991). The most diversified family of woody plants in these forests is Myrtaceae, with 26 native species (Rodríguez *et al.* 2018). The Patagonian Myrtaceae are shrubs or trees that inhabit wet or even flooded zones, such as lake and river shores of the Andean region, although they may also live at some distance from water bodies, like in forest understories. These species share quite a few morphological attributes: simple, perennial and coriaceous/sub-coriaceous leaves, small, white flowers, and, except for one species, fleshy fruits (Landrum 1988); in addition, all of them develop naked apical buds in their shoots (J. Puntieri, personal observations). These morphological resemblances often make their distinction in the field rather difficult. Nonetheless, it has been proposed that these species tend to occupy different micro-environmental niches (Weinberger 1978; Donoso 2013a).

The main objective of the present study is the comparison of the anatomical features of stems and leaves for recently extended shoots of plants belonging to five Patagonian species of Myrtaceae differing in water-logging tolerance. This work also tackles the relationships between the size of annual shoots and vessel lumen in their stems. On the basis of previous information about the link between the development of conductive tissues and the environmental affinities of plants (Davis *et al.* 1999; Vilagrosa *et al.* 2012), we predicted that those species more akin to wetter sites should develop (1) vessels with a wider lumen, (2) stems with a relatively larger conductive area, and (3) more tissues that provide aeration (aerenchyma; Kawase 1981; Parent *et al.* 2008) than those species that inhabit drier sites.

Methodology

Study species

The following species were chosen for this study: *Amomyrtus luma*, *Luma apiculata*, *Myrceugenia exsucca*, *M. ovata* var. *nannophylla*, and *Ugni molinae*. Despite their overlap in terms of habitat, *L. apiculata*, *M. exsucca* and *M. ovata* var. *nannophylla* are more frequent in areas subject to permanent or temporary waterlogging, whereas *A. luma* and *U. molinae* are more common on drier soils. All five Myrtaceae species selected for this study are native to the wet-temperate forests of Argentina. Herbarium vouchers of each species were chosen among the material that is available in an internationally recognized herbarium (BCRU), and whose identity was verified by the authors:

Amomyrtus luma (Molina) D. Legrand & Kausel. ARGENTINA. Río Negro province. Dep. Bariloche, Puerto

Blest, 10-I-1998, Puntieri, un-numbered (BCRU). Río Negro province. Dep. Bariloche, Puerto Alegre, 20-XI-1986, Puntieri & Brion, un-numbered (BCRU).

Luma apiculata (DC.) Burret. ARGENTINA. Neuquén province. Dep. Los Lagos, National route 40, 25 km from crossing with National route 237, 20-I-1996, Chiapella & Puntieri, un-numbered (BCRU).

Myrceugenia exsucca (DC.) O. Berg. ARGENTINA. Chubut province. Dep. Cushamen, Lago Puelo National Park, northern entrance from Graziano's alley, 28-I-2011, Puntieri 864 (BCRU).

Myrceugenia ovata (Hook. & Arn.) O. Berg var. *nannophylla* (Burret) Landrum. ARGENTINA. Neuquén province. Dep. Los Lagos, Nahuel Huapi National Park, stream margin in the vicinity of Espejo Chico lake. 18-II-1996, Puntieri 279 (BCRU).

Ugni molinae Turcz. ARGENTINA. Chubut province. Dep. Cushamen, Lago Puelo National Park, Los Hitos stream, 14-I-2008, Girscht 115 (BCRU).

The following sentences summarize the main ecological features of each of these species based on Weinberger (1978) and Donoso (2013a), and on unpublished observations by one of us (J.P.). *Amomyrtus luma* lives preferably under the canopy of larger perennial trees in low and cool areas and, despite its affinity for air humidity, may tolerate temporary shortages in water availability (Donoso & Escobar 2013). *Luma apiculata* is considered a thermophilic species; in Chile it may occupy sites that are rather distant from rivers and lakes, and demands canopy gaps and high humidity to regenerate. In Argentina, this species is found only in the proximities of lakes and rivers, although it may regenerate in drier and shaded areas (Donoso *et al.* 2013). *Myrceugenia exsucca* is frequent in flat areas where water accumulates at least for most of the year (swamps and lake margins); it tolerates high irradiance and summer drought as long as the substrate is wet (Donoso 2013b). *Myrceugenia ovata* var. *nannophylla* (hereafter referred to simply as *M. ovata*) inhabits low-altitude swamps and lake margins in which cool air accumulates at night; it often associates with *Nothofagus antarctica* (G. Forst.) Oerst. *Ugni molinae* is considered a thermophilic species that occupies un-flooded, well-lit and even seasonally dry sites, but does not tolerate sharp drops in night temperatures (Weinberger 1978).
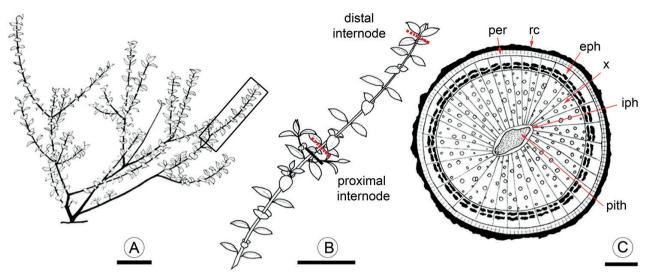
Sampling

For each of the five species, we selected between 8 and 21 plants that had developed either spontaneously, in natural habitats, or through cultivation in tree nurseries. It was assumed that the coarse anatomical traits to be investigated here would be mostly the result of evolutionary forces, so that the growth site of a plant was considered not to have a significant influence on such traits (*e.g.* see Claßen-Bockhoff *et al.* 2021). Only plants with severe signs of decay or with diebacks including more than 40 % of the aerial branching system were avoided. The areas from which the plants were sampled are described in Table 1.

Between January 2019 and August 2022, five to nine sampling campaigns were conducted for each species. We wanted to have a broad view at the intra-annual changes in shoot anatomy. Although we realized that performing samplings for all species at each time would have been ideal, we had to arrive at a compromise due, mainly, to the geographical dispersions of the species. At least one sampling took place in each season for each species. In each occasion, we cut the distal end (10-60 cm long) of one to four of the major axes of one to three plants, making sure that the most recently-extended axis portion (hereafter referred to "shoot") was entirely sampled. In the species of Myrtaceae from Patagonia, the limit between shoots of the same axis extended in two successive years may be recognized by the presence of one or more of the following morphological markers: a short internode, relatively small leaves in one of the nodes close to the short internode, and branches derived from the node located proximally with respect to the short internode (Puntieri *et al.* 2018; Guenuleo *et al.* 2020; Fig. 1A). For those samples taken between January

Table 1. Coordinates and altitude of sampling sites. The species that were sampled at each site are indicated with crosses. *A.l.*: *Amomyrtus luma*, *L.a.*: *Luma apiculata*, *M.e.*: *Myrceugenia exsucca*, *M.o.*: *Myrceugenia ovata*, *U.m.*: *Ugni molinae*. NHNP: Nahuel Huapi National Park. UNRN: Universidad Nacional de Río Negro. BG: Botanic Garden. LPNP: Lago Puelo National Park.

Sitio	Latitude S	Longitude W	Altitude m a.s.l.	A.l.	L.a.	M.e.	M.o.	U.m.
Espejo lake, NHNP	40° 41' 06.45''	71° 42' 16.17''	822		X		X	
Pire lagoon, NHNP	40° 43' 30.68''	71° 48' 04.70''	776				X	
Verde lagoon, Villa La Angostura	40° 46' 33.55''	71° 39' 41.78''	817				X	
Puerto Blest, NHNP	41° 01' 07.74''	71° 49' 34.00''	809	X	X	X		
Moreno lake, Bariloche	41° 03' 15.99''	71° 33' 40.93''	778			X		
Llao-Llao forest, Bariloche	41° 04' 13.32''	71° 33' 48.16''	778		X	X		
UNRN nursery, Bariloche	41° 07' 46.76''	71° 25' 31.50''	822		X			X
BG Bariloche	41° 08' 36.46''	71° 18' 55.60''	877	X	X			
BG Cascada Escondida, Mallín Ahogado	41° 55' 41.06''	71° 32' 34.08''	432	X	X			
Lago Puelo, LPNP	42° 05' 21.08''	71° 37' 06.25''	202		X	X		X

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Figure 1. A: Semi-schematic diagram of the aerial branching system of a *Ugni molinae* plant in which the distal end of one of its main axes, like those sampled in the present study, is indicated. B: Distal end of the axis indicated in diagram A; the proximal and distal internodes from which transverse sections were made are marked with dotted lines. C: Diagram of a stem cross section showing the main tissues that were investigated. Abbreviations: eph: external phloem, iph: internal phloem, per: peridermis, rc: rest of cortex, x: xylem. Scale bars: A: 10 cm, B: 5 cm, C: 0.5 mm.

and March (summer), the recently extended shoots could still be growing in length, whereas for those samples taken between April and November all shoots had completed their extension. In all cases, the distal end of each sampled axis had not been damaged by insects or by environmental factors, and its apex was covered by fresh leaf primordia.

Morphological and anatomical observations

At each sampling time, the axis portions were transported in wet plastic bags to the laboratory, where measurements and cuts were made. The samples were kept at 5°C for up to 5 hs between sampling and measuring. The length (to the nearest mm, with a ruler), basal diameter (to the nearest 0.1 mm, with digital calipers) and number of nodes of the most recently extended shoot of each axis were recorded. For each shoot, cross sections were obtained either by hand, using razor blades, or with a rotatory microtome. These cross sections were made at the most proximal and at the most distal internodes of each sampled shoot (Fig. 1B); by this means, equivalent shoot portions were represented for all plants. The proximal and distal ends of the shoots were fixed in formol-acetic acid for 48 hs, and dehydrated with *Deshidratante histológico* BIOPUR® S.R.L. (González & Cristóbal 1997), embedded in paraffin (HISTOPLAST® PLUS w/DMSO; Johansen 1940), and serially sectioned (12-15 µm thick) with a rotatory microtome (Microm, Walldorf, Germany). Sections were stained with safranin-astra blue (Luque *et al.* 1996) and mounted with synthetic Canadian balsam. Hand-made cross sections as well as longitudinal stem sections were stained with Lugol's stain (so as to visualize starch grains), safranin (1% solution in 70% ethanol) or toluidine blue (0.05% solution in distilled water; Peterson *et al.* 2008, Novikov & Sup-Novikova 2021),

or left unstained to distinguish naturally colored structures (suber and chloroplasts). Sections were observed under a light microscope (Leica DM500) and images were taken using a microscope attached camera (Leica ICC50W).

On images of stem cross sections observed at 400x magnification, the lumen of all vessels is included in a 70,000 μm^2 xylem field (Fig. 1C). These measurements were made exclusively on sections taken from proximal internodes, so as to make sure that the vessels had completed their radial growth, which was confirmed by cross sectioning shoot portions of different ages. Those shoots that, because of their size, would not allow the location of a 70,000 μm^2 field entirely in the xylem at 400x, were not included in these quantitative evaluations. For each shoot, the mean lumen area per vessel and the sum of all vessel lumens were computed after measuring all vessels in two to four xylem fields per shoot. The percentage of the total xylem area included in the lumen of all vessels was also computed for each cross section. All area measures were made using the Image J software (Rasband 1997-2018; González 2018).

In the case of leaves, anatomical comparisons were only qualitative. It is widely known that leaf anatomy exhibits notable quantitative variations depending on environmental as well as ontogenetic factors, so that inter-specific comparisons in this regard would have demanded a leaf sampling effort that could not be afforded. Inter-specific leaf anatomical comparisons included mainly the structures of the mesophyll and midvein, and the presence, type and position of crystals.

Statistical analyses

Species were compared regarding mean vessel lumen area and the percentage of the xylem area occupied by

the added lumen areas of all vessels. Both variables were normally distributed (after log-transformation in the case of vessel lumen area; Kolmogorov-Smirnov's test) and their respective variances were homogeneous among species (Levene's test), so that analysis of covariance (ANCOVA) was applied for each of these variables including species as fixed factor. In each analysis, one of the descriptors of shoot size (length, basal diameter and number of nodes) was included as covariable. Since these size descriptors were closely correlated with each other, they were not all included in the same ANCOVA. These comparisons were performed with Minitab version 14. A 5% significance level was adopted in all comparisons.

Results

Stem anatomy

The anatomical features of both distal and proximal internodes were similar for all species, so that a general anatomical description is provided. The main inter-specific differences are indicated further below.
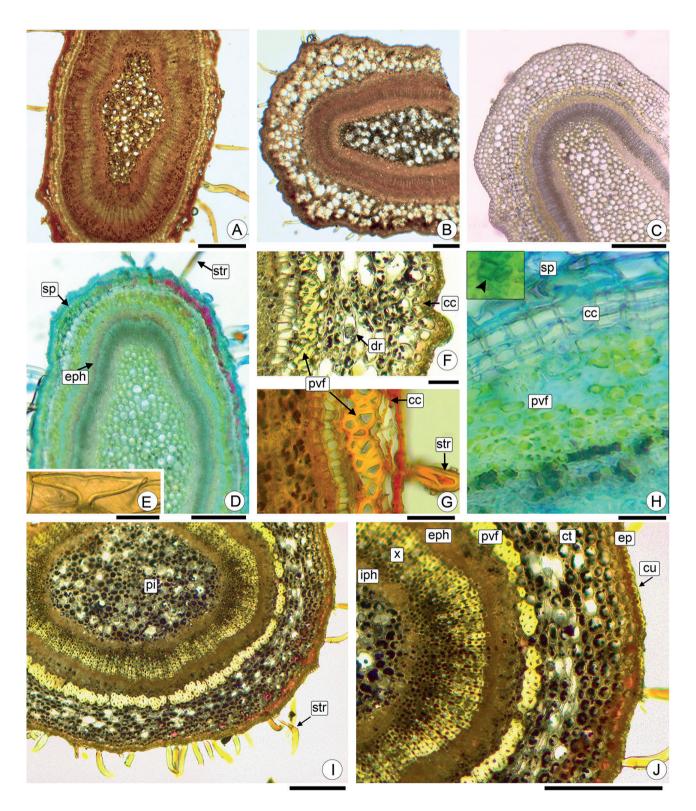
General attributes

The distal internodes – those that completed their extension more recently – are, in cross section, oblong or tend to have a four-sided outline; cortex and pith occupy the largest proportion of the stem's cross section, and the pith reaches its largest expansion in the direction of maximum stem diameter (Fig. 2A-D). Epidermis and primary cortex persist, even though secondary growth may be initiated by early summer. The epidermis is unistratified and thin (12 - 16 μm thick), includes non-glandular unicellular trichomes, and is covered by a smooth cuticle layer (Fig. 2E-G). The cortex consists of a subepidermal collenchyma with chloroplasts and druse crystals (Fig. 2F), and 4-8 layers of chlorenchyma conformed by relatively large cells (Fig. 2B-C). A cylinder of perivascular fibers arranged in 2-5 layers is present between the chlorenchyma and the secondary phloem (Fig. 2F, G-J). The vascular cylinder consists of a complete ring of secondary xylem and phloem derived from the cambium. Inside the xylem, the internal phloem of primary origin is visible (Fig. 2J). Uniseriate parenchyma rays with starch grains are present in the secondary xylem (Fig. 2J). Cells containing prismatic crystals, often constituting a continuous cylinder, are found in the secondary phloem (Fig. 2H). The ground tissue of the pith has thick-walled starch-storing parenchyma cells (Fig. 2I); intercellular spaces are evident in this tissue. Druses are present in scattered cells of the cortex and pith (Fig. 2F). During the period of primary growth of a shoot (especially from January to March), the initiation of cork cambium close to the perivascular fibers becomes evident. The cork cambium may be distinguished

by the radial arrangement of its cells, which have a clear content and thin walls (Fig. 2F-H).

Proximal shoot internodes – those that extended earlier in the period of shoot growth – have a circular cross section (Fig. 3A). The epidermis (including some unicellular simple trichomes) may still be seen, although it may appear discontinuous and compressed (Fig. 3A, C, F). The radial compression and suberization of cortex cells (Fig. 3H, I) is more generalized than in distal internodes (Fig. 2I, J). The cork cambium and its derived cell layers, phellem and phelloderm, are distinguished from about one month after the initiation of a shoot's primary growth (Fig. 3B, C, H, I inset). At this stage, the perivascular fibers either form an almost continuous cylinder or are arranged in bundles with parenchyma cells in between. One month after the beginning of their extension, proximal shoot internodes have a high proportion of secondary vascular tissues compared to distal internodes. Secondary phloem and xylem form a continuous cylinder separated by cambium. In proximal stem internodes, the secondary xylem constitutes a significant proportion of the cross section, and includes vessels, fibers and uniseriate ray parenchyma. In the direction of maximum radial development of the proximal internodes, the xylem is thinner and the internal phloem may be very thin (Fig. 3D). The presence of starch grains was verified for the external phloem, ray parenchyma, xylem parenchyma and pith (Fig. 3A, G, H, I).
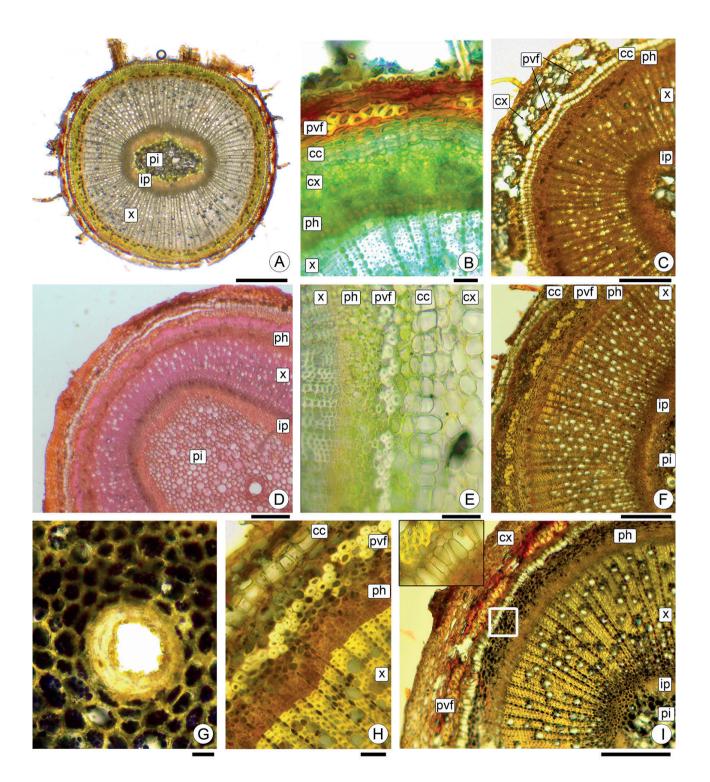
Inter-specific comparison in stem anatomy

Shoots with alternate, subalternate and verticillate phyllotactic patterns were found in several plants of *M. ovata* and *U. molinae*; in the other three species phyllotaxis was almost invariably opposite-decussate. Deviations from the opposite-decussate phyllotaxis affect the outline of the stem pith, which is triangular or irregular in the cross section of stems with verticillate phyllotaxis.

Myrceugenia exsucca and *M. ovata* have both simple and dibrachiate trichomes (Fig. 2D-E) in the epidermis, whereas *A. luma*, *L. apiculata* and *U. molinae* have simple trichomes (Fig. 2A, G-I). In *M. exsucca*, *M. ovata*, *A. luma* and *L. apiculata*, the sub-epidermal collenchyma is rather thin (with 1 or 2 cell layers) and it is well differentiated from the underlying parenchyma, which consists of larger cells. In *U. molinae* there is a gradual increase in cell size from the sub-epidermal collenchyma to the cortical parenchyma. The stem cortex of *L. apiculata*, *U. molinae* and *A. luma* includes two clearly distinct cell types: larger cells devoid of plastids (some of them with druses), and smaller cells with plastids (Fig. 2B, F, I, J). This differentiation is sharper in the former species than in the latter two, and was not observed in either of the two *Myrceugenia* species. Secretory cavities that are lysigenous in nature, characterized by ruptured cells inside, are present in both the cortex and the pith of *L. apiculata* and *M. exsucca*, as well as in the pith of *M. ovata* (Fig. 3G). Relative to the perivascular fibers, the first cork cambium

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Figure 2. Images of stem cross sections taken from distal internodes of the most recently extended shoot. A, G: *Amomyrtus luma* (May 2021). B: *Luma apiculata* (Mar 2021). C, H: *Myrceugenia exsucca* (Jan 2021), detail of prismatic crystal in inset (arrowpoint). D-E: *Myrceugenia ovata* (Mar 2021). E: dibrachiate trichome. F, I-J: *Ugni molinae* (Oct 2021, Sep 2021). Abbreviations: cc: cork cambium, ct: cortex, dr: druses, ep: epidermis, eph: external phloem, iph: internal phloem, pi: pith, pvf: perivascular fibers, sp: spongy parenchyma, str: simple trichomes, x: xylem. Sections A-C, E, G, I, J: stained with Lugol. D and H: stained with toluidine blue. Scale bars: A-D, I, J: 200 µm; E-G, J: 50 µm.

Figure 3. Images of stem cross sections taken from proximal internodes of the most recently extended shoot. A-B: *Amomyrtus luma* (May 2021, Jan 2021). C: *Luma apiculata* (Jul 2021). D-E: *Myrceugenia exsucca* (May 2022, Jan 2021). F-H: *Myrceugenia ovata* (May 2022). I: *Ugni molinae* (May 2021). A, C, D, F, I: general views. B, E, H: details. G: lysigenous cavity in pith. I: the inset box shows a detail of the cork cambium in the white boxed area. Sections A, C, F-I stained with Lugol. Section B stained with toluidine blue. Section D stained with safranin. Section E unstained. Abbreviations: cc: cork cambium, cx: cortex, iph: internal phloem, ph: phloem, pvf: perivascular fibers, pi: pith, pvf: perivascular fibers, x: xylem. Scale bars: A: 0.5 mm; C, D, F and I: 200 μm; B, E, G, H: 50 μm.

develops internally in *A. luma* (Fig. 3B), *L. apiculata* and *U. molinae*, and externally in *M. exsucca* and *M. ovata* (Fig. 3E, H), in all cases from parenchyma cells, either from the cortex or from the external phloem.

Vessel lumen size

Irrespective of the covariable that was included in the statistical analyses, inter-specific differences were minor with regard to mean vessel lumen area (mainly *M. ovata* < *U. molinae*), and major regarding the percentage of xylem area occupied by the added areas of its vessels: [*A. luma = U. molinae*] < [*L. apiculata = M. exsucca = M. ovata*] (Fig. 4, Table 2 and 3). These analyses also indicate that mean vessel area increases with shoot size, whereas there is little evidence of an effect of shoot size on the percentage of xylem area corresponding to its vessels (Fig. 4).

Leaf anatomy

General attributes

In all species, leaf cross sections presented several attributes in common (Fig. 5). Both adaxial and abaxial epidermis are unistratified. The abaxial epidermis consists of cells that are smaller than those of the adaxial epidermis, and includes the stomata that are at the same level as the other epidermal cells. The cuticle of the abaxial epidermis is also thinner than that of the adaxial epidermis.

The mesophyll is dorsiventral and the palisade parenchyma consists of two or three cell layers, the inner one less compact. Druses are present in the outer two palisade parenchyma layers (Fig. 5B, F, J), but may also be present in the spongy parenchyma. Cells of the spongy parenchyma are loosely arranged and have a lower density of chloroplasts than those of the palisade parenchyma. The vascular system of each leaf's midvein has a bicollateral structure, and is surrounded by an external parenchymatous sheath and an internal fiber sheath (Fig. 5A, C, E, G, I). Collenchyma separates the parenchymatous sheath from the upper and lower epidermis. Secretory cavities with loose and broken epithelial cells were observed in all species (Fig. 5A, D).

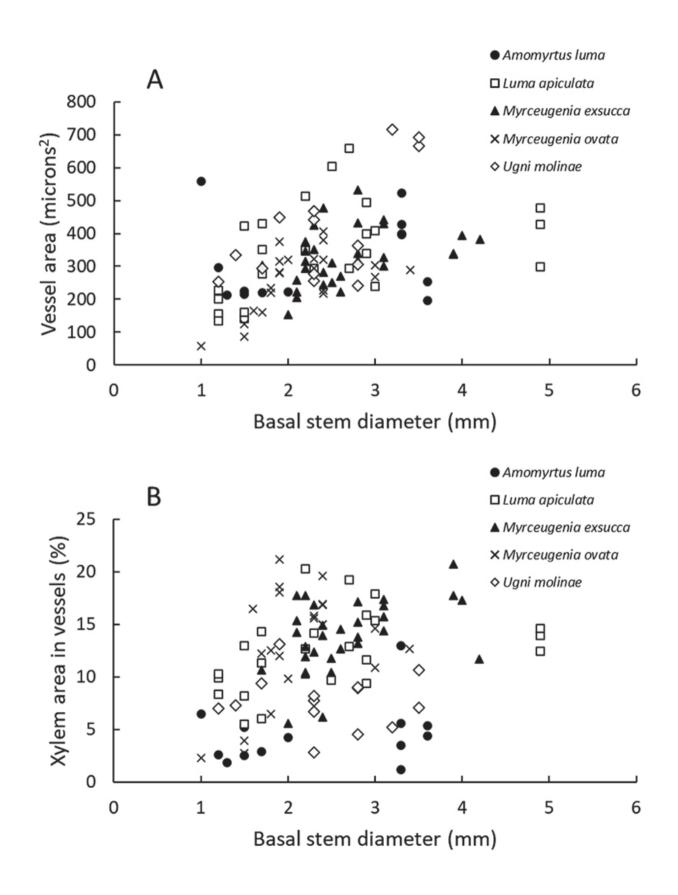### Inter-specific variations

A notable inter-specific difference in leaf anatomy concerns the structure of the midvein. In *A. luma* the midvein protrudes slightly on the adaxial side and very little on the abaxial side (Fig. 5A, C). In the other four

Table 2. Mean and standard error (SE) of cross-sectional area of the lumen per vessel and percentage of the xylem area included in the area of the lumen of all vessels for *A. luma*, *L. apiculata*, *M. exsucca*, *M. ovata* and *U. molinae*. The cross-sections were observed under light microscope at 400 x. The numbers of selected trees, sampled axes and stem cross-sections on which the measures were based are indicated for each species.
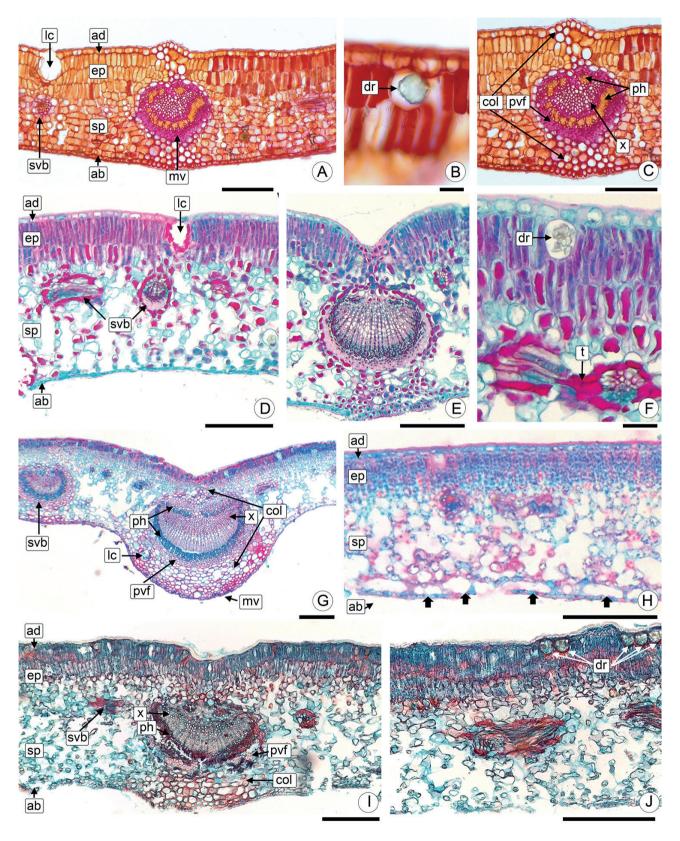
	Numbers of Trees-axes-sections	Area per vessel (μm^2)		Percentage of the xylem in vessels	
		Mean	SE	Mean	SE
Amomyrtus luma	8 - 13 - 13	318	35.1	4.5	0.83
Luma apiculata	8 - 36 - 24	345	26.3	12.3	0.78
Myrceugenia exsucca	20 - 31 - 31	353	15.7	13.7	0.74
Myrceugenia ovata	12 - 22 - 22	257	19.8	13.1	1.13
Ugni molinae	19 - 23 - 14	411	45.2	7.7	0.69

Table 3. Results of analysis of covariance to evaluate the effects of species (fixed factor: *A. luma*, *L. apiculata*, *M. exsucca*, *M. ovata* and *U. molinae*) and shoot size (covariable: length, basal diameter and number of nodes) on the vessel lumen area (log-transformed) and on the percentage of the xylem area included in the area of the vessels. For each independent variable, the value of Fisher's statistic (F) and its significance level (p) are indicated. The percentage of the variance that was explained in each analysis is also indicated (R^2). In all comparisons, the number of degrees of freedom was 4, that corresponding to the covariable 1, and the total 103.

	Species		Covariable		R^2
	F	p	F	p	
Vessel area					
Length	3.1	0.020	26.6	< 0.001	28.1
Basal diameter	3.1	0.020	25.4	< 0.001	27.4
Number of nodes	7.1	< 0.001	20.0	< 0.001	24.1
% of xylem in vessels					
Length	20.2	< 0.001	5.3	0.023	42.8
Basal diameter	19.9	< 0.001	12.6	0.001	46.5
Number of nodes	20.4	< 0.001	6.5	0.012	43.4

Figure 4. A. Relationship between mean vessel area and basal stem diameter per shoot. **B**. Relationship between the percentage of xylem area occupied by vessel lumen and basal stem diameter.

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Figure 5. Images of leaf cross sections through the midvein and mesophyll. **A–C**: *Amomyrtus luma*. **D–F**: *Luma apiculata*. **G**: *Myrceugenia exsucca*. **H**: *Myrceugenia ovata*. **I–J**: *Ugni molinae*. Scale bars: A, C-E, **G-J**: 200 μm; B, **F**: 50 μm. Abbreviations: ad: single-layered adaxial epidermis, ab: abaxial epidermis, col: collenchyma, ep: palisade parenchyma, dr: druse, t: tanniferous cells, s: lateral vascular bundle, mv: mid vein, ph: phloem, pvf: perivascular fiber sheath, sp: spongy parenchyma, x: xylem, black arrows: broad substomatal cavity.

species, the midvein protrudes on the abaxial side whereas on the adaxial side the midvein is marked by a groove that is deep and sharp in *U. molinae*, less deep in *L. apiculata* and *M. ovata*, and barely noticeable in *M. exsucca* (Fig. 5E, G, I). Anatomically, this difference is mainly related to the development of collenchyma under the adaxial epidermis. In *A. luma* this collenchyma is more developed than in the other species, forming a notable gap between the palisade parenchymas on both sides of the midvein. In *U. molinae* and *L. apiculata* the subepidermal collenchyma along the midvein is barely developed and the gap in the palisade parenchyma is quite narrow.

Interspecific variation in the spongy parenchyma was observed, as the intercellular spaces were narrower in *A. luma* and *U. molinae*, than in *L. apiculata*, *M. exsucca* and *M. ovata* (Fig. 5A, D, H, J). In both *Myrceugenia* species, the spongy parenchyma gets in contact with the lower epidermis by means of anticlinally arranged column-like cells that delimit extensive substomatal cavities (Fig. 5H).

Another feature that contributes to the distinction among species is the presence of tannins in the leaves, far more evident in *L. apiculata* than in the other species (Fig. 5D-F).

Discussion

Anatomical stem and leaf traits in the Myrtaceae family

In the present study, we verified several of the anatomical traits that had been recorded previously for stems and leaves of Myrtaceae species. Among these traits were, for stems, the development of a primary vascular tissue with internal phloem, secondary thickening developing from a conventional cambial ring, uniseriate rays in the secondary xylem, presence of crystals, and cortical chlorenchyma (Metcalfe & Chalk 1957; Al-Edany & Malik Al-Saadi 2012). Regarding leaves, the species investigated here share with others of the Myrtaceae family: the presence of a uniseriate epidermis (Tantawy 2004; Donato & Morretes 2007; Gomes *et al.* 2009; Retamales & Naulin 2010), the development of stomate only in the abaxial epidermis (James *et al.* 1999; Watson & Dallwitz 2007), dorsiventral mesophyll, secretory cavities (Donato & Morretes 2007; Alves *et al.* 2008; Gomes *et al.* 2009), idioblasts with druses (Cardoso *et al.* 2009; Gomes *et al.* 2009; Carneiro da Costa *et al.* 2020) and the presence of vascular bundles with adaxial phloem (Takhtajan 1980; Cronquist 1984; 1988; Retamales & Naulin 2010). One inconsistency with a previous study on leaf anatomy may be pointed out. Villaseñor *et al.* (2013) cited the presence of cystoliths in the palisade parenchyma of *M. exsucca*; however, the illustrations of the cited publication show the presence of druses rather than cystoliths.

Microscopic observations of distal and proximal internodes of recently extended shoots allowed us to point out several other anatomical traits of Patagonian Myrtaceae species that had not been considered in previous

studies. One of these features is the initiation of both vascular cambium and cork cambium between one and two months after the onset of stem extension. This means that, for each shoot, stem thickening and the formation of the insulating suber take place during the period of shoot extension, thus providing shoots with extra physical support and resistance against biotic and abiotic stresses (Ragni & Greb 2018; Campilho *et al.* 2020). Another result worth remarking is the anatomical location of the first cork cambium. It has been stated that the first cork cambium of a stem may be formed immediately below the epidermis or from more internal tissues, closer to the phloem (Esau 1985; Tantawy 2004; Crang *et al.* 2018). Our results were in accordance with those of a previous study on Myrtaceae (Cook *et al.* 1980), since the depth of origin of the first cork varied among species and was not placed immediately under the epidermis. Armstrong *et al.* (2012) reported the initiation of the periderm in external parts of the cortex in *Eugenia pyriformis* Cambess., a Myrtaceae species native to subtropical zones of South America, and Van Wyk (1985) indicated that the beginning of the first periderm takes place immediately under the epidermis in *Eugenia* spp. The discrepancies between these two studies and the present one could be related to the developmental stages of the cross-sectioned stems included in each investigation, or be taxon-specific. Due to the early activation of the lateral meristems, and the subsequent radial compaction and suberization of the cortex, the cross section of a proximal internode of a shoot in an advanced stage of extension may suggest that the cork cambium initiated from outer layers of the cortex. Like in other woody plants, radial cortex-compression as the periderm develops is accompanied by suberization and the progressive detachment of outer cortex cells (Cortadi *et al.* 1996; Tantawy 2004; Wunderling *et al.* 2018); as a consequence, the cork cambium is positioned close to the stem surface soon after the extension of a shoot. For some of the shoots that we analyzed after the end of their primary growth, the most recently extended internodes had a continuous epidermis and a broad and non-suberified cortex, as the periderm was still undeveloped. This could mean greater sensitivity to low temperatures (Lenz *et al.* 2016; Montgomery *et al.* 2020), and justify the apex deaths reported for some shoots in some of these species (Puntieri *et al.* 2018; Guenuleo *et al.* 2020). It can be hypothesized that the number of internodes of a distal shoot that would be affected by low temperatures would depend on the level of development reached by the periderm.

Taking all five species as a group, we found a clear tendency towards the increase in the lumen of single vessels with shoot size. This seems a reasonable outcome if we consider that bigger shoots develop a larger transpiration surface, and agrees with previous results on *Fagus sylvatica* L. shoots (Cochard *et al.* 2005). It has been proposed that the development of narrow-lumen vessels may be beneficial for plants living under conditions of water deficit (Jacobsen

et al. 2019), which may imply that the development of shoots of small size would mean a reduction in the risk of suffering cavitation under high-transpiration and water deficit conditions. The Andean zone of Patagonia is characterized by high levels of microenvironmental heterogeneity due to variations in altitude, distance to water sources, substrate origin and topography. Despite the morphological resemblances among the species of Myrtaceae that inhabit this region, they exhibit some, albeit inconspicuous, anatomical differences that may determine different capacities to adjust to specific sets of microenvironmental conditions.

Inter-specific anatomical comparisons among Patagonian Myrtaceae

The species that were included in the present contribution are among the most characteristic species of Myrtaceae from the temperate wet forests of South America; they occupy low-altitude river and lake margins of the Andean region, but their habitat preferences (Weinberger 1978) may suggest the existence of specific adaptations in each of them. The major inter-specific contrasts in shoot anatomy that were recognized in this study are synthetized in Tab. 4. A clear difference in stem anatomy between both *Myrceugenia* species and the remaining three species involves the relative positions of the cylinder of perivascular fibers and the first periderm: the latter is outside the perivascular fibers in *Myrceugenia* spp. and inside in the remaining three species. Unpublished information concerning other Myrtaceae species from this region (*Metrosideros stipularis* (Hook. & Arn.) Hook. f., *Myrteola nummularia* (Poir.) O. Berg and *Myrceugenia chrysocarpa* (O. Berg) Kausel) suggest that, within Patagonian Myrtaceae, the development of the first cork cambium externally with respect to the perivascular fibers is exclusive of *Myrceugenia* spp. (J.P. unpublished

data). Although the possibility that the initiation of the first cork cambium be affected by environmental factors cannot be ruled out, our results so far are in agreement with evolutionary studies that separate the genus *Myrceugenia* (also present in subtropical and tropical regions of South America) from the other genera of Myrtaceae present in Patagonia (Murillo *et al.* 2012). The external position of the first periderm in *Myrceugenia* spp. implies that the perivascular fibers are not affected by the expansion of the phellem in these species. On the other hand, in the cases of *A. luma*, *L. apiculata* and *U. molinae* the internal position of the first periderm mean that the perivascular fibers become more detached from the cortex as new phellem layers are added. Under the assumption that perivascular fibers play important support and protective roles during primary growth, the stem of *Myrceugenia* spp. shoots would be better able to withstand stressful environmental conditions at the end of their extension periods than those of the other Myrtaceae species in Patagonia. This hypothesis could be evaluated experimentally. Another anatomical difference between *Myrceugenia* spp. and the other three species is the presence of dibrachiate tricomes in the former, a feature that seems to be apomorphic in few species of Myrtaceae, including *Myrceugenia* spp. (Gomes *et al.* 2009).

Among the inter-specific differences in leaf anatomy, two are worth noticing. One of them is the development of extensive substomatal cavities limited by columnar cells, which was observed in both *Myrceugenia* species but not in the other three species. We found no reference to this particular attribute in previous studies on the leaf anatomy of Myrtaceae (Gomes *et al.* 2009; Al-Edany & Malik Al-Saadi 2012; Retamales & Scharaschkin 2015; Oliveira Thomaz Lemos *et al.* 2018; Almeida Bezerra *et al.* 2020). The presence of extensive subestomatal cavities is also in accordance with the increased development of

Table 4. Summary of the anatomical differences between species of Myrtaceae as observed in recently-extended shoots.

	Stem anatomy						Leaf anatomy		
	Trichomes	Collenchyma	Cortical parenchyma	Secretory cavities	Cork cambium	% of xylem area in vessels	Midvein	Spongy parenchyma	Subestom. cavities
Amomyrtus luma	Simple	Thin and differentiated	Two cell types	Not seen	Internal to perivascular fibers	Low	Protruding	Narrow	Absent
Luma apiculata	Simple	Thin and differentiated	Two cell types	In cortex and pith	Internal to perivascular fibers	High	Groove	Wide	Absent
Myrceugenia exsucca	Dibrachiate	Thin and differentiated	No distinction	In cortex and pith	External to perivascular fibers	High	Slight depression	Wide	Delimited by column-like cells
Myrceugenia ovata	Dibrachiate	Thin and differentiated	No distinction	In pith	External to perivascular fibers	High	Groove	Wide	Delimited by column-like cells
Ugni molinae	Simple	Gradual change to cortical parenchyma	Two cell types	Not seen	Internal to perivascular fibers	Low	Groove	Narrow	Absent

the intercellular spaces of the spongy parenchyma. These characteristics would be directly related to the environment in which these species grow. The *Myrceugenia* species that were included in the present study are found growing mostly on flooded ground, and often have partially waterlogged stems, and the development of large airspaces in their leaves could provide extra oxygen to sustain root respiration (see Cook *et al.* 1980). A well-developed spongy parenchyma is also present in *L. apiculata*. In contrast, *L. apiculata*, which is also found quite frequently along seasonally flooded lake shores, does not develop as large substomatal cavities in the leaves as *Myrceugenia* spp. In a study performed on two Myrtaceae species from New Zealand, a greater development of a tissue (described as aerenchyma by the authors) that favors oxygen supply to the roots was found for the most flood-tolerant species only when the water level in the substrate was experimentally increased (Cook *et al.* 1980). This suggests that inter-specific anatomical differences more profound than those described here for Patagonian Myrtaceae may arise through acclimation to specific environmental conditions, *e.g.* water availability, flooding, frost, etc. Thus, the differential capacity of some species to respond to particular environmental conditions may explain the different habitat specializations of each Myrtaceae species. This idea could be tested through experimental studies on young plants. The second leaf-anatomy trait that distinguishes the Patagonian Myrtaceae species from one another is the structure of the midvein and, in particular, that of the tissues that separate the midvein from the mesophyll. Notable differences were found in the extent to which the midvein protrudes or corresponds to a groove on the leaf surface. This trait provides a tool for telling apart four of these species which develop, in general terms, similar-looking leaves: *L. apiculata*, *A. luma*, *M. ovata* and *U. molinae*. Since leaves are rather plastic structures in many plant species (*e.g.* de Kroon *et al.* 2004), further studies on intraspecific variations in leaf anatomy would be necessary to evaluate the effects of genetic and environmental factors on this and other anatomical leaf traits.

The inter-specific differences found here regarding the percentage of the xylem that was included in the vessels indicate that *M. exsucca*, *M. ovata* and *L. apiculata* would have higher hydraulic capacities per unit of xylem cross section area than *A. luma* and *U. molinae*. This contrast, which becomes more notable for larger shoots (Fig. 4B), would be in agreement with the higher affinity of the former three species for waterlogged areas and provides support to the idea that some anatomical features are involved in determining the ecological affinities of related species. The Patagonian species of Myrtaceae investigated here had rather small vessel lumens (< 30 μm in lumen diameter) compared to other Dicotyledons (Metcalfe & Chalk 1957), and to Myrtaceae species from other regions, like *Eucalyptus* spp. from Australia (Barotto *et al.* 2017), and *Eugenia* spp. from southern Brazil (Soffiatti & Angyalossy-Alfonso 1999).

The development of relatively narrow vessels would be consistent with the xeromorphic tendency attributed to the Myrtaceae species from southern Chile and Argentina (Weinberger 1978), and would allow them to reach high levels of water retention, at the cost of being unable to increase their hydraulic capacity even under high water availability. All five species investigated here exhibited similar levels of variation in vessel size, so that the size of single vessels would not be a key feature for the ecological affinities of each of these species.

Conclusions

This study provides some new information about the anatomical features of stems and leaves of five Patagonian species of Myrtaceae, while endorsing the results of the majority of other studies in this regard. Some anatomical traits of recently extended shoots differ among the five species that were investigated here. The most notable differences refer to the cellular structure of the cortex before the initiation of the lateral meristems, and the site of initiation of the cork cambium relative to other tissues. Concerning leaf anatomy, the structure of the spongy parenchyma and, in particular, that of the substomatal chambers are informative with regard to inter-specific differences. All these characteristics separate *Myrceugenia* spp. from the other species studied, which agrees with the results of some previous studies (*e.g.* Murillo *et al.* 2012) and contrasts with those of others (Lucas *et al.* 2005). Our hypothesis concerning the connection between plant anatomy and habitat specificity is supported by the higher proportion of xylem surface devoted to water conduction, i.e. sum of vessel lumens, and by the greater increases in vessel lumen area with shoot size in those species that inhabit waterlogged areas.

Acknowledgements

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