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Comparative vegetative anatomy of Neotropical Goodyerinae Klotzsch (Orchidaceae Juss.: Orchidoideae Lindl.)

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ABSTRACT

The Neotropical genera *Aspidogyne* and *Microchilus* (Goodyerinae, Cranichideae, Orchidaceae) comprise ca. 200 rainforests terrestrial species. Although species of Goodyerinae are described with similar anatomy to other taxa of Cranichideae, some anatomical characteristics appear to be specific to the subtribe. Our goal was to characterise the anatomical structure of the vegetative organs of *Aspidogyne* and *Microchilus* to identify specific characters of Goodyerinae. Root, stem and leaf samples from eleven species were analysed using light and scanning electron microscopy. The leaves are hypostomatic and glabrous with predominantly anisocytic or tetracytic stomata, thin cuticle, homogeneous mesophyll with chromoplast and raphides, and collateral vascular bundles with parenchyma sheath and collenchyma in the midrib. Spiranthosomes were cofirmed for all species and some specific characteristics were identified in the root, such as the presence of collenchyma in both the cortex and the stele, vascular tissue with fibre-tracheids in the centre of the vascular cylinder, and the presence of a true endodermis with Casparian strips in the stem. Therefore, the root and stem were the organs that showed more taxon-specific characteristics for Goodyerinae, which can be used to better delimited the subtribe.

Keywords: *Aspidogyne*, jewel orchids, *Microchilus*, neotropical orchids, vegetative anatomy

Introduction

The Goodyerinae, one of the eight Cranichideae subtribes (Orchidoideae, Orchidaceae), is characterised by terrestrial herbs with fleshy roots either clustered or scattered along a rhizome and leaves usually arranged in a basal rosette. This subtribe is widely distributed in tropical and subtropical regions of the Old and New World, especially in the tropics and subtropics (Pridgeon *et al.* 2003). Goodyerinae is composed of 33 genera and approximately 750 species (Ormerod 2008; 2009; 2013; Chase *et al.* 2015). The genera in the New World are represented by *Aspidogyne* (71 spp.), *Microchilus* (135 spp.), and *Kreodanthus* (14 spp.) (Meneguzzo 2012; Chase *et al.* 2015; Smidt *et al.* 2016). Two genera, *Aspidogyne* (18 spp.) and *Microchilus* (19 spp.), are recorded in Brazil and are found in the phytogeographic domains of the Atlantic rainforest, Amazon rainforest and Cerrado biomes (Brazilian Neotropical Savannah) (BFG 2015; Meneguzzo 2012; Smidt *et al.* 2016), in addition to the Asian introduced species *Zeuxine strateumatica* (Menini-Neto *et al.* 2011; Engels *et al.* 2016). This group of humicolous herbs grows in humid cloud forests. These jewel orchids, as they are popularly known, are recognisable by their leaves with different variegation patterns and for their similarity to species of the Commelinaceae Mirb family. The species are morphologically characterised by their distinctive erect leafy stem and a less-developed horizontal, root bearing portion of the stem (Engels *et al.* 2016) or rhizome (Pridgeon *et al.* 2003) (Fig. 1).

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The members of the Goodyerinae are anatomically characterised by leaf anatomy with the same features as other subtribes of the tribe Cranichideae, *i.e.*, the cuticle is generally thin and rough in species of *Goodyera* and *Zeuxine* and micropapillate in *Goodyera oblongifolia*, and papillose epidermal cells are present in *Goodyera macrophylla*, *G. oblongifolia*, *G. grandis*, *Microchilus arietinus* and *Z. strateumatica*. The leaves are hypostomatic, with several types of stomata, and the mesophyll is generally homogeneous with collateral vascular bundles that lack sclerenchyma sheaths (Pridgeon *et al.* 2003; Stern 2014; Andreota *et al.* 2015).

However, the stem and root of Goodyerinae seem to present some unique anatomical character as the stem has been reported to have collenchyma or thick-walled sclereids occurring among cortical parenchyma cells in *Goodyera rubicunda* and *Goodyera pubescens*, respectively (Stern 2014). The root epidermis of Goodyerinae is sometimes referred to as a simple rhizodermis (Stern 2014), a single-layered velamen (Figueroa *et al.* 2008; Andreota *et al.* 2015), or both a *Calanthe*-type velamen and simple epidermis (Porembski & Barthlott 1988; Pridgeon *et al.* 2003). In general, the velamen in species of Goodyerinae is characterised by the

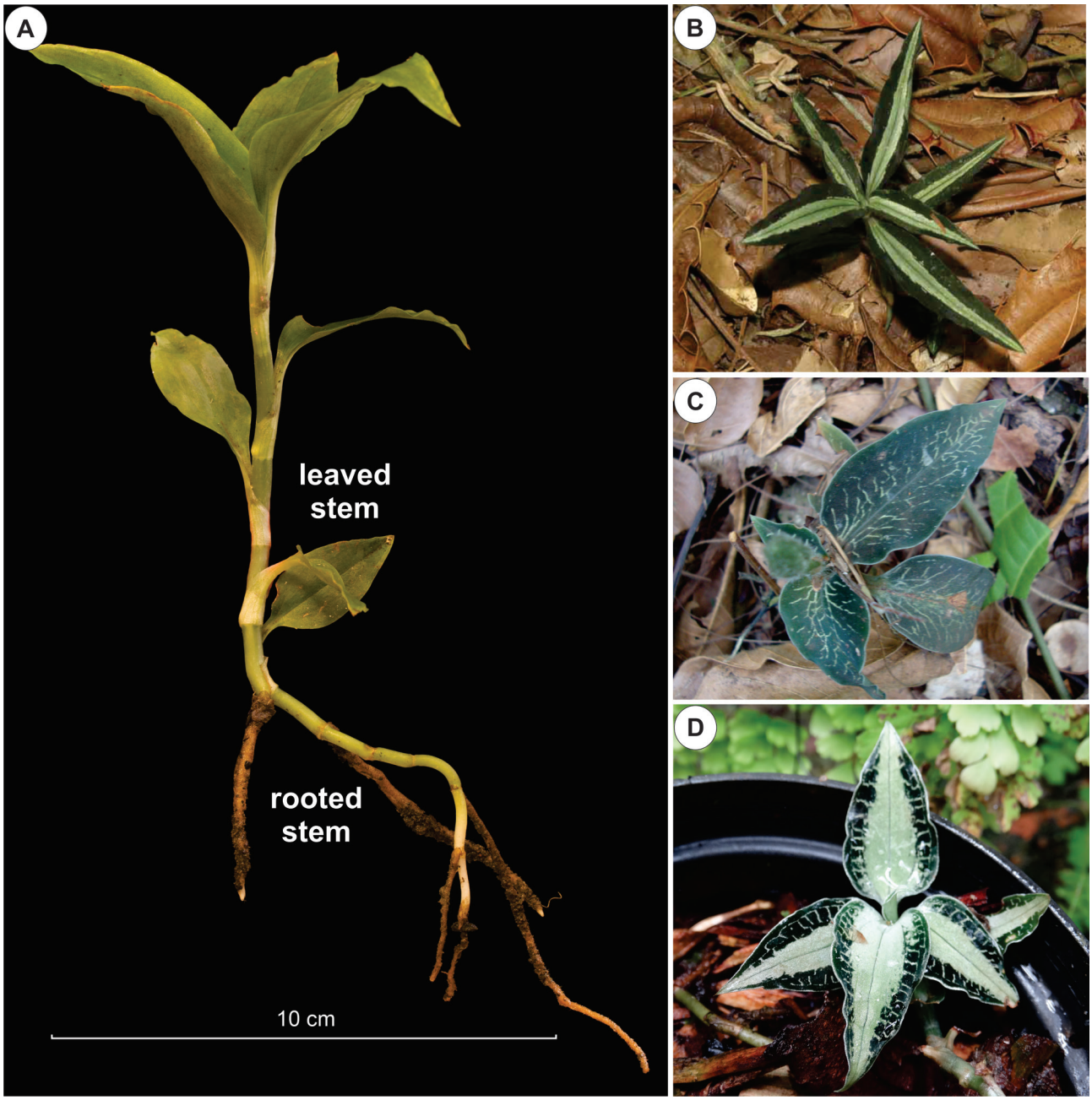

Figure 1. A. Habit of *Microchilus arietinus*. **B**. *Aspidogyne fimbrillaris*. **C**, **D**. *A. kukzynskii*. Photo author: Nicolás Gutiérrez Morales (**A**), Mathias E. Engels (**B**, **C**), Eric C. Smidt (**D**).

absence of thickening in the walls and the presence or absence of pores (Figueroa *et al.* 2008).

The presence or absence of a true endodermis in the stem of Goodyerinae species is also controversial. The boundary layer between the cortex and vascular cylinder is defined as the endodermoid layer in *G. oblongifolia*, *G. pubescens*, *Erythrodes hirtella*, *E. plantaginea*, *Ligeophila clavigera*, *Platysthelys vaginata*, *Vrydagzynea pachyceras* (Pridgeon *et al.* 2003; Stern 2014), and *Microchilus hirtellus* in which Stern (2014) reported an endodermis with Casparian strips in rhizomes and an endodermoid layer in aerial stems.

Another open question, which was investigated here, is the type of starch grain present in stem and root of Goodyerinae. Spiranthosomes were noted in *Vrydagzynea pachyceras* (Pridgeon *et al.* 2003); however, Stern (2014) referred to the presence of cruciate starch granules in *G. oblongifolia* that differed from the usual structures (spiranthosomes) found in other Cranichideae (Pridgeon *et al.* 2003; Bernal *et al.* 2015). Andreota *et al.* (2015) mentioned only the presence of starch in neotropical *M. arietinus*, and in the invasive Asian species *Z. strateumatica* without specifying the type of starch.

Two features, a central parenchymatous vascular tissue and the lack of a sclerified tissue in the vascular cylinder of roots, are common for Goodyerinae (Pridgeon *et al.* 2003; Andreota *et al.* 2015). However, the presence of conducting tissue interspersed with the central parenchyma in the vascular cylinder of *M. hirtellus* root (Stern 2014) was not widely investigated in the group and may be a specific characteristic of Goodyerinae, because it is a characteristic that has not been registered for species of the other Cranichideae subtribes.

It is hypothesised that the Goodyerinae have specific characters, which are an unstratified velamen, the presence of conducting elements embedded in the central root parenchyma, the presence of collenchyma and sclereids, and the presence of a true endodermis in the stem. The present study aims to characterise the anatomical structure of the vegetative organs of two Neotropical genera of Goodyerinae to identify specific characters that could help to better circumscribe the Goodyerinae and differentiate it from member of the Spiranthinae (as other Cranichidinae) with the vegetative anatomy.

Materials and methods

Samples were collected from vegetative organs (Fig. 1A-D) of eleven species of Goodyerinae, *Aspidogyne argentea* (Vell.) Garay (MEE 529); *A. bidentifera* (Schltr.) Garay (MEE 600); *A. commelinoides* (Barb. Rodr.) Garay (TFS and AC 266); *A. foliosa* (Poepp. & Endl.) Garay (MEE 673); *A. fimbrillaris* (B.S. Williams) Garay (MEE 610) (Fig. 1B); *A. juruenensis* (Hoehne) Meneguzzo (MEE 599); *A. kuczynskii* (Porsch) Garay (MEE 527) (Fig. 1C-D); *A. longicornu* (Cogn.) Garay (MEE 510) (Fig. 1D); *A. rosea* (Lindl.) Meneguzzo (MEE 672); *Microchilus arietinus* (Rchb.f. & Warm.) Ormerod (MEE 528, 530) (Fig. 1A); *M. austrobrasiliensis* (Porsch) Ormerod (MEE 383). All voucher material is deposited at the UPCB herbarium in the Departamento de Botânica da Universidade Federal do Paraná.

The analyses were made on fresh and fixed material. The samples were collected and fixed in formaldehyde, acetic acid and 50 % ethanol (FAA 50) (Johansen 1940), and subsequently stored in 70 % ethanol. Samples were analysed near the apex (3-10 mm) and in the medium region of the root, rooted and leaved parts of the stem and completely expanded leaves. Semi-permanent microscope slides were prepared with free-hand section with a disposable razor and stained with 1 % Astra Blue (363405 Sigma-Aldrich) and 0.1 % Safranine (84120 Sigma-Aldrich) in aqueous solution, mounted in glycerin-gelatin (Kaiser 1880). For better visualization of Casparian bands, the sections were clarified in 30 % sodium hypochlorite, washed in distilled water and stained with 0.1 % Astra Blue and 0.01 % Safranin in distilled water. When necessary, permanent slides were prepared with material embedded in 2-hydroxyethyl methacrylate (Leica® historesin, nº 10695-0003) according to manufacturer's information and stained with 0.5 % Toluidine Blue O (89640 Sigma-Aldrich) in aqueous solution. Histochemical tests for the identification of starch, lipids and lignin were carried out, respectively, with Lugol solution (L6146 Sigma-Aldrich), 0.5 % Sudan III (S4136 Sigma-Aldrich) in 80 % ethanol and acidified phloroglucinol (P3502 Sigma-Aldrich). The control was done with freehand sections of the fixed or fresh samples mounted in semi-permanent microscope slides with glycerinated-gelatin.

The photomicrographs were obtained using an Olympus microscope (BX41) with a CS30 Olympus digital camera, with analySIS getIT Olympus software. The calibration was done with a micrometer calibration slide (ZEISS 5 + 100/100 mm, Oberkochen, Germany). For scanning electron microscope (SEM) analyses, samples were fixed in 2.5 % glutaraldehyde and 4 % paraformaldehyde solution in 0.2 M sodium phosphate buffer at pH 7.2 for 12 h (Karnovsky 1965), dehydrated in an ethanol series and submitted to critical point drying with CO_2. They were then affixed to metal supports with adhesive copper tape, sputter-coated with gold (BALZERS SCD030), and analysed with a JEOL JSM – 6360LV Scanning Electron Microscope (JEOL Ltd, Tokyo, Japan) at the Centre for Electron Microscopy of the Universidade Federal do Paraná (UFPR, Curitiba, Brazil).

Results

Root

Single-celled root hairs are present in all species (Fig. 2A). In all species analysed, the epidermis has one layer of velamen. Young velamen cells (at protodermal stage), with evident nuclei, are present near the apex of the root (Fig. 2A).

The velamen cells are isodiametric or wider than high in cross-section, have thin or slightly thickened walls, and are lignified to approximately 3 mm from the apex with visible pits on the walls in all species (Fig. 2B). The exodermis is a single layer with juxtaposed hexagonal-shaped cells, thin walls, and conspicuous suberin lamellae in all species examined (Fig. 2B, C). These lamellae can be clearly noted in the portions of the root where the velamen was often broken, indicating a region with restricted absorption. Internally, the cortex is parenchymatous (Fig. 2D) or has lacunar collenchyma (Fig. 2E) in most species, though these features may be present or absent in the same species (Tab. 1). Endodermal cells have thin walls and Casparian strips in all species (Fig. 2E-G). Raphides are present in thin-walled idioblasts, usually in external layers of the cortex (Fig. 2D).

The stele has a non-lignified, parenchymatous pericycle in all species (Fig. 2E-I) and is polyarch, with six to 13 protoxylem poles in most species (Fig. 2F, H, I) or up to 16 poles in *A. longicornu* (Tab. 1). The number of poles varies within the same species, and xylem and phloem elements are interspersed among the central parenchymatous tissue (Fig. 2H-I, Tab. 1). The phloem elements, both at the periphery and in the central region, are small, sometimes smaller than the companion cells, with transverse or slightly oblique sieve plates (Fig. 3A, B). Phloem and xylem elements occur both in the peripheral and central regions of the vascular cylinder (Fig. 2H-I, 3C, D). The companion cells and their adjacent sieve tubes are visible in the centre of the stele (Fig. 3D-F). The outermost xylem elements in *A. rosea* are thick-walled tracheids; the central elements are fibre-tracheids with thicker walls than the peripheral elements (Fig. 2I). The central portion of the stele, when delimited, is usually parenchymatous but may contain collenchymatous tissue (Fig. 3C), which may be present or absent in the same species (*A. rosea, A. longicornu*) (Tab. 1). Spiranthosomes, specialised amyloplasts that store starch, (Fig. 3G; see Stern *et al.* 1993), and hyphae (Fig. 3H) are present in the cortex of all species. The results described previously are summarised in Table 1.

Stem

The epidermis is single-layered, with thin walls and cuticle (Fig. 4A) or slightly thickened on the external periclinal wall in *Microchilus* spp. and *A. foliosa* (Fig. 4B). The cortex has nine to 23 cell layers (Tab. 1), with angular, sub-epidermal collenchyma in all species, and the number of layers varies from one to four (Fig. 4A-C). Slightly more pronounced collenchyma is present in leafy stem portions (Fig. 4B, C). The cortical parenchyma is below the collenchyma (Fig. 4C, D) with raphides idioblasts (Fig. 4E). Spiranthosomes are abundant (Fig. 4D, F) and more concentrated in the innermost layer of the cortex (endodermis) or evenly distributed throughout the cortex and central cylinder (*A. argentea, A. foliosa, A. kuczynskii,* and *A. longicornu*). There are more spiranthosome in the rooted-stem portions (Fig. 4D-H) than in the leaved-stem portions (Fig. 4C-I). The endodermis with Casparian strips is distinct in all species, and its cells are smaller and more flattened than the other cortical cells (Fig. 4G-K); it surrounds the central cylinder (stele) composed of vascular bundles arranged in an atactostele. The central cylinder has 10 to 56 collateral vascular bundles (Tab. 1) without a sclerenchyma sheath, with a single xylem band, or with two bands separated by parenchyma (Fig. 4J). The ground tissue is parenchymatous (Fig. 4G, J, K). Vascular bundles are dispersed in the ground tissue of the central cylinder in two to four layers, generally larger in the centre and smaller in the periphery of the cylinder (Fig. 4I-J). Medullar tissue is usually not clearly delimited (Fig. 4I).

Table 1. Anatomical characters of root, stem and leaves of neotropical Goodyerinae. CC = cortical collenchyma; CV = collenchyma in the vascular cylinder; XS = xylem vessels and sieve elements embedded in the centre of the vascular cylinder (X = xylem, Ph = phloem); PX = number of protoxylem poles; LC = layers of cortex cells; VB = number of vascular bundles; ADE = adaxial surface epidermis in cross-section (D = domed, C = convex, F = flat); ABE = abaxial surface epidermis in cross-section (D = domed, C = convex, F = flat); ID = pigment idioblast; (+), present; (-) absent; (?) unknown.

Species	Root	Stem	Leaves						
	CC	CV	XS	PX	LC	VB	ADE	ABE	ID
Aspidogyne argentea	+	-	Ph	7	15	14-20	C	C	+
A. commelinoides	+	+	-	12	?	28	?	?	?
A. bidentifera	-	-	-	10	14	10-11	C	C, F	-
A. fimbrillaris	-	-	Ph	6-8	9	13-20	C	C	+
A. foliosa	+	-	Ph	12-13	23	22-30	C	F	+
A. juruenensis	+	+	X, Ph	11-14	14	10-17	F	F	+
A. kuczynskii	- +	-	Ph	7	15	15-20	D	C	-
A. longicornu	- +	- +	Ph	13-16	16	38-56	C	C, F	-
A. rosea	+	- +	X, Ph	8-12	23	35-45	C	C	+
Microchilus arietinus	+	+	X, Ph	9-11	17	25-35	C	C	+
M. austrobrasiliensis	+	-	Ph	6-8	16	12-30	C	F	-

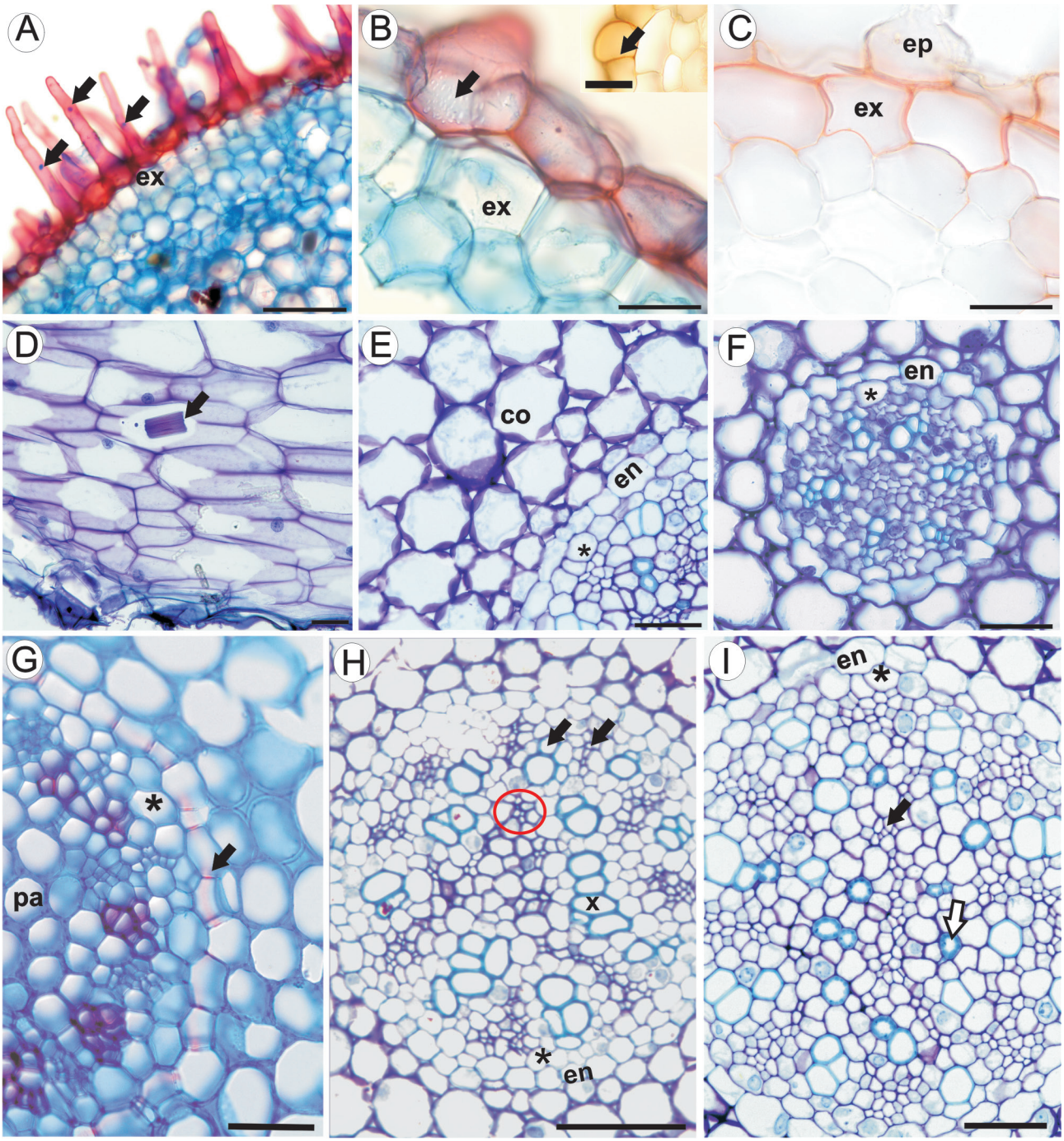

Figure 2. Roots of Goodyerinae: cross-sections (**A-C, E-I**) and longitudinal section (**D**). Free hand sections of fresh material (**A-C, G**). *Aspidogyne longicornu* (**A, B, C, G**), *A. juruenensis* (**D**), *A. rosea* (**E, I**) and *Microchilus austrobrasiliensis* (**F, H**). **A.** 5 mm from the apex, staining with Astra Blue and Safranin, young epidermis showing cells with lignified walls and nucleus present (arrows). **B**. Staining with Astra Blue and Safranin showing pits on the walls of epidermal cells (arrow) and with acidified phloroglucinol test (detail above), showing lignified walls (arrow). **C.** Sudan III test highlighting the suberin lamella at exodermis. **D.** Parenchymatous cortex showing an idioblast with raphides (arrow). **E.** Lacunal collenchyma present in internal cortex. **F.** Root with six protoxylem poles. **G.** Staining with Astra Blue and Safranin, endodermis with Casparian strips (arrow) and parenchymatous pericycle (*). **H.** Vascular cylinder (stele) evidencing phloem and protoxylem externally (arrows), phloem (red circle) and xylem internally. **I.** Vascular cylinder evidencing the centre of stele with phloem (black arrow) and xylem in central region, containing fibre-tracheids (white arrow). Epidermis (ep), exodermis (ex), endodermis (en), collenchyma (co), pericycle (*), xylem (x), parenchyma (pa). Bars: **A** = 200 µm, **B**, **C**, **D**, **E**, **F**, **G**, **I** = 50 µm, **H** = 100 µm.

Leaf

The leaves are hypostomatic and glabrous (Fig. 5A-D). The epidermis, in surface view, has polygonal cells on the adaxial surface, with straight anticlinal cell walls (Fig. 5A). The abaxial surface has irregularly shaped cells, with straight and thin (Fig. 5B) to curved or slightly sinuous anticlinal cell walls, as in *M. austrobrasiliensis* (Fig. 5C). Stomata are predominantly anisocytic or tetracytic with two to four subsidiary cells in most species, rarely five (*A. argentea*, *A. rosea*) (Fig. 5B, C), at the same level as other epidermal cells. The single-layered epidermis presents a thin and striated cuticle in *M. arietinus* (Fig. 5D) or is smooth on both sides of the leaf; the external periclinal wall is usually thin (Fig. 5E) to slightly thickened in *A. rosea* (Fig. 5F) and in the epidermal cells on the midrib. The epidermis cells are clear and larger than the cells of the mesophyll, usually larger and domed or look like conical papillae on the adaxial surface of *A. kuczynskii* (Fig. 5E), and convex in most species (Fig. 5F, G) or flat in *A. juruenensis* (Fig. 5H) (Tab. 1) with small and sparse chloroplasts. The mesophyll is homogeneous, with four to six layers of regular chlorenchyma (Fig. 5G-I). Idioblasts with raphides are visible in the mesophyll due to the absence of chloroplasts (Fig. 5I). Collateral vascular bundles, in one row, are surrounded by a barely defined parenchyma sheath with chloroplasts (Fig. 5I). The tracheary elements of the midrib are paired side by side and separated by the parenchyma (Fig. 5I). Collenchyma cells are in the outer border of the phloem (Fig. 5I) and subepidermally in the midrib. Some mesophyll

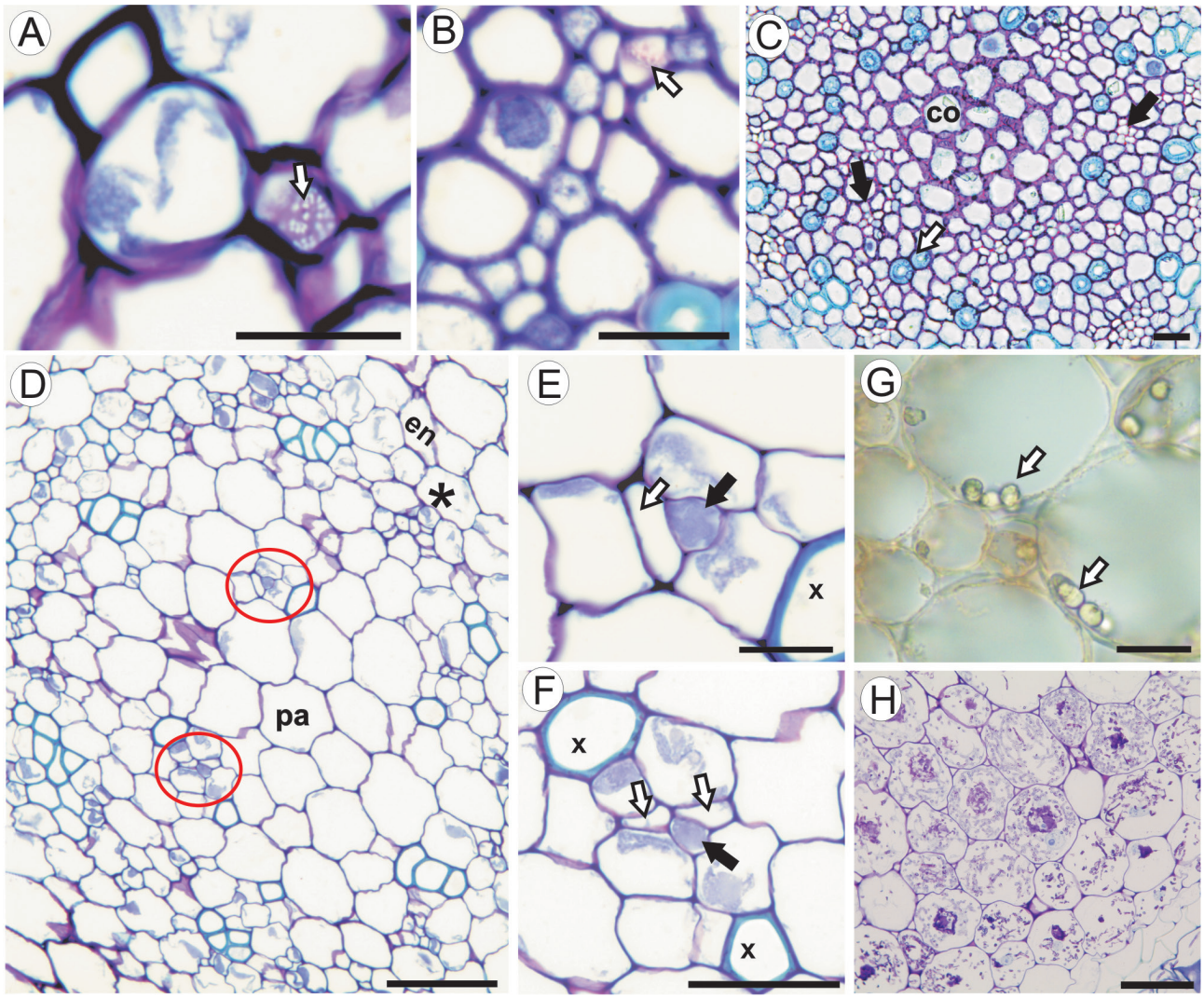

Figure 3. Goodyerinae, root, cross-sections. *Aspidogyne rosea* (**A, B, C**), *Microchilus austrobrasiliensis* (**D-G**). *M. arietinus* (**H**). **A.** Sieve tube with sieve plate (arrow) in the peripheral phloem. **B.** Sieve tube with sieve plate (arrow) in the internal phloem. **C.** Colenchyma in the centre of the stele, fibre-tracheids (white arrow) and phloem (black arrow). **D.** Phloematic elements embedded in the central vascular tissue (circles). **E, F.** Detail of the circles of figure (**D**), sieve tubes (white arrows) and companion cells (black arrows). **G.** Spiranthosomes (arrows). **H.** Cortex with fungi hyphae. Endodermis (en), pericycle (*), xylem (x), parenchyma (pa), collenchyma (co). Bars: **A**, **B**, **E** = 20 µm, **C**, **F**, **G** = 50 µm, **D**, **H** = 100 µm.

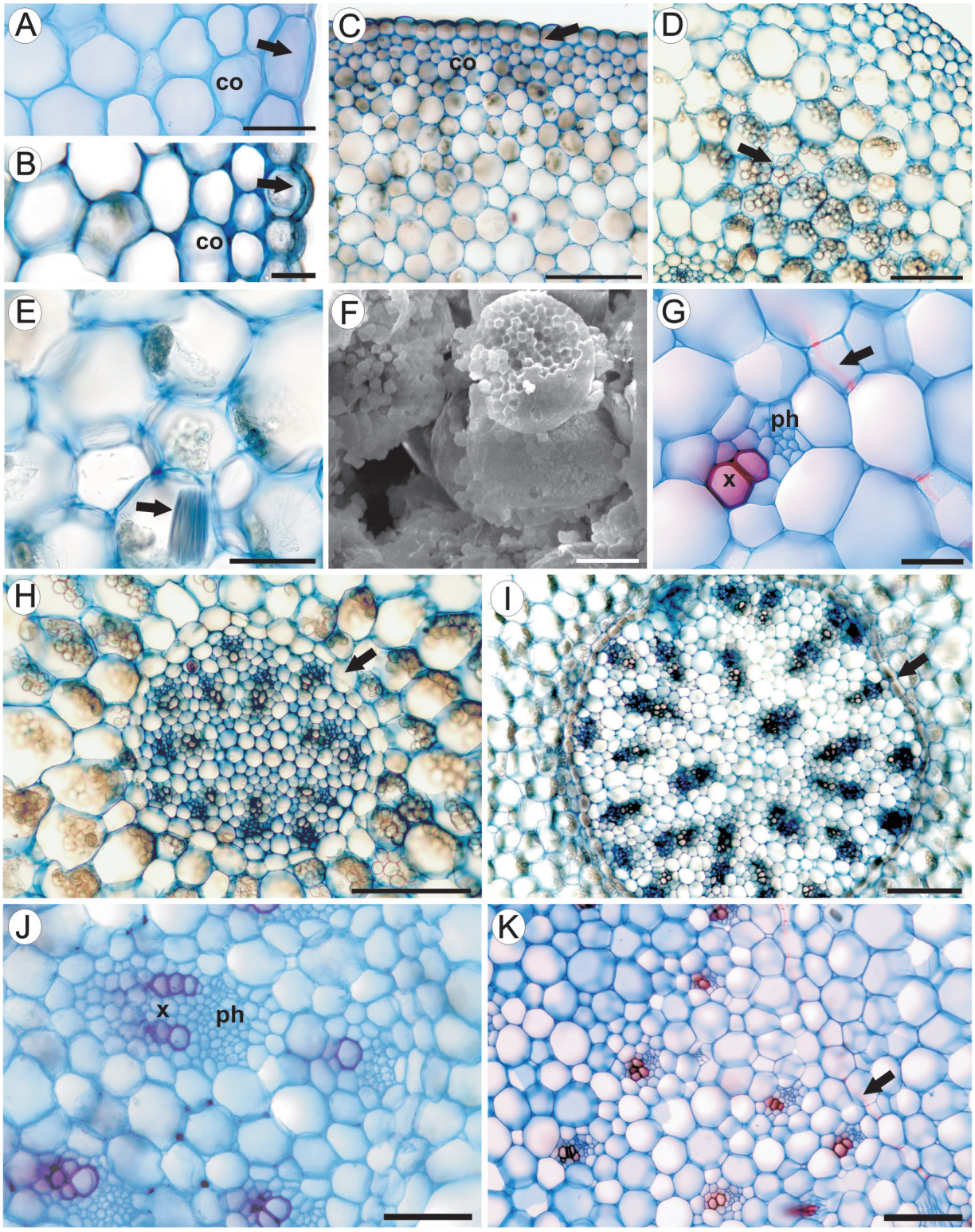

Figure 4. The stems of Goodyerinae: horizontal (rooted) (**A, D, E, G, H, J**) and erect (leaved) (**B, C, F, I, K**). *Aspidogyne longicornu* (**A, C, G, K**), *A. foliosa* (**B**), *A. kuczynskii* (**E**), *A. jurueneensis* (**D, H**), *Microchilus austrobrasiliensis* (**F, I**) and *A. rosea* (**J**). **A, B.** Epidermal cells with thin walls (**A** arrow), slightly thickened walls (**B** arrow) and subepidermal collenchyma (co). **C.** Cortex with peripheral collenchyma (co; arrow on epidermis). **D.** Spiranthosome in cortex tissue (arrow). **E.** Cortex idioblast with raphides. **F.** Detail of spiranthosome in SEM. **G.** Endodermis with Casparian strips (arrow) and vascular bundle. **H.** Central cylinder with 14 vascular bundles, endodermis (arrow). **I.** Central cylinder with 28 vascular bundles, endodermis (arrow). **J, K.** Detail of vascular bundle (**J**) and central cylinder delimited by endodermis with Casparian strips (arrow) (**K**). Clarified samples stained with Astra Blue and Safranin (**G, K**). Phloem (ph), xylem (x). Bars: **A**, **B**, **G** = 50 µm, **F**, **J** = 100 µm, **C**, **D**, **H**, **I**, **K** = 200 µm, **E** = 5 µm.

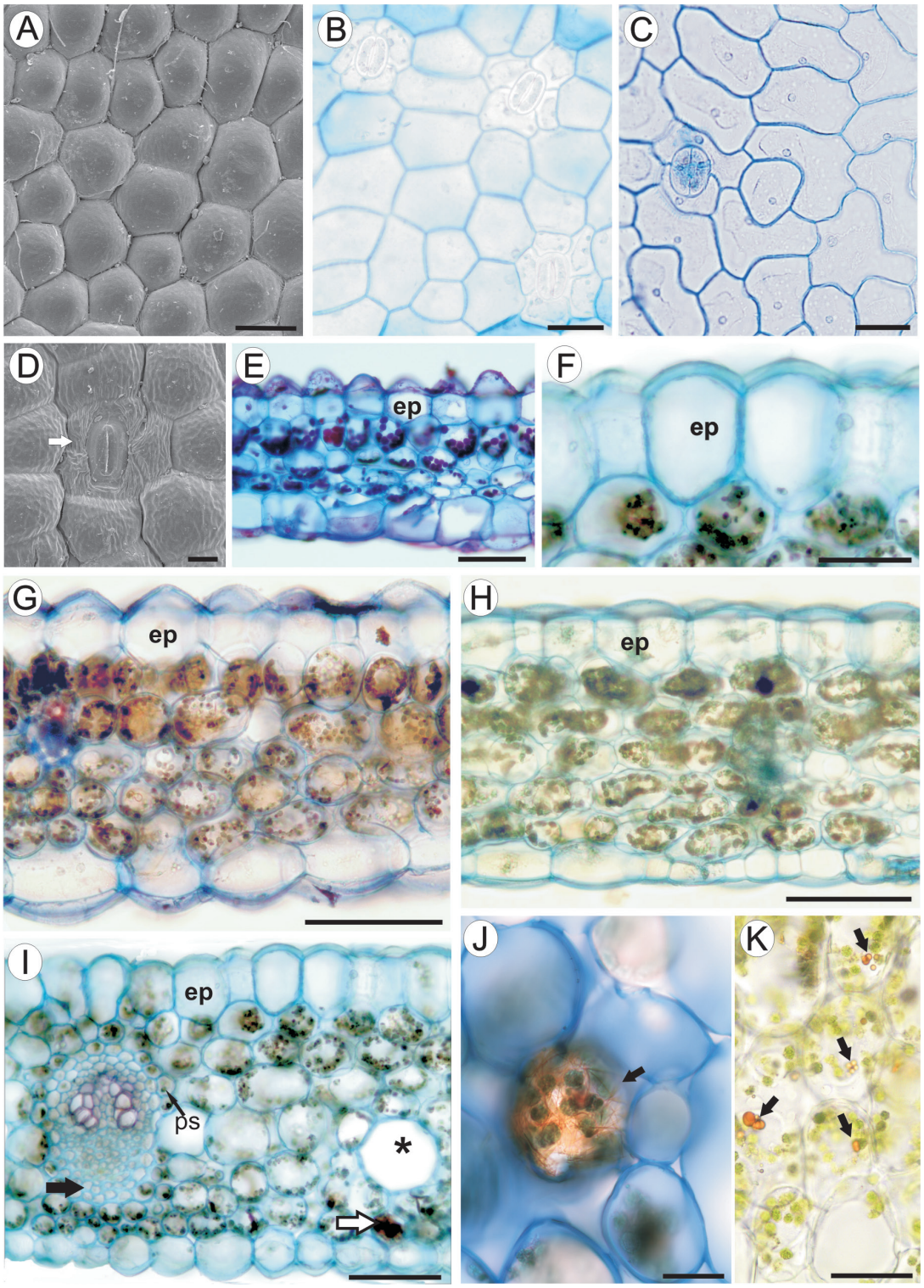

Figure 5. Goodyerinae leaf, epidermis in frontal view (**A-D**) and cross-sections (**E-K**). *Microchilus arietinus* (**A, D, G, J, K**). *Aspidogyne rosea* (**B, F, I**). *M. austrobrasiliensis* (**C, D**). *A. kuczynskii* (**E**). *A. juruenensis* (**H**), *A. longicornu* (**N**). **A.** Adaxial surface in SEM, showing straight anticlinal walls and convex periclinal walls. **B, C.** Abaxial surface, evidencing stomata with 2, 3, 4 and 5 subsidiary cells (*). **D.** Abaxial surface in SEM showing epicuticular grooves (arrow). **E.** Domed adaxial epidermal cells and flat abaxial cells. **F, G.** Convex epidermal cells, slightly thickened in (**F**), and homogeneous mesophyll (**G**). **H.** Flat epidermal cells. **I.** Vascular bundle with two band of xylem and parenchymal sheath with chloroplasts, collenchymatous phloematic cells (black arrow), pigment cell (white arrow) and crystal idioblast lacking raphides (*). **J, K.** Detail of mesophyll cells with orange pigment, 70% ethanol fixed sample and stained with Astra Blue and Safranin (**J**) and live sample without staining (**K**). Adaxial surface epidermis (ep), parenchyma sheath (ps). Bars: **A**, **B**, **F**, **K** = 50 µm, **C** = 250 µm, **E**, **G**, **H**, **I** = 100 µm, **D**, **J** = 20 µm.

cells have filamentous brownish-orange chromoplasts in the fixed material and yellow microspheres in the fresh material. These chromoplasts were observed in several species of *Aspidogyne* and *M. arietinus* (Fig. 5J, K, Tab. 1).

Discussion

The analysed species of *Aspidogyne* and *Microchilus* present a very similar vegetative anatomy, with a few variations from what has already been described for other representatives of Cranichideae and/or Goodyerinae (Stern *et al.* 1993; Pridgeon *et al.* 2003; Stern 2014; Andreota *et al.* 2015). The presence of collenchymatic tissue in the root cortex and vascular cylinder and of fibre-tracheids in the stele are reported for the first time for this group. The presence of phloem and xylem conducting elements in the central vascular tissue was recorded for *Microchilus* species and in *Aspidogyne* species confirms Stern's reports (2014). The presence of collenchyma and fibre-tracheids in the root stele represents new features for Goodyerinae.

The roots of analysed species of *Aspidogyne* and *Microchilus* have a rhizodermis with a Calanthe type velamen as described previously by Porembski & Barthlott (1988) and later by Stern *et al.* (1993) for *Goodyera macrophylla*, *G. oblongifolia* and Andreota *et al.* (2015) for *M. arietinus*. All analysed species in this work present a typical thin-walled exodermis and the absence of tilosomes, as previously reported for some Goodyerinae (Stern *et al.* 1993; Stern 2014). The spiranthosomes characteristic for Cranichideae (Pridgeon *et al.* 2003; Stern 2014) and considered a synapomorphy for this tribe (Stern *et al.* 1993; Salazar *et al.* 2003) were confirmed for all analysed species of Goodyerinae.

The presence of collenchyma in the root cortex and stele is frequent in the studied species and provides support and flexibility in an unstable substrate. This feature is reported by several authors (Turner 1934; Fleet 1950) for aerial roots associated with mechanical stress (Walker 1957; Moysset & Simón, 1991; Leroux 2012). Although collenchyma was notobserved in *A. bidentifera* and *A. fimbrillaris* in our studies, it could be present. Root collenchyma has not been recorded for Cranichideae (Pridgeon *et al.* 2003; Stern 2014) or the Goodyerinae already studied (Stern 2014; Andreota *et al.* 2015), but it seems to be a common feature in the species of this subtribe that grows upon an unstable substrate.

The root stele in Goodyerinae is characterised by a variable number of xylem poles, parenchymatic ground tissue, and vascular cells interspersed in the central parenchyma in some species (Stern *et al.* 1993; Stern 2014). Vascular elements among cells of the central parenchyma, which had already been reported for *Goodyera*, *L. clavigera, M. hirtellus* and *Z. strateumatica* (Stern 2014), were also observed in most of the species analysed in this study (see Tab. 1). These may be only peripheral or may be found throughout the central parenchyma, as described for nine of the eleven analysed species. According to Stern (2014), the xylem tissue is composed of tracheids of varying degrees of thickness; however, the presence of fibre-tracheids (fusiform, with distinctively thickened walls and pits with small borders) amongst the parenchymatous central tissue in *A. argentea* stands out.

Anatomically, there are few distinctions between Goodyerinae stems and the other Cranichideae (Pridgeon *et al.* 2003; Stern 2014). The presence of collenchyma in outer layers (*G. rubicunda* (cited as *G. grandis*)) and a true endodermis with Casparian strips in *Microchilus hirtellus* (as *Erythodes hirtella*), *Platystelys vaginata* and *Vrydagzynea elongata* (Stern 2014), as well as all species analysed in this study, seems to be a constant character for Goodyerinae. The endodermis with Casparian strips was observed in both the rooted-stem and the leafy stem in all analysed species. Stern (2014) cites both the presence of an endodermoid layer with tangentially flattened cells (*Goodyera oblongifolia, G. pubescens, Ligeophila clavigera*) and the presence of a true endodermis in *M. hirtellus*, *M. plantagineus* and in the rhizome of *M. hirtellus*. We observed that if there is no specific staining to detect Casparian strips, they may not be observed because they are very thin and the layer is identified as an endodermoid layer. The number of vascular bundles was also more variable than the number previously recorded for the tribe, from 15–48 (Stern 2014) to 10–58. The similar structure observed in both kinds of stems was expected since the leafy stem eventually loses leaves, becomes prostrate and produces roots as the plant grows. Therefore, the rooted stem still presents an aerial structure, although it acquires more storage functions, accumulating more starch.

The leaf anatomy of Goodyerinae is typical for all Cranichideae (Pridgeon *et al.* 2003; Stern 2014) concerning the epidermis, mesophyll and vascular tissue. Hypostomatic leaves are the rule, as in most Orchidaceae (Silva & Milaneze-Gutierre 2004), as is the occurrence of several morphological types of stomata (Zanenga-Godoy & Costa 2003; Silva & Milaneze-Gutierre 2004). The presence of two to five subsidiary cells per stoma in Cranichideae was interpreted by Pridgeon *et al.* (2003) as diacytic, anisocytic, tetracytic and anomocytic, depending on the number of cells. However, the anomocytic type is defined by the lack of morphological distinction between subsidiary cells and surrounding epidermal cells (Metcalfe & Chalk 1950), which was not observed in Goodyerinae. In the species studied here, it is possible to observe that subsidiary cells are smaller than the other epidermal cells, and therefore do not fit in the description of anomocytic stomata. We thus believe it is correct to characterise Goodyerinae stomata using the predominant pattern (anisocytic or tetracytic) or by indicating the number of subsidiary cells (two to five).

Orange chromoplast in mesophyll cells were recorded in six of the eleven species in this study (the leaves of *A. commelinoides* were not analysed due to the lack of material). Samples from most species came from fixed

material and were stored in ethanol. In these samples, filamentous structures of orange or brown colour, similar to crystalline chromoplasts (Mohr 1979), were recorded inside isolated cells. This type of chromoplast is defined by the crystalline inclusions of carotene (Mohr 1979; Evert 2006) and is often found in yellow, brown, tan or golden petals in Orchidaceae (Kay & Daoud 1981; Mudalige *et al.* 2003). In the fresh *M. arietinus* material without staining, only globose chromoplasts of homogeneous orange colour were present.

Conclusions

The anatomy of the vegetative organs of the analysed Goodyerinae is typical for plants found in mesophytic environments and is associated with the humicole life habit of these species, such as the thin cuticle and the absence of sclerenchyma on the leaf and collenchyma on both stem and root. Although they have very similar anatomy to the other Cranichideae, our results indicate a few characteristics that are specific to Goodyerinae, such as the presence of conducting elements and fibre-tracheids embedded in the root central parenchyma and the presence of Casparian strips in the stem endodermis, which can be used as reliable taxonomic character. The anatomical similarity between representatives of Goodyerinae supports the monophyly of the group. Further studies of comparative anatomy with other subtribes are necessary to identify potential synapomorphies for these lineages.

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