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COMPARATIVE ANATOMY OF *CALOLISIANTHUS* SPECIES (GENTIANACEAE – HELIEAE) FROM BRAZIL: TAXONOMIC ASPECTS

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This work aims to characterise the morphology and anatomy of roots, stems and leaves of *Calolisianthus* species (Gentianaceae – Helieae) to assist in the taxonomy and understanding of some adaptive responses to high luminosity, prolonged water deficit and nutritional stress in their environment. Samples of *Calolisianthus speciosus* and *C. pendulus* were collected in *campo rupestre* (rocky land) and samples of *C. amplissimus* were collected in *cerrado* (savanna) areas in southeastern Brazil. The roots have a cortex with *Arum*-type arbuscular mycorrhizae. The three species have winged and square stems and in *Calolisianthus amplissimus* the stem is hollow. *Calolisianthus pendulus* and *C. speciosus* have a pair of conspicuous extrafloral nectaries at the leaf base, which are absent in *C. amplissimus*. *Calolisianthus pendulus* has a dorsiventral mesophyll and a round leaf margin with parenchymatic cells. *Calolisianthus amplissimus* has a homogeneous mesophyll and a leaf margin with collenchyma. *Calolisianthus speciosus* leaves have a homogeneous mesophyll and a margin with sclerenchyma and collenchyma. Our results demonstrate that some anatomical characters are useful for the identification of *Calolisianthus* species and might be used to elucidate evolutionary relationships among *Calolisianthus* and their adaptive responses.

Keywords. Anatomy, *Calolisianthus*, extrafloral nectaries, mycorrhizae, systematics.

INTRODUCTION

The Gentianaceae is the third largest family of the order Gentianales, comprising about 1700 species (Molina & Struwe, 2009), with two-thirds of the genera being distributed in the tropics (Meszaros & De Laet, 1996). Phylogenetic studies on Gentianaceae suggest six tribes should be recognised: Exaceae, Chironieae, Gentianaceae, Helieae, Potalieae and Saccifolieae (Struwe *et al.*, 2002). The tribe

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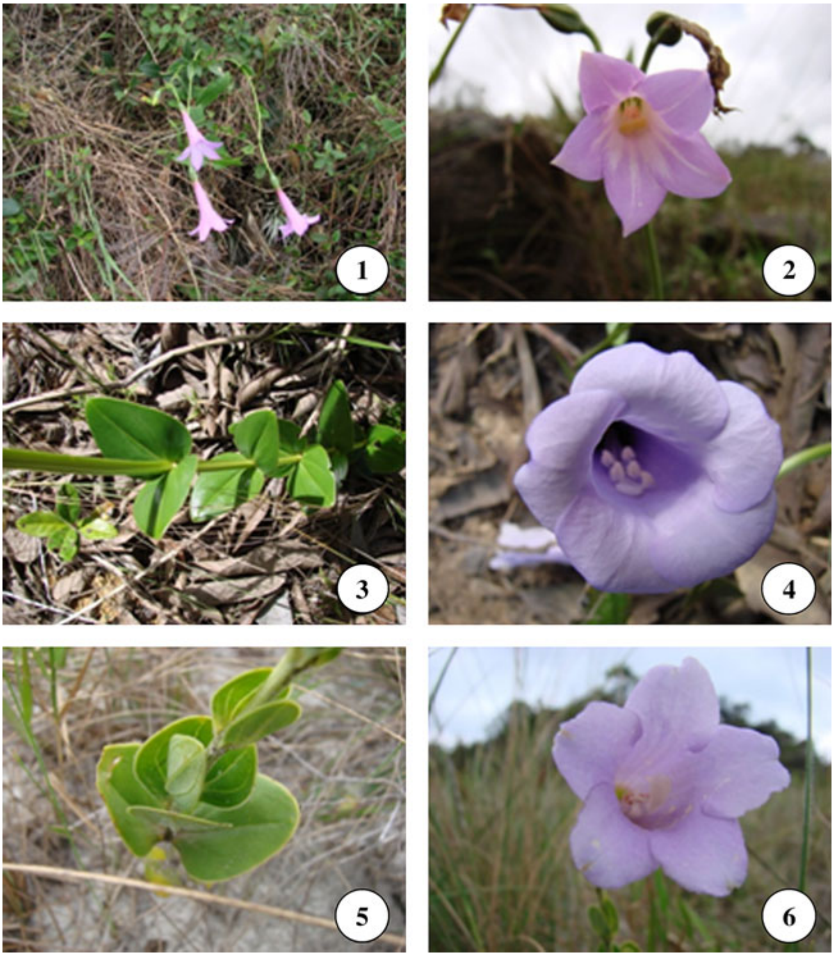
Helieae is strictly neotropical, and comprises 22 genera and 184 species. Phylogenetic studies on Helieae, combining molecular and morphological data, strongly support the monophyly of several genera and two major subclades: the *Macrocarpaea* (Griseb.) Gilg and *Symbolanthus* G.Don subclades. The phylogeny of the *Symbolanthus* subclade, however, has not been completely clarified (Struwe *et al.*, 2009). The genus *Calolisianthus* (Griseb.) Gilg, in the *Symbolanthus* subclade (Struwe *et al.*, 2009), is comprised of largely unbranched perennial herbs with sessile oblong-elliptic leaves and 5-merous flowers with large pink corollas in terminal few-flowered cymes (Struwe *et al.*, 2002). This genus consists of 6–10 species distributed in Bolivia and southeastern Brazil, occurring in Brazilian savannas (*cerrado*) and rocky field (*campo rupestre*) formations (Struwe *et al.*, 2002).

The *cerrado* is a savanna physiognomy characterised by strong rainfall seasonality, coupled with constantly high diurnal air temperatures and deep and well-drained soils (Furley, 1999). The nutrient-poor, acid soils are an additional limiting factor for plant growth in this region (Haridasan, 2000). *Campo rupestre* vegetation is found on the slopes of hills in rocky outcrops, being characterised by a predominant physiognomy of grassland and shrubs (Gottsberger & Silberbauer-Gottsberger, 2006). The *campo rupestre* is also characterised by seasonal rainfall, high temperatures during the day and low temperatures during the night, frequent winds (Scarano *et al.*, 2001), low soil fertility, and soil with sandstone and quartz (Giulietti *et al.*, 1987). Because of low fertility and water deficits (Dickison, 2000), morpho-anatomical adaptations, such as root-mycorrhizal associations that increase the water and nutrient absorption (Herrman *et al.*, 2004), may facilitate, or even be essential to, the survival of plants in these two environments.

The comparative anatomy of vegetative organs of *Calolisianthus* species may be important in our understanding of anatomical evolution and variation in the family. Leaf anatomy has always played an important role in plant taxonomy and may be useful for infrageneric delimitation (Metcalfe & Chalk, 1950; Kocsis *et al.*, 2004; Matias *et al.*, 2008; Erxu *et al.*, 2009; Moon *et al.*, 2009), as well as being important in our interpretation of adaptation of plant species to ecological constraints. Such detailed anatomical analysis is necessary for an understanding of the effects of environmental constraints on *Calolisianthus* ecology in *cerrado* and *campo rupestre* in Brazil.

Materials and Methods

The anatomy of vegetative organs was studied in three species: *Calolisianthus pendulus* (Mart.) Gilg (Figs 1–2), *C. amplissimus* (Mart.) Gilg (Figs 3–4), and *C. speciosus* (Cham. & Schltdl.) Gilg (Figs 5–6). Voucher specimens are housed at VIC (*Index Herbariorum*: http://sweetgum.nybg.org/ih/): *Calolisianthus pendulus* (*M.N. Delgado, A.A. Azevedo & G.E. Valente* VIC 31.361), *C. amplissimus* (*M.N. Delgado, A.A. Azevedo & G.E. Valente* VIC 31.366), *C. speciosus* (*M.N. Delgado, A.A. Azevedo & G.E. Valente* VIC 31.362).



FIGS 1–6. Morphological diversity in species of *Calolisianthus* studied. 1–2: *Calolisianthus pendulus*. 3–4: *C. amplissimus*. 5–6: *C. speciosus*.

Samples of *Calolisianthus speciosus* and *C. pendulus* for the anatomical study were collected in *campo rupestre* areas of the Ouro Branco Mountain Range (Giulietti & Pirani, 1988; Giulietti *et al.*, 1997) and samples of *C. amplissimus* in *cerrado* areas of the Paraopeba National Forest (Balduino *et al.*, 2005; Souza *et al.*, 2008), in Minas Gerais, Brazil. The *campo rupestre* area where *Calolisianthus pendulus* specimens were collected is a slope, located at 20°29′35.5″–20°29′35.7″S and 43°42′36.1″–43°42′36.8″W, with altitudes ranging from 1517 to 1540 m. The area is sunny, windy and rocky, but the soil is soft. Specimens of *Calolisianthus speciosus* were collected at 20°30′07.6″–20°30′20.02″S and 43°38′39.9″–43°38′21.6″W, with altitudes ranging from 1321 to 1352 m. The area is sunny and dominated by grasses and the soil is hard and sandy. *Calolisianthus amplissimus* specimens were collected at

19°15′15.13″–19°15′21.4″S and 44°24′04.4″–44°24′06.3″W, with altitudes ranging from 763 to 769 m. This area is a *cerrado* with some trees and shrubs and the soil is soft and clayey.

Leaf, stem and root samples were fixed in FAA_{50} and then stored in 70% ethanol (Johansen, 1940). Samples were collected from the basal, medial and apical portions and margins of leaf blades. Stems were sampled at the second and third internodes and roots were sampled in the area of secondary growth.

Samples of root, stem and leaf were processed and embedded in Historesin (Leica Embedding Kit), following standard procedures. Longitudinal and cross-sections, 6–8 µm thick, were obtained using an automatic rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA) and stained with Toluidine Blue in MacIlvaine buffer for 15 minutes (O'Brien & McCully, 1981). In extrafloral nectaries and colleters the histochemical test PAS was utilised to detect carbohydrates (Maia, 1979), Ruthenium Red for acid polysaccharides, and Sudan Red B for total lipids (Johansen, 1940). Histological slides were prepared to study epidermal characteristics. Entire leaves were cleared by immersion in a 10% NaOH solution for 1 hour and transferred to 20% sodium hypochlorite for approximately 72 hours until they became translucent. Samples were stained in Basic Fuchsin for 1 or 2 hours, dehydrated in an ethanol-xylol series and mounted in Permount (Berlyn & Miksche, 1976). Photographs were taken using an Olympus AX70 microscope equipped with a U-Photo photographic system.

Leaf samples of the species studied were also collected for scanning electron microscopy (SEM) analysis, fixed in Karnovsky's solution (Karnovsky, 1965) in the field, dehydrated in an ethyl alcohol series, and dried to the critical point with CO_2. The samples were gold coated as described by Bozzola & Russel (1992) and examined by scanning electron microscope SEM 1430 LEO VP (Cambridge, UK) at 5–15 kV.

RESULTS

Anatomical description

Roots (*Table 1*). All the roots studied exhibited secondary growth (Figs 7–9, 11). However, we observed that a uniseriate epidermis remained in thin roots. This epidermis consisted of irregularly shaped cells in cross-section (Figs 7, 9). In all species, the cortex is parenchymatous and colonised by *Arum*-type arbuscular mycorrhiza (AM) (Figs 7, 10). Only cortical cells of *Calolisianthus amplissimus* contain starch.

In thicker roots of *Calolisianthus pendulus* and *C. speciosus* the epidermis and some layers of cortical parenchyma with *Arum*-type AM are discarded and are replaced by elongate cortical cells in the periclinal direction and irregular-shaped cells in the anticlinal direction (Figs 8, 11). Phellogen does not occur in secondary roots of the species studied. Sclereids were found scattered in the cortex of *Calolisianthus*

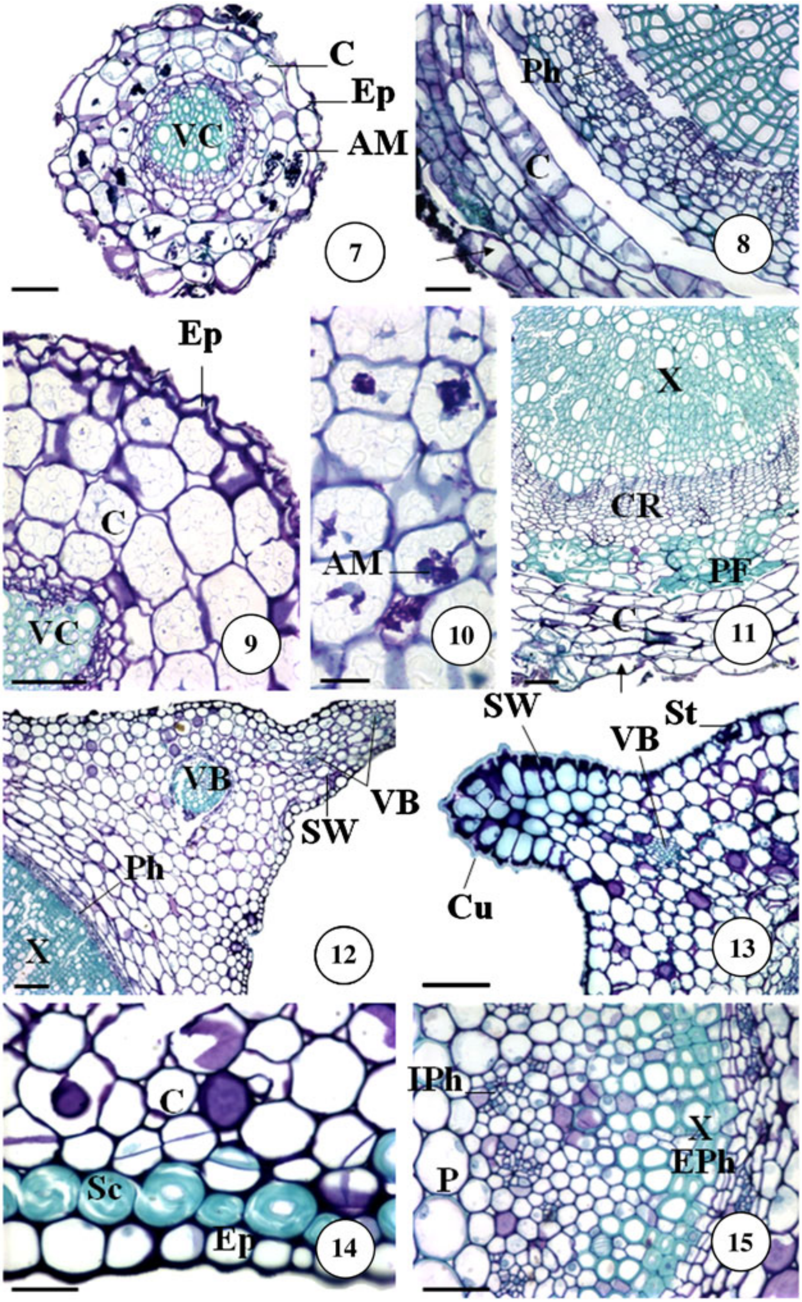
TABLE 1. Root and stem characters of *Calolisianthus pendulus*, *C. amplissimus* and *C. speciosus*

Anatomical root and stem diagnostic characters	*C. pendulus*	*C. amplissimus*	*C. speciosus*
Root			
Parenchymatous cortex with arbuscular mycorrhiza	1	1	1
Cortex with sclereids	1	0	0
Cortex with fibres	0	0	1
Stem			
Quadrangular stem	1	1	1
Unistratified epidermis	1	1	1
Tabular epidermis cells	0	1	0
Elongated epidermis cells	1	0	1
Thick external periclinal walls	1	1	1
Smooth cuticle	0	1	0
Decorated cuticle	1	0	1
Parenchymatous cortex	1	1	1
Subepidermal sclereids	0	0	1
Undifferentiated bundle sheath	0	1	0
Endodermis with Casparian strips	1	0	1
Pericycle with sclereids	1	0	0
External and internal phloem	1	1	1
Parenchymatous pith	1	1	1
Fistulous pith	0	1	0

1 = presence; 0 = absence.

pendulus, primarily in the innermost layers. In *Calolisianthus speciosus* there are several layers of fibres arranged between the cortical parenchyma and the secondary phloem (Fig. 11). We found two types of vessel perforation in the root secondary xylem: in *Calolisianthus pendulus* plates are foraminate and simple, while in *C. speciosus* and *C. amplissimus* we observed only simple plates.

Stems (*Table 1*). The epidermis is unistratified with thick external periclinal walls that are cutinised. *Calolisianthus amplissimus* has a smooth and fine cuticle, whereas in *C. pendulus* (Fig. 13) and *C. speciosus* the cuticle is decorated and thick. Epidermal stem cells in the cross-section are elongated in *Calolisianthus pendulus* (Fig. 13) and *C. speciosus* (Fig. 14) and tabular in *C. amplissimus* (Fig. 12). Stem cortex is parenchymatous and chlorophyllous with subepidermal sclereids in *Calolisianthus speciosus* (Fig. 14). Three to four cortical bundles are observed towards the stem wings in *Calolisianthus amplissimus* (Fig. 12) and *C. speciosus*, but only one is present in *C. pendulus* (Fig. 13). The endodermis of *Calolisianthus pendulus* and *C. speciosus* has clear Casparian strips that are not observed in the material of *C. amplissimus* that was analysed. The stem vascular system has external and internal phloem in the



FIGS 7–15. Transverse sections of root and stem. 7–8: Root of *Calolisianthus pendulus*. 9–10: Root of *C. amplissimus*. 11: Root of *C. speciosus*. 12: Stem of *C. amplissimus*. 13: Stem of *C. pendulus*. 14–15: Stem of *C. speciosus*. Ep – epidermis, C – cortex, AM – *Arum*-type arbuscular mycorrhiza, VC – vascular cylinder, X – xylem, Sc – sclereids, PF – perivascular fibre, CR – cambial region, Arrow – cortical cells that replace epidermis, SW – stem wing,

second and third internodes (Fig. 15). There is an initial secondary growth with the addition of secondary xylem. The vascular cylinder consists of secondary xylem, secondary phloem, vascular cambium and pith. The vessels found in the stem are the same as those in the roots. Parenchymatous pith is fistulous only in *Calolisianthus amplissimus*.

Leaves (*Tables 2 and 3*). In *Calolisianthus pendulus* the epidermis is unistratified with cells that have straight anticlinal walls (Figs 16, 18). *Calolisianthus amplissimus* and *C. speciosus* have straight anticlinal walls on the adaxial surface (Fig. 17) and sinuous walls on the abaxial surface (Fig. 19). All species have cuticular ornamentation visible under light microscopy (Figs 16–17) which is rather thick in *Calolisianthus pendulus* (Fig. 27) and *C. speciosus* (Fig. 31) but thinner in *C. amplissimus* (Fig. 29). All species of *Calolisianthus* studied have hypostomatous leaves (Figs 27, 29, 31), often with anomocytic stomata (Figs 18–19). We have observed variation in the shape of epidermis cells in cross-section at the basal, medial and apical portions of the same leaf.

A variable number of non-vascularised colleters are found on the adaxial surface and close to the leaf axis in all three species (Figs 20–22). The colleters are very small and glossy and can only be observed using a stereomicroscope. Micromorphological studies on leaf colleters show that the colleter heads are undulate and uneven (Figs 20–21), possessing a large quantity of mucilage between the secretory structures (Fig. 21). Colleters are formed from large cells with a prominent nucleus, dense cytoplasm and thin cell walls covered with a thin cuticle (Fig. 22). Colleters involved in secretion have the central region separated from the most peripheral cell layers by a space filled with a secretion (Fig. 22) that reacts intensely to the PAS test. The peripheral layer cells are not different from inner cells in shape, but stained more intensively with Ruthenium Red (Fig. 22) and PAS. The Sudan Red B test is negative for the secreted substance and the neck region, staining red only in the thin cuticle that covers the colleter. In *Calolisianthus speciosus* and *C. pendulus*, two conspicuous extrafloral nectaries (EFNs) have been observed at the base of the abaxial surface of the leaves. These large extrafloral nectaries, which are described for the first time, are formed by the adhesion of several secretory units. Each secretory unit is a very small nectary that is formed by a few cells (Figs 23–24, 26). Furthermore, a set of secretory units forms an apical nectary at the leaf apex of the three species. These secretory units react positively with PAS (Fig. 26), suggesting a secretion of soluble sugars, and are scattered throughout the leaf on both adaxial (Fig. 25) and abaxial (Figs 24, 26) surfaces.

VB – vascular bundle, Ph – phloem, Cu – cuticle, St – stomata, EPh – external phloem, IPh – internal phloem, P – pith. Scale bars = 70 µm (7, 8, 9, 11), 50 µm (10, 14, 15), 100 µm (12, 13).

TABLE 2. Leaf blade characters of *Calolisianthus pendulus*, *C. amplissimus* and *C. speciosus*

Anatomical leaf blade diagnostic characters	*C. pendulus*			*C. amplissimus*			*C. speciosus*		
	BP	MP	AP	BP	MP	AP	BP	MP	AP
Adaxial surface with tabular epidermis cells	0	1	0	1	0	0	0	1	0
Adaxial surface with elongated epidermis cells	1	0	1	0	1	1	1	0	1
Abaxial surface with tabular epidermis cells	0	0	1	1	1	1	1	0	1
Abaxial surface with elongated epidermis cells	1	1	1	0	0	0	0	1	0
Adaxial surface with thick external periclinal walls	1	1	1	1	1	1	1	1	1
Abaxial surface with thick external periclinal walls	1	1	1	0	0	0	0	0	0
Adaxial surface with smooth cuticle	0	0	0	0	0	0	0	0	0
Adaxial surface with decorated cuticle	1	1	1	1	1	1	1	1	1
Abaxial surface with smooth cuticle	0	0	0	0	0	0	0	0	0
Abaxial surface with decorated cuticle	1	1	1	1	1	1	1	1	1
Hypostomatous leaves	1	1	1	1	1	1	1	1	1
Anomocytic stomata	1	1	1	1	1	1	1	1	1
EFNs formed by several secretory units in abaxial surface	1	0	1	0	0	1	1	0	1
Scattered secretory units in adaxial surface	0	1	0	0	1	0	1	1	0
Scattered secretory units in abaxial surface	0	1	0	0	1	0	1	1	0
Colleters in adaxial surface	1	0	0	1	0	0	1	0	0
Midrib with bicollateral bundle	1	1	1	1	1	0	1	1	0
Midrib with collateral bundle	0	0	0	0	0	1	0	0	1
Midrib with subepidermal collenchyma in abaxial surface	0	0	0	1	1	0	0	1	1
Undifferentiated bundle sheath in bundle of midrib	0	0	0	1	1	1	1	1	1
Endodermis with Casparian strips in bundle of midrib	1	1	1	0	0	0	0	0	0
Bundle of midrib with collenchymatous parenchyma	1	1	1	0	0	0	1	1	1
Homogeneous mesophyll	0	0	0	1	1	1	1	1	1
Dorsiventral mesophyll	1	1	1	0	0	0	0	0	0

1 = presence; 0 = absence; EFNs = extrafloral nectaries; BP = basal portion; MP = medial portion; AP = apical portion.

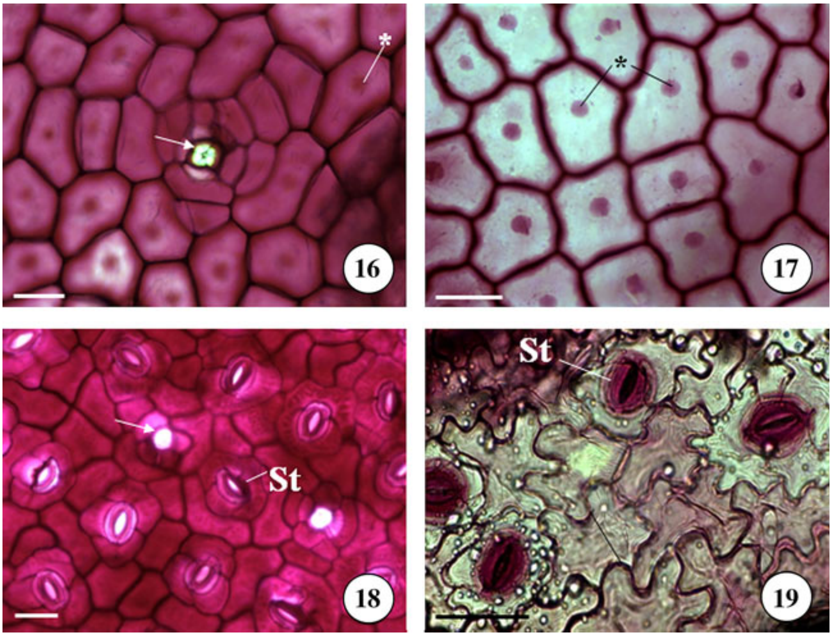
The laminas are dorsiventral with a mesophyll that has three layers of palisade parenchyma in *Calolisianthus pendulus* (Figs 27, 33) but is homogeneous in *C. amplissimus* (Figs 29, 36) and *C. speciosus* (Figs 31, 38). The leaf margin structure varies between the three species: in *Calolisianthus pendulus* (Fig. 28) the leaf margin is rounded and formed from parenchymatous and collenchymatous cells, in *C. amplissimus* (Fig. 30) it is acute, curved, and formed only from collenchyma, and in *C. speciosus* it is curved and formed from collenchyma containing some sclereids (Fig. 32).

TABLE 3. Leaf margin characters of *Calolisianthus pendulus*, *C. amplissimus* and *C. speciosus*

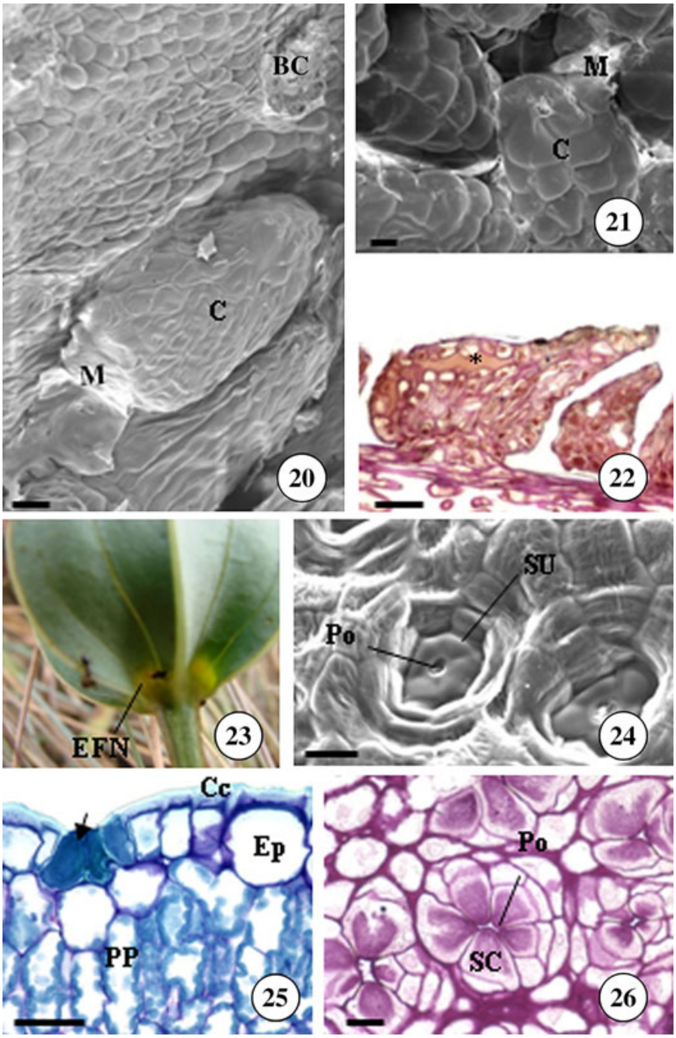
Anatomical leaf margin diagnostic characters	*C. pendulus*	*C. amplissimus*	*C. speciosus*
Acute and curved leaf margin	0	1	1
Rounded leaf margin	1	0	0
Leaf margin with collenchyma	0	1	0
Leaf margin with collenchyma and sclereids	0	0	1
Leaf margin with collenchyma and parenchyma	1	0	0

1 = presence; 0 = absence.

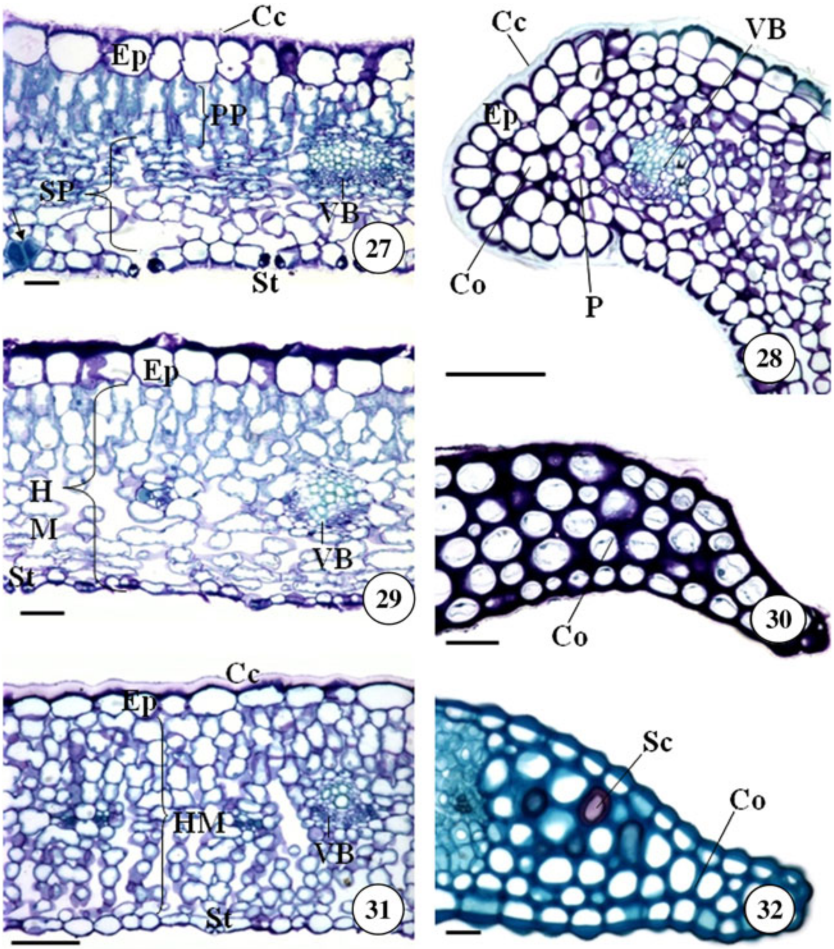
Differences in leaf midrib anatomy have been found between the three species. The vascular bundle of the midrib is bicollateral at the base and in the middle of the leaf (Figs 34, 37, 39) but is always collateral at the leaf apex in all three species. In *Calolisianthus pendulus*, the bicollateral bundle initiates secondary growth (Fig. 34), with an endodermis with Casparian strips around the midrib (Figs 34–35). *Calolisianthus pendulus* (Fig. 34) and *C. speciosus* (Fig. 39) have collenchymatous



FIGS 16–19. Leaf epidermis. 16: Adaxial surface of a *Calolisianthus pendulus* leaf. 17: Adaxial surface of a *C. speciosus* leaf. 18: Abaxial surface of a *C. pendulus* leaf. 19: Adaxial surface of a *C. amplissimus* leaf. Arrow – secretory unit of nectar, * – cuticular ornamentation, St – stomata. Scale bars = 50 μm (16, 17, 18, 19).



FIGS 20–26. Secretory structures. 20: Colleters, by scanning electron microscopy, on the adaxial surface of the leaf base of *Calolisianthus pendulus*. 21–22: Colleters on the adaxial surface of the leaf base of *C. speciosus*: 21: Colleters under scanning electron microscopy. 22: Colleters under light microscopy, stained with Ruthenium Red. 23: Two conspicuous extrafloral nectaries on the abaxial surface of the leaf base of *C. speciosus*. 24: Nectar secretory unit of *C. speciosus* on the abaxial surface of the leaf base, under scanning electron microscopy. 25: Nectar secretory unit (arrow) of *C. pendulus* at the apex of the leaf, adaxial surface. 26: Nectar secretory unit of *C. speciosus* at the base of the leaf, abaxial surface, stained with PAS. C – colleter, M – mucilage, BC – broken colleter, * – mucilage accumulated in apoplastic space of colleter head, EFN – extrafloral nectary, SU – secretory unit, Po – secretory pore, Arrow – nectar secretory unit, Cc – cuticle, Ep – epidermis, PP – palisade parenchyma, SC – secretory cell. Scale bars = 10 μm (20), 50 μm (21, 22), 20 μm (24, 25, 26).

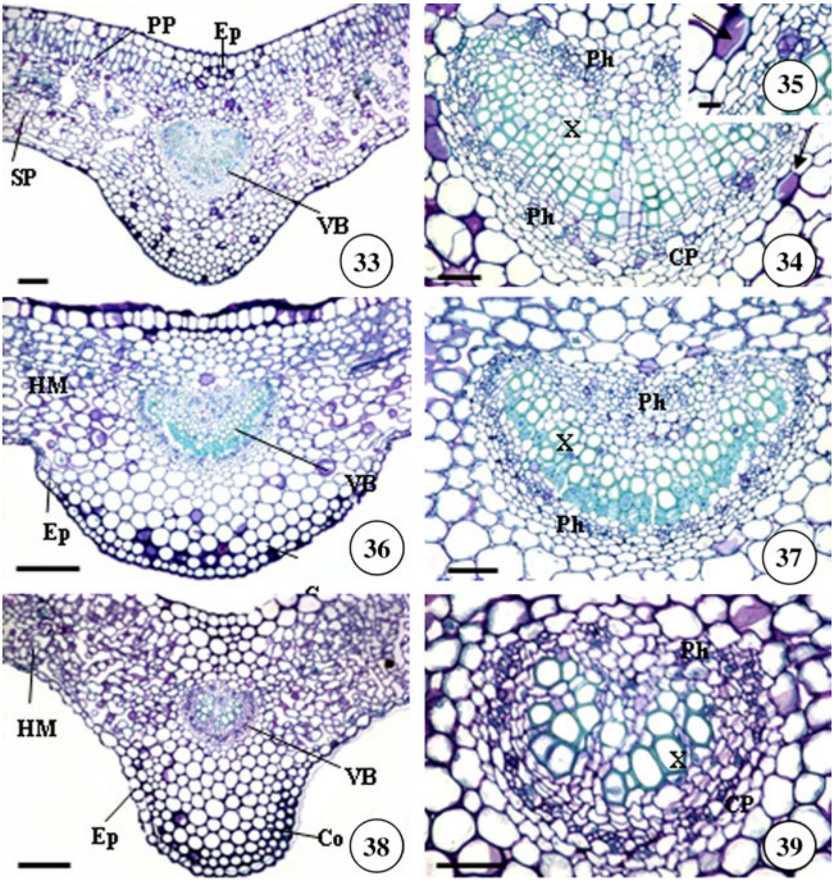


Figs 27–32. Mesophyll and margin. 27: Mesophyll of *Calolisianthus pendulus*. 28: Leaf margin of *C. pendulus*. 29: Mesophyll of *C. amplissimus*. 30: Leaf margin of *C. amplissimus*. 31: Mesophyll of *C. speciosus*. 32: Leaf margin of *C. speciosus*. Cc – cuticle, Ep – epidermis, PP – palisade parenchyma, SP – spongy parenchyma, St – stomata, VB – vascular bundle, Arrow – nectar secretory unit, P – parenchyma, Co – collenchyma, HM – homogeneous mesophyll, Sc – sclereids. Scale bars = 50 μm (27, 29, 30, 32), 150 μm (28), 100 μm (31).

parenchyma associated with the midrib bundle. *Calolisianthus amplissimus* (Fig. 36) and *C. speciosus* (Fig. 38) have subepidermal collenchyma on the abaxial surface.

DISCUSSION

A comparison of the anatomy of vegetative organs in *Calolisianthus pendulus*, *C. speciosus* and *C. amplissimus* shows that *C. amplissimus* and *C. speciosus* share



FIGS 33–39. Leaf midrib. 33–35: *Calolisianthus pendulus*. 36–37: *C. amplissimus*. 38–39: *C. speciosus*. Ep – epidermis, PP – palisade parenchyma, SP – spongy parenchyma, VB – vascular bundle, HM – homogeneous mesophyll, Co – collenchyma, Ph – phloem, X – xylem, CP – collenchymatous parenchyma, Arrow – endodermis. Scale bars = 150 μm (33, 36, 38), 50 μm (34, 37, 39), 20 μm (35).

a great number of characters, although there are also unique characters in each species. *Calolisianthus pendulus* has more unique diagnostic characters than *C. amplissimus* and *C. speciosus*. Some of the characters shared by *Calolisianthus amplissimus* and *C. speciosus* are: leaf midrib with collateral bundle and sub-epidermal collenchyma in the abaxial surface, homogeneous mesophyll, and an acute and curved leaf margin. In *Calolisianthus amplissimus* the diagnostic characters are: roots with tabular epidermis cells, stem epidermis cells with smooth cuticle, presence of fistulous pith in the stem, the lack of EFNs at the base of the leaf, and the leaf margin formed from collenchyma. In *Calolisianthus speciosus* the diagnostic characters are: root cortex with fibres, stem with subepidermal sclereids, and leaf margin formed from parenchyma and sclereids. In *Calolisianthus pendulus* the unique characters are: root cortex with sclereids, stem pericycle with sclereids, leaf epidermis cells with thick external periclinal walls on the adaxial surface, endodermis with Casparian strips around the midrib, dorsiventral mesophyll, and a rounded leaf margin formed from parenchyma.

Some root traits are diagnostic and may be used to identify the species studied, such as the presence of sclereids or fibres or absence of both. Other characters, like the presence of mycorrhizae, may be closely related to ecological conditions and are not diagnostic. Mycorrhizae are found in many *cerrado* herbs (Detmann *et al.*, 2008), these being crucial for increased water absorption in the dry season. Furthermore, the soil of *cerrado* and *campo rupestre* is poor in some nutrients, especially phosphorus (Giulietti *et al.*, 1987; Furley & Ratter, 1988; Furley, 1999), and mycorrhizae can decrease the nutritional stress (Siqueira *et al.*, 2002).

Arum-type arbuscular mycorrhizae (AMs) are found in the parenchymatous cortex of the roots of all three species. *Arum*-type AM consists of hyphae that grow across the cortex apoplast before forming arbuscules within cells (Peterson *et al.*, 2004). Although we observed *Arum*-type AM in *Calolisianthus* species, intracellular hyphal coils that are named *Paris*-type AM have already been described for several Gentianaceae species (Demuth & Weber, 1990).

An important and interesting observation is the absence of hairs on the root epidermis of the three species studied. This absence could be an artefact of the root collection method or be due to the fast secondary growth that induces hair senescence. However, this absence could also be due to the intense mycorrhizal colonisation in roots (Imhof, 1999). These mycorrhizae increase access to water and nutrients, being more efficient than root hairs (Herrman *et al.*, 2004; Peterson *et al.*, 2004).

Anatomical data can contribute to our understanding of adaptive strategies in plants (Dickison, 2000; Matias *et al.*, 2008; Delgado *et al.*, 2009). For example, anatomical xeromorphic characteristics, such as adaptive responses to high luminosity, a stress factor in areas of *campo rupestre* and *cerrado* (Gottsberger & Silberbauer-Gottsberger, 2006), have been identified in stems and leaves of the species studied (e.g. *Calolisianthus pendulus* and *C. speciosus* that were collected in sunny areas). There is a thick cuticle and the epidermal cells are elongated in the stems of *Calolisianthus pendulus* and *C. speciosus*. *Calolisianthus pendulus* occurs in the Ouro Branco Mountain Range, on a windward cliff, where strong winds and high light intensities predominate. *Calolisianthus speciosus* occurs in the Ouro Branco Range in an open field area, consisting mainly of tall grasses that generally shade plants of *C. speciosus* which are shorter than them. *Calolisianthus amplissimus* is found in shaded areas of *cerrado* and has a thinner cuticle, whilst *C. pendulus* and *C. speciosus* that occur in full sun in the Ouro Branco Range have a thicker cuticle. The thickness of the cuticle might be influenced by solar radiation (Ashton & Berlyn, 1992). Since the cuticle can reflect intense sunlight (Morais *et al.*, 2004) thicker cuticles prevent excessive water loss (Sarmiento *et al.*, 1985; Oliveira *et al.*, 2003), thereby avoiding excessive increases in temperature within the mesophyll.

The shape of epidermal cell walls may also be an environmental adaptation in these species. Plants growing in more shaded areas, such as those of *Calolisianthus amplissimus*, have more sinuous epidermal cells, whereas plants that grow in full sunlight have straight epidermal cells on the adaxial surface (*C. speciosus*) or on both leaf surfaces (*C. pendulus*). Straight or curved walls are generally characteristic of species growing in drier or brighter conditions, whereas undulating walls are found in species growing in more humid or shaded conditions (Stace, 1965).

The anatomical study of secretory structures is of fundamental importance for solving taxonomic problems (Solereder, 1908; Metcalfe & Chalk, 1979). For example, some species of Rubiaceae can be distinguished by the arrangement of colleters (Klein *et al.*, 2004). Furthermore, secretory structures can also help in our understanding of phylogenetic relationships between related species (Pascal *et al.*, 2000).

Colleters are found on the adaxial basal part of the leaf of all three *Calolisianthus* species and their presence is a general trait in the Gentianaceae (Struwe & Albert, 2002). Colleters are likely to be associated with protection of vegetative meristems against dehydration and herbivory (Fahn, 1979; Evert, 2006). They are an adaptive strategy to xeric conditions since the secretion of mucilage helps to decrease water loss by cuticle transpiration (Dell, 1977). The environmental conditions found in *cerrado* and *campo rupestre*, where we sampled *Calolisianthus* species, could select for colleters in these species, although these colleters occur throughout the tribe Helieae (Struwe *et al.*, 2002).

Conspicuous extrafloral nectaries on the abaxial surface at the leaf base may be a useful taxonomic attribute, as it is present in *Calolisianthus pendulus* and *C. speciosus* but absent in *C. amplissimus*. Extrafloral nectaries at the abaxial leaf base can easily be seen in the field without a magnifying glass which makes its use practical and fast in field surveys. The EFNs found at the abaxial leaf base of *Calolisianthus pendulus* and *C. speciosus* consist of several small and inconspicuous secretory units called nectarioles which together form a conspicuous nectary. This character was first identified in sepals of *Calolisianthus pendulus* (under the synonym *Irlbachia pendula* (Mart.) Maas) by Vogel (1998). Nectarioles spread throughout both surfaces of the leaf blade and are observed in all three species. At the leaf apex of all three species a protuberance consisting of an isolated EFN formed from several nectarioles can be viewed without a hand lens. This is the first time that these EFNs have been described.

Some mesophyll anatomical characteristics are also taxonomically informative. The consistently dorsiventral mesophyll in *Calolisianthus pendulus* differentiates it from *C. amplissimus* and *C. speciosus*. A bicollateral bundle is always present in all three species. Only *Calolisianthus pendulus* has a sheath with Casparian strips in the midrib bundle, constituting typical endodermis. The presence of an endodermis in leaves of *Calolisianthus pendulus* may be a diagnostic attribute for this species. The leaf margin structure also differentiates the three species. The presence of collenchyma and sclereids in the margin adds stability and support against high winds in open areas (Castro & Menezes, 1995).

Anatomical characters such as subepidermal sclereids and a hollow stem, conspicuous nectaries at the leaf base, an endodermal layer in the midrib bundle of the leaf blade, and the margin structure are all characters that could be useful to distinguish between *Calolisianthus* species and could contribute substantially to taxonomic and phylogenetic studies in the Gentianaceae, especially in the *Symbolanthus* clade. Our results show that the anatomy of vegetative organs is useful for the identification of *Calolisianthus* species and might play a role in elucidating evolutionary relationships in *Calolisianthus*. Further research is necessary.

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