Evolução e desenvolvimento dos órgãos vegetativos aéreos em *Euphorbia* sect. *Brasilienses* V.W. Steinm. & Dorsey (Euphorbiaceae)

Gustavo Arévalo Rodrigues
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Tese apresentada ao Instituto de Pesquisas Ambientais da Secretaria do Meio Ambiente, Infraestrutura e Logística, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Vasculares em Análises Ambientais.

SÃO PAULO

2023

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Dr. Rivete Silva de Lima

Dedico à minha orientadora Dra. Inês Cordeiro, por todo carinho e cumplicidade durante o desenvolvimento desta tese.

Temos raízes como a grande árvore
Que nos conecta aos antepassados
Somos os ramos, somos os frutos, somos as folhas que cantam no ar
Sussurrando as histórias dos que já foram
O que é lembrado, para sempre viverá

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RESUMO

Euphorbia é considerada o maior gênero em número de espécies dentre as angiospermas. Sua diversidade morfológica é impressionante, ocorrendo como ervas, geófitas e árvores, no entanto, sua maior diversidade é em espécies xerófitas. A seção *Brasilienses* (subg. *Euphorbia)* é um grupo monofilético do gênero com espécies de caule suculentos e costados, os quais variam em número e disposição dentre as espécies. Além disso, suas folhas são sempre reduzidas e cedo caducas. O principal objetivo desta tese foi o de descrever o desenvolvimento vegetativo e propriamente caracterizar as estruturas secretoras associadas a estes órgãos em dezesseis espécies do gênero, com o objetivo de descrever a anatomia e morfologia dos caules das espécies dessa seção. Aqui foram usadas técnicas usuais e inovadoras para estudos anatômicos, com o intuito de descrevemos o desenvolvimento e a vascularização caulinar, bem como a micromorfologia, anatomia e histoquímica dos coléteres. Os principais resultados obtidos referem-se a uma nova proposta de interpretação do desenvolvimento das costas caulinares em *Euphorbia*, particularmente nas espécies de *Euphorbia* sect. *Brasilienses*. As costas nessas espécies se desenvolvem a partir do incremento dos tecidos basais foliares, onde uma projeção parenquimática ocorre em cada traço foliar. Logo após o alongamento do entrenó, estes traços foliares são observados como feixes corticais caulinares, constituindo, juntamente com a projeção parenquimática, um componente axial. Esse componente é observado em todas as espécies com caules suculentos, apresentando seis diferentes padrões de vascularização. Coléteres são identificados com quatro morfotipos diferentes, e são encontrados na axila e lâmina foliar, bem como constituindo estípulas. E por fim, o padrão de vascularização dos caules e os tipos de coléteres das espécies de *Euphorbia* sect. *Brasilienses* permitiram propor uma possível sinapomorfia para a seção.

Palavras-chave: adnação foliar; coléteres; estípulas; feixes corticais; parênquima.

ABSTRACT

Euphorbia is considered as one of the biggest genera in number of species in angiosperms. The morphological diversity of the group is outstanding, occurring as herbs, geophytes, and trees, however, they are mostly recognized by their xerophytes species. The sect. *Brasilienses* (subg. *Euphorbia*) is a monophyletic group in the genus, with species having succulent and ribbed stems, which varies in number and disposition among species. Moreover, their leaves usually are reduced and early deciduous. Our primary goal was to describe the vegetative development and properly characterize the secretory structures associated to these organs in sixteen species of the genus. We also provide some possible phylogenetic relationships of the anatomical features between the sect. *Brasilienses* species and their close-related phylogenetically lineages. Here, we use conventional and innovative anatomical techniques to analyze the vascularization of the succulent stems, as well to describe the micromorphology, anatomy and histochemistry of colleters. The key results obtained are related to a new interpretation of the stem ribs development in *Euphorbia*, mainly in species of the sect. *Brasilienses*. The ribs in these species originated from the increment of the basal leaf portion tissues, where a parenchymatic projection occurs in each leaf trace. Soon after the internode elongation, these traces are observed as collateral cortical bundles, and together with the parenchymatic projection compose an axial component. These component is observed in all species with succulent stems, having six distinct vasculature patterns. Colleters are identified with four morphotypes, being found in the axile and foliar lamina, and also composing stipules. Finally, the investigations related to the vascularization allowed us to propose a synapomorphy to the sect. *Brasilienses*.

Keywords: colleters; cortical bundles; foliar adnation; stipules; parenchyma proliferation.

LISTA DE ABREVIATURAS

AP: Amiliferous parenchyma

Eu: Eustele

L: Leaf

LM: Light microscopy

LP: Leaf primordium

LT: Leaf traces

PP: Palisade parenchyma

P: Medullary parenchyma

PL: Spongy parenchyma

SAM: Shoot apical meristem

SEM: Scanning electron microscopy

VB: Vascular bundles

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Introdução Geral

Euphorbia L. é o maior gênero de Euphorbiaceae e está entre os maiores dentre as angiospermas (Frodin 2004), com cerca de 2000 espécies (Dorsey et al. 2013). Sua distribuição geográfica é extensa, entretanto é especialmente diversa em ambientes áridos e semiáridos nos trópicos e subtrópicos de todo o globo (Dorsey et al. 2013), sendo a região Indomalaia seu principal centro de diversidade e o secundário na região neotropical (Govaerts et al. 2000). Apresenta diversas formas de vida, entre elas, geófitas, ervas, arbustos, árvores e muitas espécies xeromórficas suculentas.

Apesar dessa diversidade morfológica vegetativa, sua única sinapomorfia é a presença do ciátio, uma inflorescência do tipo pseudanto, composta por quatro a cinco címulas de flores estaminadas reduzidas a um único estame dispostas em torno de uma flor pistilada central, também reduzida a um único pistilo, sendo esse conjunto protegido por um invólucro com glândulas nectaríferas com ou sem apêndices de morfologia variada (Prenner & Rudall 2007, Horn et al. 2012). No que se refere a sua expressão gênica, o ciátio possui características intermediárias entre uma flor e uma inflorescência (Prenner & Rudall 2007).

Graças a recentes estudos filogenéticos (Steinmann & Porter 2002, Bruyns et al. 2006, Zimmermann et al. 2010, Horn et al. 2012) *Euphorbia* é atualmente o único gênero da tribo Euphorbiineae, destacando-se o trabalho de Steinmann & Porter (2002) que reconheceram para o gênero quatro clados, posteriormente categorizados como subgêneros (Bruyns et al. 2006). As relações filogenéticas entre estes subgêneros foram estabelecidas por Horn et al. (2012), sendo *E.* subg. *Esula* o clado mais basal, seguido de *E.* subg. *Athymalus*, e em seguida do clado formado por *E.* subg. *Chamaesyce* e *E.* subg. *Euphorbia*.

O maior deles é *E.* subg. *Euphorbia* com 661 espécies (Dorsey et al. 2013), que inclui quatro clados de alto suporte filogenético: Novo Mundo (América tropical), Velho Mundo I (Madagascar), Velho Mundo II (África, Arábia e Ásia) e Pacífico (Pacífico Sul), distribuídos em 21 seções (Horn et al. 2012; Dorsey et al. 2013). O clado do Novo Mundo se destaca com 11 seções, entre as quais *E.* sect. *Brasilienses* com cinco espécies suculentas exclusivamente brasileiras distribuídas pela caatinga e campos rupestres (Hurbath et al. 2018): *E. attastoma* var. *attastoma* Rizzini, *E. attastoma* var. *xanthochlora* Rizzini, *E. holochlorina* Rizzini, *E. phosphorea* Mart., *E. sipolisii* N.E.Br. e *E. tetrangularis* Hurbath & Cordeiro. Essas plantas são arbustos com caules suculentos, fotossintetizantes, ramificados, 4-6 costados, com folhas reduzidas e cedo caducas, com glândulas do ciátio providas de apêndices em forma de chifres.

O seu grupo irmão, segundo Dorsey et al. (2013), é *E.* sect. *Stachydium*, com morfologia bastante distinta (Figura 1), constituída por seis espécies: *E. comosa* Vell., *E. gollmeriana*

Klotzsch ex Boiss., *E. heterodoxa* Mull.-Arg., *E. invaginata* Croizat, *E. lagunillarum* Croizat e *E. vervoorstii* Subils. Essas plantas são herbáceas, de caule clorofilado, cilíndrico, ramificado, com folhas sésseis ou pecioladas, decíduas, com exceção de *E. comosa* Vell. que possui folhas perenes; as sinflorescências possuem brácteas bem desenvolvidas e imbricadas entre sí e as glândulas do ciátio não possuem apêndices. As espécies dessa seção ocorrem principalmente no norte da América do sul e nordeste do Brasil, em florestas decíduas e afloramentos rochosos (Dorsey et al. 2013). Embora as características morfológicas vegetativas e florais das seções de *Euphorbia* sejam razoavelmente bem conhecidas, estudos anatômicos são escassos e restritos a poucos grupos desse gênero e mesmo de Euphorbiaceae como um todo. (veja detalhamento adiante).

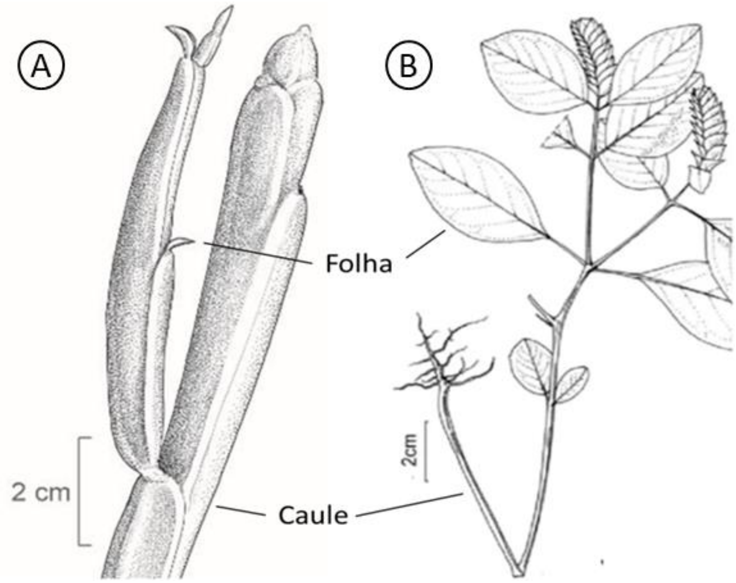

Figura 1: A- *E. tetrangularis* Hurbath & Cordeiro (*E*. sect. *Brasilienses*) e B- *E. comosa* Vell. (*E*. sect. *Stachydium*) . Ilustração adaptada respectivamente de Hurbath et al. (2018) e Carneiro-Torres et al. (2017).

Caules suculentos favoreceram a conquista de diferentes ambientes com restrição hídrica em muitas linhagens de plantas (von Willert et al. 1992), essa inovação evolutiva provavelmente ocorreu em mais de 30 famílias dentre as angiospermas (Jacobsen 1978; Arroyo-Leuenberger et al. 2001; Albers & Meve 2002; Eggli 2002, 2003). A suculência e outras adaptações xeromórficas estão intimamente relacionadas a casos de convergência evolutiva, como entre *Euphorbia* do Velho Mundo e Cactaceae do Novo Mundo (Raven et al. 1986; Futuyma 1997; Niklas 1997; Stearns & Hoekstra 2005; Hearn et al. 2009), que são clássicos exemplos de evolução paralela. Entretanto, os processos de desenvolvimento e evolução destas adaptações são pouco conhecidos, principalmente em grupos com ancestrais não suculentos (Hearn et al. 2009), que reconstruções de estados de caráter mostraram ser o caso de *Euphorbia* (Horn et al. 2012).

Em Euphorbiaceae, análises da estrutura caulinar foram realizadas por Gales & Toma (2006, 2007), Thakun & Patil (2012) e Nassar et al. (2010), enquanto que sobre seu desenvolvimento há os estudos de Swingle (1930) e Hayden & Hayden (1994). Sobre as espécies suculentas de *Euphorbia* há apenas os trabalhos de Trumpke (1913) e Carlquist (1970) que estudaram a anatomia de espécies do Velho Mundo, sendo as do Novo Mundo analisadas unicamente por Steinmann (2001). Apesar da importância dos caules suculentos e das raízes de reserva para a evolução e o sucesso adaptativo de várias famílias em ambientes áridos, segundo Hearn et al. (2009), a maioria dos estudos com estes órgãos refere-se à família Cactaceae. E ainda neste cenário, poucos trabalhos analisaram aspectos particulares da anatomia de órgãos de reserva, como fez Hearn et al. (2009) que relacionou a presença de cordões vasculares ao aumento de tecidos de reserva no gênero *Adenia*, das Passifloraceae, cujas espécies possuem caules suculentos e raízes de reserva. Essa característica anatômica também foi observada por Steinmann (2001) em caules suculentos de espécies neotropicais de *Euphorbia*.

Embora haja descrições do desenvolvimento e vascularização de gemas em plântulas de Euphorbiacae (Raju 1975; Hayden 1988), a relação destas estruturas no desenvolvimento do caule e folhas em *Euphorbia* ainda é desconhecida. Vale ressaltar a importância da caracterização apropriada destas estruturas, como foi feito em Cactaceae, onde a aréola é considerada um ramo lateral modificado (Salgado & Malseth 2002) e uma importante sinapomorfia dessa família. Sua estrutura e desenvolvimento são foco de vários trabalhos (Boke 1941, 1953, 1957; Hamilton 1970; Arruda 2010) e também em outros grupos filogeneticamente relacionados, entretanto as estruturas anatômicas descritas nestes últimos se referem a um estabelecimento precoce da periderme e ainda ao desenvolvimento de estruturas foliares derivadas da gema axilar que podem, segundo os autores, serem homólogas às aréolas de Cactaceae (Hernandes-Lopes 2010).

A estrutura foliar de Euphorbiacae tem sido descrita com ênfase na epiderme (Ndukwu & Okoli 2005; Illohe & Udoh 2012; Bercu & Popoviciu 2015; Kalaskar et al. 2017; Talebi et al. 2017) e em poucos trabalhos na sua vascularização (Sehgal & Paliwal 1973,1974). Swingle (1930) estudou *Euphorbia intisy* Drake, um arbusto do Velho Mundo, cujas folhas reduzidas brotam de emergências do caule, que possuem um único traço vascular, e que ocorrem na maioria dos nós. Embora o autor não tenha observado esta estrutura em todos os indivíduos dessa espécie, ele interpretou que a mesma não se origina a partir de uma gema lateral, mas sim unicamente caulinar.

Estudos anteriores em *Euphorbia* sect. *Brasilienses* evidenciaram cordões vasculares incomuns em sua estrutura caulinar (Steinmann 2001; Hurbath 2018). Tais cordões foram interpretados por Steinmann (2001) como responsáveis pelo transporte de foto-assimilados da região clorenquimática para o câmbio vascular e também pela sustentação nos tecidos de armazenamento de água. Além desta estrutura vascular única, Steinmann (2001) destacou no caule a presença de fibras semelhantes às encontradas no floema, mas dispersas por todo o córtex, grandes câmaras subestomáticas e diferentes tricomas em sua epiderme, que não puderam ser devidamente caracterizados.

Além dos tecidos de reserva, estruturas secretoras ou glândulas também são importantes na conquista de ambientes com restrição hídrica, como p. ex. as responsáveis pela produção de mucilagem em espécies xerófitas (Zaman & Padmesh 2009), composto que está relacionado à redução do dano tecidual (Dickinson 2000). Entre as glândulas intimamente relacionadas a tecidos meristemáticos encontram-se os coléteres, que produzem mucilagem e/ou compostos lipofílicos, e em menor quantidade terpenos, proteínas e compostos fenólicos (Fahn 1979). Sua função pode estar relacionada à lubrificação dos órgãos em desenvolvimento (Thomas 1991), bem como sua proteção contra microrganismos e insetos, funcionando como uma barreira química (Miguel et al. 2006; Muravnik & Kostina 2011; Ribeiro et al. 2017). Estas estruturas foram descritas em diversas famílias de dicotiledôneas e se destacam por sua importância taxonômica em diferentes níveis (Thomas 1991).

Coléteres foram identificados em órgãos vegetativos e florais de Euphorbiacae (Vitarelli et al. 2015; Machado et al. 2015; Feio et al. 2016; Gagliardi et al. 2016). Entretanto, há controvérsias quanto a sua caracterização, sendo confundidos com nectários extraflorais ou glândulas de resina (Thomas 1991), como discutido por Vitarelli et al. 2015. Contudo trabalhos acerca destas glândulas em *Euphorbia* são escassos.

Diante da escassez de trabalhos sobre a anatomia caulinar e estruturas secretoras em *Euphorbia* e da disponibilidade de filogenias moleculares no gênero, como as de Dorsey et al. (2013), Horn et al. (2012) e Hurbath et al. (2018), a presente tese analisou a ontogênese caulinar e estruturas glandulares vegetativas em *Euphorbia* sect. *Brasilienses*, em uma abordagem filogenética, como contribuição para o conhecimento deste gênero tão diverso e rico em espécies.

Esta tese analisou a ontogênese caulinar e das estruturas glandulares vegetativas de *E*. sect. *Brasilienses*, de seu grupo irmão sect. *Stachydium*, bem como de outras espécies pertencentes a grupos filogeneticamente relacionados segundo a filogenia de Dorsey et al., (2013), de maneira a responder às seguintes questões:

(1) Como é o desenvolvimento dos órgãos vegetativos aéreos?
(2) Como é a vascularização caulinar durante o desenvolvimento?
(3) Como são as estruturas glandulares vegetativas?
(4) Há diferença na ontogênese caulinar entre as seções?
(5) O tipo de vascularização do caule em *E*. sect. *Brasilienses* é uma sinapomorfia da seção ou é encontrada em outros representantes do clado neotropical de *E*. subg. *Euphorbia*?

O texto desta tese esta organizado em três capítulos. O primeiro capítulo contempla o artigo que descreve a ontogênese caulinar de duas espécies da sect. *Brasilienses*, o segundo corresponde ao estudo comparativo da morfologia e anatomia da suculência caulinar. O terceiro capítulo reporta detalhadamente o primeiro estudo comparado de coléteres em *Euphorbia*.

Capítulo I

Adnate Leaf-Base and the Origin of Ribs in Succulent Stems of *Euphorbia* L.

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Article

Adnate Leaf-Base and the Origin of Ribs in Succulent Stems of *Euphorbia* L.

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Abstract: Stem succulence evolved independently in many plant lineages as an adaptation to arid environments. One of the most interesting cases is the convergence between Cactaceae and *Euphorbia*, which have anatomical adaptations mostly to increase photosynthetic capability and water storage. Our goal was to describe the shoot development in two succulent species of *Euphorbia* using light microscopy coupled with high-resolution X-ray-computed tomography. Collateral cortical bundles were observed associated with the stem ribs in both species. The analysis of vasculature demonstrated that these bundles are, in fact, leaf traces that run axially along a portion of the internode. That structural pattern is due to an ontogenetic alteration. During shoot development, the leaf-bases remain adnate to the stem near the SAM, forming an axial component. When the internode elongates, the leaf bundles stretch as cortical bundles. The meristematic activity associated with the bundles forms the stem ribs, as leaf veins near the node, and induce rib formation along the entire internode even in the portion where the leaf traces join the stele. In addition, heterochronic shifts are also involved in the evolution of the shoot system in these *Euphorbia*, being related to early deciduous reduced leaves and the transference of the main photosynthetic function to the stem. This study demonstrates for the first time the influence of leaf developmental shifts and stem rib formation in *Euphorbia* and sheds new light on the evolution of stem succulence.

Keywords: plant development; vasculature; leaf traces; structure; microtomography; Euphorbiaceae

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1. Introduction

Among the numerous adaptive strategies found in arid environments, stem succulence plays a pivotal role in plant survival. This evolutionary innovation probably occurred in more than 30 families [1–5], as an important convergence which favored the occupation of environments lacking water by distinct angiosperm lineages [6]. This strategy involves organ thickening by means of the expansion of parenchyma (chlorophyll and water or starch storage tissues) in the cortex or pith [6–9]. A most remarkable convergence case is observed comparing cactiform species of *Euphorbia* (Euphorbiaceae) and cacti (Cactaceae) [10–14], but it is not restricted to these two families. Stem succulence based on parenchyma expansion has also been reported for several unrelated genera, such as *Caralluma*, *Duvalia*, *Echidnopsis*, *Hoodia*, *Huernia*, *Larryleachia*, *Pachypodium*, *Plumeria*, *Pseudolithos*, and *Stapelia* in Apocynaceae, *Othonna* and *Senecio* in Asteraceae, *Tylecodon* in Crassulaceae, *Pelargonium* and *Sarcocaulon* in Geraniaceae, *Dorstenia* in Moraceae, and *Cissus* in Vitaceae [1,2,4,5,9,15–17].

Despite the fact that many valuable data on the origin and structure of the succulence are available for leaves of some families [18–20] and stems of Cactaceae [9,21–24], little is known about the succulent stems of *Euphorbia* (Euphorbiaceae). Even though some xeromorphic characters related to succulence are apparently of the same morphological nature, their development and evolutionary shifts are practically unexplored, particularly in groups with non-succulent ancestors [14], such as *Euphorbia* [25]. The need for further studies to determine the origin of succulence in different lineages is reinforced by a study of Mauseth [9], who reported the lack of many features typically considered xeromorphic in succulent stems of 28 species from seven families other than Cactaceae.

Euphorbia L. is the largest genus of Euphorbiaceae and one of the largest within angiosperms [26], with about 2000 species occurring worldwide, especially in arid and semiarid environments in the Tropics [27]. The genus has many life forms, such as herbs, geophytes, trees and shrubs, which encompass the major diversity of succulent species of the genus and are morphologically characterized as xerophytic cactiform species and pencil-stem species [25]. The cactiform species usually have organs modified into thorns and succulent and/or ribbed stems which are photosynthetic, at least in the young parts. The pencil-stem species have similar features, but the stems are thinner and permanently photosynthetic along the entire shoot system [25]. Remarkably, species of *Euphorbia* with those morphologies usually have highly reduced leaves but some other species retained large foliage leaves, decreasing the dependence on the stem photosynthesis, a trait much rarer in Cactaceae [25,28,29].

The subgenus *Euphorbia* is recognized by the most intricate evolution of xeromorphic growth form with five independent origins in the genus [25] and a wide diversity of species found in Africa and Madagascar. Fifteen species occur in the Neotropics [30], whose main monophyletic section is *Euphorbia* sect. *Brasilienses* V.W. Steinm. & Dorsey with five succulent pencil-stem species: *E. attastoma* var. *attastoma* Rizzini, *E. attastoma* var. *xanthochlora* Rizzini, *E. holochlorina* Rizzini, *E. phosphorea* Mart., *E. sipolisii* N.E.Br. and *E. tetrangularis* Hurbath & Cordeiro. These five species are shrubs, usually ramified, with succulent photosynthetic stems with 4-8 ribs, bearing reduced, early deciduous leaves. They are endemic to Brazil, occurring in environments with sandy soils such as "caatinga" (shrubland vegetation common to the arid climate of northeast Brazil) and rocky uplands [31].

Succulent stems usually have abundant water-storage tissue and chlorenchyma [6–8,32–35] and may have vascular modifications since the succulence is recurrently associated with anatomical changes in the vascular tissues [19,20,36–40]. Although the xeromorphic features of the cactiform *Euphorbia* are widely known, anatomical investigations of their stems and early deciduous leaves are lacking.

Among the few anatomical studies performed in the genus, the presence of cortical vascular bundles stands out as one of the striking unexpected features found in some species [9,30,32,41,42], also reported for some Cactaceae [9,43–47]. The main function assigned to these bundles is to improve the transport of photo-assimilates from the chlorenchyma to secondary vascular system and to provide mechanical support to the shoot [30,46,48]. Additionally, Solereder [32] and Metcalfe & Chalk [33] reported a high development of the cortex in succulent stems, mainly due to the activity of marginal meristem. However, its action mechanism remains unclear.

Our study aimed to investigate the development and vascular architecture of pencil stems and reduced leaves in *Euphorbia* in order to provide a model to understand the origin of some xeromorphic features in succulent stems of the genus.

2. Results

Euphorbia attastoma and *E. tetrangularis* are candelabriform shrubs with succulent branches of pencil-stem type. The leaves are reduced and restricted to the apex with early abscission (Figure 1A). Both species have ribbed stems, showing six ribs and spiral phyllotaxis in *E. attastoma* (Figure 1B) and four ribs and alternate, distichous leaves in *E. tetrangularis* (Figure 1C). The ribs are found only in the internodes, arranged in alternate groups of three projections, which coincide with the base of the leaves, which are simple and sessile.

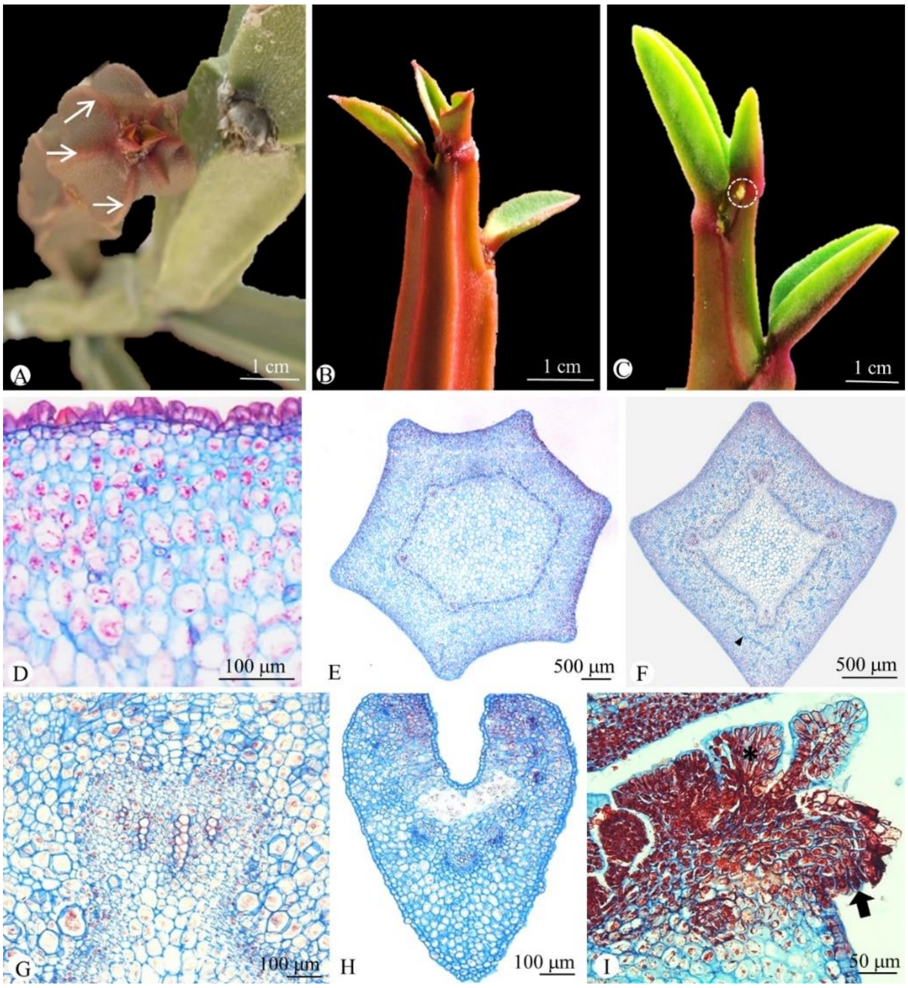

Figure 1. Stem morphology and anatomy of *Euphorbia attastoma* (**A**,**B**,**D**,**E**) and *E. tetrangularis* (**C**,**F**–**I**). Cross sections (**D**,**E**,**F**–**I**). (**A**) Stem ribs in frontal view (arrows) with leaves restricted to the shoot apex. (**B**,**C**) Note the spiral leaves in (**B**) and alternate, distichous leaves with stipules in the leaf base (dashed circle) in (**C**). (**D**) Detail of a rib showing many layers of chlorenchyma and a papillate epidermis. (**E**,**F**) Stem with a prominent vascular bundle in the eustele in each radius of the ribs, numbering six in *E. attastoma* (**E**) and four in *E. tetrangularis* (**F**). Note the numerous branched laticifers (arrowhead in (**F**)). (**G**) Detail of a vascular bundle of the eustele. (**H**) Median portion of the leaf with chlorenchyma and vascular bundles. (**I**) Detail of the leaf axil with colleters (asterisk) and one of the glomeriform stipules (arrow).

2.1. Anatomy

The stem of both species has a uniseriate papillate epidermis with stomata and a cortex particularly thicker in the ribbed region due to the presence of dozens of layers of parenchyma (Figure 1D). The cortex is divided in two regions: an outer cortex formed by chlorenchyma with elongate cells near the epidermis and an inner cortex containing several layers of parenchyma with starch grains. The vascular system is arranged in an eustele of irregular shape (polygonal), whose angles vary according to the number of ribs (Figure 1E,F). Each angle has a large bundle, which is considerably larger than the other bundles (Figure 1G). In addition to the stele, vascular bundles are observed in the cortex, opposite to the ribs, whose number varies according to the number of ribs (Figure 2A–F). Branched laticifers occur throughout parenchyma and vascular bundles (Figure 1F).

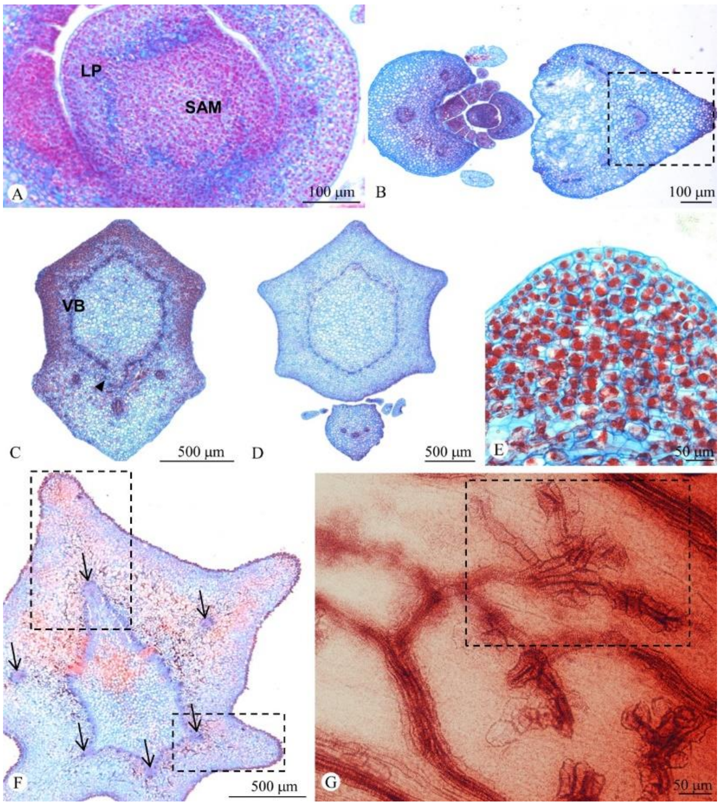

Figure 2. Ontogeny and vasculature of the shoot in *Euphorbia attastoma* (**A**,**C**,**D**,**F**) and *E. tetrangularis* (**B**,**E**,**G**). (**A**) Origin of the leaf primordium (LP) in the flank of the shoot apical meristem (SAM). (**B**) Leaves from distinct nodes in different developmental stages. Note the parenchymatic expansion of the midrib of the leaf (dashed square). (**C**) Nodal region showing three leaf traces associated with their respective ribs and the leaf gap (arrowhead). (**D**) Reduced leaf with three bundles and the ribbed stem. (**E**) Detail of the anticlinal and periclinal division of the ground meristem cells during rib development. (**F**) Divergence of leaf traces from the eustele, evidencing their relationship with the stem ribs (arrows). Note the profuse multiplication of parenchyma in front of each trace, forming the ribs (dashed squares). (**G**) Leaf terminal venation with areoles composed of tracheids (dashed square). VB = vascular bundle.

The leaves are relatively small, varying from 6.7–11 x 2.2–3 mm and have a uniseriate epidermis with stomata and chlorenchyma (Figure 1H). They have three vascular bundles in the base, which subsequently branch in five to nine in the median portion (Figure 1H). Laterally to the base of the leaf blade, a pair of glanduliform stipules with papillate epidermis are formed together with a profusion of sessile colleters, which extend from one stipule to the other along the leaf axil (Figure 2B). These colleters are formed by a secretory palisade epidermis and a non-secretory parenchyma core (Figure 1I).

2.2. Shoot Ontogeny and Vasculature

The leaf primordium originates from the peripheral zone in the flank of shoot apical meristem (SAM). The initiation occurs in regular plastochrons, which vary according to the phyllotaxis (Figure 2A). During the expansion of the leaf primordium, its base remains united to the stem primordium, becoming part of the axis. The subsequent intercalary growth of the stem primordium elongates this adnate region, and the leaf-base is stretched along a portion of the internode, where the leaf traces are observed as stem collateral cortical bundles.

In this second stage of the shoot morphogenesis, the ground meristem in front of the collateral cortical bundles (leaf bundles) generates numerous layers of parenchyma (Figure 2E), which correspond to leaf veins. Ontogenetically, these veins are the ribs of the stem, formed by the cortical bundle and a large amount of parenchyma, which doubles the thickness of the cortex. In regions without ribs, the cortex of both species is 1.5–2 mm thick, but in the rib radius the cortex is about 4–4.5 mm. This relationship of the leaf base as an axial component can also be perceived through the analysis of vasculature (Figures 2 and 3), but it is not restricted to the portion of leaf-base adnation. In fact, since the leaf-base becomes an axial component in the shoot apex, its incorporated vasculature induces the proliferation of the ground meristem basipetally, both in the region with cortical bundles and in the region without bundles, which, actually, corresponds to the most length of the internode.

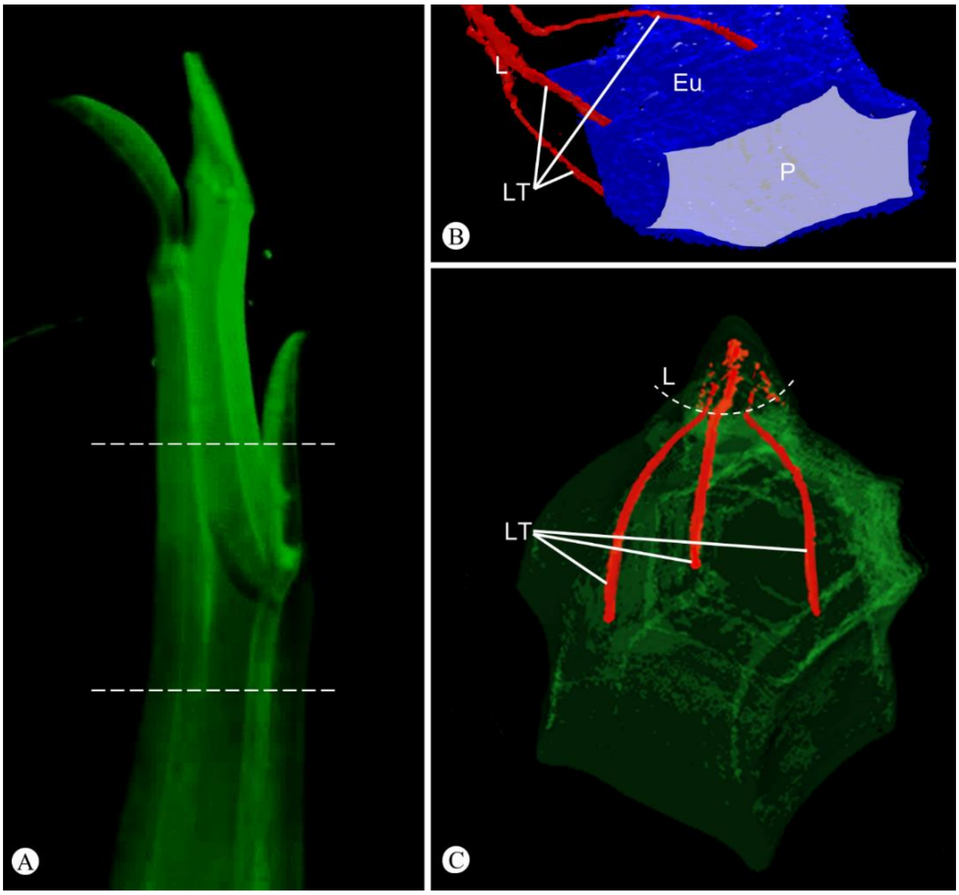

Figure 3. Microtomography with 3D-reconstruction of the shoot of *Euphorbia attastoma*. (**A**) General view of the shoot. (**B**,**C**) Reconstructions of the area enclosed by the dashed lines in (**A**). (**B**) Detail of the leaf traces (LT in red) connected to the eustele (Eu in blue) surrounding the medullary parenchyma (P in gray). Note that the three leaf traces do not branch until reach the free portion of the leaf (L). (**C**) Nodal region showing the leaf traces (LT in red).

Leaf morphogenesis is rapid. The free portion of the leaf, found around 1.5–2 cm above the node, is comprised exclusively of the leaf blade. Early in leaf development, meristems differentiate into mature tissues, stopping the leaf expansion. As a consequence, the leaves remain small and are also early deciduous, being restricted to the apex portion of the shoot. Thus, the photosynthesis of the plant is supplied almost entirely by the stem with adnate leaf-bases.

Shoot vasculature varies according to evolutionary developmental shifts. In the nodal region, three leaf traces diverge from the stele and occupy a peripheral position in the cortex in the region of the stem rib, running parallel to the surface along the internode, profoundly altering the stem morphology. These traces remain unbranched until the base of the free portion of the leaf (Figure 3, Supplementary Material Video1). In the leaf blade, the three bundles ramify, forming secondary and tertiary veins, which terminate with tracheids in the areoles (Figure 2G).

3. Discussion

The present study revealed the origin and nature of the ribs of the succulent stems in two Neotropical species of *Euphorbia* for the first time. Using developmental analysis and 3D-reconstruction techniques, we were able to demonstrate that the leaf-base is congenitally adnate to the stem, as an axial component, and the development of the three main veins of this base induces rib formation along the entire internode of the succulent pencil stems.

Succulent stems are common in *Euphorbia*, exhibiting ribs with reduced, early deciduous leaves. Rudimentary leaves occur in other New World lineages with ribbed stems of the subgenus *Euphorbia*, such as in *E. pteroneura* A. Berger (sect. *Euphorbiastrum* (Klotzsch & Garcke) Boiss.), a Mexican species (sensu Dorsey et al. [27]). Ribbed stems also occur in herbs, particularly in *E.* sect. *Stachydium* Boiss., the sister group of *E.* sect. *Brasilienses*, e.g., *E. heterodoxa* Müll. Arg., an endemic species from rocky outcrops in Northeastern of Brazil. Other succulent lineages within the genus also have rudimentary leaves with succulent ribbed and/or tuberculate stems. Those species belong to the Old World and are currently classified in the subgenus *Euphorbia* and *E.* subg. *Athymalus* Neck. ex Rchb. [27,49]. Given the diversity of species bearing ribbed stems in unrelated lineages, the main common trait is the presence of rudimentary leaves. This fact is certainly involved in the stem organogenesis in *Euphorbia* since the stem ribs are concomitantly produced with the leaf primordium, exhibiting an easily recognizable relationship between both organs.

Restriction of leaf blade development and transference of function to stem, which becomes the main photosynthetic organ of the plant, is usually related to succulent stems in arid environments [9,48,50–53]. The enlargement of the cortex of these stems appears to be related to the expansion of the photosynthetic tissue, in addition to the increment in water and starch storage [9,48,51,54–58].

The cortex may be extremely broad in Cactaceae due to cortical bundles which produce intrafascicular secondary phloem and xylem in many species [9,48,50]. On the other hand, we did not observe any expansion of the stem tissues in *Euphorbia* due to vascular proliferation of the cortical bundles. Actually, the non-separation of the leaf-base from the SAM flank during the leaf primordium development seems to be the reason for the succulence of the pencil stem in the genus. The maintenance of the leaf-base forming an axial component has already been reported for plants of other families [59].

Fusion of the leaf adaxial side to the stem is not an uncommon process for bud protection in xerophytes since it reduces the water loss to the environment. This process usually involves expansion of the leaf-base. In succulent species of Aizoaceae and Amaranthaceae, a large expansion of the leaf-base around the SAM and a reduction in the leaf blade have previously been reported [18,20,60]. This foliar characteristic appears to have evolved multiple times in these two families, coinciding with the aridification of southern Africa in the Late Miocene [60–62]. However, there is no fusion of the leaf-base to the stem, as observed in *Euphorbia*. Conversely, leaf fusion may be total in other lineages. In Podostemaceae, the leaf-base is completely united to the promeristem, originating shoots apparently devoid of SAM in the subfamily Podostemoideae [63].

In general, main xeromorphic features of the succulent stems are the increase in parenchyma tissue of the cortex or pith, reduction of leaf size and number, and establishment of mechanisms for water protection and its storage [9]. Regardless of the origin of expanded cortical tissues of the succulent stems, the enlargement is associated with the presence of cortical bundles. The emergence of cortical bundles was a key innovation in the evolution of Cactoideae (Cactaceae), which allow some species to develop cortices up to 30 cm thick keeping all their tissues hydrated [48,64]. If the cortex is unvascularized, the slow transport of water by diffusion appears to limit the increase in cortex thickness [55,64].

Extrastelar vascular bundles, such as the collateral cortical bundles (leaf traces) observed in the Neotropical *Euphorbia* sect. *Brasilienses*, have also been reported for *E. weberbaueri* Mansf. (*E.* sect. *Euphorbiastrum*) (Klotzsch & Garcke) Boiss. [30], another Neotropical species with ribs very similar to those described herein, and also for African species as *Euphorbia fortuita* A.C. White, R.A. Dyer & B. Sloane, *Euphorbia horrida* Boiss., *Euphorbia obesa* Hook. F. and *Euphorbia officinarum* L. [9]. Those additional bundles have been reported for 55 eudicot families, extending along the internodes, as cortical bundles or medullary bundles [33]. In the case of cortical bundles, Howard [65] observed that their relationship with the leaves varies depending on the group analyzed and can be found in the stem (1) unrelated to the leaf vasculature, (2) partially related to leaf vasculature or (3) completely related to leaf vasculature. This third condition is the one discovered here in *Euphorbia attastoma* and *E. tetrangularis*. This variation indicates that the mere occurrence of cortical bundles in the stem does not necessarily imply adnation of the leaf to the stem. The main example is the cacti, whose extensive set of cortical bundles, observed in addition to the leaf/bud traces, are truly cauline, derived from procambial cells originated in the cortical area near the shoot apical meristem [44,48,64].

Despite the fact that the origin of ribbed stems has not been investigated in other species of *Euphorbia*, further anatomical studies may reveal similarities in relation to our results, indicating the likely evolution of this character in other clades of the genus. Mauseth [9] found that the leaf traces are related to the stem ribs in nine species of *Euphorbia*. If this relationship exists between this type of stem succulence and leaf traces in other species, we can assume that the number of ribs and their disposition depends on the phyllotaxis and plastochron. This hypothesis is supported by the difference observed between *E. attastoma* (six ribs) and *E. tetrangularis* (four ribs). This is related to the three leaf traces that diverge from the eustele in different times during development, indicating a case of heterochrony in closely phylogenetically related species. The ribs are surely associated with the stem thickness, allowing the candelabriform morphology observed in *E.* sect. *Brasilienses* and seems to be important to accommodate seasonal expansion and contraction of stem [66].

The evolutionarily shift of plants from one form into another involves modifications of the developmental patterns [48]. In this study, we verified that during the initial development of leaf primordia, the leaf-base remains adnate to the developing stem in *Euphorbia*. This congenital fusion of tissues displaces the orientation of the leaf traces, which are oblique/horizontal and run directly to the leaf-base at the nodes in the vast majority of angiosperms [65], to run roughly parallel to the surface of the stem along a short portion of the internode (Figure 4).

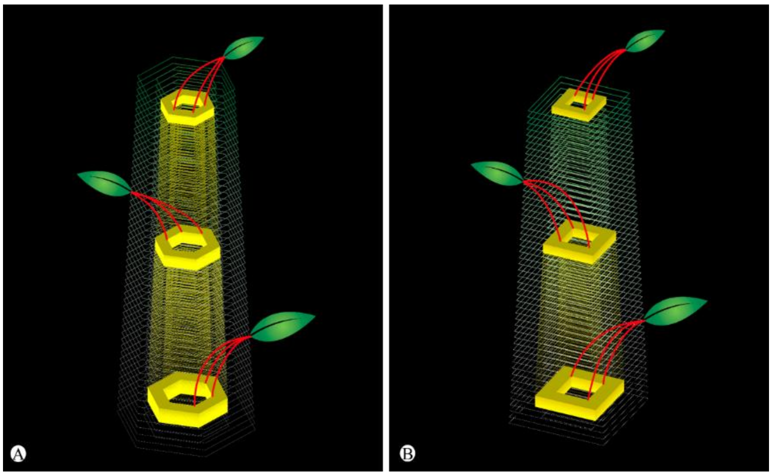

Figure 4. Diagrams showing the leaf traces diverging from stele in *Euphorbia attastoma* (**A**) and *E. tetrangularis* (**B**). Note that the leaf traces (red) diverge in groups of three in both species.

Fusion of the leaf-base to the stem is not an uncommon event in angiosperms [67], but the developmental process observed in *Euphorbia* appears to be an evolutionary novelty and is described for the first time in this study. Despite the fact that the general leaf development is well known and widely analyzed in textbooks and articles, the origin of the stem is often neglected. After the formation of the leaf primordia in the shoot apex and the establishment of the nodal regions, there is an elongation of the internode by multiplication and elongation of the cells of the primary meristems [65]. This second stage of the stem morphogenesis has been altered in the *Euphorbia* pencil stem. When the internode elongates, the leaf-base united to the stem also elongates (Figure 5), stretching the leaf traces along the internode. Consequently, the leaf is "displaced" to another region above its point of origin (Figure 4).

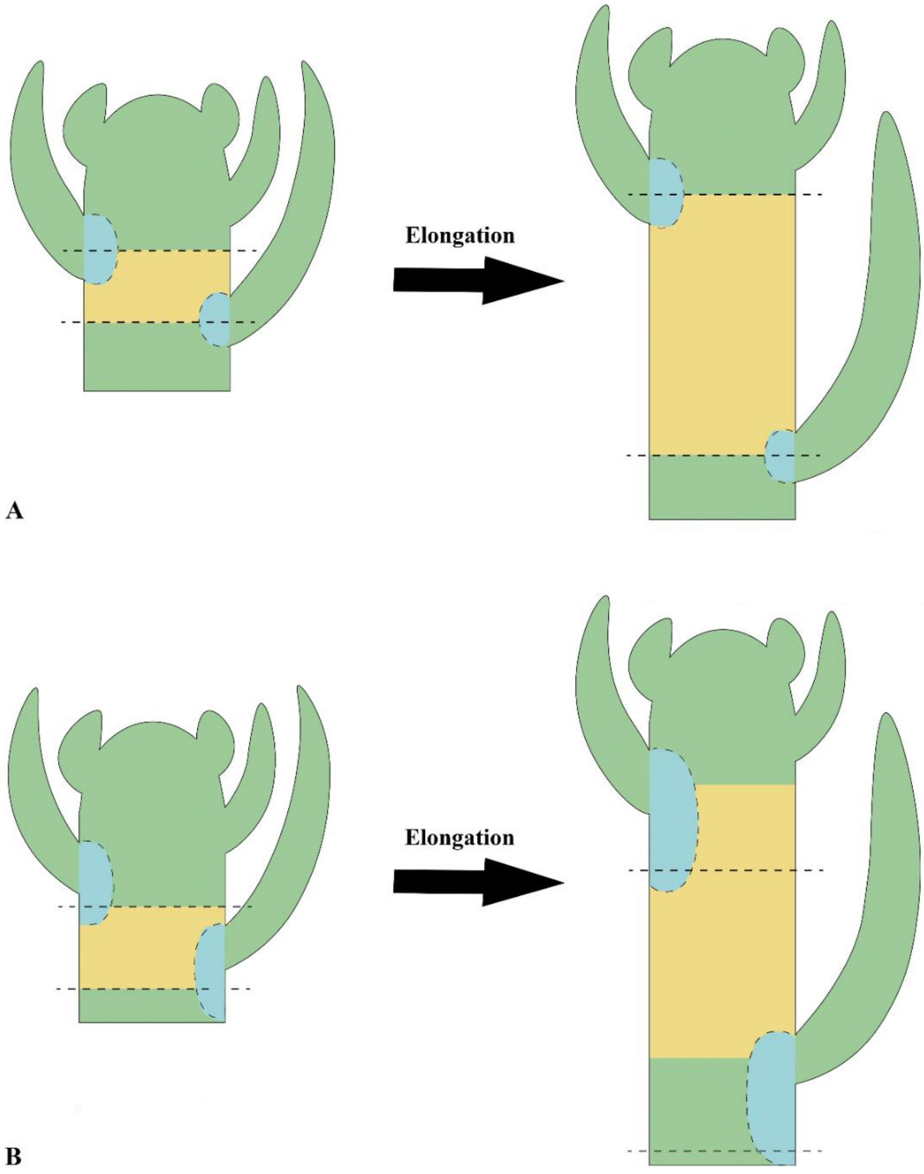

Figure 5. Schematic drawings of two stages of shoot development. (**A**) Regular shoot development. Note that the leaf-base participation in the axis is restricted to its point of origin at the node, even after the internode elongation. (**B**) Shoot of *Euphorbia attastoma* and *E. tetrangularis*. Note that adnate leaf-base forms an axial component, displacing the leaf to a region above the node. The fused portion elongates along with the internode. The origins of axial tissues were colored in one plastochron. Dashed line: node; blue: leaf-base component; yellow: cauline component; green: indiscriminant components.

The increment of foliar tissues into the stem considerably expanded the cortex, increasing its photosynthetic capacity (Figure 5). Additionally, there is an early maturation of the leaf meristems, interrupting the leaf blade development and producing very reduced leaves. This type of developmental shift of the tissue differentiation timing is known as heterochrony and has been shown to be one of the main processes in plant evolution [68]. In addition, some heterochronic changes may lead to transfer of function (heterotopy) due to spatial relationships that change over time [69]. This can be observed in the cactiform species of *Euphorbia*, whose stem becomes the main photosynthetic organ of the plant due to the early leaf abscission.

Despite our observation that cortical bundles (leaf traces) elongate for a short region of the stem above the node, these vascular bundles undoubtedly stimulate parenchyma proliferation, forming the ribs continuously along the entire internode. To date, it is not possible to infer which endogenous signal is responsible for this change in stem tissues. However, previous studies show that the hormonal stimulus responsible for the differentiation of procambium, and consequently the vascular bundles in shoots, is the auxin. Polar auxin gradients induce procambium differentiation along the path of its flow and may regulate vascular adaptation to the plant's environment [70,71]. Thus, if auxin is related to the differentiation of cortical bundles, we can hypothesize that auxin is also related to rib differentiation regardless of the presence of cortical bundles from one node to the other, deviating from the expected transport pathway due to the adnation of the leaf base to the stem. Further immunocytochemical studies are needed to definitively verify this hypothesis and clarify the physiological regulation of succulence evolution in *Euphorbia*.

4. Materials and Methods

For this study two species of *E.* sect. *Brasilienses* were selected: *E. attastoma* (Hurbath 853, 854) and *E. tetrangularis* (Hurbath 844). The samples were collected from three individuals of each species cultivated in glasshouse at Instituto de Biociências at the Universidade de São Paulo. Vouchers of the species were deposited at SP Herbarium.

Shoots were fixed in FAA for 24 h (formalin, acetic acid, 50% ethanol 1:1:18 *v:v*) [72] and then stored in 70% ethanol. Entire leaves were first cleared using 100% ethanol, then treated with 10% sodium hydroxide for 2 h, followed by 5% sodium hypochlorite. The leaves were stained with 1% safranin and mounted in Kaiser's glycerin gelatin [73]. For anatomical analyses, shoot apices were isolated, dehydrated in a butyl series [72], embedded in Paraplast® (Leica Microsystems, Wetzlar, Germany) and transversely or longitudinally sectioned using a Leica RM2145 rotary microtome. Sections 12 μm thick were stained with astra blue and safranin [74], and the slides were mounted in Permount® (Fisher Scientific, Pittsburgh, PA, USA). The photomicrographs were taken using a Leica DMLB light microscope coupled with a digital camera.

For high-resolution X-ray-computed tomography (HRXCT), we used the fixed shoots of *E. attastoma*, which were treated with phosphotungstic acid in 70% ethanol for one week. Subsequently, the samples were dehydrated in an ascending ethyl series containing 1% phosphotungstic acid (1:1; *v:v*) and wrapped with parafilm in a tube filled with 100% ethanol. Finally, the samples were scanned using a SkyScan 1176 microtomograph (Bruker, Billerica, MA, USA). The exposure time was approximately 4 h per sample and for the 3D reconstruction we used the software CTVox and CTVol, 3D Doctor (Able Software Corp., Lexington, KY, USA) and 3D Slicer. Image sequences and the video (Supplementary Material) were segmented automatically and manually.

The vasculature diagrams were made based on the microscopic observations and the high-resolution X-ray-computed tomography, using the software Cinema 4D (Friedrichsdorf, Hesse, Germany) and Adobe Photoshop (San Jose, CA, USA).

5. Conclusions

We demonstrate for the first time that the succulence of the pencil stem in *Euphorbia* is due to the incorporation of the leaf-base as an axial component during shoot development. This joining of tissues doubles the thickness of the cortex of stem and its amount of photosynthetic tissue. The elongation of the internode joined to the leaf-base transforms the leaf traces into cortical bundles that are associated with proliferation of the parenchyma, forming the stem ribs. A likely hormonal signal from the leaf is transported basipetally along the entire internode, inducing rib formation regardless of the presence of cortical bundles. Heterochronic shifts are also involved in the evolution of the very small leaves of *Euphorbia*, their early abscission and the transference of the main photosynthetic function to the stem. A major sampling is needed to evaluate the specific role of these features in the adaptive success of the *Euphorbia* and its diversification in arid environments.

Supplementary Materials: The following supporting information can be downloaded https:

//www.mdpi.com/article/10.3390/plants11081076/s1, Video S1: Microtomographic reconstruction of the shoot of *Euphorbia attastoma*.

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Capítulo II

Stem succulence diversity in *Euphorbia* L.

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ABSTRACT

Succulence has an outstanding morphological diversity and occur in many unrelated lineages of plants. Here, our main focus was to report the relationship of ribs and the leaf trace/cortical bundles in the succulent stems of *Euphorbia*, as well as to identify some evolutionary transitions of xeromorphic features. Conventional developing anatomical techniques were used in the shoot apices of sixteen succulent species in order to describe their main morphological and anatomical features. The leaf base constitutes an axial component in the stem of all succulent *Euphorbia*. The elongation of the adnate-leaf base in the species of the sect. *Brasilienses* occurs by means of anticlinal divisions that poorly occur in the other succulent species. The vascularization found in *E.* sect. *Brasilienses* is an essential model to understand the other pencil-stems of *Euphorbia*. The stem vasculature diversity in the genus is remarkable and has six unprecedent types. Ribs composed by three leaf traces/ cortical bundles with only one parenchymatic projection were described here for the first time in the cactiform species of *E.* subg. *Euphorbia* of the Old World and *E.* subg. *Athymalus* and could be a notable feature of these habit in the genus. Alternate and distichous leaves, together with a 6-ribbed pencil stem are highly affected by the prolonged plastochron, which are exclusive of *E. holochlorina*. Overall, ribbed stems are the most common type of pencil stem, being certainly homoplastic in the genus.

Keywords: anatomy – sect. *Brasilienses* – ribs – rudimentary leaves – vasculature

INTRODUCTION

Succulent plants occur in at least 12,000 species from about 690 genera in 83 families (Nyffeler and Eggli, 2010). The morphological similarity observed between the cactiform *Euphorbia* species (Euphorbiaceae), and cacti (Cactaceae) is considered as one of the most interesting cases of succulence convergence in flowering plants (Raven et al., 1986; Futuyma, 1997; Niklas, 1997; Stearn and Hoekstra, 2005; Hearn, 2009). Succulence occurs in species of many morphological forms (Evans et al., 2014), being intimately associated to the portion of the organ, the location and nature of water-stored tissues (Jürgens, 1990). Water-stored tissues could occur with all cells having chlorophyl in order to improve the photosynthetic load (Grace, 2019), or having a non-photosynthetic portion particular to water-storage known as hydrenchyma (Lüttge, 2008). These water-storage tissues are considered as an evolutionary innovation to plant survival in water restriction environments (Grace, 2019).

Euphorbia is one of the largest genera among the flowering plants with almost global distribution (Frodin, 2004). The genus is especially found in arid and semi-arid regions, comprising herbs, geophytes, trees, shrubs, and an impressive diversity of succulent plants (Dorsey et al., 2013). Succulent species occur in Old and New World lineages of the four recognized subgenera in *Euphorbia* (see Yang et al., 2012; Riina et al., 2013). The subgenus *Euphorbia* and *Athymalus* are known to have the major variety of succulent species (Peirson et al., 2013), being the first widely regarded as with the most complex evolution of xeromorphic growth form (Horn et al., 2012).

Succulent stems in *Euphorbia* have been referred as terete, pencil-stem, spiny/cactiform, ribbed, medusoid and tuberculate (Peirson et al., 2013; Horn et al., 2012; Dorsey et al., 2013). According to Horn et al., 2012, the pencil-stemmed species generally possess an entire photosynthetic shoot, while the cactiform are mainly photosynthetic at the young parts. The medusoid and tuberculate succulent stem types always are highly swollen (see Peirson et al., 2013), which strongly modify the stem morphology. In general, succulent stems of other families lack usual xeromorphic features found in the stems of cacti (Mauseth, 2004), and little is known regarding the development and anatomical features of those succulent stems.

In *Euphorbia* the succulent ribbed stems are linked to the proliferation of parenchymatic tissue due to the distinct leaf development during internode elongation (Arévalo-Rodrigues et al., 2022). Indeed, a marginal meristem seems to be connected to the high development of cortex in succulent plants (Solereder, 1908; Metcalfe and Chalk, 1950), and albeit a parenchymatic proliferation has been reported in the succulent stems of many families (Mauseth and Kiesling, 1997; Mauseth, 2000; Anderson, 2001; Terrazas and Mauseth, 2002), the mechanisms related to parenchymatic development in succulent stems are still obscure (Arévalo-Rodrigues et al., 2022).

In two Brazilian species of *Euphorbia* the stem axial component is also constituted by a leaf basal portion, during development part of the leaf primordium remains united to the stem (see Arévalo-Rodrigues et al., 2022). Leaves essentially occur in the succulent species of the genus, being in some cases quickly deciduous, while in other species large foliage are kept during maturity (Horn et al., 2012), while in cacti the occurrence of leaves is considered rare (Cactaceae; Edwards et al., 2005; Hernández-Hernández et al., 2011; Horn et al., 2012). The relationship of leaves in the increment of the succulent stem tissues are still poorly understood in both groups (Mauseth, 2004; Arévalo-Rodrigues et al., 2022).

In *Euphorbia* the succulent stems have collateral cortical bundles that are in fact leaf traces in the internode due to an ontogenetic alteration (Arévalo-Rodrigues et al., 2022). Cortical vascular bundles have been reported in other species of the genus as well (Kniep, 1905; Solereder, 1908; Trumpke, 1914; Steinmann, 2001; Mauseth, 2004). These bundles may add some advantages in the transport of photo-assimilates from the chlorenchyma to secondary vascular system and also to provide mechanical support to the stem (Mauseth and Sajeva, 1992; Steinmann, 2001; Schwager et al., 2015). Moreover, each rib possesses its respective leaf trace/cortical vascular bundle, which are connected to the phyllotaxis and plastochron in *Euphorbia* (Arévalo-Rodrigues et al., 2022). The relationship of these leaf traces to the stem ribs has already been mentioned by Mauseth, (2004) in nine species of *Euphorbia*, but a comparative study considering the main xeromorphic lineages are needed in order to clarify the evolutionary patters of succulent stems in the genus.

Here, we aimed to describe the relationship of ribs and the leaf trace/cortical bundles in the succulent stems of *Euphorbia*, as well as to identify some evolutionary transitions of xeromorphic features in succulent stems of the group. So, we selected thirteen species representing the variety of succulent stem type found in the subgenus *Euphorbia*, in addition three species of other subgenera as an outgroup.

MATERIAL AND METHODS

Shoot apices of sixteen species of *Euphorbia* were collected from cultivated plants at Instituto de Biociências at the Universidade de São Paulo and Instituto de Pesquisas Ambientais (Table 1). Vouchers of the species were deposited at SP and SPF Herbarium.

Table 1. List of *Euphorbia* species selected for this study with provenance and voucher numbers. They are listed following the lineages currently recognized in the genus (Horn et al., 2013).

Species	**Collection site (Brazil)**	**Voucher**
Euphorbia subg. *Euphorbia*		
sect. *Brasilienses*		
E. attastoma Rizzini	Universidade de São Paulo, SP (Garden)	Hurbath 824; Hurbath 837 (SP)

E. phosphorea Mart.	Universidade de São Paulo, SP (Garden)	Hurbath 629; Hurbath 645; Hurbath 654 (SP)
E. sipolisii N.E.Br	Universidade de São Paulo, SP (Garden)	Hurbath 828 (SP)
E. holochlorina Rizzini	Universidade de São Paulo, SP (Garden)	Hurbath 835; Hurbath 834 (SP)
E. tetrangularis Hurbath & Cordeiro	Universidade de São Paulo, SP (Garden)	Hurbath 839 (SP)
sect. *Stachydium*		
E. heterodoxa Müll.Arg.	Universidade de São Paulo, SP (Garden)	França 1940 (SP)
sect. *Crepidaria*		
E. tithymaloides L.	Universidade de São Paulo, SP (Garden)	Cordeiro 3585 (SP)
E. peperomioides Boiss.	Campos do Jordão, SP	Cordeiro 3331 (SP)
sect. *Euphorbiastrum*		
E. pteroneura A. Berger	Universidade de São Paulo, SP (garden)	Pirani 6621 (SPF)
sect. *Monadenium*		
E. guentheri (Pax) Bruyns	Instituto de Pesquisas Ambientais, SP (collection)	
sect. *Euphorbia*		
E. trigona Haw.	Instituto de Pesquisas Ambientais, SP (collection)	
sect. *Deuterocalli*		
E. alluaudi Drake	Universidade de São Paulo, SP (garden)	Pirani 6722 (SPF)
sect. Tirucalli		
E. tirucalli L.	Universidade de São Paulo, SP (garden)	D.P.A. Paz 1 (SPF)
subg. Chamaesyce		
E. sobolifera Silva & Braun	Universidade de São Paulo, SP (garden)	Brito, BG 11 (SP)
E. appariciana Rizzini	Universidade de São Paulo, SP (Garden)	Carneiro, 53 (SP)
subg. Athymalus		
E. ferox Marloth	Instituto de Pesquisas Ambientais, SP (collection)	

The material was fixed in FAA (formalin-acetic acid-alcohol) for 24h (Johansen, 1940), in BNF (buffered neutral formalin) for 48 h (Lillie, 1965), and in Karnovsky solution for 24h (Karnosvky, 1965, modified by Kraus and Arduin, 1997). The samples were washed in the same buffer in which they were prepared, dehydrated in an ethanol series, and stored in 70% ethanol.

The stored material (at least three shoot apices) was dehydrated, embedded in Historesin® (Technovit 7100, Heraus Kulzer, Germany) or isolated, dehydrated in a butyl series, embedded in Paraplast® (Leica Microsystems, Wetzlar, Germany; Johansen, 1940) and transversely or longitudinally sectioned at 7-12 mm thickness using a rotary microtome. Sections prepared with resin were stained with toluidine blue O (Sakai, 1973) and mounted in water, while those prepared with Paraplast were stained with safranin and astra blue (Gerlach, 1969). Both slices were mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA), while some sections were stained in 0.01% calcofluor white for 10 min for cellulose identification, rinse shortly in distilled water and mounted in water (Demarco, 2022).

The sections stained by calcofluor white were analyzed using a Leica DMLB fluorescence microscopy under UV light. In general, the observations and digital images were acquired using the light microscope coupled with a digital camera.

The vasculature diagrams were manually made using the software Adobe Illustrator 23.02, while the photo boards were built using Adobe Photoshop (San Jose, CA, USA).

Stem succulence diversity

The phylogenetic tree here presented was manually assembled using the Adobe Illustrator version 23.0.2 (San Jose, CA, USA). and the investigated species were plotted according to the main lineages recognized in *Euphorbia* (Horn et al., 2012).

RESULTS

Morphology

The studied species are shrubs and subshrubs and possess succulent stems with early caducous, simple, and sessile/subsessile leaves restricted to the shoot apex (Table 2; Fig. 1), except *E. peperomioides* that have leaves entire and perennial and also a non-succulent stem. The phyllotaxis is alternate and spirally arranged in *E. attastoma*, *E. phosphorea*, *E. heterodoxa*, *E. pteroneura*, *E. guentheri*, *E. trigona* and *E. alluaudii*, alternate and distichous in *E. tetrangularis*, *E. holochlorina*, *E. sipolisii*, *E. tithymaloides*, *E. tirucalli*, *E. ferox*, *E. sobolifera* and *E. appariciana*, being opposite in *E. peperomioides*.

Except *E. peperomioides* that is an herb, all species have succulent stems (see Table 2). Ribbed stems are mostly identified and varies in number and arrangement within species (Table 2; Fig. 1A-D). The most ribbed in the sect. *Brasilienses* is *E. phosphorea* with six to eight ribs (Fig. 1A), while in the other lineages the least ribbed is *E. trigona* with three ribs (Fig. 1C) and the most one with twelve ribs is *E. ferox* (Fig. 1D). In *E. tithymaloides*, *E. alluaudii*, *E. tirucalli* the ribs are absent (Fig. 1E). In *E. guentheri* the stem possess conical tubercles throughout the organ and the leaves are found at the apex of these structures (Fig. 1B). Modified branches comprising spines solely occur in *E. ferox* (Fig. 1D).

Anatomy

All species have the stem with the epidermis constituted by a uniseriate epidermis (Fig. 2A). The cortex has many layers of chlorenchyma particularly in the ribbed region (Fig. 2B), being the pith composed by an amiliferous parenchyma. In *E. peperomioides*, *E. tithymaloides*, *E. alluaudi*, *E. tirucalli*, the stems are terete and shows a circular shape in cross-section (Fig. 1F), with the chlorenchyma homogeneous all around the organ.

The eustele has an irregular shape (polygonal) and diversify according to the number of ribs in all the species of sect. *Brasilienses*, *E. heterodoxa*, *E. pteroneura*, *E. sobolifera* and *E. appariciana* (Fig. 2B; Table 2). Each rib possesses respectively a large bundle in the eustele. On the other hand, in *E. trigona* and *E. ferox* each rib is entirely vascularized by three leaf traces and one branch trace (Fig. 2D–E). In *E. guentheri* each tubercles are vascularized by 5 to 8 leaf traces (Fig. 2F). The three latter species the eustele is circular (Fig. 2D).

The leaves mostly possess a uniseriate epidermis and chlorenchyma homogeneous, while in *E. heterodoxa*, *E. peperomioides* and *E. appariciana* the leaf is notably dorsiventral (Fig. 2G). Laticifers occur in the mesophyll and around the vascular bundles (Fig. 2H). In all species the leaves are vascularized by three leaf traces, which ramifies in five to six in the median region. (Fig. 2G).

Ontogeny and vascularization

The leaf primordium develops laterally by diffuse cellular divisions in the shoot apical meristem and are strongly connected to the phyllotaxis and plastochron of each analyzed species. The increment of the marginal meristem in the leaves of *E. heterodoxa, E. peperomioides, E. tithymaloides* and *E. trigona* remains active much longer when compared to species with early caducous and rudimentary leaves in the maturity. In all species the midrib stablishes earlier when considered the leaf lateral bundles, being the latter usually constituted by five to six bundles in the median portion of the leaf blade (Fig. 3A–B).

During leaf expansion their constitutive base remains united to the stem, forming an axial component in the succulent species (Fig. 3C). After the establishment of the nodal regions, the axial component composed by the adnate leaf base, extends by virtue of anticlinal divisions basipetally in the cortex during intercalary growth in all the succulent species (Figs. 3D–E). Anticlinal divisions are mostly noted in all species with ribbed stem of the sect. *Brasilienses* (Fig. 3D), which poorly occur in the other species, such as *E. alluaudi* (Fig. 3E). Collateral cortical bundles occur in the stem of all the succulent species after internode elongation (Fig. 3F, 4A).

Concomitantly to the intercalary growth, the ground meristem also proliferates centrifugally in the same radius of the median leaf trace in the developing stem of some species with ribs and tubercles (Fig. 4A). For instance, in *E. trigona, E. guentheri* and *E. ferox*, the group of three leaf traces possess only one parenchyma proliferation (Fig. 4B). In general, each rib/tubercle are respectively constituted by leaf traces/cortical bundles and a great portion of parenchymatic tissue (Figs. 4A–B). Sections just above the internode demonstrate that parenchyma proliferation can also be perceived in regions without bundles particularly in the ribbed stems (Fig. 4C). Periclinal divisions mostly occur in these parenchymatic region, which mostly constitutes the internode length in the ribbed species (Fig. 4D). Periclinal divisions are also observed in the rib’s intersections in *E. guentheri* and *E. ferox* (Fig. 4F). On the other hand, in *E. tithymaloides, E. alluaudi, E. tirucalli,* anticlinal divisions occur about three rows of parenchyma in the cortex (Fig. 4G).

Here we identified six different types of stem vasculature in *Euphorbia* (Fig. 5). In all species three leaf traces diverge from the eustele and gradually are observed oblique/horizontal, directly vascularizing the free portion of the leaf-base (Fig. 5). They normally did not ramify, but in *E. guentheri* these leaf traces/cortical bundles ramified between five to eight bundles in the tubercles (Fig. 5D). Three leaf traces vascularized the base of the leaf free portion, which subsequently ramifies in the median region of the leaf blade.

Table 2. Vegetative morphological and anatomical features of *Euphorbia*.

Species	**Phyllotaxis**	**Leaf type**	**Stem type I**	**Stem type II**	**Stem type III**	**Eustele shape**
E. attastoma	spirally arranged	sessile	succulent	Pencil-stem	6-ribbed	polygonal
E. tetrangularis	distichous	sessile	succulent	pencil-stem	4-ribbed	polygonal
E. phosphorea	spirally arranged	sessile	succulent	pencil-stem	6/8-ribbed	polygonal
E. holochlorina	distichous	sessile	succulent	pencil-stem	6-ribbed	polygonal
E. sipolisii	distichous	sessile	succulent	pencil-stem	4-ribbed	polygonal
E. heterodoxa	spirally arranged	subsessile	succulent	pencil-stem	6-ribbed	circular
E. tithymaloides	distichous	subsessile	succulent	pencil-stem	terete	circular
E. peperomioides	opposite	petiolate	non-succulent	herbaceous	terete	circular
E. pteroneura	spirally arranged	subsessile	succulent	pencil-stem	6-ribbed	polygonal
E. guentheri	spirally arranged	sessile	succulent	cactiform	tuberculate	circular
E. trigona	spirally arranged	subsessile	succulent	cactiform	3-ribbed	circular
E. alluaudii	spirally arranged	sessile	succulent	pencil-stem	terete	circular
E. tirucalli	distichous	sessile	succulent	pencil-stem	terete	circular
E. sobolifera	distichous	sessile	succulent	pencil-stem	6-ribbed	polygonal

E. appariciana	distichous	sessile	succulent	pencil-stem	6-ribbed	polygonal
E. ferox	distichous	sessile	succulent	cactiform	12-ribbed	circular

Fig 1: Shoot apex morphology of *Euphorbia*. (A) *E. attastoma*. (B) *E. guentheri*. (C) *E. trigona*. (D) *E. ferox.* (E) *E. tirucalli*. (A) The ribs possess a reddish color. (B) Tubercles are found throughout the stem (red arrowhead). (D) Modified branches constituting spines (white arrowhead). (E) Terete stem without any rib. Scale bars: 1cm.


A
B
C
D
E

Fig 2: Shoot apex anatomy of *Euphorbia*. (A) *E. pteroneura*. (B) *E. holochlorina* (C) *E. alluaudi* (D) *E. trigona.* (E) *E. ferox.* (F–G) *E. guentheri*. (H) *E. heterodoxa*. (A–H) Cross-sections. (A) Uniseriate Epidermis. (B) Chlorenchyma with many layers in the ribbed region (arrow). (C) Terete stems with homogeneous chlorenchyma. Note the larger bundle respectively to each rib comprising the eustele (*). (B–C) Amiliferous parenchyma in the inner cortex. (D–E) Ribs vascularized by three leaf traces and one branch trace (dashed circle). (F) Tubercles vascularized by 5 to 8 leaf traces (arrowheads). (G) Homogeneous mesophyll with laticifers (arrowhead). (H) Dorsiventral leaf. (A, C–H) Toluidine blue. (B) Astra blue and safranin. (AP) Amiliferous parenchyma; (PP) Palisade parenchyma. (PL) Spongy parenchyma. Scale bars: 200mm (B–F); 100mm (G–H); 20mm (A).

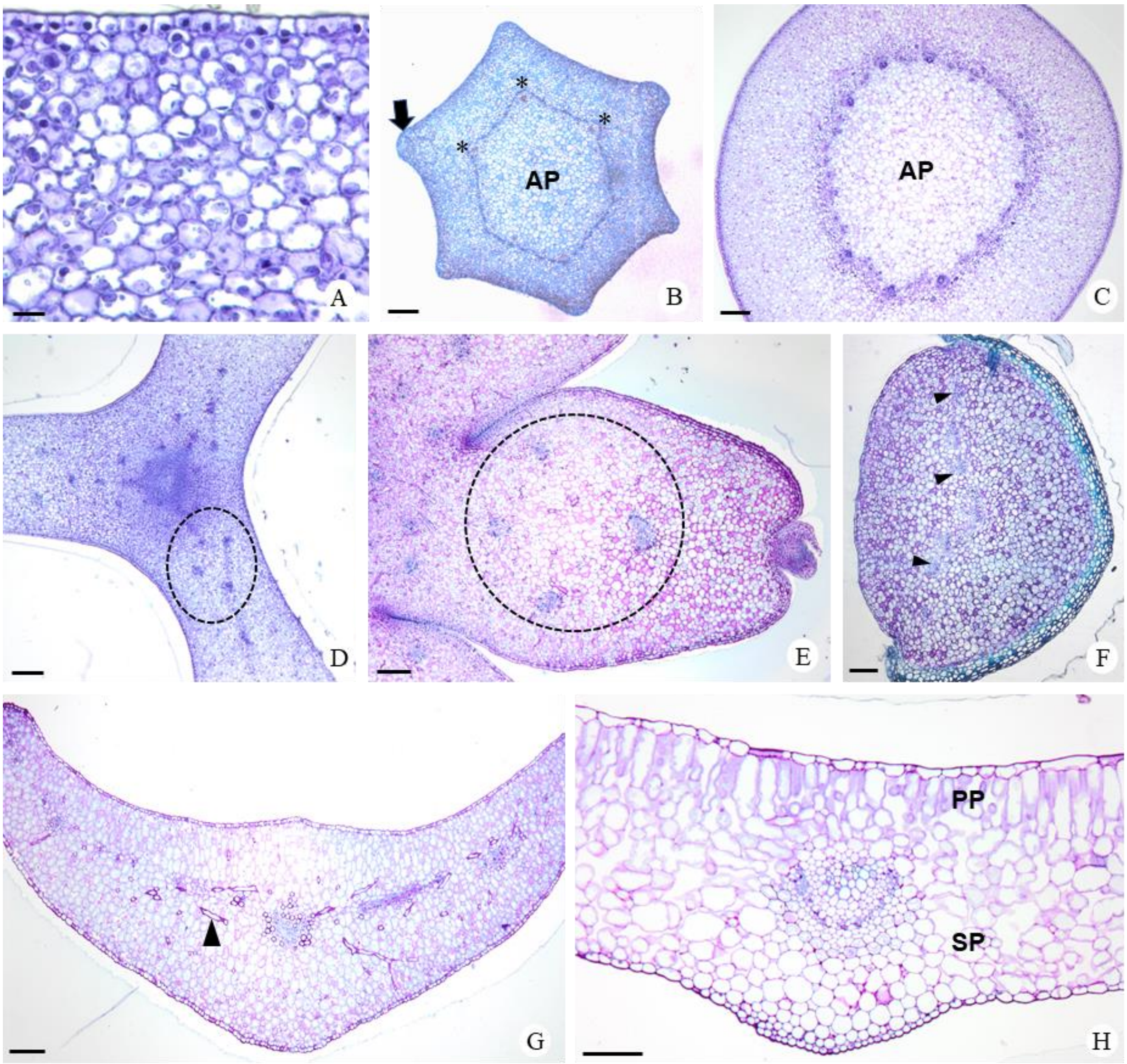
A
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Fig 3: Ontogeny and vascularization of *Euphorbia*. (A) *E. trigona*. (B) *E. sobolifera* (C) *E. tirucalli* (D) *E. phosphorea.* (E) *E. alluaudi*. (F) *E. appariciana*. (A–C, F) Cross-sections. (D–E) Longitudinal section. (A) Meristematic cells in the marginal leaf portion of all developing leaves (arrows). (B) Midrib ramified in five to six bundles in the median portion of the leaf blade. (C) Detail of the axial component constituted by three adnate leaf base. (D) Cells with anticlinal divisions along the internode (red arrowheads). (E) Terete stems with few anticlinal divisions (red arrowheads). (F) Leaf traces/ collateral vascular bundles in the internode (*). (A–B, F) Toluidine blue. (C) Astra blue and safranin. Calcofluor White (D–E). Scale bars: 500µm (D); 200µm (C, E); 100µm (A–B); 50µm (F).

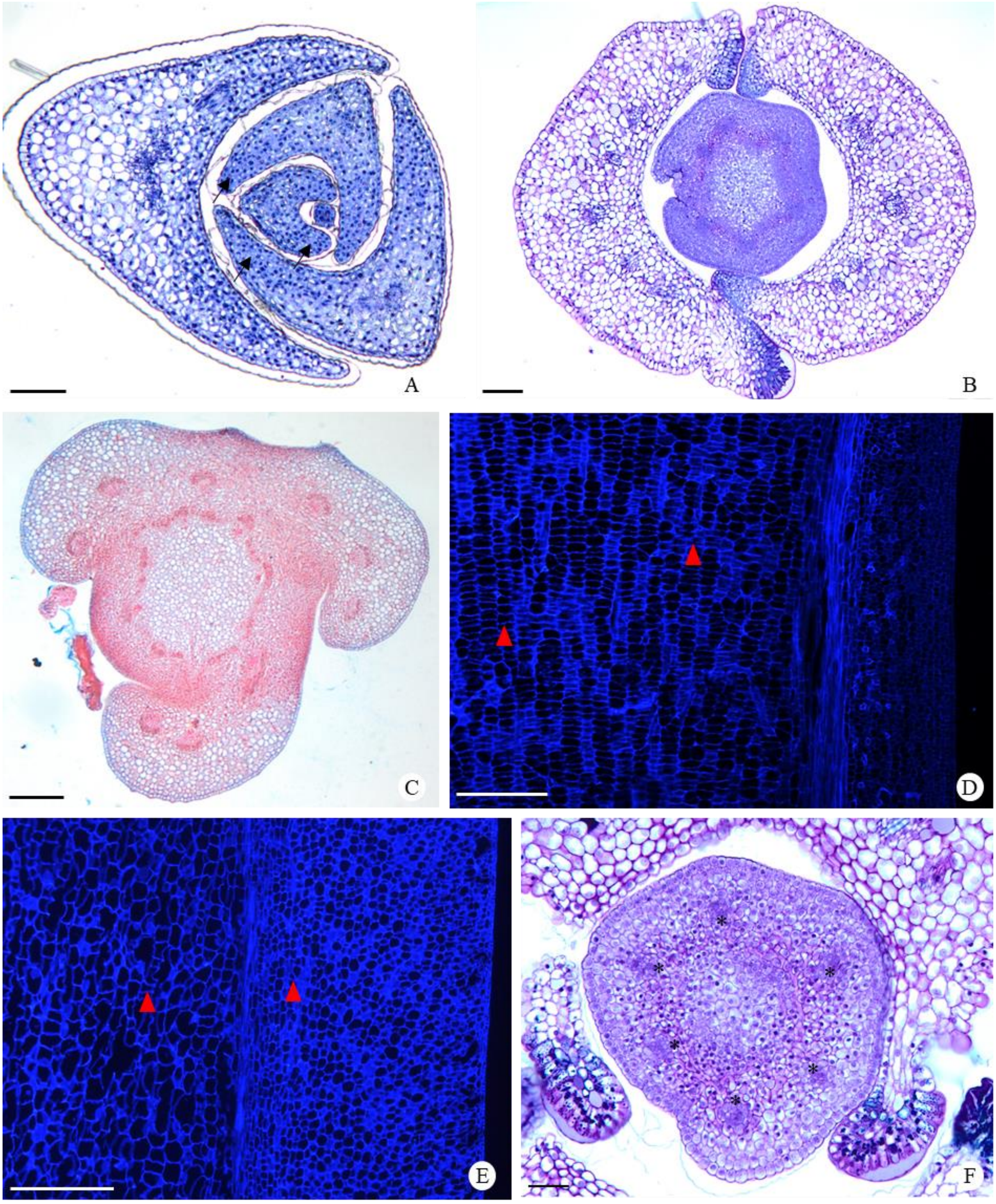
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Fig 4: Ontogeny and vascularization of *Euphorbia*. (A) *E. appariciana*; (B, E) *E. ferox*. (C) *E. attastoma*. (D) *E. holochlorina*. (F) *E. alluaudi.* (A–F) Cross-sections. (A) Note that each leaf trace/cortical vascular bundle possess their respective parenchymatic projection (dashed rectangular square). (B) Groups of three leaf traces/colateral vascular bundles and one branch trace with one parenchymatic projection (dashed circle). (C–D) Cells with periclinal divisions in the rib (dashed rectangle). (E) Periclinal divisions in the rib's intersections (red arrowhead). (F) Terete stems with cells in anticlinal divisions about three rows of parenchyma in the cortex (red arrowheads). (A, C, E, F) Toluidine blue. (B, D) Astra blue and safranin. Scale bars: 200µm (B, D); 100µm (A, C); 50µm (E) 20µm (F).

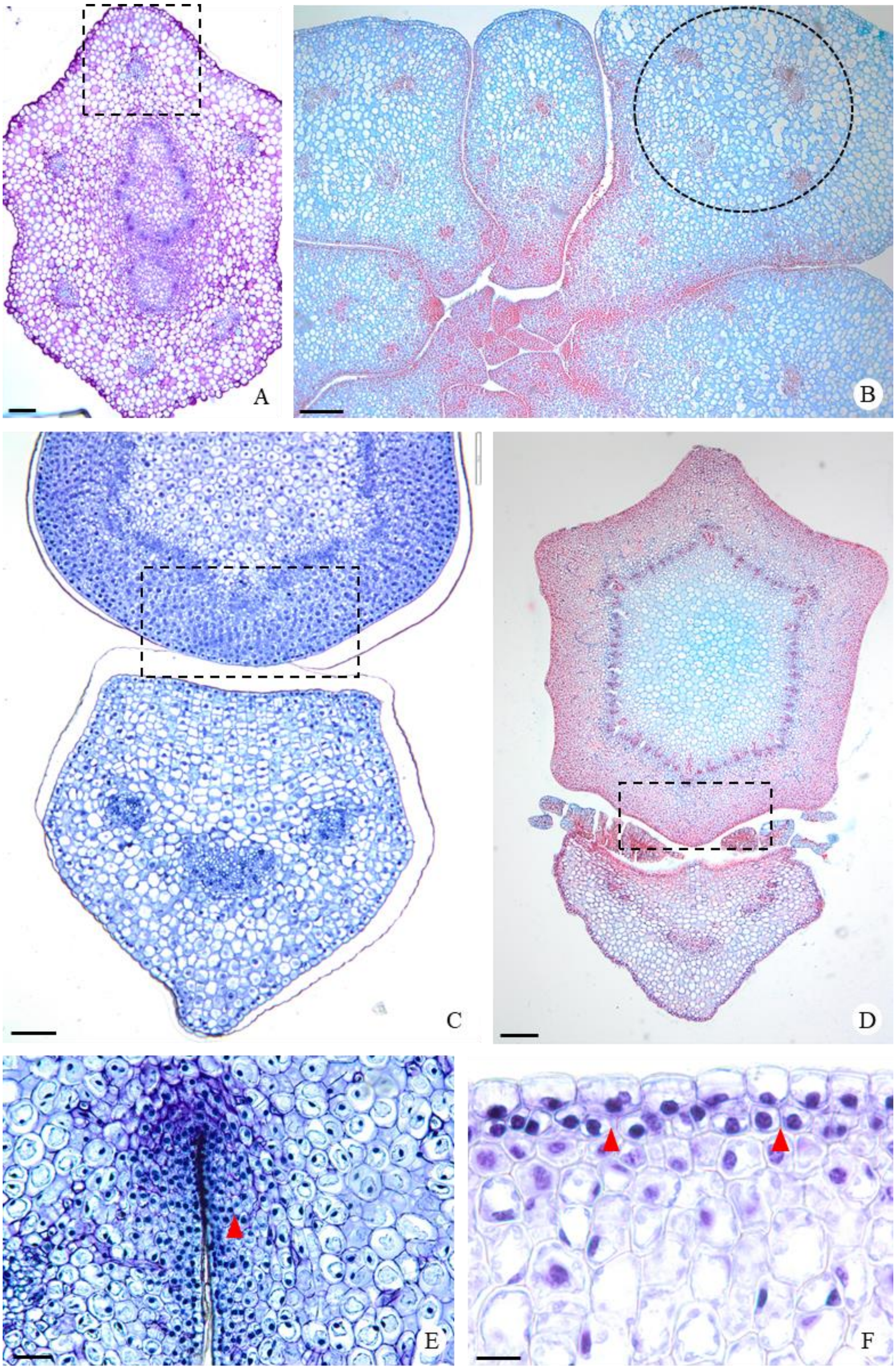
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Fig. 5: Diagrams of stem vasculature. (A) Terete-distichous. (B) Terete-spirally arranged. (C) 6-Ribbed-distichous. (D) 3-Ribbed-spirally arranged. (E) Tubercles. (F) 12-Ribbed-distichous. The leaf traces diverge in groups of three in both species, and only ramifies in the tubercles (E). Only in (C) the eustele are polygonal.

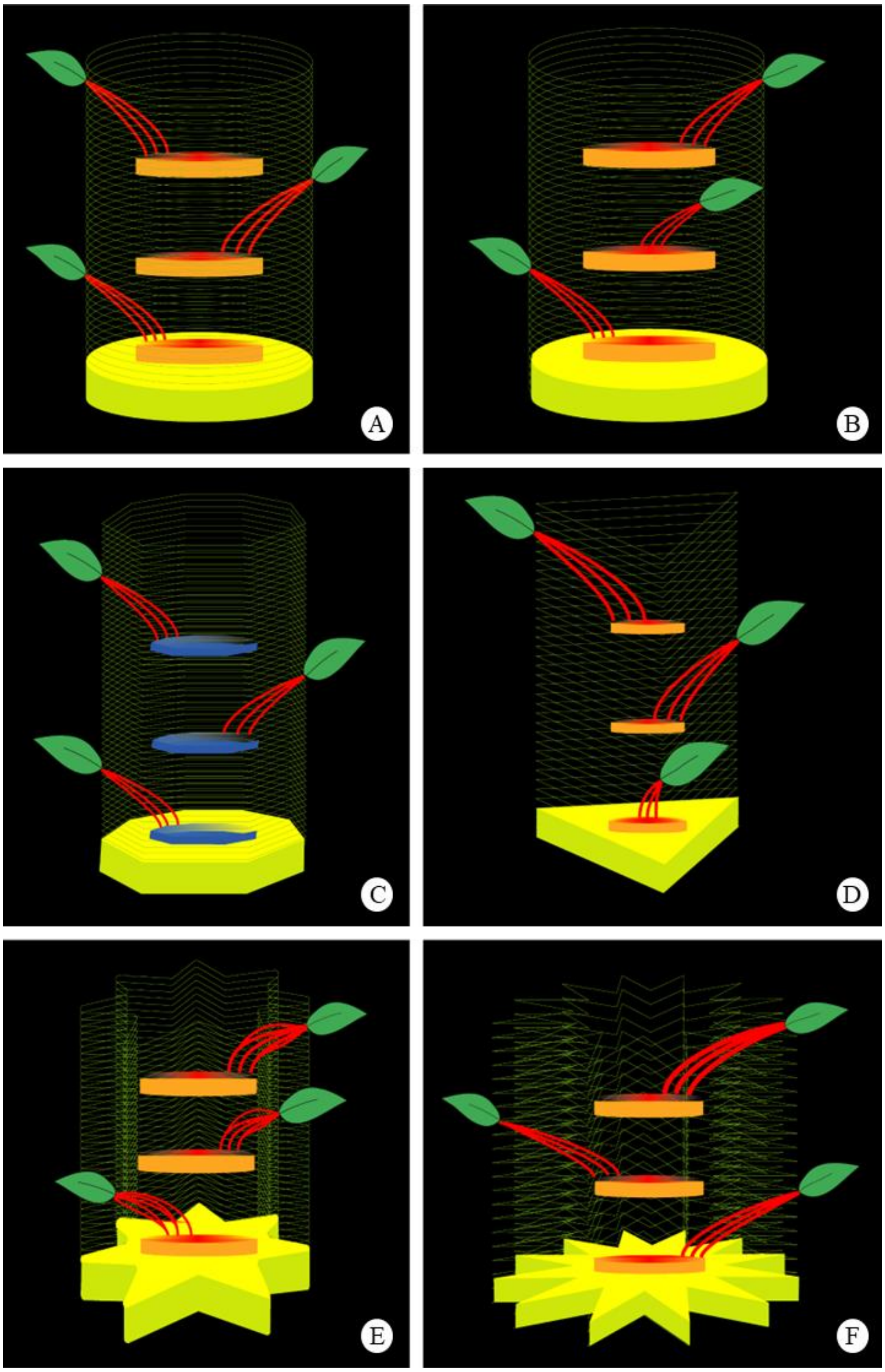
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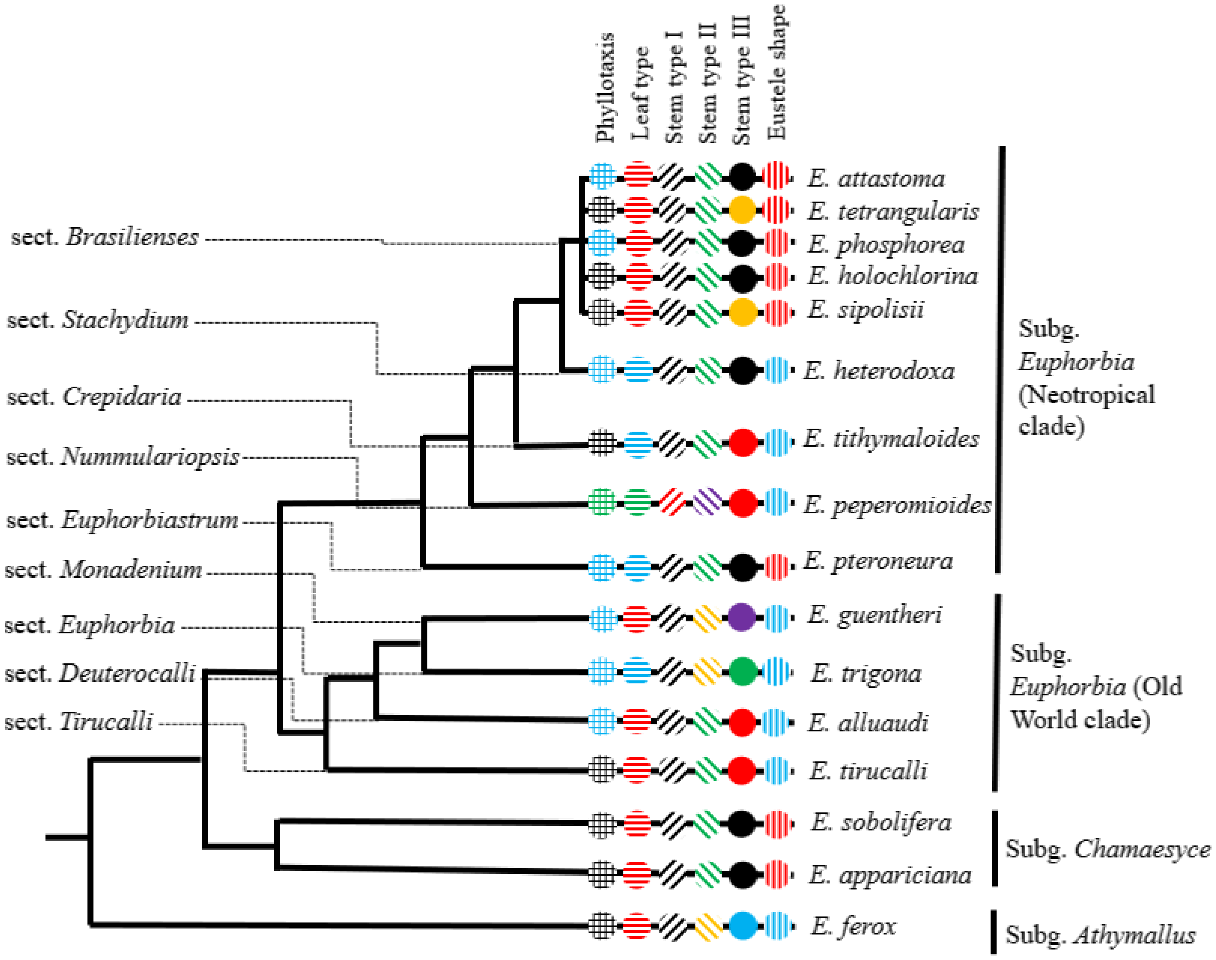
Fig. 6: Simplified phylogenetic tree of the *Euphorbia* species investigated, based on Horn et al., 2012. The circles represent the character state of each species. Phyllotaxis: ▦/ spirally arranged, ▦/distichous, ▦/opposite. Leaf type: ▤/sessile, ▤/subsessile, ▤/petiolate. Stem type I: ▨/succulent, ▨/non-succulent. Stem type II: ▧/ pencil-stem, ▧/cactiform, ▧/herbaceous. Stem type III: ⚫/6-8 ribbed, 🟡/4-ribbed, 🟢/3-ribbed, 🔵/12-ribbed, 🔴/terete, 🟣/tuberculate. Eustele shape: ▥/polygonal, ▥/circular.


Phyllotaxis
Leaf type
Stem type I
Stem type II
Stem type III
Eustele shape
E. attastoma
E. tetrangularis
E. phosphorea
E. holochlorina
E. sipolisii
E. heterodoxa
E. tithymaloides
E. peperomioides
E. pteroneura
E. guentheri
E. trigona
E. alluaudi
E. tirucalli
E. sobolifera
E. appariciana
E. ferox
sect. *Brasilienses*
sect. *Stachydium*
sect. *Crepidaria*
sect. *Nummulariopsis*
sect. *Euphorbiastrum*
sect. *Monadenium*
sect. *Euphorbia*
sect. *Deuterocalli*
sect. *Tirucalli*
Subg. *Euphorbia* (Neotropical clade)
Subg. *Euphorbia* (Old World clade)
Subg. *Chamaesyce*
Subg. *Athymallus*

DISCUSSION

This investigation demonstrate that all *Euphorbia* species here analyzed have a portion of the leaf-base adnate to the stem as previously described for two species of the section *Brasilienses* (Arévalo-Rodrigues et al., 2022) and in other families of plants (Mitra and Majumdar, 1952).

Stems of the pencil-stem type with 6 ribs are found in *E.* sect. *Brasilienses, Stachydium, Euphorbiastrum,* of *E.* subg. *Euphorbia* and in *E.* sect. *Crossadenia* of the *E.* subg. *Chamaesyce*. These 6-ribbed stems are mainly developed by means of two different types of phyllotaxis, which is alternate and distichous in the two species of the *E.* subg. *Chamaesyce* and alternate and spirally arranged in the two species of the subg. *Euphorbia*, particularly in the species of the sect. *Brasilienses*. Additionally, the 6-ribbed type is the most common type of pencil stem described in this investigation, being certainly homoplastic in the genus (Fig. 6).

In the species with sessile leaves an earlier maturation of the leaf meristems maintains the leaf blade fairly reduced (see Arévalo-Rodrigues et al., 2022). On contrary, in the subsessile species the marginal meristems remain active much longer increasing the leaf blade length to some extent. In any case, these variations in the leaf development led to the transference of the main photosynthetic function (heterotopy) to the stem. A feature that are commonly found in the succulent species of *Euphorbia*, in which the stem become the main photosynthetic organ (Horn et al., 2012, Arévalo-Rodrigues et al., 2022).

Euphorbia heterodoxa, *E. pteroneura* and *E. trigona* have ribbed stems concomitantly with subsessile leaves, while in the two species of the sect. *Brasilienses* the leaves are sessile, both groups have a portion of the leaf base adnate to the stem (Arévalo-Rodrigues et al., 2022). Except *E. peperomioides,* that did not have any distinguishable leaf base adnate to the stem, all succulent species have a portion of the leaf-base adnate to the stem, comprising an axial component. The incidence of the fusion of the leaf base to the stem was earlier identified in the angiosperms by Majumdar (1956), but the evolutionary implications regarding the meristematic portion of the leaf that will be adnate to the stem is still poorly reported (Arévalo-Rodrigues et al., 2022). In *Euphorbia* is evident that there is a meristematic distinction regarding the portion of the leaf base that will constitute the axial component in the sessile and the subsessile leaves. In species with subsessile leaves, it is possible to identify a clear distinction between the leaf blade and a minute petiole, suggesting that these tissues differentiation occurs early during leaf development.

The occurrence of rudimentary and early caducous leaves is also a shared feature in the terete pencil-stem species. These rudimentary leaves have an essential role in the stem developing tissues in *Euphorbia* (Arévalo-Rodrigues et al., 2022). Here, *E. tithymaloides, E. alluaudi* and *E. tirucalli* also have during the stem organogenesis the leaf-adnate to the stem, but differently their stems did not have any rib. These species have the parenchymatic projection in the ground meristem by means of anticlinal divisions mostly in the three layers of parenchyma below the stem epidermis, while the cactiform species have periclinal divisions also in the rib intersection. Therefore, it is possible to assume that the maintenance of rudimentary leaves in species with terete, ribbed and/or tuberculate stems is a noticeable adaptative strategy to improve the stem parenchymatic tissue in the succulent *Euphorbia* species.

Together with the elongation of the leaf-adnate base during the intercalary growth, the parenchymatic proliferation by means of anticlinal divisions occurs basipetally, being remarkable in all species of the *E.* sect. *Brasilienses*. Indeed, some species of the section can reach 6m tall (Dorsey et al., 2013). The great parenchymatic proliferation increases the stem length and is the main tissue which composes the ribs of these stems. Certainly, these unusual development is the main responsible for the behavior to lean in the neighboring plants, which has been described in species belonging to this section (Hurbath, 2018). According to Gibson and Horak (1978), the ribs in cacti could additionally facilitate seasonal expansion and contraction in the stems. In *Euphorbia*, besides these abundance of parenchyma, the absence of sclerenchyma may improve these tissue accommodation during dry and wet season.

The proliferation of parenchyma in the cortex of *Euphorbia* species certainly improve the photosynthesis and water storage as proposed to cacti by Boke (1951), Barcikowski and Nobel (1984), Mauseth (1988, 1989, 1990, 2004), Mauseth and Sajeva (1992). Moreover, the association of parenchymatic proliferation with cortical bundles could be an important adaptation to keep them hydrated as proposed by Mauseth and Sajeva (1992, 2006) to cacti, once these bundles facilitate the water movement laterally from eustele to the external cortex.

The types of vasculature found in the sect. *Brasilienses* gather the major variations of ribbed pencil-stem species studied in this investigation and could be an important model to understand the plastochron change in *Euphorbia* (Fig. 5). Usually, three leaf traces diverge from the eustele and vascularize the leaf in *Euphorbia*, however, the phyllotaxy and plastochron highly alter the disposition of these traces inside tissues of the succulent stems, as previously reported to *E. attastoma* and *E. tetrangularis* (Arévalo-Rodrigues et al., 2022).

Euphorbia holochlorina is the only species that have alternate and distichous leaves and a pencil-stem with 6 ribs, the other two species with this same phyllotaxis have only 4 ribs. This distinction occurs because the modified lateral bundles in the eustele do not share the vascularization of the subsequent leaf, as earlier described in *E. tetrangularis* (Arévalo-Rodrigues et al., 2022). The vascularization of *E. holochlorina* follows the pattern of *E. attastoma* but with the phyllotaxis alternate and distichous (Fig. 5). Alternate and distichous leaves, together with a 6-ribbed pencil stem are highly affected by the prolonged plastochron, which are exclusive of *E. holochlorina*.

The relation between ribs and the leaf traces was previously mentioned by Mauseth (2004) to nine species of *Euphorbia*. These species occur in Africa, and are currently classified in two different subgenera, more precisely in the *Athymallus* and *Euphorbia* (*sensu* Dorsey et al., 2012; Peirson et al., 2013). Additionally, similar cortical bundles have also been described for *E. weberbaueri* Mansf. (*E.* subg. *Euphorbia*; Steinmann, 2001). Ribs constituted by three leaf traces/ cortical bundles with only one parenchymatic projection were described here for the first time to the cactiform species of *E.* subg. *Euphorbia* of the Old World and *E.* subg. *Athymalus* (Fig. 6) and could be a notable feature of these habit in the genus.

Polygonal eustele and cortical vascular bundles/leaf traces found in the ribbed pencil-stem *Euphorbia* species are uncommon among angiosperms. In our previous investigation we speculate that parenchyma proliferation and the cortical vascular bundles/leaf traces could be intimately connected to the gradient of auxin during procambium differentiation in the developing shoots (Arévalo-Rodrigues et al., 2022). The flow generate by the polar auxin transport in branches is known to be connected to the vascular tissue differentiation and its adaptations to different environments (Aloni, 2010, 2015). Auxin could be connected to the differentiation of these vascular tissues, however, the endogenous hormonal signals that results in these uncommon pattern of vasculature is still obscure.

CONCLUSION

Our findings revealed that the leaf base constituting an axial component in the stem is a common feature in all succulent *Euphorbia*. The elongation of the adnate-leaf base in the species of the sect. *Brasilienses* occurs through a great amount of periclinal divisions that poorly occur in the other succulent species of the genus. The vascularization found in the section *Brasilienses* is an essential model to understand the other pencil-stems of *Euphorbia*. The stem diversity in the genus is impressive and six types are here described for the first time: Terete-distichous, Terete-spirally arranged, 6-Ribbed-distichous, 3-Ribbed-spirally arranged, Tuberculate-spirally arranged, 12-Ribbed-distichous. Ribs constituted by three leaf traces/ cortical bundles with only one parenchymatic projection were described here for the first time in the cactiform species of *E.* subg. *Euphorbia* of the Old World and E. subg. *Athymalus* and could be a notable feature of these habit in the genus. Alternate and distichous leaves, together with a 6-ribbed pencil stem are highly affected by the prolonged plastochron, which are exclusive of *E. holochlorina*. Finally, ribbed stems are the most common type of pencil stem, being certainly homoplastic in the genus.

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Capítulo III

Comparative systematic study of colleters and stipules of *Euphorbia* L.

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ABSTRACT

Colleters are secretory structures mostly found in the developing organs and possess a wide occurrence in many unrelated lineages within angiosperms. Stipules are leaf appendages that share with colleters the function to protect buds. Our main focus is to describe colleters distribution, anatomy, and exudate nature, looking to evaluate the stipular origin of colleters in *Euphorbia* for the first time. Scanning electron and light microscopy were employed to examine the glands located on the developing shoots in sixteen species of the genus. Colleters possess four morphotypes, standard, reduced standard, bifurcated and protrusion type. These glands were observed in stipules, leaf axil and lamina. Colleters of the standard type mostly occur in *Euphorbia*, while reduced standard type of colleters constitutes a profusion along the leaf axil in all species of *E.* sect. *Brasilienses* and could be considered as a synapomorphy to the section. Stipular colleters cooccurring with multicellular unbranched non-secretory trichomes are probably homoplastic in the genus. All colleters release an exudate largely constituted by mucilage, lipophilic compounds, phenolics and proteins, exclusively in the *sect. Brasilienses* mostly composed of lipophilic compounds. Future studies sampling species from the others subgenus of *Euphorbia* and other groups of Euphorbiaceae will provide insights about the evolutionary patterns of colleters in the family.

Keywords: anatomy – lipophilic compounds – sect. *Brasilienses* – morphotypes – mucilage – synapomorphy

INTRODUCTION

Secretory structures broadly related to buds and meristematic tissues, with the timing of secretion related to organ development, are known as colleters (Thomas, 1991). These glands appear in approximately sixty unrelated phylogenetically families in angiosperms (Thomas, 1991). Euphorbiaceae, a much-recognized family for its outstanding species richness, phytochemical and morphological diversity in Malpighiales (Wurdack, Hoffman & Chase, 2005), possess colleters particularly in Crotoneae (e.g *Croton, Brasiliocroton* and *Astraea*). They occur in the reproductive and vegetative organs in Crotoneae (Machado et al., 2015; Vitarelli et al., 2015). Colleters are also found in the flowers of *Dalechampia alata* (Martins, Cunha-Neto & Pereira, 2016), and in the apical region of the stipules in *Croton* and *Mabea fistulifera* Mart. (Feio et al., 2016; Almeida & Paiva, 2019).

Stipules are inconspicuous leafy appendages normally encountered at the leaf base and could be altered into thorns and glands with primary protective role of buds (Sinnott and Bailey 1914; Simpson 2006). The glands normally found at different portions of stipules are colleters and has been focus of many investigations in Malpighiales (Thiebaut & Hoffman, 2005; Paiva & Machado, 2006; Sheue et al., 2013; Faria et al., 2019; Paiva et al., 2021). Their occurrence and anatomy are firmly determined by habitat and phylogenetic relationships in Rhizophoraceae (Malpighiales; Sheue et al., 2013). However, comparative studies in order to describe the origin and nature of stipular colleters are still scarce in Euphorbiaceae and Malpighiales as a whole, even with the great taxonomic importance observed in families of other orders in angiosperms, such as Gentianales (Bremer and Struwe 1992; Capelli, Rodrigues & Demarco, 2017).

Colleters are morphologically emergences of the standard-type in Euphorbiaceae, formed by slightly elongated or palisade cells arranged radially around central-axis; having in some instances a very short stalk (Vitarelli et al., 2013; Riina et al., 2014; Feio et al., 2016;

Gagliardi, Cordeiro & Demarco, 2016; Martins et al., 2016). In *Croton*, colleters comprises a massive body of a very large bulbous base and a narrow neck with a shape that resembles a bowling pin (Machado et al., 2015). Sometimes these glands appear as a modified apex in the bracts and sepals (Gagliardi et al., 2016), while in *Mabea fistulifera* they were considered as trichomes (Almeida & Paiva, 2019). Colleters produce an exudate mostly constituted by mucilage, lipophilic compounds, or both (Vitarelli et al., 2013; Machado et al., 2015; Feio et al., 2016; Almeida & Paiva, 2019). Other minor constituents have been recognized in the secretion, being proteins the primary one particularly involved to the plant defense (Feio et al., 2016; Almeida & Paiva, 2019). The essential function attributed to their exudate are to lubricate and protect the developing organs against desiccation (Thomas 1991, Mayer et al, 2011, 2013; Silva et al., 2017; Almeida & Paiva, 2019), as well to create a medium to allow symbiotic bacteria associations (Lersten, 1975).

Numerous investigations have been discussed the ambiguous classifications of some plant structures as colleters (Thomas, 1991; Mayer et al, 2011; Cardoso-Gustavson et al., 2014; Feio et al., 2016; Almeida & Paiva, 2019). In Euphorbiaceae, colleters were assigned as extrafloral nectaries (see Paiva, 2012; Feio et al, 2016) and filamentous structures (De-Paula et al., 2011; Feio et al., 2016). The secretory activity restricted to the development organ has been properly employed to distinguish colleters from extrafloral nectaries in the family (Riina et al., 2014; Vitarelli et al., 2015, Feio et al., 2016). However, the occurrence of the extrafloral nectaries, colleters and secretory trichomes at the base of petioles, replacing/related to stipules, at the base or on the lamina (Metcalfe & Chalk, 1950; Dave and Patel, 1975; Coutinho et al., 2010; Vitarelli et al., 2015; Feio et al., 2016), emphasizing the need to properly circumscribe the secretory structures found in the Euphorbiaceae species.

Euphorbia L. is the major genus of Euphorbiaceae and one of the largest of the flowering plants (Frodin, 2004). The genus comprises around of 2000 species, demonstrating one of the most interesting cases of life forms diversity (Yang et al. 2012; Dorsey et al 2013). The group is essentially diverse in arid and semi-arid regions of the tropics (Dorsey et al., 2017). The outstanding habit variety in *Euphorbia* range from, small, annual herbs, shrubs, large trees, and xeromorphic species, being the latter mainly characterized by cactiform and pencil-stem species (Horn et al., 2013). Despite this great vegetative diversity in the group, the unique morphological synapomorphy that unify the entire genus is the presence of cyathium- a pseudanthial inflorescence (Steinmann & Porter, 2002; Horn & al., 2012).

The subg. *Euphorbia* possess the most complex evolution of xeromorphic growth form, with five independent origins in the genus (Horn et al., 2012). The major diversity of the group occurs in Africa and Madagascar with fifteen species in the New World (Steinmann, 2001). One of the xeromorphic sections is the *E.* sect. *Brasilienses* V.W. Steinm. & Dorsey, which comprises five succulent pencil-stem species, endemic to Brazil, occurring in environments with sandy soils such as “caatinga” (shrub-land vegetation common to the arid climate of northeast Brazil) and rocky uplands (Hurbath, 2018).

Colleters in *Euphorbia* was previously analyzed in bracts (Gagliardi et al., 2016), being poorly investigated in the vegetative organs. Our previous investigation detected a profusion of sessile colleters extending from one stipule to the other along the leaf axil in some species of sect. *Brasilienses* (Arévalo-Rodrigues et al., 2022), an evidence that led us to hypothesize that some of these colleters may have stipular origin. The presence or absence of stipules is a strong taxonomic character in *Euphorbia* (see Riina et al., 2013) and also possess an impressive diversity, being recurrently mentioned as glandular, filiform, subulate, ciliate, spiny, punctiform, comb-like, triangular, reduced and caducous (Yang et al., 2012; Dorsey et al., 2013; Peirson et al., 2013).

Our main focus is to describe colleters distribution, anatomy, and exudate nature, looking to evaluate the stipular origin of colleters in *Euphorbia* for the first time. Therefore, we selected species from one succulent lineage of the New World (sect. *Brasilienses*; Dorsey et al., 2013) and other species from related lineages of these section in the genus, as well three species of other subgenera (Table 1).

MATERIAL AND METHODS

Plant material

The species selected for this investigation are listed in the Table 1. The anatomical and morphological studies were performed in the shoot apices of plants cultivated at Instituto de Biociências at the Universidade de São Paulo and in the Parque Estadual das Fontes do Ipiranga at Instituto de Pesquisas Ambientais. Vouchers were deposited at the SPF and SP herbarium.

The material was fixed in FAA (formalin-acetic acid-alcohol) for 24h (Johansen 1940), in BNF (buffered neutral formalin) for 48 h (Lillie 1965), and in Karnovsky solution (Karnosvky, 1965, modified by Kraus and Arduin, 1997) for 24h for morphological and anatomical purposes. Then the material was washed, dehydrated, and stored in 70% ethanol.

Scanning electron microscopy (SEM)

The stored material was dehydrated in a graded ethanol series and was dried with CO2 (exposure time 200s on a Balzers 107 SCD-050 sputter coater). Shoot apices were mounted on stubs, coated with gold palladium in a Leica ACE200 sputtering system (Leica, Wetzlar, Germany), and viewed using a JEOL JSM 741F scanning electron microscope at 10 kV.

Light microscopy (LM)

The stored material (at least three shoot apices) was dehydrated, embedded in Historesin® (Technovit 7100, Heraus Kulzer, Germany) and serial sectioned on a rotary microtome. Longitudinal and cross-sections of 7 μm thick were stained with toluidine blue for metachromasy (Feder and O'Brien, 1968)

The main class of compounds were detected using the following histochemical tests: Schiff's reagent (PAS) for total polysaccharides (Jensen 1962), Sudan black B for total lipids (Pearse, 1985), aniline Blue Black for proteins (Fisher, 1968), and ferric chloride for phenolic compounds (Johansen 1940). Standard controls were performed in all of the presented tests. Slides were mounted in water or glycerin and the sections were viewed with an Olympus BX53 compound microscope. Digital images were acquired with an Olympus I-Color 5 digital camera, controlled using Image Pro Express 6.3 software. Images were edited using Adobe Photoshop version 7.0 (San Jose, CA, USA).

Colleters diversity distribution

The phylogenetic tree demonstrated was manually assembled using the Adobe Illustrator version 23.0.2 (San Jose, CA, USA). and the investigated species were arranged according to the main lineages recognized in *Euphorbia* (Horn et al., 2012).

Table 1. List of *Euphorbia* species investigated with provenance and voucher numbers. They are listed according to the currently recognized lineages (Yang et al. 2012; Peirson et al 2013; Riina et al. 2013; Dorsey et al. 2018).

Species	Collection site (Brazil)	Voucher
Euphorbia subg. *Euphorbia* sect. *Brasilienses*		
E. attastoma Rizzini	Universidade de São Paulo, SP (Garden)	Hurbath 824; Hurbath 837 (SP)

E. phosphorea Mart.	Universidade de São Paulo, SP (Garden)	Hurbath 629; Hurbath 645; Hurbath 654 (SP)
E. sipolisii N.E.Br	Universidade de São Paulo, SP (Garden)	Hurbath 828 (SP)
E. holochlorina Rizzini	Universidade de São Paulo, SP (Garden)	Hurbath 835; Hurbath 834 (SP)
E. tetrangularis Hurbath & Cordeiro	Universidade de São Paulo, SP (Garden)	Hurbath 839 (SP)
sect. *Stachydium*		
E. heterodoxa Müll.Arg.	Universidade de São Paulo, SP (Garden)	França 1940 (SP)
sect. *Crepidaria*		
E. tithymaloides L.	Universidade de São Paulo, SP (Garden)	Cordeiro 3585 (SP)
E. peperomioides Boiss.	Campos do Jordão, SP	Cordeiro 3331 (SP)
sect. *Euphorbiastrum*		
E. pteroneura A. Berger	Universidade de São Paulo, SP (garden)	Pirani 6621 (SPF)
sect. *Monadenium*		
E. guentheri (Pax) Bruyns	Instituto de Pesquisas Ambientais, SP (collection)	
sect. *Euphorbia*		
E. trigona Haw.	Instituto de Pesquisas Ambientais, SP (collection)	
sect. *Deuterocalli*		
E. alluaudi Drake	Universidade de São Paulo, SP (garden)	Pirani 6722 (SPF)
sect. Tirucalli		
E. tirucalli L.	Universidade de São Paulo, SP (garden)	D.P.A. Paz 1 (SPF)
subg. Chamaesyce		
E. sobolifera Silva & Braun	Universidade de São Paulo, SP (garden)	Brito, BG 11 (SP)
E. appariciana Rizzini	Universidade de São Paulo, SP (Garden)	Carneiro, 53 (SP)
subg. Athymalus		
E. ferox Marloth	Instituto de Pesquisas Ambientais, SP (collection)	

RESULTS

Morphology

Euphorbia peperomioides is the only herb and evergreen species investigated, all the other ones have succulent stems with quickly deciduous leaves restricted to the apex (Fig. 1). The phyllotaxis is alternate and spirally arranged in *E. attastoma*, *E. phosphorea*, *E. heterodoxa*, *E. pteroneura*, *E. guentheri* and *E. alluaudii,* alternate and distichous in *E. tetrangularis*, *E. holochlorina*, *E. sipolisii*, *E. tithymaloides*, *E. tirucalli*, *E. trigona*, *E. ferox*, *E. sobolifera* and *E. appariciana*, being opposite in *E. peperomioides*. Excepting *E. alluaudii* and *E. ferox* all species have a pair of secretory stipules, that vary from reddish (Fig 1A–B, F), greenish (Fig. 1C) to orange (Fig. 1D, F). Non-secretory stipules are filiform in *E. heterodoxa* and absent in *E. ferox*. In *E.trigona* and *E. guentheri* the stipules are modified into spines, being in the latter ochreate-like, comprising three to five spines in its distal portion (Fig. 1F).

The localization of colleters is listed in the Table 2. All the species of sect. *Brasilienses* have a profusion of colleters extending from one stipule to the other along the leaf axil (Fig. 1A). In the sect. *Brasilienses*, *E. peperomioides*, *E. tythymaloides*, *E. pteroneura*, *E. tirucalli*, *E. sobolifera* and *E. appariciana* the stipules are reduced to colleters (Fig. 1A–D), while in *E. tythymaloides* colleters additionally occur in the apex of the leaf blade (Fig. 1E). In *E. guentheri* and *E. trigona* these glands occur in the proximal portion of stipules (Fig. 1F–G).

Table 2. Colleters position and type in *Euphorbia*

Species	Colleter position	Colleter type
E. attastoma	St, Ax	S, Rs
E. tetrangularis	St, Ax	S, Rs
E. phosphorea	St, Ax	S, Rs
E. holochlorina	St, Ax	S, Rs
E. sipolisii	St, Ax	S, Rs
E. heterodoxa	-	-
E. tithymaloides	St, Lm	S
E. peperomioides	St	S
E. pteroneura	St	Bs
E. guentheri	St	P
E. trigona	St	Bs

E. alluaudii	*	*
E. tirucalli	St	S
E. sobolifera	St	S
E. appariciana	St	S
E. ferox	-	-

Note: St, stipular colleter; Ax, along the axil; Lm, Laminar colleter; S, standard type; Bs, bifurcated standard; P, colleters in protrusion; Rs, reduced standard type.

Anatomy

We identified four types of colleters in the sixteen species investigated (Table 2). In all species of sect. *Brasilienses* a co-occurrence of two types of colleters has been observed in the shoot node, the stipular standard type and the reduced standard type ones (Fig. 2). The stipular colleters quickly diverge during leaf development (Fig. 2A). They develop laterally by means of periclinal and anticlinal divisions from two groups of meristematic cells, being the epidermis constituted by cells in palisade (Fig. 2B–C). Emergences comprising sessile colleters of the reduced standard type have also, as the stipular ones, their origin due to two groups of meristematic cells, but in these case the majority of cells originate from protoderm (Fig. 2D–G).

At the third node, the profusion of colleters cover completely the lateral bud along the leaf axil (Fig. 2G). Reduced standard type colleters have a constriction at the base and the epidermal cells remain much longer with a dense protoplast and a basal nucleus when compared to the stipular ones (Fig. 2H). Stipular colleters also occur in *E. sobolifera* and *E. appariciana* (Fig. 2I–L), while in *E. peperomioides*, *E. tythymaloides* and *E. tirucalli* they occur together with multicellular unbranched non-secretory trichomes (Fig. 3A–D).

The stipules bifurcate during their development and constitutes two emergences in *E. pteroneura* (Fig. 3E–F). The emergences that compose the bifurcated colleters type share the same base in cross-section (Fig. 3F).

In *E. guentheri* the stipules are ochreate and adnates do the stem soon during their development (Fig. 3G). The ochreate stipules are constituted by four to eight layers of sclerenchyma (Fig. 3H). Spines were found laterally and in the median region of the ochreate-like stipules, varying from 3 to 5 along their length (Fig 3G). A protrusion of meristematic cells occurs in the abaxial proximal region of these spines, but only in the lateral ones (Fig. 3I). Colleters protrusions occur at the same location previously occupied by the meristematic cells and comprise almost half of stipule length (Fig. 3J). *E. trigona* also possess spines showing this same protrusion of meristematic cells, however in this species the bifurcated type colleters were found at the proximal portion of the spines (Fig. 4K–L).

Particularly in *E. tithymaloides* laminar colleters were found at the apex of the leaf blade together with many multicellular unbranched non-secretory trichomes, which occur all over both leaf surfaces (Fig. 4A). These glands are related to enations of restricted points of the leaf apex, being active mostly during leaf expansion (Fig. 4B).

All colleters are sessile and are active during early stages of the vegetative organ’s ontogeny, having cells with a smooth cuticle (Fig. 4C). The exudate produced is profuse, with an emulsion-like and dense appearance in the extracellular space (Fig. 4C–D). The uniseriate secretory palisade epidermis covers a multicellular axis of a non-secretory parenchyma and have a dense protoplast with a globular content (Fig. 4E–H). Vascular traces were also absent in any of colleters identified (Fig. 4E). In the species belonging to the sect. *Brasilienses* the colleters become plasmolyzed at the end of secretory phase (Fig. 4I).

Histochemical testes identified the occurrence of lipophilic compounds composing the secretion inside all colleters (Fig. 5A–B), besides polysaccharides (Fig. 5C–F), proteins (Fig. 5G–I) and phenolic compounds (Fig. 5I) comprising a heterogeneous secretion in the investigated species. All species of the sect. *Brasilienses* have the uniseriate palisade secretory epidermis with a hyaline protoplast in the samples fixed with FAA (Fig. 5J–K).

Euphorbia alluaudi have a profusion of sessile emergences covering all around the leaf petiole (Fig. 6A). The non-secretory emergences have a smooth cuticle, having cells with the protoplast hyaline and huge vacuole (Fig. 6B–C).

Fig. 1: Diversity of colleters morphology of *Euphorbia* species. (A) *E. attastoma*. (B) *E. holochlorina*. (C) *E. pteroneura*. (D) *E. tirucalli*. (E) *E. tythymaloides*. (F) *E. guentheri*. (G) *E. trigona*. (A) Colleters extending from one stipule to the other along the leaf axil are observed in species of sect. *Brasilienses* (blue arrow). (A–D) Each pair of stipules are reduced to colleters (red arrows). (E) Laminar colleters (red arrow). (F) Ochreate stipules (white arrow). (G) Stipules modified into spines (green arrows). (F–G) Colleters occur in the stipular proximal portion (red arrows). Scale bars = 1cm.


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Fig. 2: Colleters in *Euphorbia* species. (A–D) *E. sipolisii*. (E–F) *E. holochorina*. (G) *E. phosphorea*. (H) *E. tetrangularis.* (I–J) *E. sobolifera*. (K–L) *E. appariciana*. (B–C, E, H, J, L) Cross-section. (A–D; G–L) Stipular colleters of standard type (green arrows). (A, D, G–L) Reduced standard colleters (red arrows). (A) The stipular colleter develops early during leaf primordia development (arrow). (B–C) Two groups of meristematic cells promoting periclinal and anticlinal cell divisions are involved in the stipular colleters development (arrows). (D) The epidermis of the stipular colleters have some scattered depressions on their surface. (E–F) Reduced standard type of colleters have their origin mostly related to protoderm derivatives. (G) At the third node a profusion of colleters of the reduced standard type cover completely the lateral buds. (H) Reduced standard colleters possess a constriction at the base. (A, D, G, I, K) Scanning electron microscopy (SEM). (B–C; E–F, H, J, L) Light microscopy. (B, C, E, H) Astra blue and safranine. (F) PAS reaction. (J–L) Toluidine blue. Scale bars = (A, G) 500µm; (I, K) 300µm; (J, L) 100µm; (D) 150µm; (F, H) 50µm; (B, C, E) 20µm.

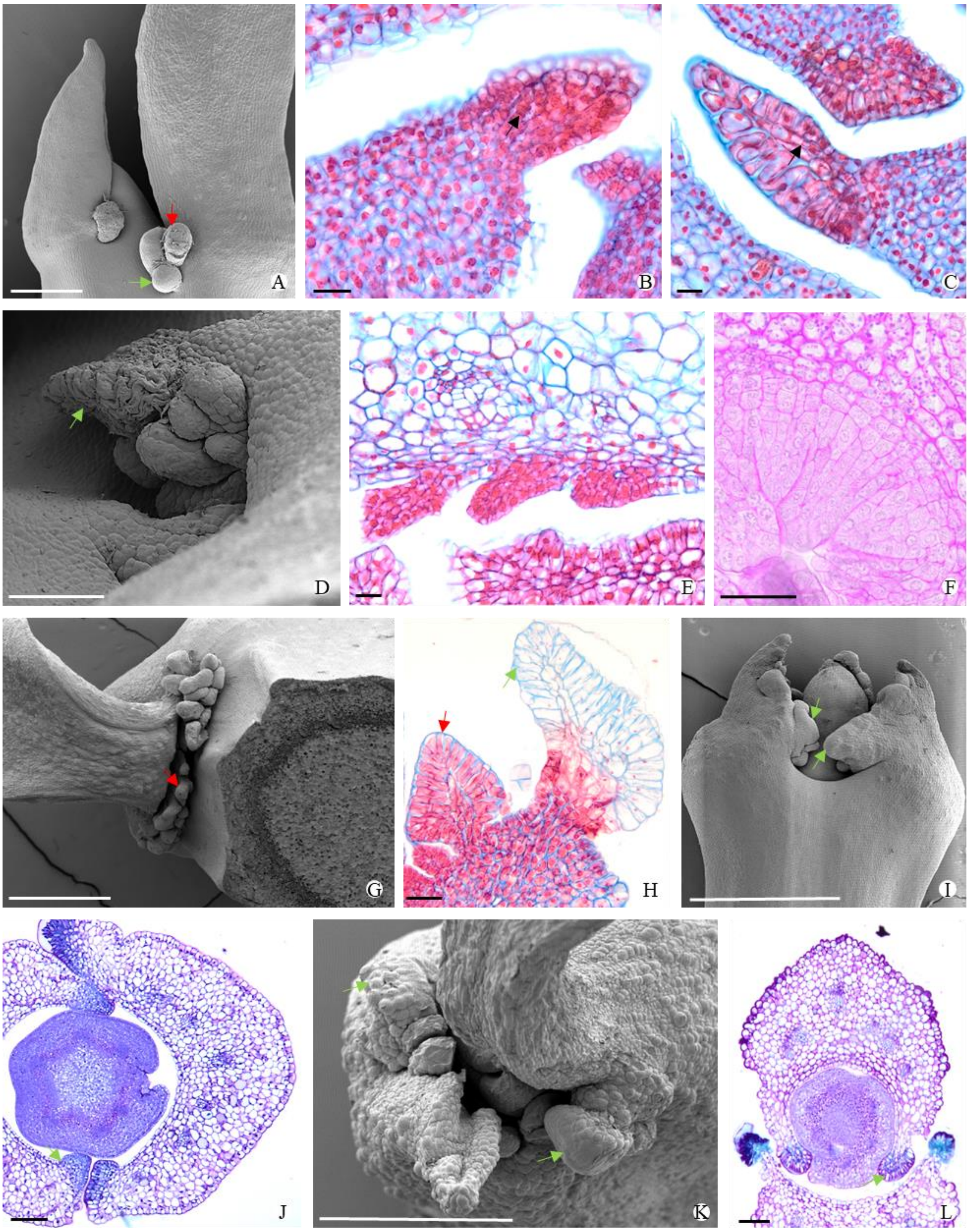
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Fig. 3: Colleters of *Euphorbia* species. (A) *E. peperomioides*. (B) *E. tythymaloides*. (C–D) *E. tirucalli*. (E–F) *E. pteroneura*. (G–J) *E. guentheri*. (K–L) *E. trigona*. (D, F, H, L) Cross-section. (A–D) Stipular colleters of the standard type. (E–F, K–L) Stipular colleters of the bifurcated type. (G–J) Colleter protrusion (A–D) Multicellular unbranched non-secretory trichomes cooccur with colleters (red arrows). (B) The exudate is firmly attached to the profuse secretion (*). (G) Ochreate-like stipules adnate to the stem (blue arrows), they are composed by four to eight layers of sclerenchyma (H). (I) Protrusion of meristematic cells occurs in the abaxial proximal region of spines. (J) Colleters comprise more than half of the spines during their secretory phase (arrowhead). (K–I) Colleters were found at the proximal portion of the spines (red arrowhead). (A–C, E, G, I, J–K) SEM. (D, F, H, L) Toluidine blue. Scale bars = (G, K) 1mm, (A–C) 300µm (I–J, H) 200µm; (L–E, D) 100µm; (F) 50µm.

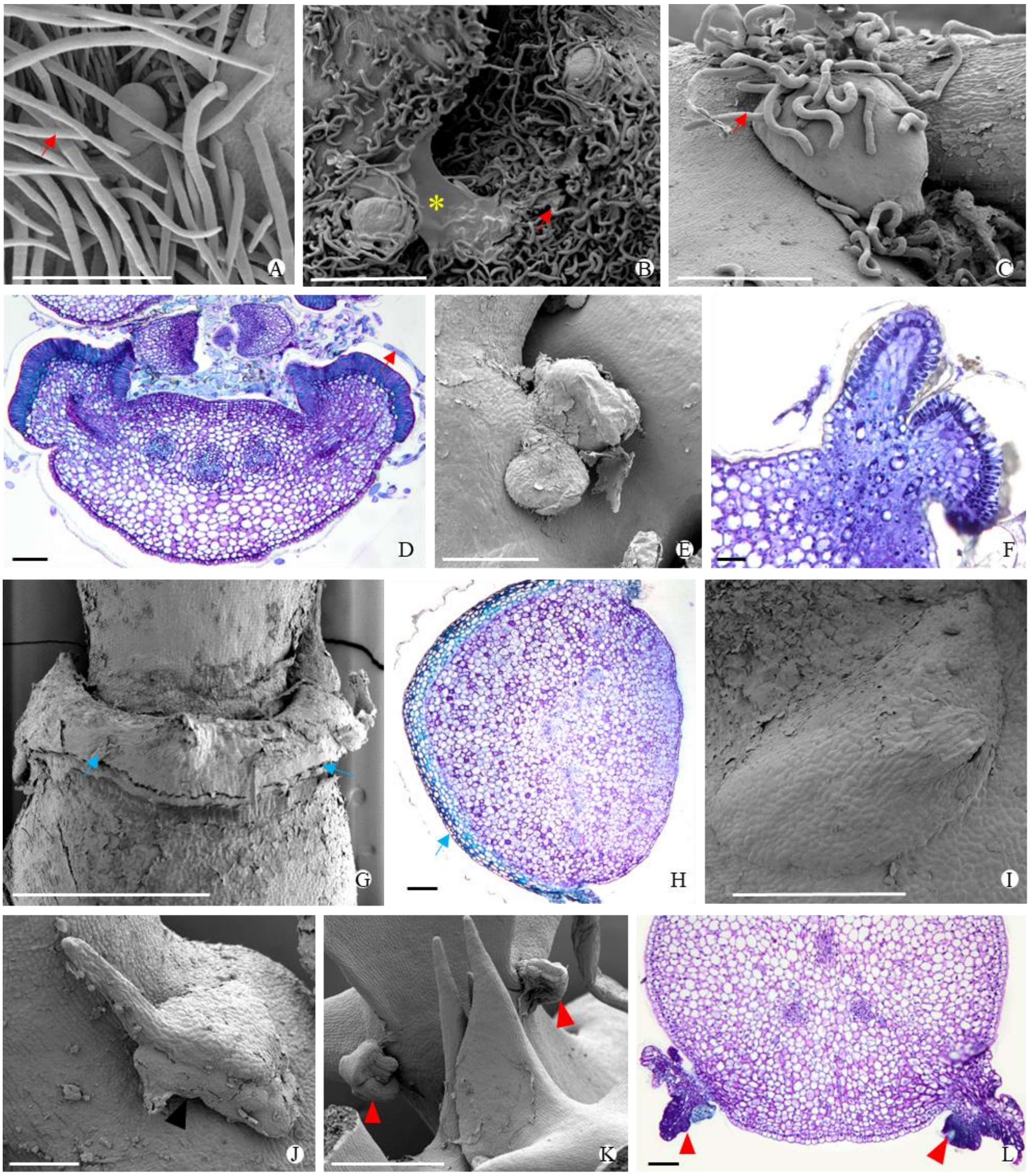
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Fig. 4: Colleters of *Euphorbia* species. (A–B) *E. tythymaloides*. (C) *E. holocholorina*. (D) *E. trigona*. (E) *E. pteroneura.* (F) *E. tirucalli*. (G–I) *E. phosphorea.* (B, E–H) Cross-section. (A–B) Laminar. (C, black arrow in H) Reduced standard type. (D, E) Bifurcated. (E–I) Standard type. (A) Colleters occur at the apex of the leaf blade together with many multicellular unbranched non-secretory trichomes all over both sides of leaf surface. (B) Structurally, these colleters also possess a uniseriate secretory epidermis that cover an axis of multicellular non-secretory parenchyma. (C–D, E, G–H) The exudate is profuse, with an emulsion-like and dense appearance in the extracellular space (*). (E–F) The uniseriate secretory palisade epidermis covers a multicellular axis of a non-secretory parenchyma and have a dense protoplast with a globular content. (I) The cells become plasmolyzed at the end of secretory phase. (A, C, D, I) SEM. (B) PAS reaction. (E–F) Toluidine blue. (G–H) Astra blue and safranine. Scale bars = (A, C) 150μm (H) 100μm, (D, I, B, G) 50μm, (E–F) 20μm.

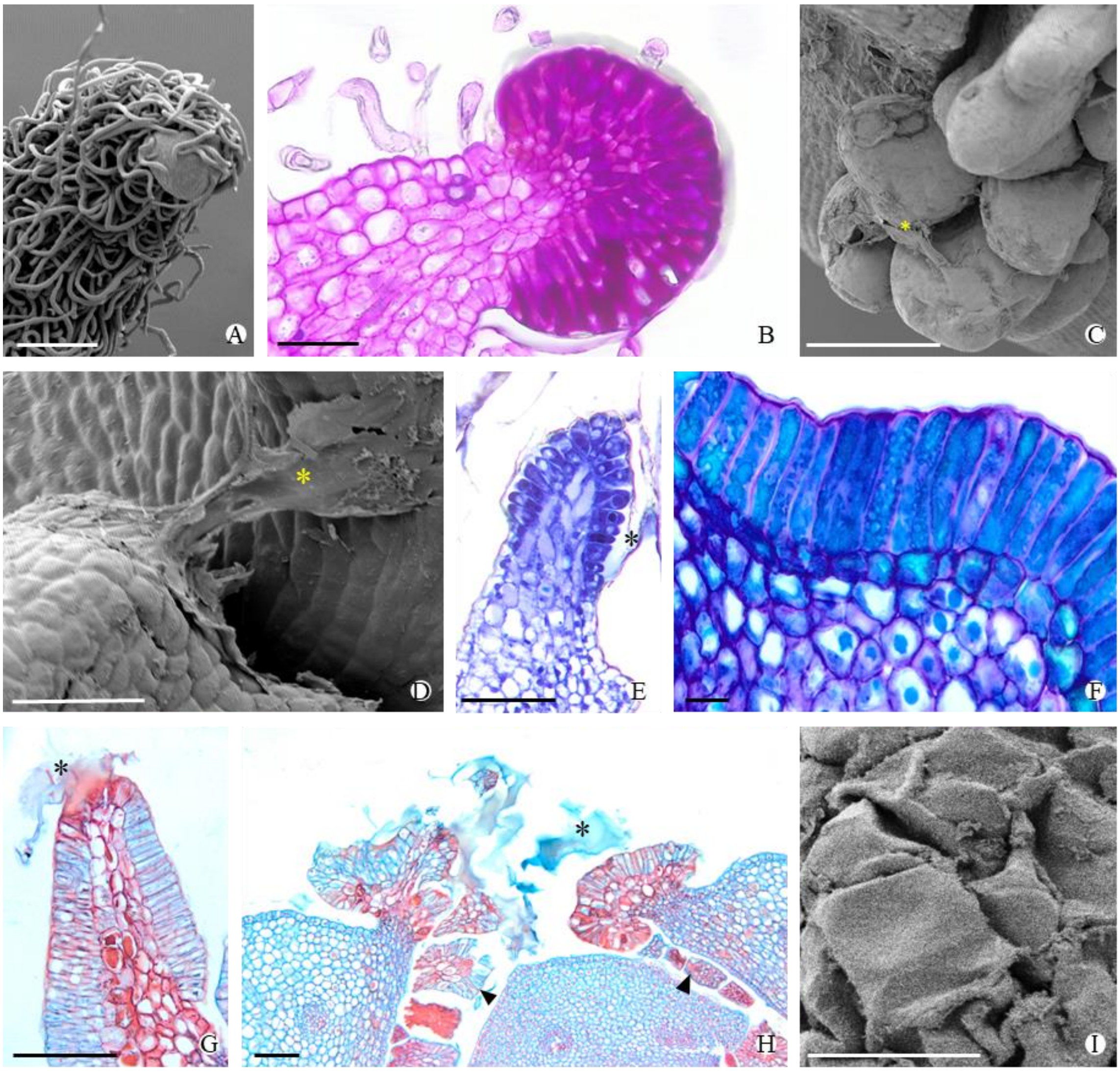
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Fig. 5: Histochemical characterization of colleters in *Euphorbia*. (A) *E. appariciana*. (B, H) *E. tirucalli*. (C, G) *E. pteroneura*. (D–E) *E. tythymaloides*. (F) *E. peperomioides*. (J) *E. phosphorea*. (I) *E. guentheri*. (K) *E. holochlorina*. (A–B) A lipophilic secretion constitutes the exudate inside cells. (C–F) Polysaccharides inside cells and in the extracellular space indicate that the released secretion is heterogeneous. (G–H) Proteins comprises the secretion as well. (H–I) Phenolic compounds constitute the secretion within the vacuole, note the greenish content in H. (J–K) The content inside cells of the samples fixed with FAA is hyaline. (A–B) Sudan Black B. (C–F, J) PAS reaction. (G–H, K) Aniline blue black. (I) Ferric chloride. Scale bars = (A–B) 20µm, (C,D–E, J) 100µm, (G–H, I, K) 50µm, (F) 10µm.

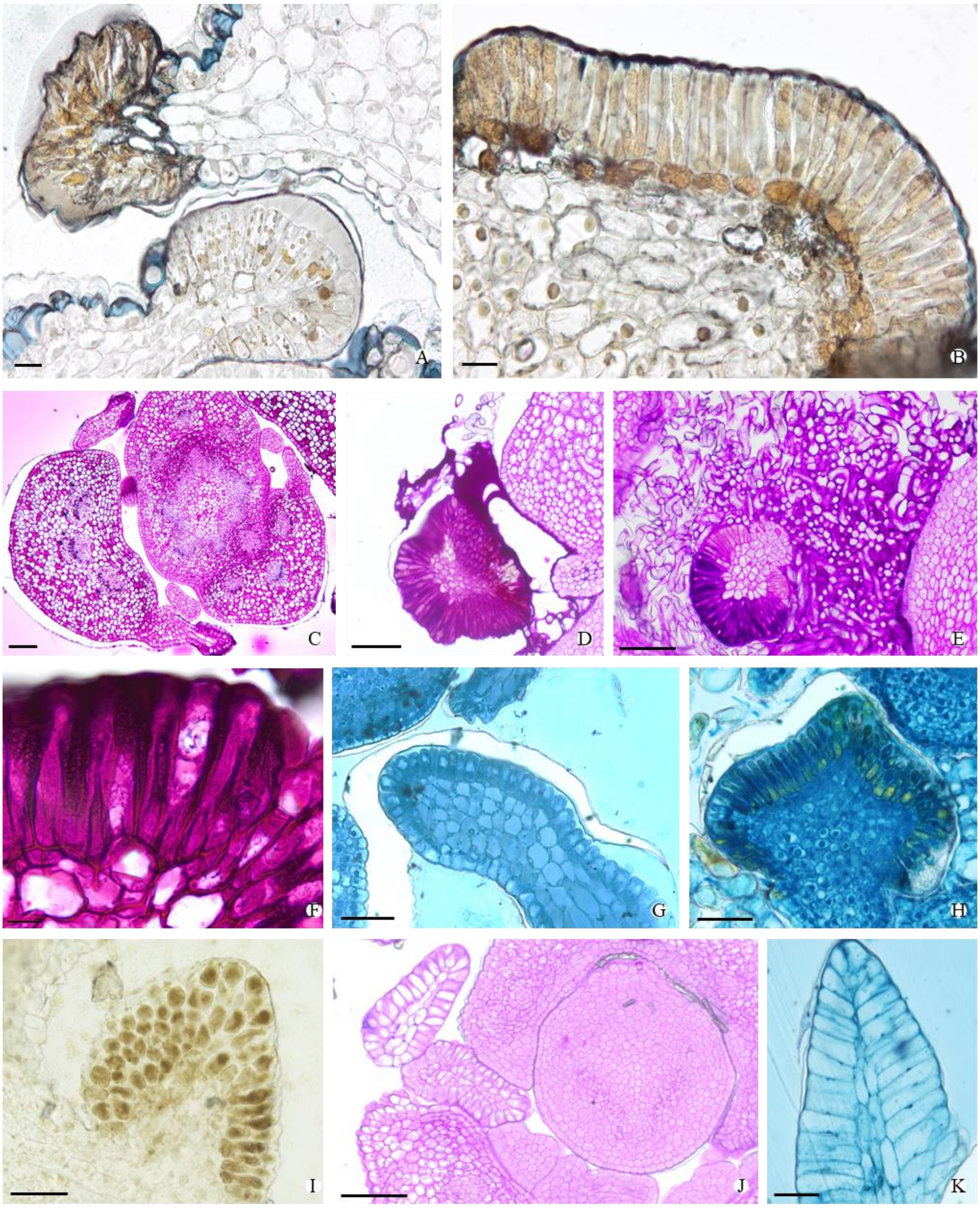
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Fig. 6: Emergences of *E. alluaudi.* (A) The profusion of sessile emergences covers all the leaf petiole. (B) The cuticle is smooth with a soft appearance. (C) All the uniseriate palisade epidermis is hyaline in cross-section. (A–B) SEM. (C) Toluidine Blue. Scale bars = (A–B) 300 μm, (C) 100μm.

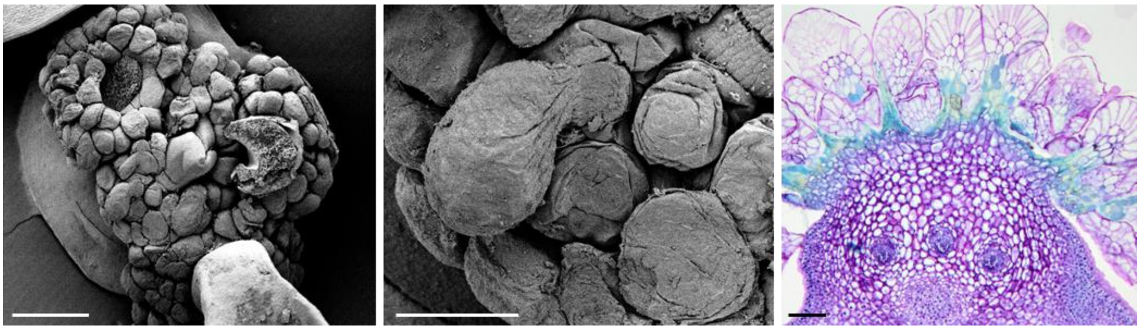
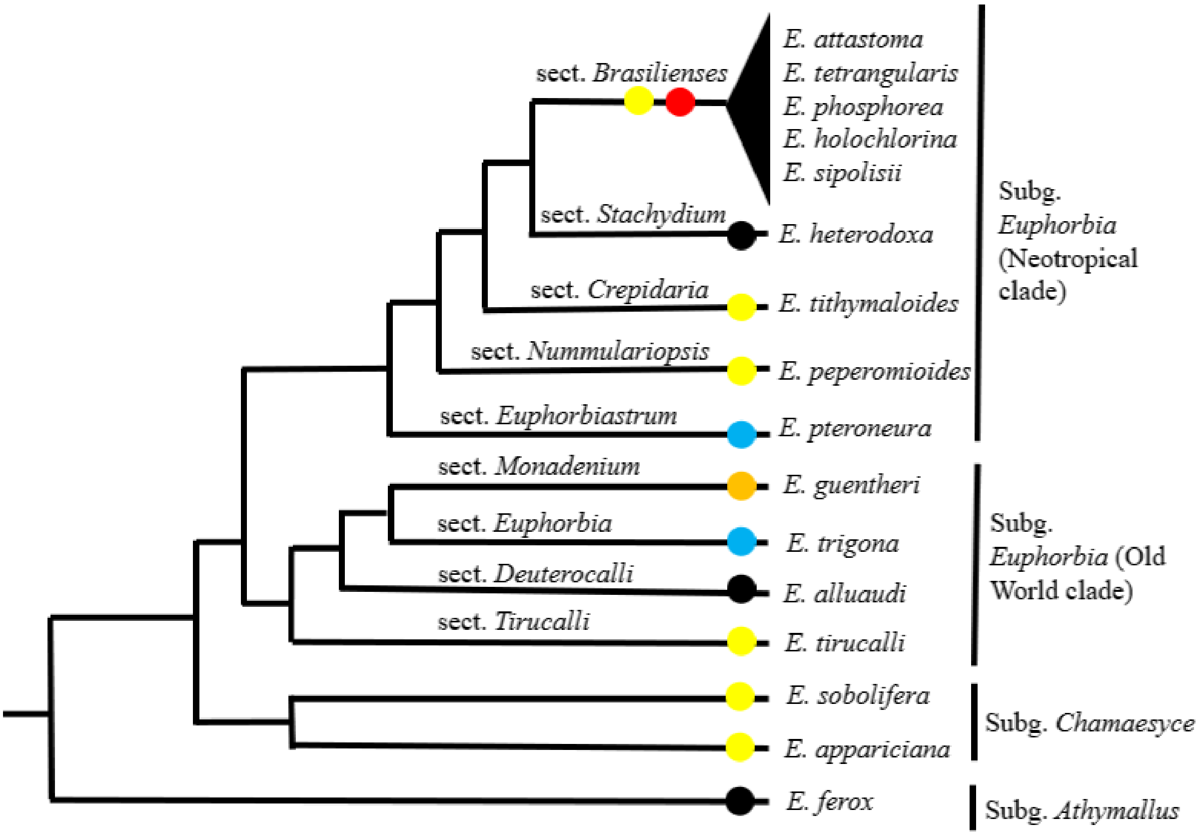


Fig. 7: Simplified phylogenetic tree of the *Euphorbia* species investigated, based on Horn et al., 2012. Yellow circle represents standard type of colleters, red circle represents reduced standard type, blue circle represents the bifurcated type, orange circle represents the colleters in protrusion and black circle represents the absence of these glands.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E. attastoma
E. tetrangularis
E. phosphorea
E. holochlorina
E. sipolisii
sect. Stachydium
E. heterodoxa
sect. Crepidaria
E. tithymaloides
sect. Nummulariopsis
E. peperomioides
sect. Euphorbiastrum
E. pteroneura
Subg. Euphorbia (Neotropical clade)
sect. Monadenium
E. guentheri
sect. Euphorbia
E. trigona
sect. Deuterocalli
E. alluaudi
sect. Tirucalli
E. tirucalli
Subg. Euphorbia (Old World clade)
E. sobolifera
E. appariciana
Subg. Chamaesyce
E. ferox
Subg. Athymallus

DISCUSSION

All the glands here investigated are considered as colleters based on their location, composition of the secretion, and the secretory phase occurring properly during the shoot development (see Fahn, 1979; Thomas, 1991).

These colleters result of anticlinal e periclinal divisions of protodermal and meristem fundamental cells. The colleters in *Mabea fistulifera* (Euphorbiaceae) were described as trichomes, having their development restricted to the protodermal derivatives. These glands also did not have any cell distinction, showing the epidermal and the central axis cells with the same anatomical structure (Almeida & Paiva, 2019). Those descriptions of colleters contrast to results found in this investigation, in which all the colleter' cells possess a secretory palisade epidermis covering radially a multicellular axis of a non-secretory parenchyma, analogous to the overall description of colleters in Euphorbiaceae (Machado et al., 2015; Vitarelli et al., 2015; Feio et al., 2016, 2018; Gagliardi et al., 2016; Martins et al., 2016). It is important to highlight that these distinction regarding the epidermal cells and the non-secretory parenchyma is a strong evidence of a conservative trait in the colleters of *Euphorbia*.

Due to the exclusive occurrence of the reduced standard type of colleters in the sect. *Brasilienses*, we propose it as a synapomorphy of the section (Fig. 7). Additionally, the occurrence of reduced (leaf axil) and the standard type (stipules) colleters with different periods of secretory activity during shoot development, undoubtedly improve the period of secretion upon the developing vegetative organs. Despite *E. pteroneura* (sect. *Euphorbiastrum*; *sensu* Dorsey et al., 2013) has similar stems to those found in sect. *Brasilienses*, lack the co-occurrence of those types of colleters. However, only the species of the sect. *Brasilienses* the stems could notably reach 6m tall (Dorsey et al., 2013), while *E. pteroneura* reaches up 0,5m. We assume that the increase of period of exudate secretion of colleters may provide additional advantages to the stems in species of sect. *Brasilienses*, which may facilitate the plants to reach these length.

The stipules in mostly *Euphorbia* species are entirely modified into colleters of the standard type (Fig. 7), which has been assigned to be the widespread type in angiosperms (Thomas, 1991). In Euphorbiaceae standard type of colleters is poorly understood but has been mentioned to different portions of the leaf in *Crotoneae* (Vitarelli et al., 2015). The leaves of *Croton* species are folded during development and the exudate produced by colleters at the leaf margins during leaf expansion insulate the adaxial region of the organ. According to Vitarelli et al., 2015, these exudate may protect the developing leaves against desiccation, which may occur similarly in *E. tithymaloides,* since the colleters found at the leaf apex are mostly active during leaf expansion.

Another common morphological trait found cooccurring with stipular colleters is the multicellular unbranched non-secretory trichomes (Fig. 7). They occur in *E. peperomioides* (sect. *Nummulariopsis*), *E. tirucalli* (sect. *Tirucalli*) and in *E. tithymaloides* (sect. Euphorbiastrum) also in their leaves. *E. peperomioides* and *E. tithymaloides* are lineages from the Neotropical clade, while *E. tirucalli* belongs to an Old World clade of the subg. *Euphorbia*. These unrelated occurrence are certainly homoplastic in the genus.

The occurrence of bifurcated colleters comprising stipules of *E. pteroneura* (sect. *Euphorbiastrum*; Fig.9) and in the proximal portion of the stipules of *E. trigona* (sect. *Euphorbia*) are unprecedent in Euphorbiaceae. Bifurcated colleters has been assigned in Rubiaceae (Coelho et al., 2013) Gentianaceae (Dalvi et al., 2014), and Apocynaceae (see Simões et al., 2006).

The ochreate stipules of *E. guentheri* (sect. *Monadenium*) have three to five spines in its length, while in *E. trigona* (sect. *Euphorbia*) the stipules constitute spines. The protective role of stipules and their shifts into glands has been discussed by some authors (Lubbock, 1891;

Sinnott & Bailey 1914; Simpson 2006), but the evolutionary shifts to keep spines and colleters in the shoot node in *Euphorbia* lineages is still obscure. Apart of mechanical protective role of spines against herbivores, they may shade the photosynthetic epidermis from the sun irradiation stress in succulent plants (Nobel, 1983; Gibson & Nobel, 1986; Mauseth, 2006). Other functions assigned to spines is related to camouflage and vegetative dispersal (Benson, 1982; Gibson & Nobel, 1986).

In *E. guentheri* (sect. *Monadenium*) a colleter protrusion is remarkable observed and comprise almost half of stipule length. These morphology was only described before for Rhizophoraceae (Malpighiales; Sheue et al., 2013) and Rubiaceae (Gentianales; Coelho et al., 2013), being rarely reported in other groups of angiosperms. According to Sheue et al., 2013, these structures were considered as vestigial colleters in *Pellacalyx axillaris* (Rhizophoraceae; Fig. 7). Vestigial colleters were observed solely in *E. alluaudi* (sect. *Deuterocalli*), showing a morphology extremely similar to the standard-type of colleters. The identification of two different non-functional vestigial colleters morphology in two families of Malpighiales emphasized the diversity of the evolutionary shifts related to colleters in the order.

The histochemical tests performed reveled a mucilaginous nature of the exudate of colleters. According to Fahn (1979), mucilage is mostly constituted by acid and neutral polysaccharides, which have water retention properties (Nobel et al., 1992). So, the colleters mucilage are certainly an essential adaptation to *Euphorbia* species to grow in environments with water restriction. Moreover, *Euphorbia* species have unbranched non-secretory trichomes co-occurring with stipular colleters. Indeed, these trichomes have been also related to protection against desiccation in *Crotoneae* (Vitarelli et al., 2015), and in *Mabea* related to mechanical protection in order to retain a humidity gradient on meristematic cells (Paiva & Almeida, 2019).

The secretion is mostly the same in all morphotypes of colleters identified, besides mucilage, they are constituted by lipophilic compounds, phenolic compounds, and proteins, indicating a conservative nature of the exudate in *Euphorbia* species. The lipophilic portion are probably related to the protection against pathogens and herbivory (Fahn, 1979), and seems to be an important feature in the colleters of the sect. *Brasilienses*. The exudate of these species reacted to FAA solution, and due to the chemical properties of the fixative this could be considered as a strong evidence of a great amount of lipophilic compounds. The lipids in stipular colleters of Rubiaceae have been related to the response to environmental conditions, such as periods of drought and high rates of solar radiation (Tresmondi et al., 2015). Phenolics may act as a chemical barrier, protecting against potential pests (Lattanzio et al. 2008; Castro and Demarco 2008), while the proteins, as assigned to the ones found in the latex of *Euphorbia*, may avoid the development of fungi (van Deenen et al., 2011) and protection against herbivory (Kitajima et al., 2016).

CONCLUSION

Our data reported for the first time a comparative investigation regarding stipules and colleters in *Euphorbia*. These glands possess four morphotypes in the genus and could be identified in stipules, leaf axil and lamina. Stipular colleters are the most morphological diverse, mainly in the species belonging to the New World Neotropical clade. Colleters of the standard type is probably widespread in *Euphorbia*. Reduced standard type of colleters constitutes a profusion along the leaf axil in all species of sect. *Brasilienses* and could be considered as a new synapomorphy to the section. Stipular colleters cooccurring with multicellular unbranched non-secretory trichomes are certainly homoplastic in the genus, adding some important advantage in maintain a humid gradient in the organ of these spurge species. All colleters release the exudate mostly composed of mucilage, lipophilic compounds, phenolics and proteins, being in the *sect. Brasilienses* mostly composed of lipophilic compounds. Future studies sampling species from the others subgenus of *Euphorbia* and other groups of Euphorbiaceae will shed some light into the evolutionary patterns of colleters in these groups.

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Considerações Finais

Os resultados obtidos durante o desenvolvimento da pesquisa permitiram responder satisfatoriamente às questões inicialmente levantadas; as perguntas e suas respectivas respostas são detalhadas adiante:

(1) Como é o desenvolvimento dos órgãos vegetativos aéreos?

Com excessão de *E. peperomioides*, a única espécie herbácea estudada, todas as espécies suculentas possuem desenvolvimento caulinar semelhante. Na morfogênese do ápice apical caulinar, a base foliar permanece adnata ao meristema caulinar, formando um componente axial. Durante o alongamento do entrenó, os traços foliares são observados como feixes corticais caulinares devido a uma alteração ontogenética. Essa constituição basicamente se dá por proliferação parenquimática basípeta.

(2) Como é a vascularização caulinar durante o desenvolvimento?

As análises da vascularização caulinar das dezesseis espécies estudadas puderam constatar que os traços foliares, são também feixes corticais em todas as espécies suculentas. Estes feixes divergem em grupos de três e puderam ser categorizados em seis diferentes tipos de vascularização: Terete-distichous, Terete-spirally arranged, 6-Ribbed-distichous, 3-Ribbed-spirally arranged, Tuberculate-spirally arranged, 12-Ribbed-distichous. Somente nas espécies com caule tuberculado estes feixes ramificam antes de vascularizar a porção livre da folha.

(3) Como são as estruturas glandulares vegetativas?

As únicas estruturas secretoras observadas nos órgãos vegetativos aéreos são os coléteres. Essas glândulas foram observadas apresentando quatro tipos diferentes, o padrão, o padrão reduzido, bifurcado e em protrusão. A ocorrência varia entre as espécies estudas, no entanto, ocorrem sempre na base foliar, axila e constituindo estípulas. O exudato liberado pelos coléteres é composto majoritariamente por mucilagem, compostos lipofílicos, fenólicos e proteínas.

(4) Há diferença na ontogênese caulinar entre as seções?

Sim, especialmente nas espécies da sect. *Brasilienses*. O meristema caulinar nesse grupo possui um incremento das divisões anticlinais no desenvolvimento intercalar, fato que, facilita o alongamento do entrenó. Além disso, particularmente nas espécies cactiformes estudadas (*E. guetheri*, *E. trigona*, e *E. ferox*) as costas caulinares ou os tubérculos são formados integralmente por três traços foliares/ feixes corticais, inseridos em uma única projeção parenquimática. Enquanto as espécies do tipo "pencil-stem" possuem costas constituídas por um único traço foliar cada.

(5) O tipo de vascularização do caule em E. sect. Brasilienses é uma sinapomorfia da seção ou é encontrada em outros representantes do clado neotropical de E. subg. Euphorbia?

O tipo de vascularização encontrada nas espécies da sect. *Brasilienses* não pôde ser considerado como uma sinapomorfia do grupo. Nesse sentido, essa característica é homoplásica em *Euphorbia*, ocorrendo em diversas linhagens. No entanto, a sinapomorfia aqui descrita, refere-se aos coléteres, pois a profusão dessas glândulas recobrindo a axila foliar foi exclusivamente observada nesse grupo.

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