Israel Lopes da Cunha Neto

Diversidade e evolução do sistema vascular

em Nyctaginaceae (Caryophyllales)

Diversity and evolution of the vascular system

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo (USP), para a obtenção do título de Doutor em Ciências, na Área de Botânica.

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São Paulo

2021

Comissão Julgadora:

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Prof. Dr.   Prof. Dr.

_____   _____

Prof. Dr.   Prof. Dr. Veronica Angyalossy

Dedico este trabalho

Aos mestres que me iniciaram no mundo da ciência e que primeiro sonharam comigo o sonho de me tornar um botânico: Ana Cristina Fermino Soares e Fabiano Machado Martins

À minha família, porque sempre me encorajam a ir além e porque sentirão um orgulho enorme por esta conquista.

"The dominant position which the Dicotyledons unquestionably hold among existing forms of vegetation is probably due in a greater degree to their method of secondary growth in stem and root, than to any other single character. The ability to increase indefinitely the amount of mechanical, conducting and storing tissues in the axial organs, in proportion to the increasing development of the foliage, has more or less generally existed in all the most successful classes of plants; but it is in the Dicotyledons that the highest differentiation of the secondary tissues is attained… The study of the modifications in the secondary formation of tissue in this class is therefore an important branch of biological inquiry.

Dukinfield H. Scott & George Brenner
On the Anatomy and Histogeny of Strychnos, 1889

"The central question in every morphological investigation became twofold: it was no longer simply *what is*? it was also *how came it to be*? And this second question, be it observed, is not properly a speculative matter at all, but an historical one; it related not to an ideal or hypothetical mode of origin, but to a real process that has actually taken place in the past and is to be determined like any other historical event."

Ronald A. Jenner
Natural History Museum, London, United Kingdom
The origin of evolutionary storytelling / Perspectives on Evolutionary and Developmental Biology, 2019

Agradecimentos

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Prefácio

Este trabalho foi projetado como uma investigação sobre a diversidade dos **padrões anatômicos** encontrados nos **caules** adultos de espécies de plantas da família **nictaginácea** (ou Nyctaginaceae, nome em latim). A espécie mais conhecida desse grupo é a **primavera** ou **buganvília** (*Bougainvillea,* em latim), e tenho certeza que você já se encantou com a beleza de suas flores em algum momento da vida. Além do grande poder ornamental das buganvílias e da "maravilha" (*Mirabilis*), as nictagináceas são importantes quer seja por serem usadas como plantas modelos em estudos ecológicos ("sand verbena" - *Abronia,* uma erva dos desertos da América do Norte), como madeira para serragem ou construção de pequenas embarcações lá na Amazônia (maria-mole - *Neea*) ou como fonte de compostos químicos para fins diversos, inclusive farmacológicos (*Boerhavia, Boldoa, Colignonia, Mirabilis*). Conhecer os padrões anatômicos dos caules das nictagináceas (e outras plantas) é importante tanto para o conhecimento da nossa biodiversidade, como para usos em estudos da biologia e outras ciências aplicadas (comercialização de madeira, botânica forense, biotecnologia). Assim, o estudo da anatomia das plantas pode desempenhar um importante papel para o progresso da ciência e da nossa sociedade.

Além de identificar os padrões observados nos caules das nictáginaceas, um dos principais objetivos do meu estudo foi descobrir quais as alterações que ocorrem na anatomia desses órgãos durante as fases de crescimento da planta. Assim, estudos voltados para conhecer o **desenvolvimento** (ou ontogenia) são realizados para investigar a forma dos organismos (plantas ou animais) desde sua origem até o estágio adulto. No nosso caso, estudamos os caules lá na ponta dos ramos (origem) até atingirem as formas mais complexas nos caules adultos, isto é, nos troncos das árvores ou na base das ervas e arbustos, ou ao longo dos caules dos cipós. Ou seja, este trabalho consistiu em nada mais nada menos do que contar a **história** da formação da estrutura interna dos caules nessas plantas com diferentes hábitos de vida e que estão distribuídas nos mais variados ambientes, incluindo florestas, montanhas, desertos ou mesmo na beira da praia. A diversidade estrutural que observamos nesses caules está diretamente relacionada com a organização das células que compõem os **tecidos vasculares** (ou condutores), isto é, os tecidos que

contém as células que transportam a água e os açúcares. À propósito, vale dizer que esses tecidos são fundamentais para a existência das plantas terrestres que vemos ao nosso redor – e por isso conhecê-los se torna tão importante, sendo alvo dos nossos estudos em uma escala anatômica.

Embora o objetivo principal desta pesquisa estivesse relacionado com os padrões anatômicos do sistema vascular, o olhar de anatomista e a curiosidade de pesquisador não me permitiu deixar passar despercebido uma **estrutura secretora** (=glândula que produz substâncias químicas) que ocorre em algumas plantas da família. A presença desta estrutura é tão marcante que em 1909, Paul C. Standley, um pesquisador dos Estados Unidos usou esta característica para escolher o nome científico dado a essas plantas (=*Anulocaulis*). Uma dessas espécies foi utilizada aqui para investigar esta estrutura porque ninguém havia descrito como ela de fato é em sua anatomia, e nem o tipo de substância que produz. Na natureza, a substância que é secretada é muito pegajosa, e alguns insetos ficam grudados nela e acabam morrendo. Curiosamente, algo semelhante acontece com as estruturas secretoras localizadas nos frutos de algumas espécies arbóreas (*Ceodes, Pisonia*) de ilhas do pacífico; nesse caso, as estruturas e substâncias são tão poderosas que podem grudar na asa de pássaros que não conseguem mais voar e acabam morrendo, motivo pelo qual estas plantas são conhecidas como "pegadoras de passarinhos").

O presente trabalho foi desenvolvido em parte durante a pandemia de coronavírus (COVID-19), pela qual vários planos, viagens de campo e atividades foram comprometidos enquanto uma doença infecciosa disseminada desolava o Brasil e o mundo. Parte do agravamento dessa pandemia se deve à natureza do vírus, parte ao descuido dos tomadores de decisão e, claro, ao ceticismo infundado em relação ao trabalho incansável de cientistas ao redor do globo.

Enfim, a seguir eu introduzo os vários temas relacionados à minha tese e então apresento a incrível diversidade anatômica dos caules das nictagináceas na forma de artigos científicos. Caso não seja possível ler todo esse conteúdo, espero que possa ao menos folhear as próximas páginas para se encantar com as belas imagens que a anatomia das nictagináceas nos forneceu! É tão incrível que uma dessas imagens foi destaque na revista Pesquisa FAPESP, em abril de 2020, e outra estampará a capa de um dos números do International Journal of Plant Sciences, em setembro de 2021.

Foreword

This work was originally planned as an investigation on the diversity of **anatomical patterns** found in adult **stems** of species of the **four o'clock family** (or Nyctaginaceae, name in latin). The most known plants of this group are the **paperflowers** (*Bougainvillea*) which I am certain you have been enchanted by their beauty of these flowers at some point in your life. In addition to the ornamental power of the paperflowers and four o'clocks (*Mirabilis*), the nyctaginacea are important because they are used as model plants in ecological studies ("sand verbena" – *Abronia*, an herb native to the deserts of North America), as sawdust or timber for construction of canoes in the Amazon region ("maria-mole" – *Neea*) or as source of chemical compounds for various purposes, including pharmacological (*Boerhavia, Boldoa, Colignonia, Mirabilis*). Knowing the anatomical patterns of stems of the four o'clock species (and other plants) is important for our knowledge of biodiversity, as for uses in a variety of biological studies, as well as other applied sciences (timber exploitation and marketing, forensic botany, biotechnology). Therefore, the study of plant anatomy can play an important role in the progress of science and our society.

In addition to identifying the structural patterns observed in the stems of the four o'clock plants, one of the main goals of my study was to unravel the changes occurring in the anatomy of the stems during the different stages of the growth of the plant. Thus, studies seeking to understand the **development** (or ontogeny) are carried out to investigate the form of organisms (plants or animals) from their origin until they reach the adult stage. In my case, I studied the stems from their origin (at the tip of the branches) until they reached the most complex forms in the adult stems, that is, in the trunks of trees or at the base of herbaceous plants, shrubs, or along the stems of climber plants. In other words, this work consisted of nothing more than telling the **story** of the formation of the internal structure of the stems in these plants with different habits and distributed different environments, including forests, mountains, deserts or even on the shore. The structural diversity that we observe in these stems is related to the organization of the cells that make up the **vascular tissues** (or conductive tissues), that is, the tissues that contain the cells that transport water and sugars. Additionally, it is worth noting that these tissues are fundamental

for the existence of the land plants we see around us – and that is why learning of them has become an important target of my studies on an anatomical scale.

Although the main goal of this study was to understand the anatomical patterns of the vascular system, the trained eye of an anatomist and the curiosity of a researcher did not prevent me from overlooking a **secretory structure** (=gland that produces chemical substances) that occurs in some plants of the family. The presence of this structure is so incredibly remarkable that in 1909, Paul C. Standley, a researcher from the United States, used this feature to decide on the scientific name given to these plants (=*Anulocaulis*). One of these species was used in this study to investigate this structure, as no one had described what it actually looks like in its anatomy, nor the type of substance it produces. In nature, the substance that is secreted is very sticky, and some species of insects tightly bind to it causing them to dye. Interestingly, something similar happens with the secretory structures present in the fruits of some tree species (*Ceodes, Pisonia*) of the Pacific Islands; in this case, the structures and substances are so powerful that they can stick to the wing of birds impairing their flying abilities and eventually causing their death; these plants are known as "bird catchers".

This work was developed in part during the coronavirus pandemic (COVID-19) by which various plans, field trips and activities were compromised as widespread infections desolated Brazil and the world. Part of the aggravation of this pandemic is due to the nature of the virus, part due to the carelessness of decision-makers, and, of course, due to unfounded skepticism towards the tireless work of scientists around the globe.

Anyway, below I present a summary of the results of my thesis (page 15). Afterwards, I start to present the incredible anatomical diversity of stems from species of the four o'clock plants in the form of scientific articles. If you are not interested in reading all this content, I hope you can at least flip through the next few pages to be enchanted by the beautiful images that the anatomy of the four o'clock has provided us! It's so incredible that one of these images was featured in [Pesquisa FAPESP magazine](), in April 2020, and another image will be on the cover page of an issue of the International Journal of Plant Sciences in September, 2021.

General Introduction

General Introduction

Plant Morphology: a way to understand nature

Plant morphology can be described as the discipline that try to explain what each part of the plant is (Scott, 1906). This branch of botany encompasses the study of plant anatomy, which is concerned with the survey of the internal morphology of plants. It is a traditional field in plant science seeking to comprehend the structure and any part of living organisms. Plant morphology is, therefore, a complex concept since it encompasses the knowledge from macromolecules to whole organisms. The ways to study these structures is also variable and that is how morphology become a so ample and interesting subject. The morphological research can be divided in many fields (e.g., concept, process, developmental morphology, morphometrics) (Sattler, 1996; Weber, 2003; Sattler, 2019; Rutishauser, 2020). Currently, plant morphology plays an important role not only in describing the variety of forms in nature, but also to understand the evolutionary history of phenotypic characters and ontogenetic pathways within the framework of molecular phylogenies (Endress, 2003; Jaramillo et al., 2004; Rutishauser, 2020). This evolutionary developmental approach is the main method used in this study.

The evolutionary developmental biology is a field of study that aims to understand the processes involved in the generation of morphological diversity and evolutionary patterns of the organisms (Hall, 2003, 2012; Arthur, 2004). In this sense, the interpretation of ontogenetic data within a phylogenetic framework allows us to better understand the morphological diversity of organisms because the development is the process that generates the adult forms. Therefore, comparative studies integrating ontogeny and phylogeny in plants have become increasingly common, from seed morphology to pollination biology, allowing us to test whether developmental mechanisms such as heterochrony, heterotopy, novelty, or homeosis had been involved in the generation of morphological diversity (Jaramillo et al., 2004; Rudall and Bateman, 2006; Pryer and Hearn, 2009; Pace et al., 2009; Armbruster et al., 2013; Vasconcelos et al., 2018).

Exploring the structure of plants is fundamental in botanical science and offers essential information to look at the hypotheses of plant systematics and evolution. Specifically, the study of wood anatomy (secondary xylem) and bark anatomy (secondary phloem + periderm) has played a crucial role in our understanding of plant biology (Baas, 1982; Olson, 2007; Carlquist, 2009; Rosell et al., 2017; Frankiewicz et al., 2020), including in the interface with biomechanics (Rowe *et al.* 2004; Isnard *et al.* 2005; Gerolamo *et al.* 2020), hydraulics (Gerolamo and Angyalossy 2017; Lamarque *et al.* 2018; Dória *et al.* 2019), as well as ecophysiology (Feild and Isnard 2013; Jupa *et al.* 2016) and biotechnology/biomimetics (Fiorello et al., 2020; Gallentine et al., 2020; Soffiatti and Rowe, 2020).

Anatomy of vascular tissues: a major aspect of plant biology

Because of the vital importance of conducting tissues in vascular plants, the stele is one of the firstborn and key concepts in plant biology. The stele is the organization of the primary vascular system in stems and roots (Van tieghem 1884). The vascular tissue produces during this developmental stage is a result of the activity of the primary meristem, the procambium, and the different types of steles have long been showed to have pivotal systematic importance. As these organs continue to develop, additional vascular tissue is formed by the vascular cambium, which is a secondary meristem that differentiates in a continuum with the procambium (Esau 1943). Structurally, the vascular cambium is a meristem formed by two types of cells but organized generally as a single or few layers of cells along the stem surface, as seen in transverse view. Yet, the vascular cambium is the group of cells that produce most of the biomass of the planet, since its meristematic activity yields the wood (secondary xylem) and bark (secondary phloem) forming the tall, long, and robust stems/trunks of plants over the surface of the Earth.

The risen of vascular cambium was a key innovation accounting for the possibility of new growth forms, i.e., tall self-supporting plants like trees and shrubs, and enabling them to conquer different ecological niches. Evolutionarily, the vascular cambium appeared in the ancestors of a large group of plants, the lignophytes, which includes most of the tallest and largest plants ever known (Simpson, 2010; Decombeix et al., 2019). This group encompass both extinct and extant plants, the latter including

the well-known gymnosperms and angiosperms, together known as the seed plants (Simpson, 2010). Nevertheless, one must bear in mind that this meristem is in no way uniform in origin, location, structure, or nature of action. Overall, in relation to their activity, the cambium of the seed plants is usually a bifacial meristem generating xylem centripetally and phloem centrifugally (Spicer and Groover, 2010; Chiang and Greb, 2019). However, there are plants interpreted as having a unifacial cambium (e.g., Lycopsids, Decombeix et al., 2019), and plants with cambium producing secondary tissues with inverted polarity due to natural and/or experimental causes (Siebers, 1971; Terrazas et al., 2011; Cunha Neto et al., 2018; Tomescu and Groover, 2019).

In addition, the stems (and some roots) can show alternative types of vascular growth, called cambial variants, which result from the differential activity of the regular/single cambium (e.g., phloem wedges, xylem and phloem in plates) or the formation of several cambia (e.g., successive cambia, compound, divided and corded stems) (Angyalossy *et al.* 2012, 2015). The various types of cambial variants are widespread across the phylogeny of angiosperms, appearing also in the gymnosperms (e.g., Gnetales) (Angyalossy *et al.* 2012, 2015).

Unlike the evolution of the vascular cambium in the lignophytes, however, the multiple evolution of cambial variants does not seem to have impacted the life of plants on earth in the same proportion. Although much has still to be investigated, there are reasons to believe that cambial variants confer important ecological advantages to vascular plants especially in lianas (scandent plants), in which they are referred to increase their mechanical strength, conductivity and flexibility (Putz and Mooney 1991; Schnitzer *et al.* 2015). The functional role of cambial variants in self-supporting plants the functional role is still waiting for clarification.

In relation to the diverse biological aspects of the cambium, the case study presented here is certainly a good example to show how liable this meristem can be even within a small lineage as it is the family Nyctaginaceae.

Nyctaginaceae (the four o'clock family): a neotropical lineage with intriguing morphological diversity

Nyctaginaceae is a monophyletic family considered a core group within the order Caryophyllales based on both morphological and molecular evidence (Bittrich

and Kühn, 1993; Douglas and Manos, 2007; Hernández-Ledesma et al., 2015). Morphologically, the monophyletism of the family is indicated by the absence of corolla and the type of fruit, an achene, commonly known as 'anthocarp' (Levin, 2000; Douglas and Manos, 2007). Although the majority of the genera within Nyctaginaceae may be recognized based on the variation of the anthocarp (Douglas and Manos, 2007; Douglas and Spellenberg, 2010), the relationships inside the family are less evident, probably as a result of the absence of characters provided by the flowers (Levin, 2000; Douglas and Spellenberg, 2010).

Currently, the classification of Nyctaginaceae includes around ~300-400 species, distributed in ~34 genera, divided in seven tribes (e.g., Boldoeae, Bougainvilleeae, Caribeaeae, Colignonieae, Leucastereae, Nyctagineae and Pisonieae) (Douglas and Manos, 2007; Douglas and Spellenberg, 2010; Rossetto et al., 2019; Rossetto and Caraballo-Ortiz, 2020). While the tribal classification seems to be quite stable due to its recent re-assessment based on molecular data (Douglas and Spellenberg, 2010), the generic classification is undergoing new circumscriptions, as for the resurrection of earlier described genera (e.g., *Ceodes* and *Rockia*), that were segregated from the large genus *Pisonia* (Rossetto and Caraballo-Ortiz, 2020).

The species of Nyctaginaceae are distributed mostly in the tropics and subtropics of the New World (Bittrich and Kühn, 1993; Douglas and Manos, 2007; Hernández-Ledesma et al., 2015). In the Neotropics, one of the centres of distribution of the family, approximately 23 genera and approximately 180 species have been recorded (Damascena and Coelho 2009). In Brazil, there are 11 genera and more than 50 species distributed throughout the country (Sá *et al.* 2020); the genera *Andradea, Belemia, Bougainvillea, Guapira, Leucaster, Neea, Pisonia, Ramisia* and *Reichenbachia* have been recorded as native plants; *Andradea, Belemia, Leucaster* and *Ramisia* are endemic to the country, while *Boerhavia* and *Mirabilis* are considered naturalized genera (Sá *et al.* 2020). The second centre of distribution of the family is in the deserts of North America, where several native genera of herbs and subshrubs underwent a remarkable species radiation (Douglas and Manos, 2007).

Overall, the species of Nyctaginaceae comprise a wide diversity of growth forms, including herbs, subshrubs, shrubs, scandent-shrubs, trees and scandent plants, herbaceous or woody vines (lianas) (Bittrich and Kühn 1993; Douglas and

Spellenberg, 2010; Hernández-Ledesma et al., 2015). It is worth to note that several taxa within the family can be found sometimes as self-supporting (e.g., free-standing shrubs or trees) and sometimes as scandent individuals (i.e., scandent-shrubs or lianas), which is the case of some species of *Bougainvillea* and *Leucaster* (pers. obser.) and some *Commicarpus* (Friis et al., 2016; Thulin, 2021). Other taxa are remarkable as suffrutescent plants (=sufrutescens or sufruticosus) meaning that they are woody at the base and herbaceous throughout the branches, which are similar to subshrubs (Bittrich and Kühn 1993; Douglas and Spellenberg, 2010; Hernández-Ledesma et al., 2011, 2015; Friis et al., 2016; Blecher and Blecher, 2017). This is the case of genera such as *Colignonia, Commicarpus* and *Mirabilis* (Friis et al., 2016; pers. obser.), and likely other less known taxa, as the species *Cryptocarpus pyriformis* from the Galapagos and continental Ecuador. As we will see, this diversity of habits is accompanied by a variation in the degree of wood formation ('woodiness'), as well as in the developmental processes resulting in their adult forms characterized by cambial variants. This remarkable diversity is one reason why we use the family Nyctaginaceae as a model to investigate aspects of the diversity, distribution, and evolution of stem anatomical characters.

Stem anatomy in Nyctaginaceae: intricate diversity from stele to cambium and cambial variants

In Nyctaginaceae, the origin, development and activity of the vascular meristems have been debated since a long time. Different interpretations have been proposed in the literature and there has been no consensus on several aspects of stem development. Given the notorious diversity of vascular patterns in the family, as easily spotted from the presence of medullary bundles in young stems and mature stems with cambial variants, a variety of studies focuses on the presence and development of these various patterns of secondary growth (Chalk and Chattaway 1937; Rajput and Rao 1998; Carlquist 2004; Hernández-Ledesma *et al.* 2011). However, these works generally analyses few taxa, and it is possible to observe that different cambial variants are reported for the same genus or even the same species without a clear developmental understanding and classification. For example, Chalk and Chattaway (1937) reported that the cambial variant of the genera *Guapira, Neea* and

Pisonia, is interxylary phloem, whereas Carlquist (2004) states that they all have successive cambia. On the other hand, detailed studies in relation to both wood (secondary xylem) and the bark (all tissues outside the cambium) are still needed, since most information on stem anatomy for the family are restricted to classical works (Solereder 1908; Record and Hess 1943; Metcalfe and Chalk 1957; Roth 1981), or papers concentrating on a few taxa (Puglia and Norverto 1991; Rajput and Rao 1998; Hernández-Ledesma *et al.* 2011; Sonsin *et al.* 2014).

Studies integrating stem anatomy and ontogenetic data with phylogeny have never been performed for Nyctaginaceae. Because the family have a wide diversity of habits and types of cambial variants within these habits, multiple scenarios are possible to evaluate whether there have been shifts in rates of diversification and if the transitions of habit (as suggested by Gianoli, 2004, 2015) and habitat, as well as if the acquisition of new anatomies have impacted the family in terms of diversification.

Furthermore, the analysis of anatomical characters of the secondary xylem and phloem will allow us to contribute to the understanding of the evolution of these tissues in plants with transitions of habits and acquisition of cambial variants. In other words, with this study we will be able to answer several questions, such as: How many cambial variants are there in Nyctaginaceae and when did they arise? Are these characters ancestral or derived for the family? Was the cambial variant already present in Nyctaginaceae and its sister group, thus constituting a symplesiomorphy or alternatively the cambial variants appear/evolve within the group? If so, how many times? In the self-supporting plants, which are submitted to different selection pressures than lianas, what would be the possible functions of the cambial variants?

This thesis aims to bring together the aspects concerning the development and evolution of stem anatomical characters in Nyctaginaceae, with emphasis on the distribution and anatomical diversity of vascular patterns. We accomplish this by studying the origin, anatomy, and development of vascular tissues within a phylogenetic framework. By addressing the anatomical and developmental information upon a robust phylogenetic hypothesis, we are now able to identify the main aspects behind the evolutionary history of stem characters in the family.

Thesis outline

Our hypotheses and specific goals are investigated in each of the five chapters of the thesis. Chapters one to four have already been published, and chapter five will be submitted for publication after the thesis defense and due corrections of the manuscript. The organization of the articles follow the guidelines of the respective journals.

In **chapter 1**, we demonstrate the structure and histochemistry of a poorly known secretory structure found in the stems of *Anulocaulis.* With regard the vascular structure, Nyctaginaceae has presented an intriguing and complex morphological diversity concerning the formation of both primary and secondary anatomies. **Chapter 2** presents a case study to approach the complexity of alternative patterns of primary and secondary vascular anatomies in *Allionia.* It also encompasses a general study of the anatomy of vegetative organs aiming at to corroborate to the systematics of this genus. The facets of the primary vascular tissue organization are explored in a broader scale in **chapter 3**, where the presence of medullary bundles is investigated in the context of stele concept. In this paper the development and evolution of the different eustele types (i.e., regular and polycyclic) is presented, with emphasis to the diversity of medullary bundles. **Chapter 4** presents a comparative anatomical study of stems under secondary growth of species from different lineages of Nyctaginaceae. This study presents a new interpretation for the secondary growth in the family as unexpected developments confirm the presence of interxylary phloem for the family. These represent some of the most important results of this thesis due to the demystification of successive cambia as the universal type of cambial variant in the family, and perhaps other families of the Caryophyllales. In **Chapter 5**, we explore the evolution of development for the two newly delimited types of cambial variants in Nyctaginaceae (i.e., interxylary phloem and successive cambia). We demonstrate that there are different combinations on how the secondary growth is established in relation to the different subtypes of eusteles. The mode how these two types of cambial variants are constructed is not always the same but constitute different developments that may contain intermediate stages, as in a case of the so called "fuzzy morphology" (continuum morphology).

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Chapter 1

What are the "sticky rings" on stems of *Anulocaulis* and related taxa (Nyctaginaceae) from arid regions?*

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WHAT ARE THE "STICKY RINGS" ON STEMS OF *ANULOCAULIS* AND RELATED TAXA (NYCTAGINACEAE) FROM ARID REGIONS?

ABSTRACT

Anulocaulis, commonly known as "ringstem," is a small, unusual genus restricted to the Chihuahuan, Sono 'ran, and Mojave deserts of North America. Here we combined light microscopy and histochemical tests to characterize for the first time the "sticky structures" (here called secretory rings) found on the stem internodes of *Anulocaulis*. The secretory rings were shown to be groups of epidermal cells, or unicellular glandular trichomes, which largely differ from their neighboring cells both in structure and histochemistry. The cells start to differentiate in early stages of stem development. They begin as regular epidermal cells, but later their anticlinal and external tangential walls start to enlarge. At maturity the cells become remarkably elongated, even balloon-like, with dense cytoplasmic content. Although the secretory rings have been reported as "mucilaginous structures" based on morphological observations, preliminary histochemical analyses showed that its exudate is complex, including a mixture of mucilage, proteins, and phenolic compounds. Future investigations are needed to compare the anatomy of the secretory rings within related genera of Nyctaginaceae and characterize the chemical components of their exudate more specifically to search for potential homologies and adaptive functions of these structures.

RESUMEN

Anulocaulis, comúnmente conocido como "ringstem," es un género pequeño que se encuentra restringido a los desiertos de Chihuahuan, Sonoran y Mojave de América del Norte. En este estúdio, usamos microscopía óptica y pruebas histoquímicas para caracterizar por primera vez las "sticky structures" (aquí denominadas "secretory rings") que se encuentran en los entrenudos del tallo de *Anulocaulis*. Se demostró que los anillos secretores son grupos de células epidérmicas que se diferencian en gran medida de sus células vecinas tanto en estructura como en histoquímica. Las células comienzan a diferenciarse en las

primeras etapas del desarrollo del tallo. Comienzan como células epidérmicas normales, pero luego sus paredes anticlinal y tangencial externa comienzan a agrandarse. En la madurez, las células se vuelven notablemente alargadas, incluso como globos, con un contenido citoplásmico denso. Aunque los anillos secretores han sido descritos como "estructuras mucilaginosas" basadas en observaciones morfológicas, análisis histoquímicos preliminares mostraron que su exudado es complejo, incluyendo una mezcla de mucílago, proteínas y compuestos fenólicos. Son necesarias investigaciones futuras que permitan estudiar comparativamente la anatomía de los anillos secretores entre los géneros de Nyctaginaceae y así mismo caracterizar los componentes químicos de su exudado más específicamente para buscar posibles homologías y funciones adaptativas de estas estructuras.

Key Words: Anatomy, Caryophyllales, Nyctagineae, secretory structures, Chihuahuan Desert, glandular trichomes.

INTRODUCTION

Anulocaulis is a small genus of perennial herbs with just five species. It is included in tribe Nyctagineae, which is the largest and most diverse tribe in the family Nyctaginaceae (Douglas & Spellenberg 2010). The genus is endemic to arid regions of North America (e.g., Chihuahuan, Sonoran, and Mojave Deserts) and is distributed from northern Mexico to southeastern California in the United States of America (Spellenberg 1993; Douglas & Spellenberg 2010). *Anulocaulis* may be divided into two groups based on fruit morphology (Spellenberg 1993). The first encompasses *A. annulatus, A. hintoniorum,* and *A. eriosolenus,* which have smooth anthocarps. *Anulocaulis annulatus* is restricted to low, hot elevations near Death Valley in the Mojave Desert, while *A. hintoniorum and A. eriosolenus* are restricted to the Chihuahuan Desert in Mexico and Texas. The second group comprises *A. leiosolenus* (considered to have four varieties, Spellenberg 1993) and *A. reflexus.* These are characterized by their variously winged and wrinkled anthocarps and are primarily gypsum endemic plants (Spellenberg 1993). Taxa in this group are mainly Chihuahuan, with three disjunct population centers in Arizona and Nevada (Moore et al. 2014).

     Anulocaulis was segregated from the large and diverse genus *Boerhavia* by Standley in 1909 based on several morphological characteristics, including the presence of the "sticky rings." Since the recognition of the genus *Anulocaulis* by Standley (1909), several authors have noted the presence of this structure (Fig. 1) encircling a small portion of the internodes (Bittrich & Kühn 1993; Spellenberg 2003; Hernández-Ledesma et al. 2010). In fact, the name *Anulocaulis* was given by Standley in reference to the presence of such structures (*anulo* = ring; *caulis* = stem), which he described as "stems glabrous, but the middle of each internode usually provided with a reddish ring which exudes a mucilaginous fluid." For this reason, *Anulocaulis* species are popularly known as "ringstem." Various terms have been used to address this structure, such as "sticky bands" (Spellenberg, 1993, 2003), "glandular rings" (Bittrich & Kühn 1993), "sticky rings" (McClellan & Boecklen 1993), "internodal bands of viscid secretions" or "viscid bands" (Douglas & Manos 2007), and "mucilaginous rings" or "glutinous bands" (Hernández-Ledesma et al. 2010); here we will use the term "secretory rings." These secretory rings are observed not only in *Anulocaulis* but also in a several species of *Boerhavia* (e.g., *B. erecta, B. spicata,* & *B. xantii*) and

the monotypic genus *Cyphomeris* (Bittrich & Kühn 1993; Douglas & Manos 2007; Hernández-Ledesma et al. 2010)*.*

The occurrence of sticky structures/secretion onto plant surfaces have received notorious attention in the last few years due to several lines of evidence indicating their positive interactions with other organisms (Krimel & Pearse 2013; LoPresti 2015; Karban et al. 2019). Sticky exudates on plant surface can be effective on organisms, either herbivores or their predators (LoPresti 2015). In several of these plants, the sticky substances derive from glandular trichomes, which are typically considered direct defenses against herbivores (Krimel & Pearse 2013). Nevertheless, in some groups, the sticky structures/secretion work as traps to catch insects which become available to other predators that defend the plants against herbivores, working as an indirect plant defense system that ultimately increase plant fitness (Karban et al. 2019). For instance, in *Nicotiana attenuata*, where the flowers components are relatively sticky, the number of carrion found on plants were positively predicted both with the number of predator and the number of seed capsules that were produced (Karban et al. 2019). Besides *N. attenuata* (Solanaceae)*,* similar cases of sticky plants have been reported in different groups, including Asteraceae (*Maedia elegans, Hemizonia congesta* – Krimel & Pearse 2013; LoPresti et al. 2018), Ranunculaceae (*Aquilegia eximia* – LoPresti et al. 2015), as well as Nyctaginaceae (*Boerhavia spicata,* McClellan & Boecklen 1993). Such interactions between sticky plants and predators can be paralleled with better-known mutualistic cases as in those plants that provide predators with nutritious resources (e.g., nectar, pollen, protein bodies; Krimel & Pearse, 2013; Karban et al., 2019). In Nyctaginaceae, as in other groups, the occurrence of sticky substances has also been related to plant defense in another way, which are sand entrapment on the plant surface (LoPresti & Karban, 2016). Plants showing this mechanism, termed psammophory, were reported as suffering less damage from herbivore chewing, as demonstrated in *Abronia latifolia* (Nyctaginaceae) and *Navarretia mellita* (Polemoniaceae) (LoPresti & Karban, 2016).

Despite the peculiarity of the secretory rings in Nyctaginaceae species, a structural investigation of this character is still lacking. Therefore, this work aimed to analyze the anatomy and histochemistry of the secretory rings in stems of

Anulocaulis leiosolenus in order to characterize the secretory structure and determine the main chemical compounds present in the exudate. By doing this, we expect to shed light on what these secretory rings truly are so that in further investigations it would be possible to search for potential homologies and the adaptive function of these structures.

MATERIALS AND METHODS

Stem samples were obtained at four sites in the northern Chihuahuan Desert, in the states of Texas and New Mexico, United States of America, from 12–17 Sep 2018. In total, we collected five specimens of *Anulocaulis leiosolenus* var. *gypsogenus,* three of *Anulocaulis leiosolenus* var. *leiosolenus* and two of *Cyphomeris gypsophiloides* (see Appendix 1 for detailed information on the collection, collectors, localities, and herbarium vouchers).
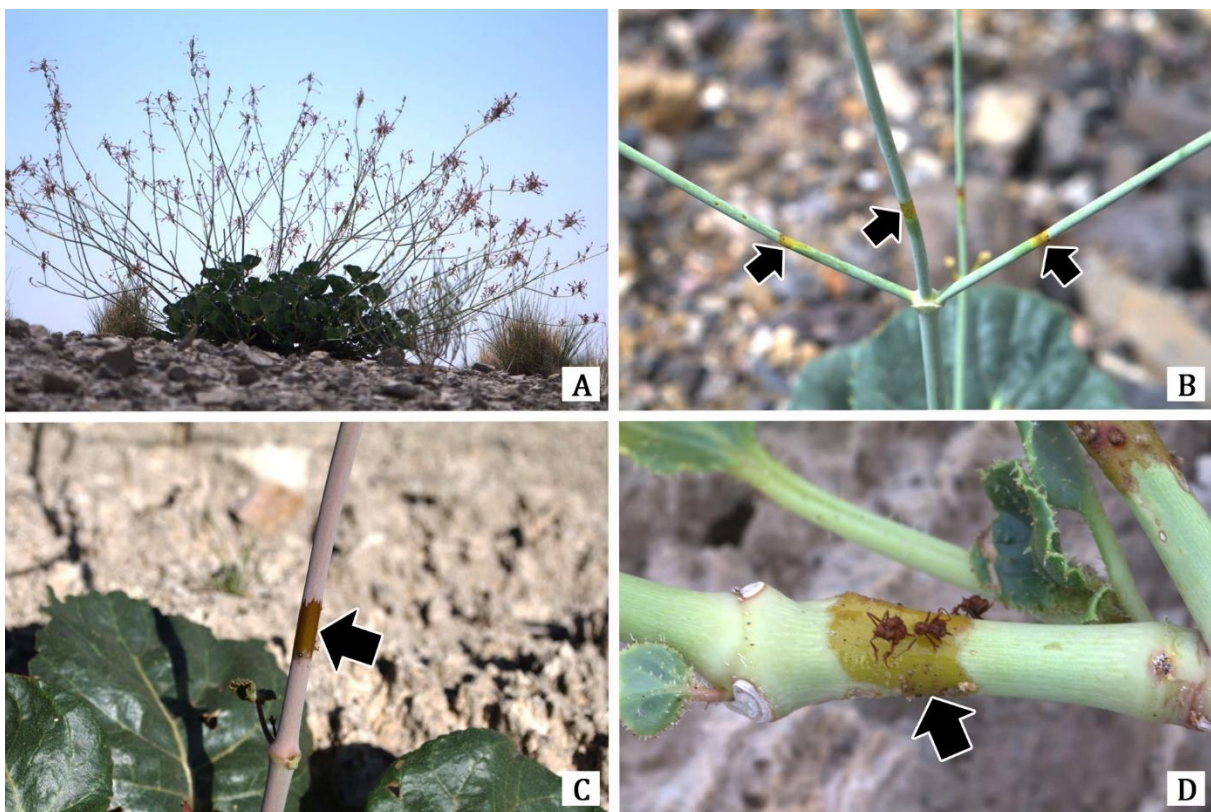
FIG. 1. Morphological aspects of the plant and secretory rings in *Anulocaulis leiosolenus* var. *gypsogenus.* 1a: Habit. 1b: Secretory rings in young internodes (arrows). 1c: Detail of secretory ring in older internode. Notice the irregular contour of the rings. 1d: Secretory ring with immobilized insects (arrow).

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The secretory rings are variable in their expression either at species or population levels. Although all the species and varieties collected have been reported with these structures, only the representatives of *Anulocaulis leiosolenus* var. *gypsogenus* from the population at Yeso Hills (Eddy County, New Mexico, USA) showed them at the time we visited the population. Therefore, specimens from this population were used for the anatomical and histochemical study.

Stems of all individuals were fixed in 70% isopropanol and later stored in 70% ethanol. For the anatomical study, freehand sections were initially performed to identify the regions with the cells forming the secretory rings. Later, the material was dehydrated in butanol series (Johansen 1940) and embedded in Paraplast or dehydrated in an ethanol series and embedded in 2-hydroxyethyl-methacrylate (historesin - Histosec, Merck). The samples were sectioned in transversal and longitudinal planes in a rotary microtome at ca. 5–10 μm thickness. Sections performed by hand or embedded in Paraplast were stained with Safrablau (Bukatsch 1972), whereas sections embedded in historesin were stained in Toluidine Blue (O'Brien et al. 1964). Permanent slides were produced using Permount (Fisher Scientific,. Pittsburgh, USA), and photomicrographs were taken using a light microscopy (Leica DMLB).

Freehand sections were obtained from fixed material, and the following tests were performed following Demarco (2017): Sudan Black and Sudan IV staining for detection of lipids; Nile blue for acid/neutral lipids; Ruthenium red, Alcian blue, and tannic acid and ferric chloride for acid mucilage and/or pectins; Lugol reagent for starch; PAS reaction (periodic acid: Schiff's reagent) for carbohydrates; Aniline Blue Black staining for proteins; NADI reaction for terpenes; ferric chloride and potassium dicromate staining for phenolic compounds; phloroglucinol staining for lignin; and Wagner's reagent for alkaloids. Standard control procedures were carried out simultaneously as required for each test.

RESULTS

In *Anulocaulis leiosolenus* var. *gypsogenus,* the secretory rings occur in young and mature stem internodes (Fig. 1). Morphologically, they appear as irregular marks that are yellowish-green to brown due to secreted material (Fig. 1B–D). In the field,

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the secretion can appear when the internode is young, and expands as the stem matures, and is evidently self-limiting, as older rings sometimes become covered in sand, insects, or other debris (Fig 1D). The secretion is apparently viscid at all times; furthermore, we have observed that herbarium specimens that are several decades old remain quite sticky.

In early stages of stem development, approximately at the second or third internode, the secretory rings have already started to differentiate (Fig. 2A–C). These structures consist of a group of differentiated epidermal cells, which can be characterized as unicellular glandular trichomes (Fig. 2A–C). Initially, they are cells with regular size and shape that later begin to elongate their anticlinal and/or distal tangential walls (Fig. 2C). As this structure continues to develop with the increase in diameter of the stem, cells with much larger size are gradually found side by side (Fig. 2C, 2F), forming either a complete ring (Fig. 2D) or an incomplete ring as seen in external morphology (Fig. 1C–D). In developed secretory rings, the epidermal cells are markedly enlarged and radially elongated into palisade-like cells, sometimes assuming an ovoid or balloon-like shape, overlying much smaller and isodiametric subepidermal cells (Fig. 2D–E).

The developed cells forming the secretory rings exhibit a large nucleus (Fig. 2I–J), a dense cytoplasm (Fig. 2D–J), and a characteristic thickened primary wall, especially in the distal tangential walls (Fig. 2I–J). In unstained sections, the content shows a brownish color (Fig. 3A). This content turns dark red when stained with safranin and Astra Blue (Fig. 2E), light to dark green when stained by Safrablau in free-hand sections (Fig. 2G–H), and light blue when stained with Toluidine Blue (Fig. 2I–J). The content secreted by these cells is the protoplasm itself (Fig. 2E, 2G–J).

The thickened primary cell walls of secretory ring cells were found to be rich in pectins, as indicated by the light pinkish color in sections stained with Toluidine Blue (Fig. 2J, 3B). The secretion reacted positively (brown color) to ferric chloride (Fig. 3C), which typically indicates the presence of general, non-structural phenolic compounds. The presence of mucilages was revealed by a positive reaction (light blue color) with Alcian blue (Fig. 3D), whereas Aniline Blue Black staining detected the presence of nonstructural proteins (dark color) (Fig. 3E). Therefore, the exudate of these cells showed a mixture of mucilage, proteins, and phenolic compounds.
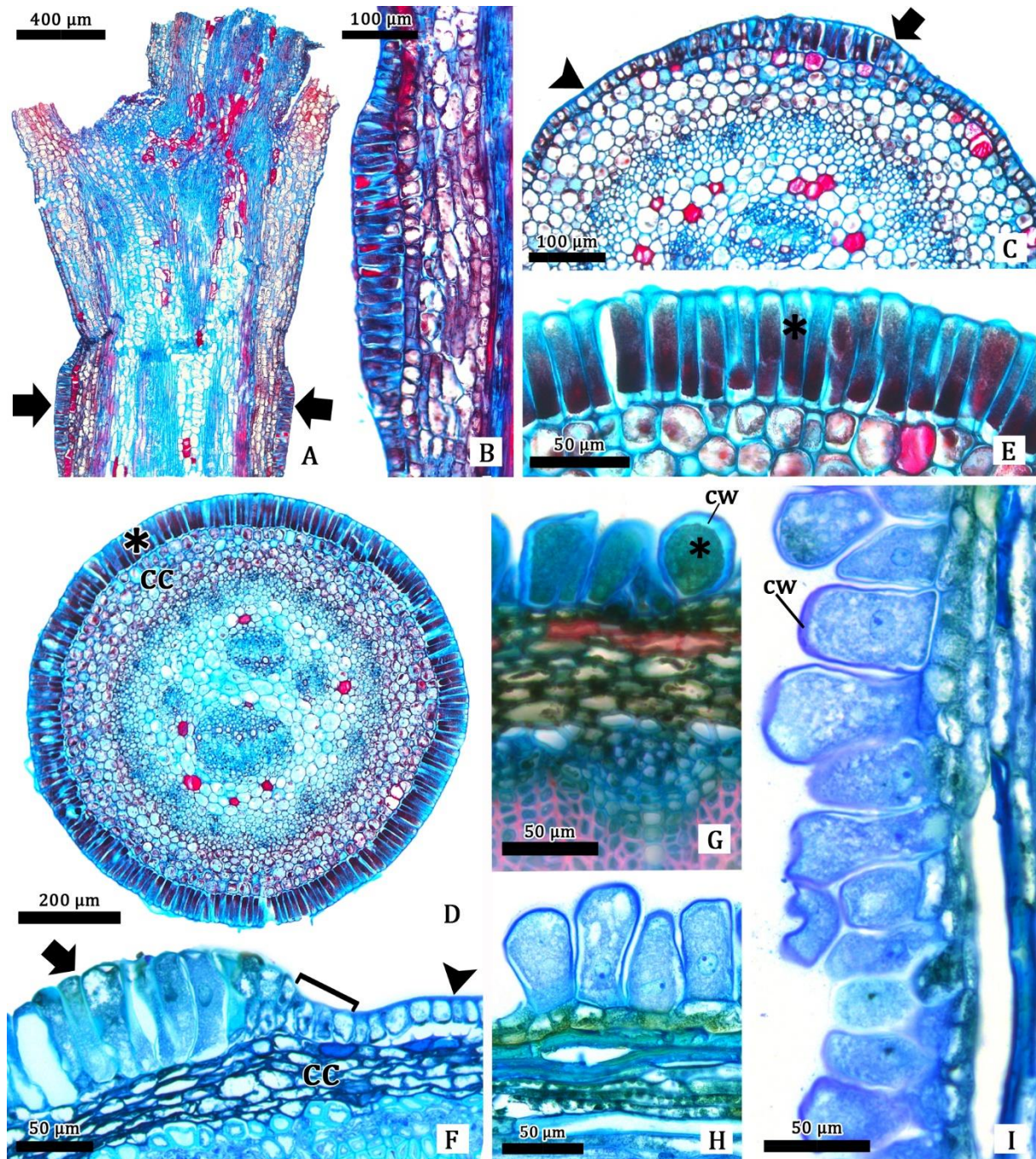
FIG. 2. Development and anatomy of secretory rings in *Anulocaulis leiosolenus* var. *gypsogenus.* A–B, J: Longitudinal sections. C–I: Transverse sections. **2a–b:** Early developmental stage of stem internodes showing the beginning on the differentiation of secretory epidermal cells (arrows). **2c:** Stem section showing that cells become gradually enlarged and radially elongated (arrow), while other cells remain still regular (arrowhead); **2d–e:** Secretory ring occupying the entire stem internode circumference. Notice the enlarged and elongated epidermal cells with dense and homogenous content (asterisk) underlying smaller and isodiametric cortical cells (cc). **2f:** Detail of secretory ring showing transition region between regular

(arrowhead), enlarging (bracket), and elongated epidermal cells (arrow). **2g–h:** Free-hand sections stained with Safrablau showing epidermal cells forming the secretory rings with dense and homogeneous content (asterisk). Notice the thickened cell wall (cw), especially in the external periclinal walls (**g**) and disrupted cells (thin arrow) (**h**), a possible mechanism for secretion releasing. **2i:** Developed epidermal cells showing dense cytoplasm, large nucleus, and thickened walls. **2j:** Detail of secretory ring in longitudinal view showing also the balloon-like shape of some cells which have also dense cytoplasm, large nucleus, and thickened walls (cw). Notice their thickened wall in pinkish color by the Toluidine Blue staining, evidence of the presence of pectins.
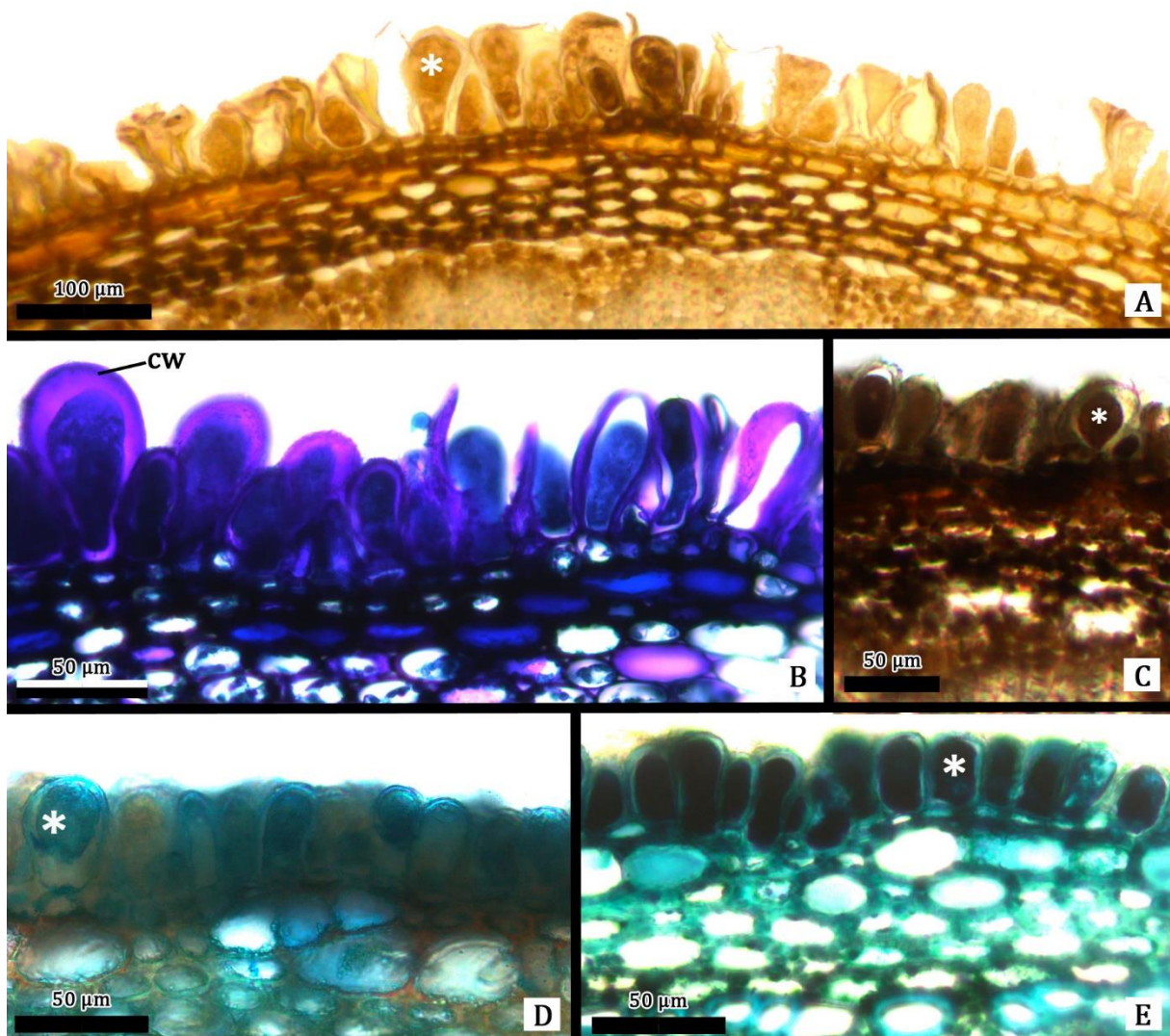
Fɪɢ. 3. Histochemical characterization of secretory rings in *Anulocaulis leiosolenus* var. *gypsogenus*. **3a:** Unstained section showing cells forming the secretory ring with balloon-like shape and yellowish to brownish content (asterisk). **3b.** Sample treated with Toluidine Blue. Thickened cell walls (cw) stain pink, thus indicating the presence of pectins. **3c:** Positive result for ferric chloride evidencing the presence of phenolic

compounds (asterisk). **3d:** Mucilage (asterisk) detected by with Alcian Blue reagent. **3e:** Positive reaction for protein (asterisk) using Aniline Blue Black.

DISCUSSION

In this study, the ontogeny, structure, and histochemistry of the secretory rings found in *Anulocaulis* are described for the first time. We found that the rings are, in fact, a group of epidermal cells that differentiate early in development and which produce, store, and release a complex secretion, consisting of mucilage, phenolic compounds, and proteins. The cells forming the secretory rings are markedly different from the surrounding cells (e.g., regular epidermal cells and subepidermical cells) both in structure and in metabolism. Structurally, the secretory rings are formed by a layer of differentiated epidermal cells, which are notably larger and elongated outwards. These cells resemble unicellular trichomes or papillose cells, which are highly variable epidermal appendages, whose distinction is not always clear (Evert 2006; Rudall 2007). Another feature that distinguishes these cells is their remarkably dense cytoplasm, which might be rich in organelles, as commonly observed in cells with active physiological roles in the secretion of secondary metabolites (Fahn 1979; Evert 2006; Tilney et al. 2014; Fernandes et al. 2017; Ballego–Campos & Paiva 2018). Also, the histochemical detection of proteins in the cytoplasm of the secretory rings may also indicate high metabolic activity within these cells (Fernandes et al. 2017).

The identification of mucilage in the exudate of the secretory rings confirms field observations predicting that these structures would contain mucilaginous components (Bittrich & Kühn 1993; Spellenberg 1993, 2003; Hernández–Ledesma et al. 2010). However, given that the secretory rings produce a complex exudate they should not be classified as "mucilaginous rings" as mentioned by Hernández–Ledesma et al. (2010). Typical mucilaginous cells, as the ones reported to occur in reproductive (e.g., flowers) or vegetative organs (e.g., leaves, wood, bark) in several eudicot lineages (Metcalfe & Chalk 1950; Gregory & Baas 1989; Matthews & Endress 2006), are interpreted as cells containing mainly polysaccharides, especially pectins (Fahn 1979; Matthews & Endress 2006), while the exudate of *Anulocaulis* is a mixture of different compounds. The presence of mucilage has been frequently related to the ability of the plant to absorb and store water when available (Gregory & Baas 1989 and references

therein). For a long time, this idea has been repeated for plants from arid and stressful environments, such as deserts (Gregory & Baas 1989), Mediterranean habitats (Christodoulakis et al. 1990) and sandy coastal areas (Ballego-Campos & Paiva 2018). However, other authors have criticized this or proposed alternative interpretations such as the potential to reduce transpiration (Gregory & Baas 1989). It may also be a self-sealing mechanism that is, to protect plants from dehydration and infections after damage by sealing wounds (Anandan et al. 2018). In any case, it has been accepted that the capacity in storage and water retention of epidermal cells may be determined by the amount and composition of pectins in thickened walls (Voragen et al. 2009; Kuster et al. 2018), as seen in *Anulocaulis* and indicated for similar epidermal cells observed in *Vangueria infausta* (Rubiaceae) (Tilney et al. 2014). Besides the similarity in shape, the epidermal cells in *V. infausta* were also reported with a cytoplasm rich in organelles, including secretory vesicles that might be involved in the production of hydrophilic and sticky substances (Tilney et al., 2014).

McClellan and Boecklen (1993) experimentally examined the role of the secretory rings in *Boerhavia spicata* and determined that such structures appear to discourage ant-aphid colonization or reduce ant or aphid density. According to the authors, the "sticky rings" work as traps, functioning in a way similar to other external secretory structures (e.g., glandular trichomes) or acting to diminish aphid populations. Indeed, these sticky plant defense syndrome, as reported to other groups, is likely to be an effective way for plants to cope with predators, especially those from arid and Mediterranean environments since their secretion are not washed due to rainstorms (Karban et al., 2019). Moreover, the presence of phenolic compounds in the exudate of *Anulocaulis* may corroborate the results found by McClellan and Boecklen (1993), since phenolics are well-known for their potential to provide chemical defense against pathogen activity and herbivory (Evert 2006). In addition, our observation that the exudate remains sticky for a long period of time supports the idea that the rings may have adaptive importance in immobilizing potential herbivores, as demonstrated in other plant-insect interactions associated with sticky secretion (Monteiro & Macedo 2014; Krimel & Pearse 2013; LoPresti et al. 2018; Karban et al., 2019), although their somewhat inconsistent expression indicates

that if the rings represent a defense mechanism it might be inducible rather than constitutive.

To the best of our knowledge there has been no report of similar epidermal cells as the one described for *Anulocaulis* for other Nyctaginaceae. Nevertheless, columnar parenchyma cells have been described for the anthocarp walls in other taxa of tribe Nyctagineae (i.e., *Acleisanthes, Boerhavia* and *Mirabilis*) by Wilson & Spellenberg (1977). Despite their structural similarity with the secretory ring cells in *Anulocaulis* (both are elongated), the cells in the fruits are located in the subepidermal region, and so seems to belong to the cortex instead of the epidermis. Although no histochemical analyses were performed for the columnar parenchyma cells, the authors observed that there is a discharge of mucilage-like material when the fruits of *Boerhavia* and *Mirabilis* were placed in water. The presence of these elongated cells and mucilage-like content was discussed as possible mechanisms to water retention, epizoochory, germination and carnivory (Wilson & Spellenberg 1977).

The occurrence of secretory rings in *Anulocaulis* and related genera have captured the attention of botanists at least since the segregation of the genus *Anulocaulis* by Standley (1909). More recently, in a morphological study to test the monophyly of *Anulocaulis* and related genera, including *Boerhavia* and *Cyphomeris*, Hernández-Ledesma et al. (2010) performed a cladistic analysis and concluded that the occurrence of the "mucilaginous rings" was not corroborated as a synapomorphy for these genera, indicating likely a convergence in the evolution of this character.

Although further experimental and ultrastructural analyses are required to elucidate the secretory activity and the mechanism of secretion, the findings obtained here shed light into the structure, development, and histochemistry of this secretory structure which was poorly known in the family. Indeed, more work is needed to compare the anatomy and chemical components of the secretory rings within related genera of Nyctaginaceae to search for potential homologies in other secretory ring-bearing species, or to the unusual viscid secretions found commonly on fruits of species in genera in different tribes of Nyctaginaceae (Wilson & Spellenberg, 1977; personal observation). Finally, additional research into the ecology of this structure (its expression, and its effects on herbivores or seed predators) is needed to understand its function.

APPENDIX 1

Information on taxa, collectors, localities and herbarium vouchers for the analyzed species.

Anulocaulis leiosolenus var. *leiosolenus* (Torr.) Standl. USA. Texas. Sierra Blanca, Malone Mountains, *Douglas & Cunha Neto 2278*, 13 Sep 2018, 31.25342, -105.598, (BRIT, FLAS).

Anulocaulis leiosolenus var. *gypsogenus* (Waterf.) Spellenb. & Wootten. USA. New Mexico, Eddy County, Yeso Hills, *Douglas & Cunha Neto 2280*, 13 Sep 2018, 32.018269, -104.433307 (BRIT, FLAS); Crest of 7 Rivers Hills, *Douglas & Cunha Neto 2277*, 14 Sep 2018, 32.487, -104.335 (BRIT, FLAS).

Cyphomeris gypsophiloides (M. Martens & Galeotti) Standl. USA. Las Cruces, New Mexico, Organ Mountains-Desert Peaks National Monument, *Douglas et al. 2287*, 15 Sep 2018, 32.33642, -106.59743, (BRIT, FLAS).

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Chapter 2

Anatomy of vegetative organs in *Allionia* (Nyctaginaceae), with emphasis on the vascular system*, **

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** This paper was developed in collaboration with *Juliana P. da Silva*, who used part of the study as her undergraduate thesis.

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ANATOMY OF VEGETATIVE ORGANS IN *ALLIONIA* (NYCTAGINACEAE), WITH EMPHASIS ON THE VASCULAR SYSTEM

ABSTRACT

Allionia is a small genus within the tribe Nyctagineae (Nyctaginaceae) which has a controversial infrageneric delimitation. Here, we investigated the two known species of *Allionia* in order to characterize the anatomy of leaves, stems and roots, with further notes on vascular system development. Additionally, the present study aimed to broaden our knowledge of stem vascular diversity and to survey for anatomical features with diagnostic value in distinguishing *A. choisyi* from *A. incarnata*. Leaf anatomy of other Nyctagineae taxa were also analysed. Anatomical and ontogenetic observations from the vegetative organs in *Allionia* revealed no diagnostic features to distinguish the two species. We illustrated the occurrence of Kranz anatomy, which in Nyctaginaceae is only known in *Allionia, Boerhavia* and *Okenia*. The stem primary vascular system was unusual in showing a polycyclic eustele (medullary bundles + continuous concentric procambium). Likewise, mature stems and roots show vascular cambial variants (successive cambia) that arise from the pericycle. The anatomy and histochemistry of multicellular glandular trichomes observed in aerial organs were presented. Raphids were seen in all organs. Although no strong xerophytic features were observed in *Allionia,* several characteristics can be associated with their arid habitats. Our findings on the vascular system of *Allionia* showed the two species to be much the same and reinforced earlier findings that the stem anatomy of Nyctaginaceae is complex and intriguing.

RESUMEN

Allionia es un género pequeño dentro de la tribu Nyctagineae (Nyctaginaceae) con delimitación infragenérica controversial. Analizamos las características anatómicas de hojas, tallos y raíces de las dos especies conocidas de *Allionia* e incluimos comentarios sobre el desarrollo del sistema vascular. El presente estudio pretende, examinar características diagnósticas entre *A. choisyi* y *A. incarnata* y de esta forma ampliar el conocimiento sobre la diversidad vascular del tallo.

Adicionalmente, analizamos la anatomía foliar de otros taxa de Nyctagineae. Las observaciones anatómicas y ontogenéticas de los órganos vegetativos en *Allionia* no mostraron características diagnósticas que permitieran diferenciaran entre las dos especies. La anatomía Kranz para Nyctaginaceae, restringida únicamente a *Allionia, Boerhavia* y *Okenia* fue ilustrada. Presentamos la anatomía e histoquímica de tricomas glandulares multicelulares observados en órganos aéreos. El sistema vascular primario del tallo era incomum al mostrar un eustele policíclico (haces medulares + procambio concéntrico continuo). Así mismo, tallos y raíces maduras mostraron variantes cambiales vasculares (cambios sucesivos) que surgen en el periciclo. Todos los órganos presentaron rafidios. No fueron observadas características xerofíticas en *Allionia*, sin embargo, varias características pueden estar relacionadas con ambientes áridos. Estos hallazgos esclarecen y corroboran la complejidad anatómica de las especies de Nyctaginaceae, y muestran la intrigante diversidad de patrones anatómicos caulinares.

KEY WORDS: *Allionia choisyi, Allionia incarnata,* Caryophyllales, development, Nyctagineae, ontogeny.

INTRODUCTION

Nyctaginaceae have about 30 genera and 400 species which include trees, shrubs, subshrubs, lianas and herbs (Douglas & Manos 2007; Douglas & Spellenberg 2010; Hernández-Ledesma et al. 2015). The species are distributed mostly in the tropics and subtropics of the New World, except for some genera that occur in the Old World (e.g. *Boerhavia*, *Commicarpus*, *Pisonia*, *Phaeoptilum* and *Mirabilis*) (Hernández-Ledesma et al. 2015). In the most recent classification, the family has been divided into 7 tribes: Nyctagineae, Boldoeae, Leucastereae, Bougainvilleeae, Pisonieae, Colignonieae and Caribeaeae (Douglas & Spellenberg 2010).

Allionia L. belongs to tribe Nyctagineae and comprises species of annual or perennial herbs with procumbent, decumbent or prostrate stems (Fig. 1). Two species are recognized *A. choisyi* Standl. and *A. incarnata* L. which are very similar morphologically, differing only in some fruit characteristics (e.g. number of lateral expansions, length of glands) (Spellenberg 2003), which makes the delimitation of infrageneric categories controversial. In general, most authors have followed Standley's work (1931) and accepted *Allionia* as having two species (Phillips 1976; Bittrich & Kühn 1993; Turner 1994; Hernandez-Ledesma & Olvera 2003; Spellenberg 2003; López & Anton 2006; Hernandez-Ledesma et al. 2015; Sandoval-Ortega et al. 2020), whereas other studies have treated the genus consisting of one variable species (Heimerl 1932; Fay 1980; Rzedowski & Rzedowski 2001; Spellenberg 2012). The two species grow in a variety of habitats, mostly in warmer, xeric regions of North America (southwestern United States of America and northern Mexico) – including on gypsum soils (Waterfall 1946; Alexander et al. 2014) – and South America (Argentina, Bolivia, Venezuela) (Phillips 1976; Bittrich & Kühn 1993; Turner 1994). The species have been found also in the Antilles (Turner 1994; Hernandez-Ledesma & Olvera 2003; Douglas & Manos 2007).

Anatomical information on *Allionia* is almost entirely restricted to the dissertation of Phillips (1976) entitled "Anatomy and developmental morphology of *Allionia* L. (Nyctaginaceae)". This work sought to understand the structural aspects of reproductive and vegetative organs, among other biological aspects of the genus. Although Phillips (1976) had described several anatomical features in *Allionia,* the taxon sampling was restricted to specimens from North America. Over the years,

additional ontogenetic data obtained for the family has promoted interest in investigating the stem vascular system within Nyctaginaceae due to its remarkable anatomical diversity.

FIG. 1. Specimens of *Allionia incarnata* in their natural habitat. 1a–c: Chihuahuan Desert, New Mexico, USA. 1a: Overview of the environment with plants growing on the ground (notice the pinkish dots from the inflorescences). 1b: Prostrate habit, leaves and inflorescence. 1c: Details of the inflorescence; 1d–f: Parque Nacional Amboró, Bolivia. 1d: Environment and habit. 1e: Details of leaves and inflorescence. 1f: Detail of primary root. Photographs: Israel L. Cunha Neto.

Whether the various vascular architectures are associated with their different habits and habitats are still uncertain. We here present further information on the anatomy and development of *Allionia,* a poorly studied group within the herbaceous representatives of the Nyctaginaceae, enhancing our understanding on the evolution of the development of vascular characteristics in the family.

Many herbs produce secondary tissues making possible the study of the "wood" (secondary xylem) and "bark" (secondary phloem + periderm) (Schweingruber, 2011; Carlquist 2012; Dória et al. 2018). Interestingly, several of these plants have anatomies known as vascular cambial variants that are found in adult stems and/or roots of species within different families across eudicots (Schweingruber et al. 2011). This is the case of *Allionia* and other species within Nyctaginaceae, such as *Boerhavia* and *Mirabilis* (Phillips 1976; Rajput & Rao 1998; Rajput et al. 2009; Hernández-Ledesma et al. 2011). In addition, most Nyctaginaceae species (including *Allionia*) present medullary bundles as a major component of their stem, resulting in an increase in the complexity and diversity of primary vascular system within the family (Cunha Neto et al. 2020).

Here we revisit the anatomy of vegetative organs in *Allionia* emphasizing the aspects related to development of stem vascular system. We studied the two currently recognized species *A. incarnata* and *A. choisyi* aiming to assess the uncertainty on the infrageneric status of the genus. In addition, the leaves of other representatives of Nyctagineae were studied and compared with the anatomical features in *Allionia.*

MATERIAL AND METHODS

Taxon sampling and material collection

In this study, the taxon sampling covered the two main distribution regions of the genus, emphasizing specimens from South America that were not investigated by Phillips (1976). Samples from *Allionia incarnata* L. with procumbent habit were collected in their natural habitats both in North and South America. For *Allionia choisyi* Standl., we obtained only one stem sample from an herbarium voucher (Table

1). Additional information for the species were obtained from Phillips' (1976). See Appendix 1 for other species studied.

Table 1. Specimens analysed with information of taxa, species authorities, location, vegetation, collector and collector numbers, and herbaria where vouchers were deposited.

Taxon	Environment	Locality	Collector (herbarium)
Allionia choisyi Standl.	Chihuahuan Desert	Mesilla Valley, New Mexico, USA	US 498327
Allionia incarnata L.	Valles secos	Parque Nacional Amboró, Santa Cruz, Bolívia	Nee, MH 64124 – 64126 (USZ)
	Chihuahuan Desert	Luna County, New Mexico, USA	Douglas, NA 2292 (FLAS)
	Chihuahuan Desert	Malone Mountains, Sierra Blanca, Texas, USA	Douglas, NA 2276 (FLAS)

Each specimen collected from natural population consisted of whole plants, including root and shoots with stems, leaves and inflorescences. Samples from these plants were fixed in FAA 70 (formaldehyde–acetic acid–ethanol) for 24 h (Johansen 1940) or in 70% isopropanol, and then transferred to 70% ethyl alcohol.

Anatomical procedures for light microscopic

Samples from different ontogenetic stages of each organ were selected. We used fully expanded leaves which were trimmed to obtain the middle portion of the petiole, midrib and leaf margin. From stems we selected samples near the apex, middle, and base in order to ensure that all developmental stages of vascular system ontogeny would be sampled. Similarly, primary and secondary roots were selected for the study.

Samples of leaves and young stems and roots were dehydrated either in an ethanol–t-butanol series and embedded in paraplast (Fisher Healthcare, Houston, Texas, USA) or dehydrated in ethanol series and embedded in Historesin (Leica; LeicaMicrosystems, Heidelberg, Germany) (Johansen, 1940; Ruzin 1999). These samples were sectioned in a rotary microtome (Leica RM2145, Nussloch, Eisfeld, Germany), typically 3–10 μm thick, and stained with 1.5% alcoholic safranin O and 1% aqueous Astra blue (Gerlach 1969) or stained with toluidine blue (O'Brien et al. 1964), respectively.

Leaves and stems of *Allionia choisyi* from dried herbarium vouchers were rehydrated in distilled water until submersion, dehydrated in ethylic series and then included in Historesin (Leica; LeicaMicrosystems, Heidelberg, Germany) and stained with toluidine blue (O'Brien et al. 1964).

Adult stem and roots were embedded in polyethylene glycol 1500 (Rupp 1964) and sectioned in transverse, longitudinal radial, and longitudinal tangential planes with a sliding microtome (Leica SM2010R, Nussloch, Eisfeld, Germany) with the aid of a styrofoam resin (Barbosa et al. 2010). These anatomical sections were double stained with safrablau (Kraus & Arduin 1997) or safranin and alcian blue (Johansen 1940).

All the sections were mounted on permanent slides with synthetic resin (Permount; Fisher Scientific, Fair Lawn, NJ).

In order to characterize the morphology of each cell type we performed macerations using Jeffrey's solution (10% aqueous nitric acid + 10% aqueous chromic acid; Johansen 1940) and mounted slides in 50% glycerine. Macerations were performed independently for the secondary xylem and secondary phloem from samples near the cambium and for the pith. The samples were obtained from adult stems.

Histochemical analyses and diaphanization

Histochemical tests were performed on hand-free sectioned samples of the stem of *Allionia incarnata* (*Douglas 2292* and *Nee 61124*), as follow: Sudan IV and Sudan Black B (Johansen 1940; Pearse 1985) to detect total lipids; Nile Blue (Cain 1947) to detect neutral and acidic lipids; ruthenium red (Gregory & Baas 1989) to detect pectates and acidic mucilage; ferric chloride to identify phenolic compounds (Johansen 1940); NADI reagent for terpenoids (David & Carde 1964), Aniline Blue Black for proteins (Fisher 1968) and lugol to detect starch (Johansen 1940). Standard control procedures were carried out as required for each test, and the sections were mounted between slides and cover slips with Kaiser's jelly glycerin (Kraus & Arduin 1997).

Fully expanded and intact leaves were treated in 5% sodium hydroxide, washed in distilled water, then cleared in sodium hypoclorite and stained with 1% aqueous safranin (Kraus & Arduin 1997). After clearing, the leaves were cut into small pieces and mounted with glycerinated gelatin.

All slides were analysed using a Leica DMBL light microscope coupled with a digital camera (Leica DFC310, Leica Microsystems, Wetzlar, Germany).

Anatomical descriptions

Anatomical descriptions for secondary xylem and secondary phloem followed the International Association of Wood Anatomists's guides, including the 'IAWA List of Microscopic Features for Hardwood Identification' (IAWA Committee 1989) and the 'IAWA List of Microscopic Bark Features' (Angyalossy et al. 2016), respectively. All measurements were performed for both regular secondary xylem and phloem and for the medullary bundles. We measured the diameter, length, frequency, and occupied area for vessels, sieve-tube elements and fibers. The wall thickness of fibers was also calculated. The cell frequencies were calculated within a grid of 0.1 mm² for the xylem and 0.01 for the phloem. The occupied areas were calculated for mature xylem and phloem using 50 points per analysis (adapted from Ziemińska et al. 2015). All measurements were performed using the free software ImageJ (ver. 1.45s; Rasband 2012), with a minimum of 30 repetitions.

RESULTS

Here we describe the anatomical aspects leaves, stems, and roots, with emphasis on the structure and ontogeny of the vascular system in the stem. The anatomical description below refers to both *A. incarnata* and *A. choisyi*.

Leaf anatomy

The epidermis shows polygonal cells with straight anticlinal walls on both surfaces (Fig. 2a–b). The leaves are amphistomatic (Fig. 2c, e) with anomocytic stomata that are scattered on the leaf blade (Fig. 2a–b). In cross section, the stomata are disposed at the same level of the ordinary epidermal cells and are underlined by a notorious substomatal chamber (Fig. 2c, e). Multicellular and uniseriate glandular trichomes occur on both surfaces of the leaves (Fig. 2g).

The epidermis is uniseriate and composed by cells with roundish or polygonal contour and variable sizes in cross view (Fig. 2c, e, 3a–d). The mesophyll is dorsiventral (bifacial), usually with two layers of palisade parenchyma, and lacunose parenchyma characterized by roundish cells of various sizes and wide intercellular spaces (Fig. 2c–d, 3a, c, d). Generally, one layer of longer palisade cells occurs

towards the adaxial epidermis, while another layer of shorter cells occurs below the vascular system, which occur in the middle of the mesophyll (Fig. 2c–e, 3a–d). The same arrangement is observed in the mesophyll near the leaf blade margin (Fig. 3d). Leaf blade margins with smaller and round cells throughout the mesophyll were also observed in one specimen collected in Bolivia (Fig. 3b). The leaf blade margins are straight (right edges) in all specimens (Fig. 3b, d).

The vascular system is composed of collateral bundles with xylem towards the adaxial surface and phloem towards the abaxial surface (Fig. 2e).
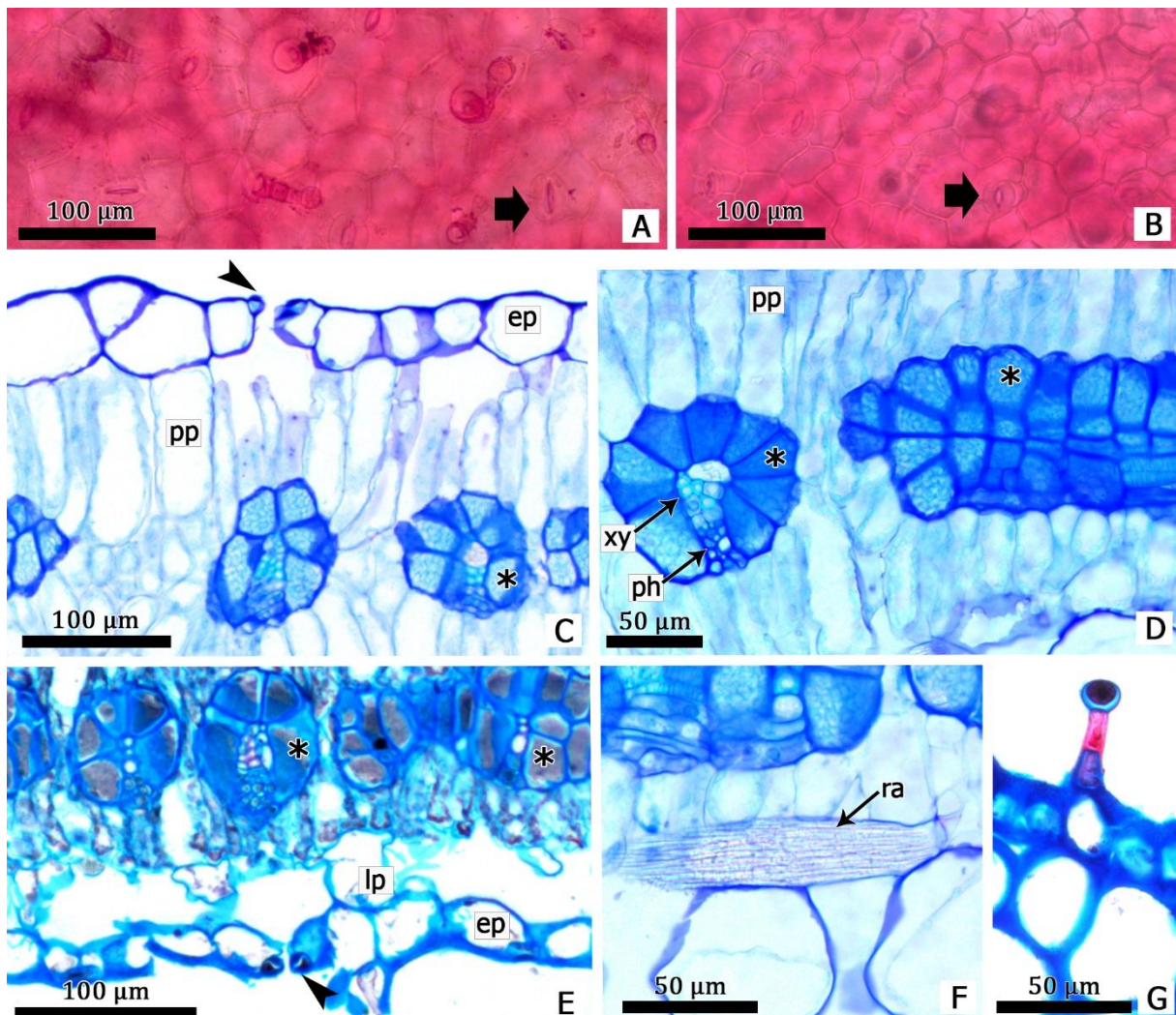
FIG. 2. Anatomy of leaf blade in *Allionia incarnata*. 2a–b: Leaf surface showing epidermal cells and anomocytic stomata. 2c: Adaxial surface with stomata (arrowhead). 2d: Bundle sheath cells (asterisks) forming Kranz anatomy. 2e: Abaxial surface with stomata (arrowhead). 2f: Crystals (raphides). 2g: Uniseriate glandular trichome. *Asterisks,* bundle sheath cells; *ep,* epidermis; *lp,* lacunose parenchyma; *pp,* palisade parenchyma; *ra*, raphides.

Within the tribe, similar anatomy is observed only in other two genera, *Okenia* and *Boerhavia* (Fig. 2c–e, 4a–h). Cells containing crystals (raphids) are found throughout the mesophyll (Fig. 2f, 3a–b).

The midrib is prominent with larger portion towards the abaxial surface (Fig. 5a, c). The epidermal cells are similar to the ones in the leaf blade (Fig. 5a, c). A few collenchyma cells occur in subepidermal layers immediately beneath the adaxial epidermis (Fig. 5c). The mesophyll is composed of round parenchymatic cells of various sizes (Fig. 5a, c). The vascular system is formed by a single unit which eventually may divide into two or more collateral bundles (Fig. 5a, c, 6a). There are one to three accessory bundles on the adaxial side of the main vascular system (Fig. 6a).
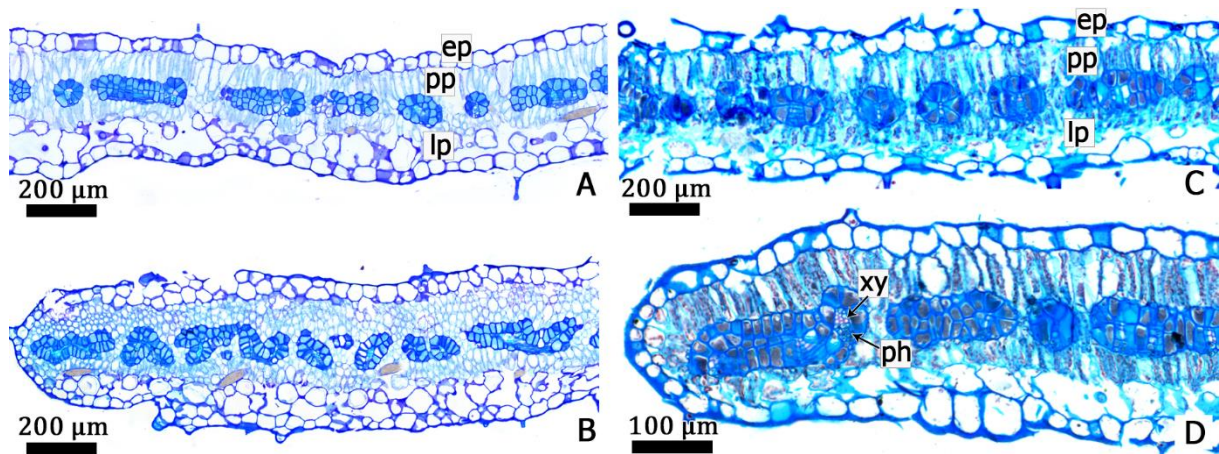
FIG. 3. Cross section of the leaf blade in *Allionia incarnata*. **3a–b**: Specimens of *A. incarnata* collected in Bolivia, Parque Nacional Amboró. **3c–d**: Specimens of *A. incarnata* collected in the Chihuahuan Desert, New Mexico, USA. **3a, 3c**: Leaf blade showing palisade parenchyma (one layer above and other below the vascular system), and one to two layers of lacunose parenchyma. **3b, 3d**: Straight leaf margins showing round cells in **3b**, and elongated palisade-like cells in **3d**. *ep,* epidermis; *lp, lacunose* parenchyma; *ph,* phloem; *pp,* palisade parenchyma; *xy,* xylem.

The petiole is roundish or slightly plane-convex in cross section (Fig. 5b, d). The epidermis is uniseriate and contains multicellular uniseriate glandular trichomes (Fig. 5b, d). The cortex is composed of round parenchymatic cells (Fig. 5b, d). The vascular system is formed by four or five collateral vascular bundles (Fig. 5b, d, 6b).
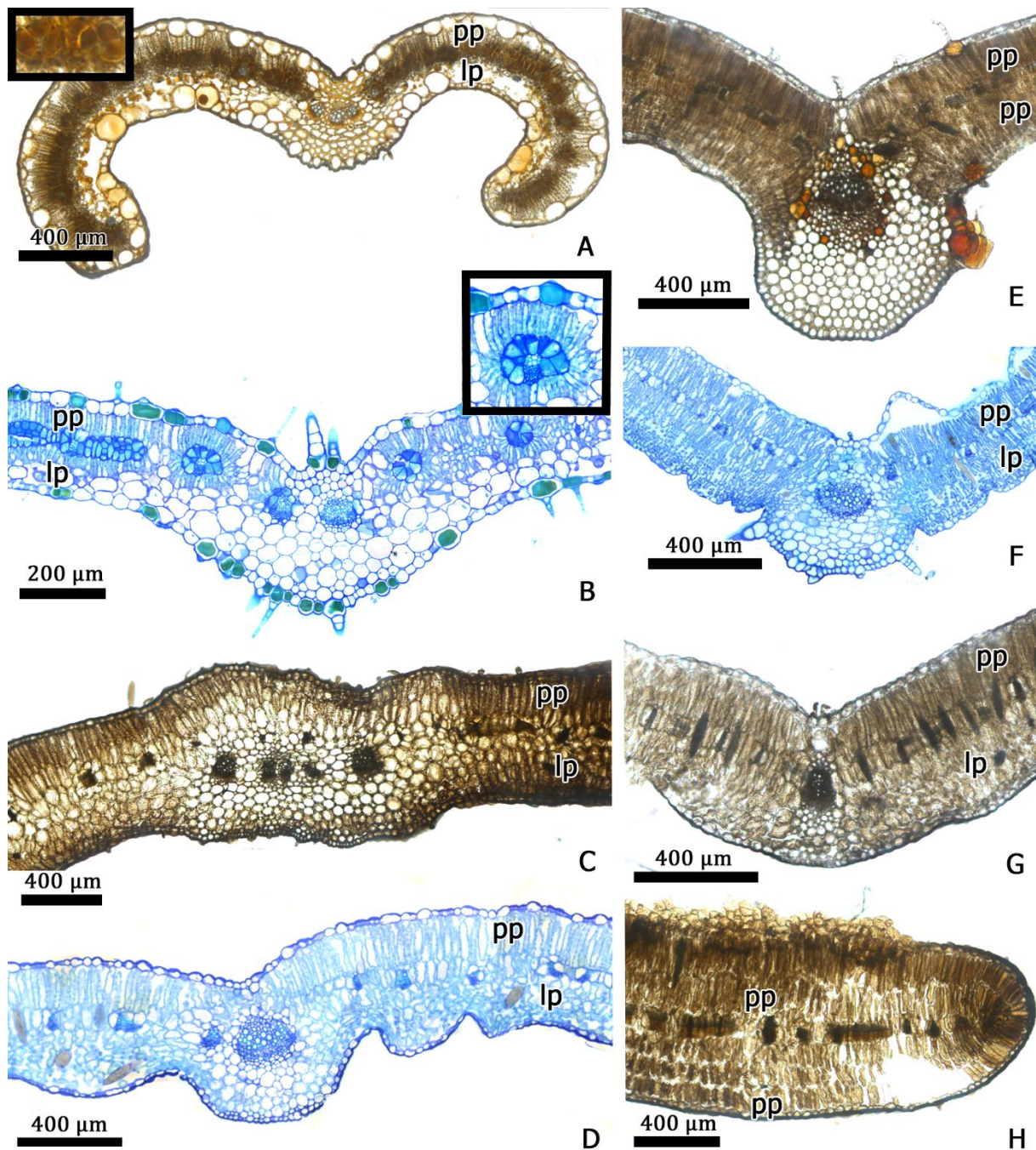
FIG. 4. Leaf anatomy in related species of Nyctagineae. **4a–b**: Species with Kranz anatomy. **4a**: *Boerhavia linearifolia*. **4b**: *Okenia hypogaea*. **4c–h**: Species without Kranz anatomy. **4c**: *Acleisanthes lanceolata*. **4d**: *Commicarpus scandens*. **4e**: *Anulocaulis leiosolenus* var*. gypsogenus*. **4f**: *Nyctaginia capitata*. **4g**: *Cyphomeris gypsophilioides*. **4h**: *Abronia nealleyi.*

Stem anatomy

Early stages of development and transition from primary to secondary vascular system. – In early developmental stages, the epidermis of the young stem is uniseriate with both multicellular, unbranched non-glandular trichomes and

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multicellular, uniseriate glandular trichomes (Fig.7a). Initially, the cortex is composed of large and round parenchyma cells (Fig.7a) which later become flattened (Fig.7b). The primary vascular system consists of six to eight medullary bundles – collateral vascular bundles located in the pith – organized in a ring and encircled by a continuous concentric procambium (CCP) (Fig. 7a, b). The CCP produces a ring of collateral vascular bundles externally to the medullary bundles and internally to the starch sheath (endodermis) (Fig. 7a, c-d). This way the CCP can be divided into a fascicular and an interfascicular portion. The pith is parenchymatous with large round cells that can store starch (Fig. 7a, b).
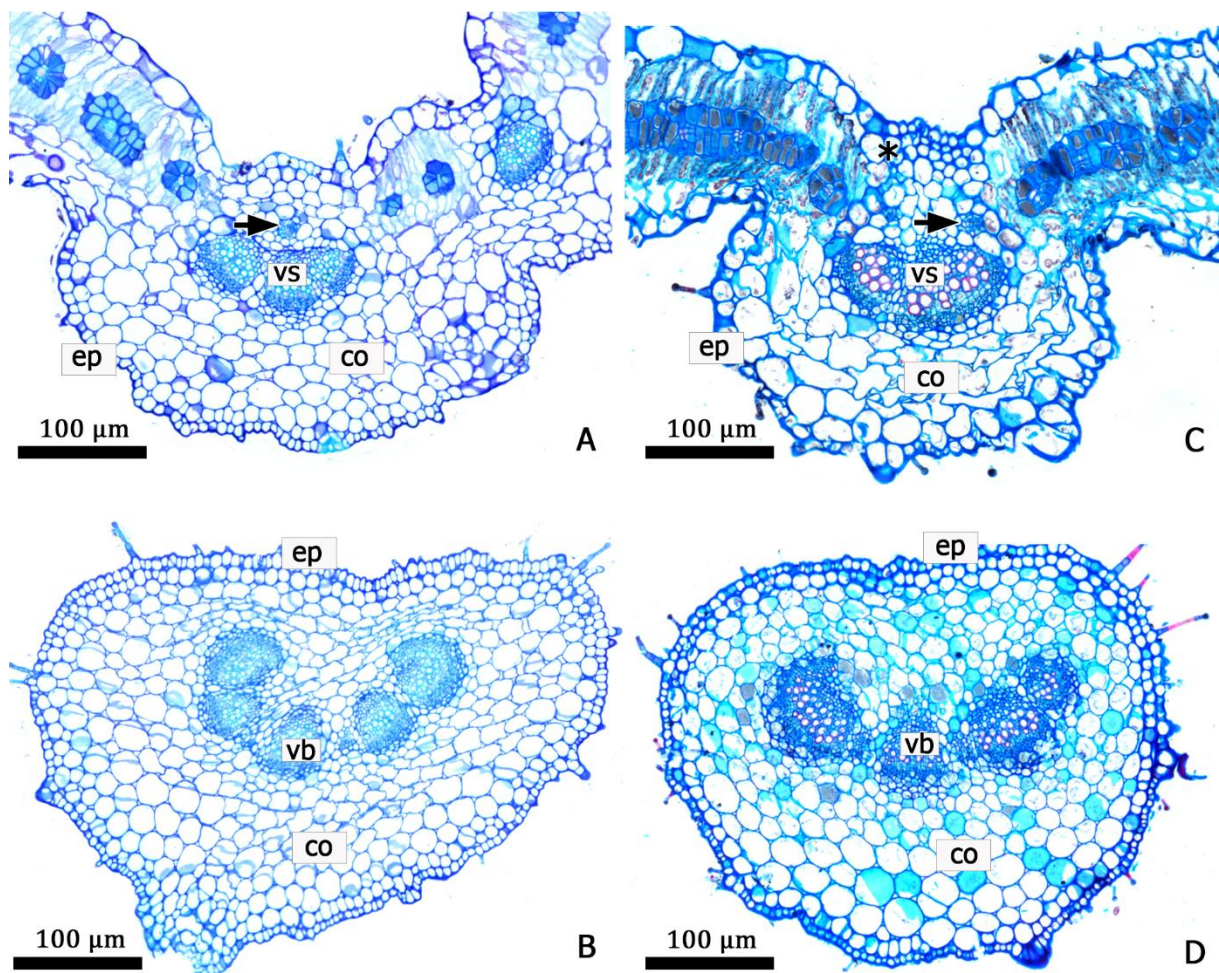
Fig. 5. Cross section of midrib and petiole in *Allionia incarnata*. **5a–b**: Specimens collected in Bolivia, Parque Nacional Amboró. **5c–d**: Specimens collected in the Chihuahuan Desert, New Mexico, USA. **5a, 5c**: Prominent midrib showing collenchyma cells (asterisk) and the vascular system (vs) including an additional accessory bundle (arrow). **5c, 5d**, Petiole showing four or five vascular bundles (vb) arranged in an arc towards the adaxial surface. *co,* cortex: *ep,* epidermis.
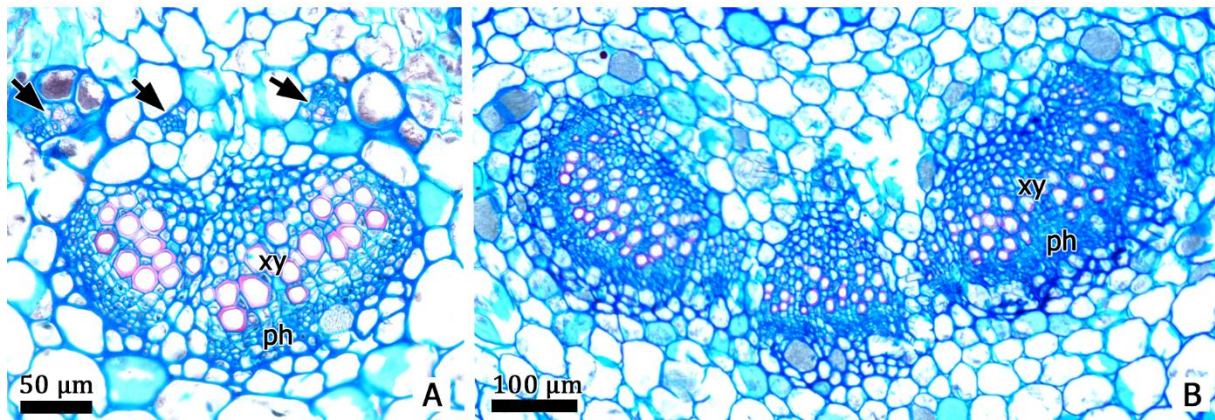
Fɪɢ. 6. Details of the vascular system in the midrib and petiole in *Allionia incarnata*. **6a**: Collateral vascular bundles in the midrib. **6b**: Three collateral vascular bundles in the petiole. *Arrow,* accessory bundles; *ph,* phloem, *xy,* xylem.

Secondary vascular system and cambial variants. – The beginning of secondary growth is marked by the establishment of the cambium, and lignification of peripheral pith cells (Fig 7b, c). The cambium is formed by the fascicular and interfascicular cambium, which are derived from the fascicular and interfascicular regions of the CCP. In addition to the residual cells of the CCP, pericyclic cells may also contribute to the formation of the interfascicular cambium. Initially, fascicular cambium forms secondary xylem centripetally and secondary phloem centrifugally containing the conducting cells, vessels and sieve-tube elements, respectively (Fig. 7b). On the other hand, the interfascicular cambium produces mainly xylem fibers internally, while the external cells remain parenchymatic (Fig. 7b). Later, conducting cells (i.e., vessels and sieve-tube elements) are also formed by the interfascicular cambium (Fig. 7b, c).

After a period of secondary growth, the pericyclic parenchyma located between the starch sheath and the primary phloem, undergoes periclinal divisions giving rise to a new meristematic zone. From this meristem, several tangential segments of new cambia differentiate along the stem circumference (Fig. 7d, e). Initially, the new cambia produce fibers and few vessels internally, whereas parenchyma and sieve-tube elements are formed externally (Fig. 7e, 8a–c). Between the new vascular increments and the regular cylinder some cells remain parenchymatic forming a conjunctive tissue (Fig. 8c). This pattern of secondary growth characterizes the stems of *Allionia* as having successive cambia (Fig. 9a) of which, however, from all analysed samples only one complete ring of new cambium was observed (Fig. 9a).

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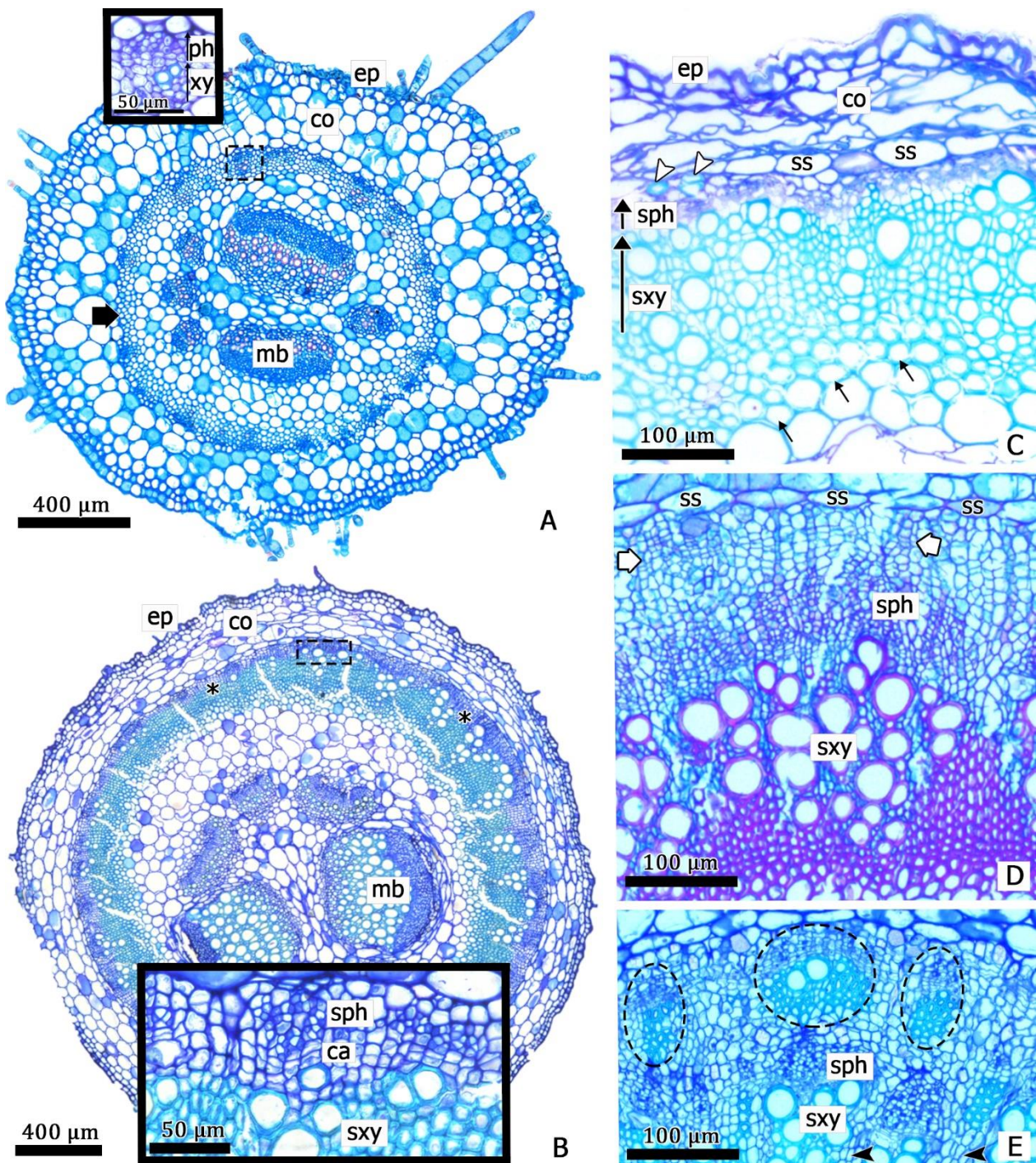
Fɪɢ. 7. Anatomy and development of the stem in *Allionia* in cross section. **7a–b, d–f:** *A. incarnata;* **7c:** *A. choisyi.* **7a**: Primary structure showing vascular system composed of medullary bundles and vascular bundles (box and inset) derived from the continuous concentric procambium (thick arrow). **7b**: Early secondary growth formed by regular activity of the vascular cambium forming xylem centripetally and phloem centrifugally (box). Asterisks indicate interfascicular region formed mainly by fibers in the xylem and parenchyma in the phloem side. Note the first vessels that are formed by the interfascicular cambium. **7c**: Detail of secondary vascular tissues, lignified pericyclic cells (white arrowheads), starch sheath (ss) and lignified pith cells (arrows). **7d**: Initiation of first ring of successive cambia derived from periclinal divisions of

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parenchymatic pericyclic cells (white arrows). **7e:** Developing arcs of successive cambia which form xylem centripetally and phloem centrifugally. Arrowheads indicate ray-like cells. *Arrow* (yellow), vessels formed by interfascicular cambium; *ep,* epidermis; *co,* cortex; *ph,* primary phloem*; mb,* medullary bundles; *sph,* secondary phloem; *ss,* starch sheath; *sxy,* secondary xylem; *xy,* primary xylem.

Qualitative and quantitative characteristics of wood and bark. – In adult stems, a periderm (the secondary protective tissues formed by the rise of the phellogen and its derivatives, which can replace the epidermis) was not observed (Fig. 9a). The cortex undergoes a dilatation process due to numerous cell divisions in periclinal and anticlinal planes (Fig. 9a).

In the vascular system, the conducting elements are usually confined to small areas of the vascular cambium, with sieve-tube elements opposing the vessels (Fig. 7b, e, 9a). The sieve-tube elements are diffuse, each one associated with a single companion cell (Fig. 7b). Sclerenchyma was not observed in the phloem. In the secondary xylem, vessels are diffuse or radially arranged (Fig. 7b–e). Vessels are solitary or in multiples, predominantly 2–4 (Fig. 7b–d). Fibers are non-septate, fusiform or with slightly straight ends, and with large lumen (Fig. 8d-e). Axial parenchyma is diffuse and represented by two to four cells per parenchyma strand (Fig. 8d). Regular rays were not observed although some regions had some radially elongated cells forming a ray-like structure – radially elongated cells in cross section (Fig. 7e). The quantitative anatomical features of both xylem and phloem are described in Table 2.

Root anatomy

In early developmental stages, the epidermis is replaced by the periderm that arises at subepidermal layers (Fig. 10a). The cortical region possesses several cells containing raphides (Fig. 10a–c). The root is diarch – with two protoxylem poles (Fig. 10a). The secondary growth is initiated by the formation of a regular vascular cambium (Fig. 10b) that produces secondary xylem and secondary phloem in the usual manner (Fig. 10b–d). Soon, a new cambium arises from the pericyclic parenchyma, forming a new ring of vascular tissue (Fig. 10c). This new cambium produces variant xylem centripetally and variant phloem centrifugally (Fig. 10c, e).
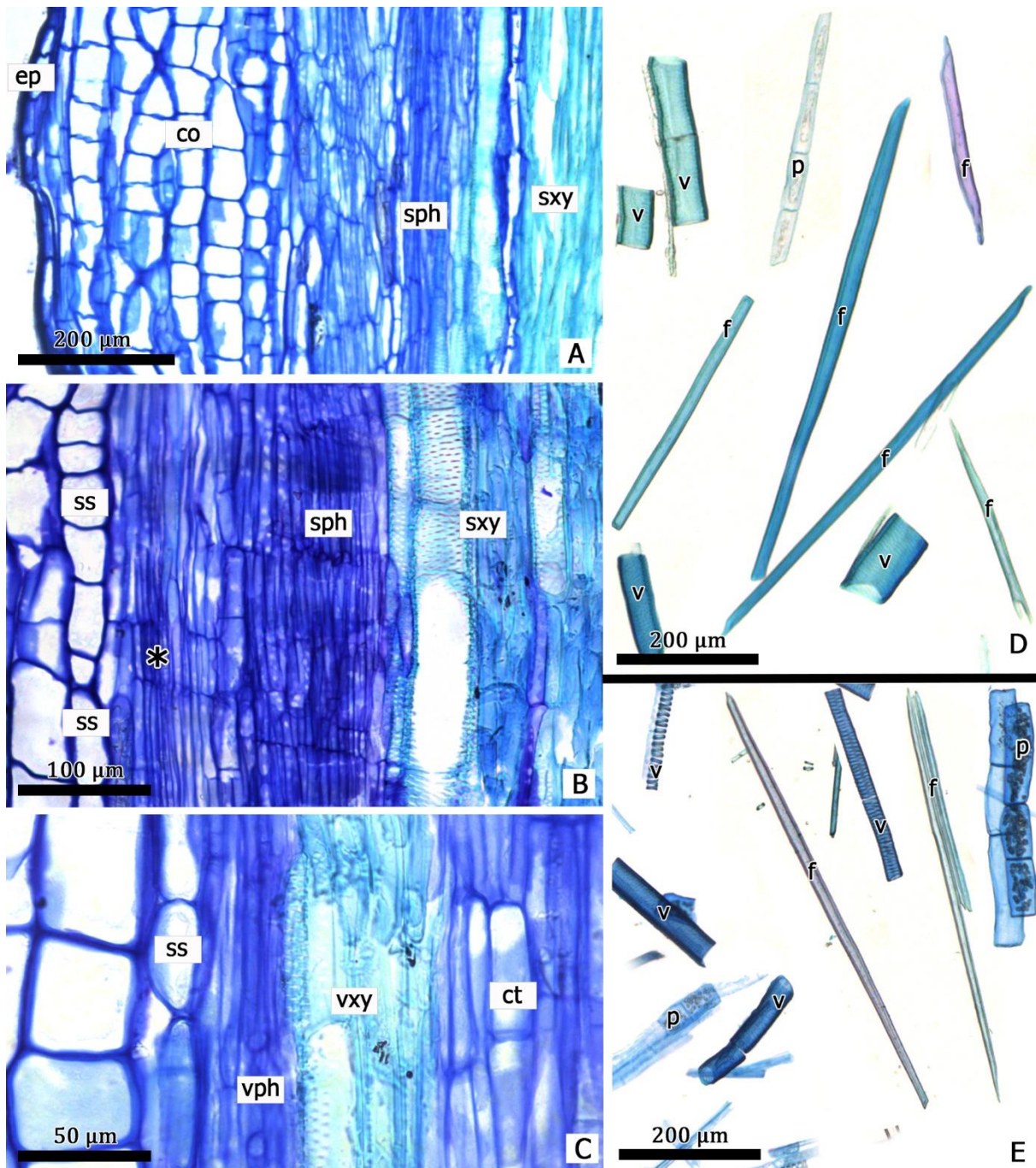
FIG. 8. Details of secondary growth, establishment of successive cambia and cell types in *Allionia incarnata*. **8a–c:** Longitudinal radial sections. **8a**: Regular secondary xylem and secondary phloem. **8b**: Divisions on pericyclic parenchymatic cells (asterisk) that give rise to the first successive cambia. **8c**: Differentiation of variant xylem (vxy) and variant phloem (vph) from the first arc of successive cambia. **8d–f:** Maceration. **8d**: Cells from the regular xylem showing vessels, axial parenchyma and fibers of different sizes and end shapes (fusiform, "u"-shaped). **8e**: Cells from xylem of medullary bundles showing vessels with different wall thickenings, parenchyma with starch and fibers. *ep,* epidermis*; f,* fiber; *co,* cortex; *ct,* conjunctive tissue; *p,* parenchyma; *ss,* starch sheath; *sxy,* secondary xylem; *v,* vessel.
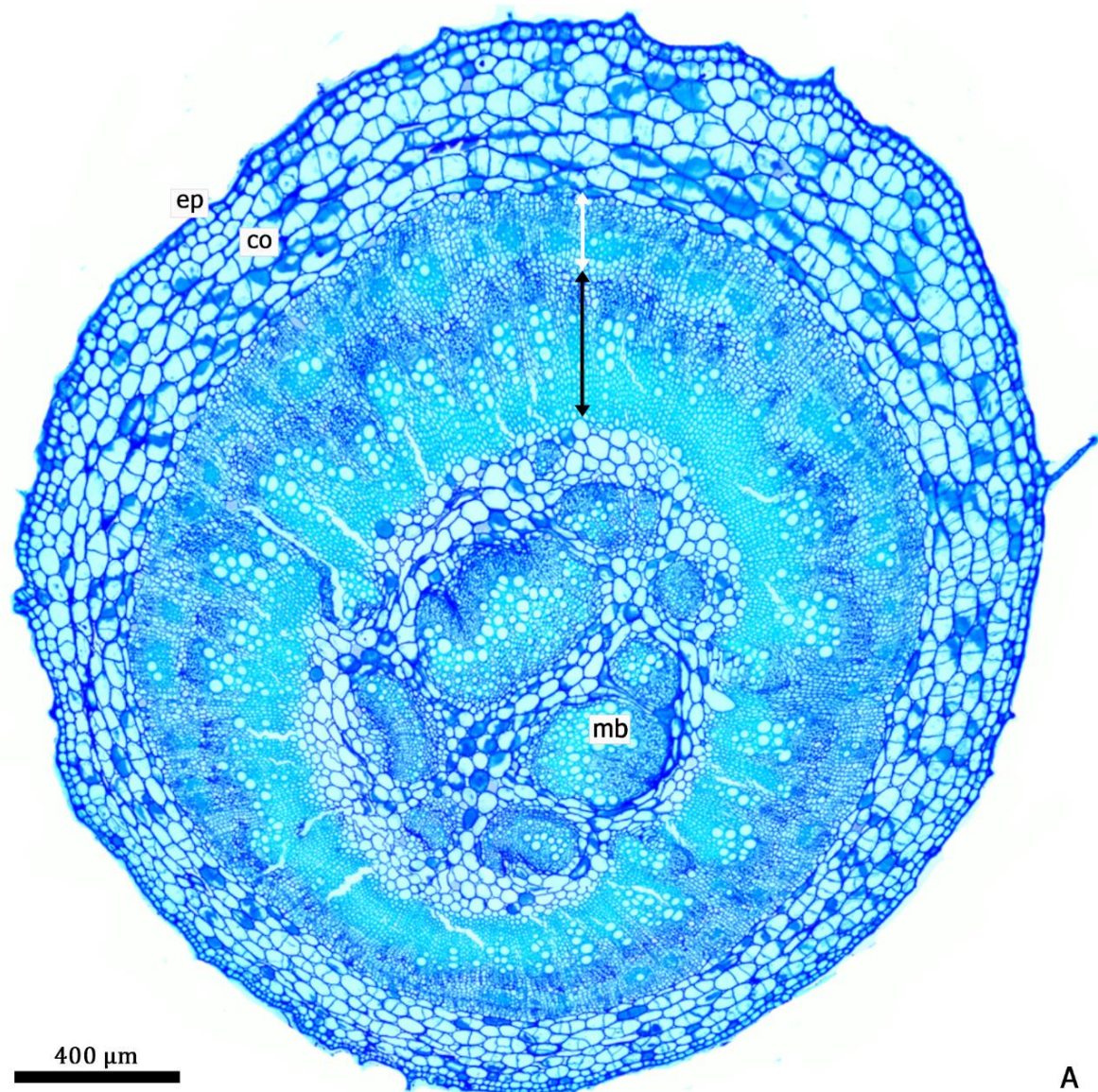
FIG. 9. General view of adult stem. **9a**: Note the regular cylinder of vascular tissues (black line) and the ring of vascular tissues produced by the successive cambia (white line) developed outwards.

Between the central cylinder and the first ring of successive cambia, some cells remain parenchymatic forming the conjunctive tissue (Fig. 10c). Subsequently, new increments are formed from remaining pericyclic parenchyma cells outside the first ring (Fig. 11a–c). This process repeats a few times producing a thick root with several increments of new cambia at maturity (Fig. 11a–c).

Anatomy and histochemistry of glandular trichomes. – Multicellular uniseriate glandular trichomes are found in both leaves and stems (Fig. 2g, 7a). There are small and large trichomes with thick walls (Fig. 12a). Trichome heads stain darkly with safranin (Fig. 2g). In fixed and non-stained sections, the secretion produced by the

trichome head is greenish in colour and is on the exterior of the head (Fig. 12a). The presence of lipids was detected by the positive reactions to Sudan IV, Sudan Black and Nile Blue (Fig. 12b–d). Lipophilic substances were also identified, such as mucilage detected by Ruthenium Red (Fig. 12e) and proteins detected by Aniline Blue Black (Fig. 12f). The Lugol reagent reacted positively only in the starch sheath (Fig. 12g).

Table 2. Quantitative anatomical features of xylem and phloem in stems of *Allionia* species.

Allionia incarnata					
	Xylem				Phloem
	Vessel elements		Fibers		Sieve-tube elements
Features / cell type	Medullary bundles	Regular cylinder	Medullary bundles	Regular cylinder	Medullary bundles	Regular cylinder
Diameter (μm)	39.9 ± 1.0 (16.2–66.4)	31.0 ± 8.2 (14–54.5)	13.4 ± 6.3 (5.7–23.1)	16.2 ± 5.8 (5.6–27.5)	14.5 ± 1.2 (12.6–18.4)	9.8 ± 2.6 (8.6–16.1)
Length (μm)	257.1 ± 1.1 (91.4–461)	127.2 ± 2.9 (36.5–201.6)	490.4 ± 192.7 (233.7–989.7)	422.4 ± 171.5 (215.3–755.0)	217.7 ± 24.3 (186.6–220.4)	111.7 ± 25 (54.7 – 140.0)
Wall thickness (μm)	–	–	3.7 ± 0.8 (1.8–5.5)	3.6 ± 0.7 (2.4–5.4)	–	–
Frequency (mm^{-2})	54.6 ± 5.8 (42.0–66.0)	35.0 ± 3.2 (22.0–38.0)	–	–	23.4 ± 4.6 (14–27)	11.7 ± 1.7 (9–15)
Area (%)	26.0 ± 5.9 (18.0–34.0)	27.0 ± 4.0 (20.0–30.0)	11.3 ± 2.5 (8.0–14.0)	22.0 ± 8.0 (14.0–31.0)	17.4 ± 5.2 (10.0–22.0)	15.0 ± 1.0 (14.0–19.0)
Allionia choisyi					
	Xylem				Phloem
	Vessel elements		Fibers		Sieve-tube elements
	Medullary bundles	Regular cylinder	Medullary bundles	Regular cylinder	Medullary bundles	Regular cylinder
Diameter (μm)	25.8 ± 1.3 (8.6–54.3)	24.6 ± 6.8 (11.5–42.9)	6.1 ± 1.1 (4.2–7.7)	7.2 ± 1.4 (5.3–8.7)	6.4 ± 1.1 (4.9–11.2)	8.6 ± 1.5 (7.6–9.8)
Length (μm)	–	–	–	–	–	–
Wall thickness (μm)	–	–	2.5 ± 1.0 (1.6–4.0)	3.9 ± 1.0 (2.0–4.6)	–	–
Frequency (mm^{-2})	28.8 ± 3.4 (25.2–36.4)	35.0 ± 3.2 (22–38)	–	–	–	–
Area (%)	16.5 ± 3.7 (18.0–34.0)	27.0 ± 4.0 (20.0–30.0)	14.6 ± 3.5 (12.0–18.0)	16.5 ± 19.0 (14.0–21.0)	–	–

DISCUSSION

Taxonomic and systematic notes.– Here we performed a comparative anatomical analysis of the two recognized species of *Allionia*. Our results showed that the vegetative anatomy of *Allionia* species is remarkably similar, and diagnostic features to distinguish the species are lacking. Although previous studies have shown the value of characteristics of the vascular system as useful traits for the distinction
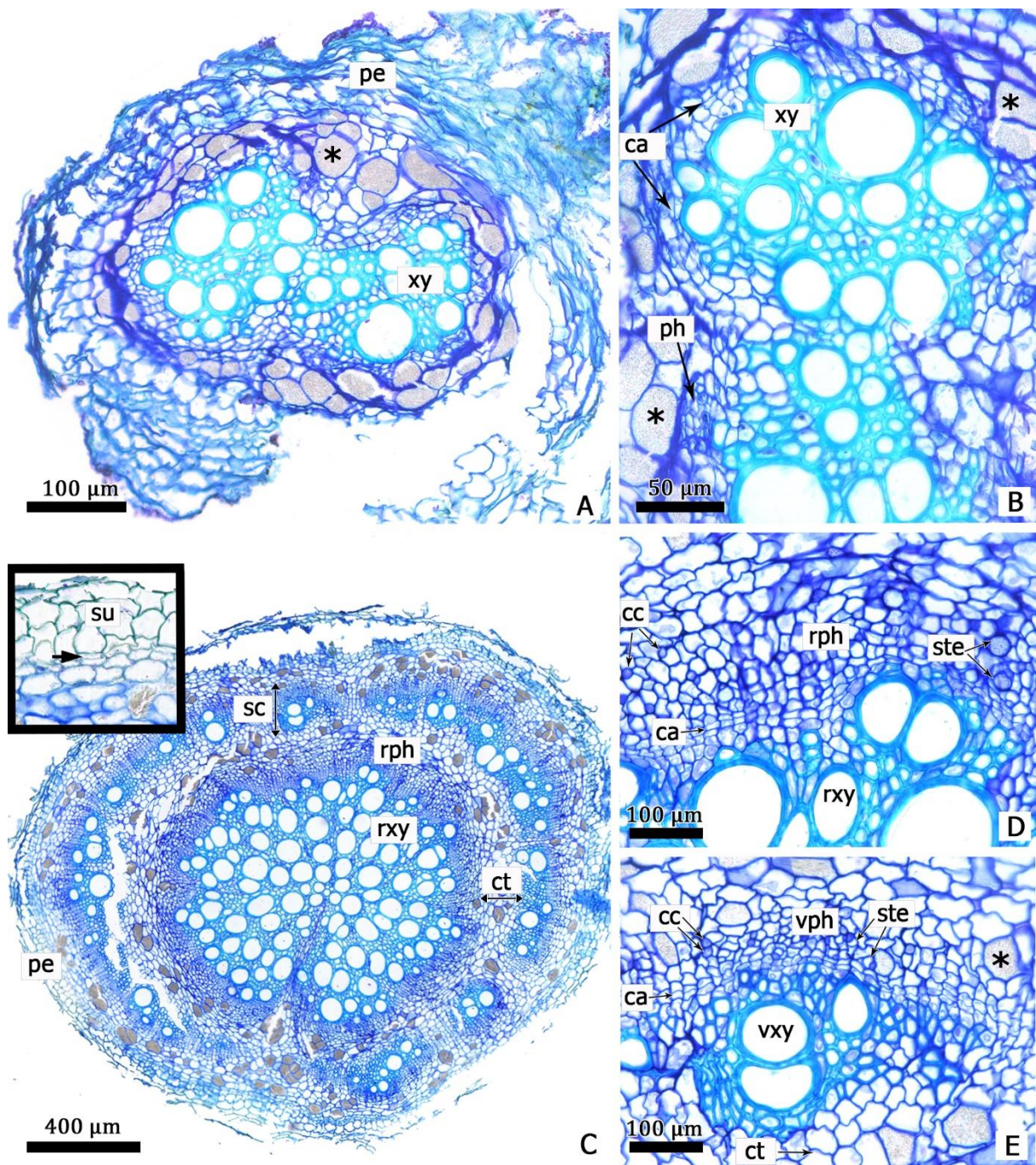
FIG. 10. Root anatomy in *Allionia incarnata* in cross section. 10a–b: Lateral root. 10a: Early secondary growth in showing diarch vascular system. 10b: Detail of previous image (right portion) showing the establishment of regular cambium. 10c–e: Primary root. 10c: Regular vascular cylinder and first ring of successive cambia. 10d: Detail of regular cambium, regular secondary xylem, and phloem. In the inset, note the phellogen (arrow) and suber. 10e: Detail of variant cambium, variant xylem, and variant phloem. *Asterisks,* crystals (raphides); *ca*, cambium, *cc*, companion cell; *ct*, conjunctive tissue; *pe*, periderm; *ph*, phloem; *rph*, regular phloem; *rxy*, regular xylem; *sc*, successive cambia; *ste*, sieve-tube element; *su,* suber; *vph*, variant phloem; *vxy*, variant xylem; *xy*, xylem.
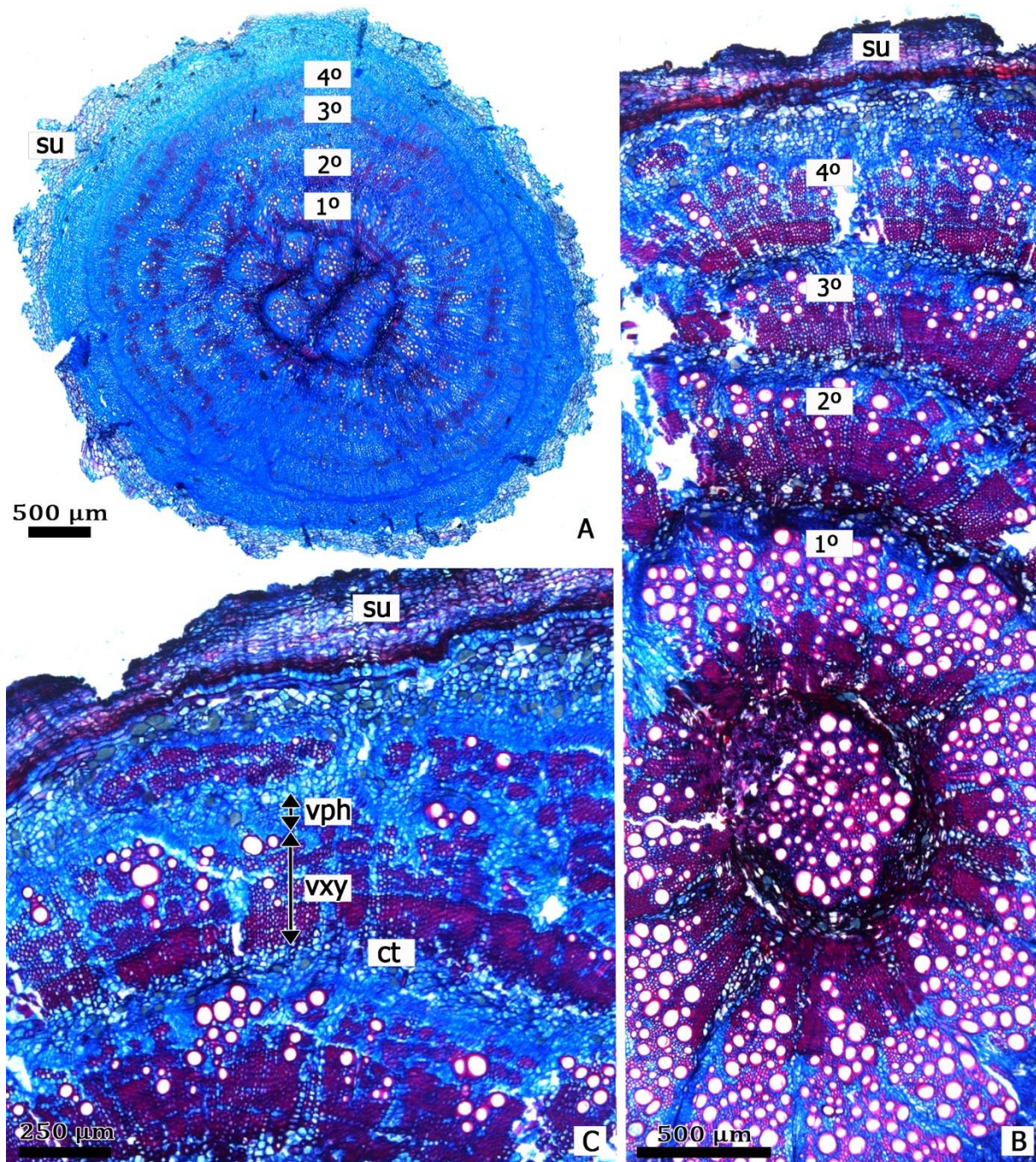
Fig. 11. Adult primary root of *Allionia incarnata* in cross section. **11a:** General cross view showing four developed rings of successive cambia (ordinal numbers). **11b:** Detail of central cylinder and rings of successive cambia. **11c:** Close-up of successive cambia showing variant xylem, variant phloem, and conjunctive tissue. *ct,* conjunctive tissue; *su,* suber; *vph,* variant phloem; *vxy,* variant xylem.

of similar species in Nyctaginaceae (e.g., arrangement of medullary bundles in the genus *Pisoniella* – Cunha Neto et al., 2020), we found that most of the variation in the anatomy of *Allionia* species is seen in size, number of cells or amount of tissue rather

than qualitative differences. Similar observations have been proposed for external vegetative and reproductive morphology (Spellenberg 2003).
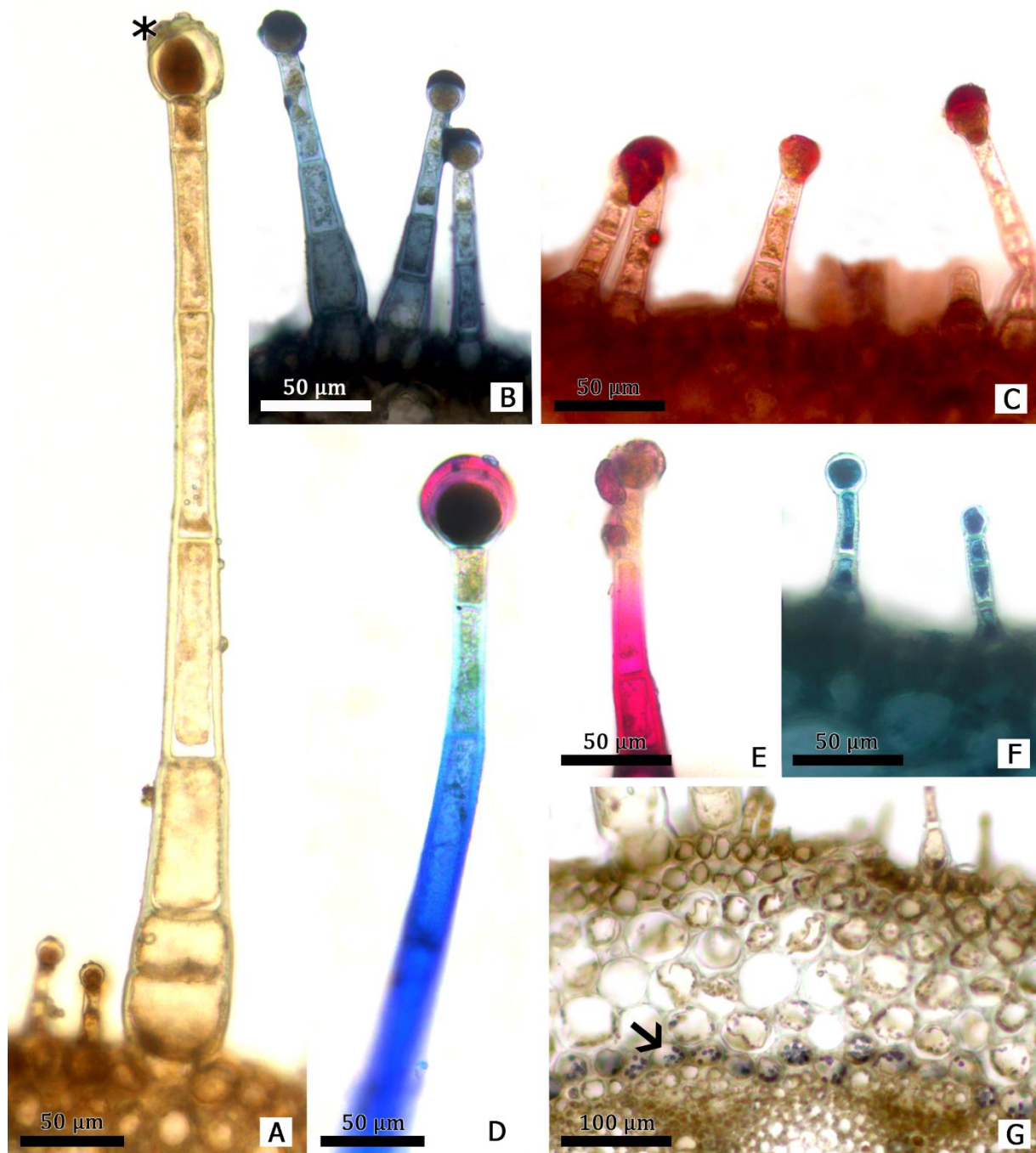
FIG. 12. Anatomy and histochemistry of uniseriate glandular trichomes in *Allionia incarnata*. **12a**: Non-stained section showing greenish secretion (asterisk). **12b–d**: Positive reactions for lipids. **12b**: Sudan Black B. **12c**: Sudan IV. **12d**: Nile Blue. **12e**: Ruthenium red for mucilage. **12f**: Aniline Blue Black for proteins. **12g**: Starch grains detected in the starch sheath by Lugol reagent.

Moreover, preliminary molecular data do not support the distinction between the two species, but additional loci and populations will need to be sampled to confirm this (Emily Humphries and Michael J. Moore, pers. comm.).

Anatomical and developmental aspects. – Leaf characteristics observed in *Allionia* confirm previous results by Phillips (1976) and consistent with that of members of the family (Metcalfe & Chalk 1950; Bittrich & Kühn 1993; Struwig et al. 2011). A striking feature found in *Allionia* is the occurrence of Kranz anatomy. In Nyctaginaceae, Kranz anatomy has been reported also in *Okenia* and some species of *Boerhavia*, all belonging to Nyctagineae (Carolin et al. 1978; Bittrich & Kühn 1993; Struwig et al. 2011; this study). Although the sheath cells are easily recognized in these taxa, they do not have a second layer of radially elongated cells, as firstly described by Haberlandt (1882) (*Kranz* = crown, in German]. In *Allionia,* the sheath cells are larger and round without intercellular spaces, while the neighbouring cells resemble regular palisade or lacunose parenchyma. These characteristics observed in *Allionia, Boerhavia*, and *Okenia* are similar to descriptions presented by Antonucci (2010), who investigated the leaf development and ultrastructure of leaves in *Gomphrena* (Amaranthaceae) species. In that study, the sheath cells were identified as the inner layer of the mesophyll, that is, the endodermis (or starch sheath).

Among the specimens analysed, the only notable difference was found in the shape of margin cells of the mesophyll (round vs. palisade). However, the round pattern was restricted to a single specimen, and such leaf characteristics are known to be strongly influenced by growing conditions, especially light intensity, water availability, temperature and nutrient supply (Dickinson 2000; Chen et al. 2010).

Both leaves, stems and anthocarps of *Allionia* are covered with trichomes that releases a viscid exudate. According to Spellenberg (2003) these glands remain sticky in specimens of Nyctaginaceae for decades in the herbarium. In the shoot of *Allionia,* these structures are glandular multicellular trichomes that were shown to produce an exudate composed by both hydrophobic and hydrophilic substances. The occurrence of secretory structures producing sticky substances has been observed in Nyctaginaceae by different authors (Willson & Spellenberg 1977; Spellenberg 2003; Struwig et al. 2011; Cunha Neto et al. 2019; Sukhorukov et al., *in press*). In the genus *Anulocaulis*, these structures are unicellular trichomes constituting secretory rings

found in each stem internode, which secrete a complex exudate (Cunha Neto et al. 2019). In *Boerhavia* and *Commicarpus,* they are multicellular and uniseriate (Struwig et al. 2011), as observed in *Allionia.* Such trichomes or similar structures are also present on the fruits of some species (Struwig et al. 2011; pers. obser.; Sukhorukov et al., *in press*), but their ecological significance are still unknown.

The structure of the stem vascular system in *Allionia* presents some unusual characteristics. The primary system is remarkable for the occurrence of medullary bundles (named "central collateral bundles" by Phillips, 1976), and vascular bundles derived from a continuous concentric procambium that delimits the pith. Together, these vascular units constitute the primary vascular system (the stele). This stele organization, named polycyclic eustele, was recently investigated in a broader scale by Cunha Neto et al. (2020). In this study, authors performed a thorough developmental and evolutionary analysis of the presence of medullary bundles for Nyctaginaceae. The polycyclic eustele was found to be ancestrally present in Nyctaginaceae and sister families (phytolaccoid clade), with a reversion to the regular eustele in one lineage of the family (tribe Leucastereae) (Cunha Neto et al. 2020).

In *Allionia,* the transition from primary to secondary stem growth follows the typical establishment of a single regular cambium resulting from fascicular and interfascicular cambium, except for the fact that it arises in a continuum with the continuous concentric procambium (CCP). Because the vascular bundles delimiting the pith comes from the CCP, previous authors had interpreted the CCP as a "secondary meristem" (De Bary 1884; Balfour 1965). However, we have found that in early developmental stages this meristem shows characteristics of a typical procambium which first form primary vascular tissue (vascular bundles) that is evidenced by wall thickenings commonly found in primary xylem (Cunha Neto et al. 2020). Concomitantly with regular cambium development, several layers of peripheral pith cells became lignified during this vascular transition. This unusual but consistent characteristic was found in *Allionia* and other genera within Nyctaginaceae (pers. obser.). The regular stem secondary growth in *Allionia* produce relatively little secondary phloem and xylem until the establishment of the successive cambia system. As reported for other taxa within the family (Carlquist 2004; Hernández-Ledesma et al. 2011), there are no typical vascular rays in *Allionia*, although some

cambial derivatives peripheral to interfascicular regions can be sometimes organized in radially oriented rows. The ray-like cells in *Allionia* and other Nyctaginaceae should be better explained as resulting from the continuity on divisions of the meristem that originates the additional cambia (Carlquist, 2004, 2007) and/or from proliferation of the conjunctive tissue.

The occurrence of cambial variants is a well-known feature for the Nyctaginaceae (De Bary 1884; Schenck 1893; Metcalfe & Chalk 1950; Carlquist 2001, 2007, 2010). However, the interpretation of the origin and development of these system has always been a matter of debate. In the case of *Allionia,* two aspects should be emphasized. First, different from Phillips' (1976) interpretation, we noticed that there is some period of regular growth in the stems of *Allionia* and only later the cambial variant arises. Second, our observation of a pericyclic origin for successive cambia (both in stems and roots) is in accordance with Phillips (1976) but differs from Carlquist's (2004) interpretation for other Nyctaginaceae – which were also described as having successive cambia. Here, we showed that the outermost layer of the stele differentiates into perivascular fibers and the innermost layer of the cortex is characterized as the starch sheath. Since we have identified these limits of the stele and the cortical region, and given that all events related to the formation of the successive cambia occur internally to these two cell layers, the additional cambia are not established in the cortex, as has been stated by Carlquist (2004, 2007, 2010) and others following him (Rajput et al. 2009; Hernández-Ledesma et al. 2011). The ontogenetic approach carried out in this study was fundamental developing our current understanding. Up to know, therefore, there has been various reinterpretations of the origin and types of cambial variants in different groups (e.g., Sapindaceae – Cunha Neto et al., 2018; Vitaceae – Pace et al., 2018; Fabaceae – Leme et al., 2020).

The formation of successive cambia can be observed also in lateral and primary roots of *Allionia*. We have detected the presence of cambial variants in the roots of several other species of Nyctaginaceae, especially in herbs and subshrubs within Nyctagineae, such as *Anulocaulis leiosolenus, Cyphomeris gypsophiloides* and *Mirabilis albida* (pers. obser). Successive cambia in roots of Nyctaginaceae have been previously reported for *Abronia latifolia* (Carlquist 2004), *Bougainvillea spectabilis*

(Esau & Cheadle 1969; Stevenson & Popham 1973; Carlquist 2004) and *Mirabilis jalapa* (Mikesell & Popham 1976). The origin and evolution of such variant anatomies is remarkable within Caryophyllales (e.g., Aizoaceae, Amaranthaceae [including the well-known beet root of *Beta vulgaris*, Chenopodiaceae], Basellaceae, Phytolaccaceae, Polygonaceae, Nepenthaceae) since they are likely a feature with multiple independent origins (Gibson 1994; Carlquist 2010; Schwallier et al. 2017).

Ecological and functional interpretations. – *Allionia* consists of annual and perennial plants. Although these plants produce stems and leaves in the summer (Phillips 1976, Mulroy & Rundel 1977), the foliage of *Allionia* is not strikingly xerophytic and only rarely is it heavily pubescent or cutinized (Mulroy & Rundel 1977). Nevertheless, according to Phillips (1976), there are some morphological traits in *Allionia* that might be associated as adaptations for harsh desert environments, they are the large taproot, anisoclady and seeds with abundant storage. Anatomically, the presence of trichomes and Kranz anatomy in relation to C_4 photosynthesis (a common adaptations for summer annual species in the Sonoran Desert, Syvertsen et al. 1976; Mulroy & Rundel 1977) that provide the ability to survive in hot, dry conditions. In addition, the formation of successive cambia, which is associated with an increase in water and starch parenchyma, can been also suggested as an adaptive feature for plants thriving in harsh environments (Carlquist 2001, 2012). In this sense, it is interesting to note that the usual thick perennial roots showed several rings of successive cambia, while the slender stems had only one or two additional rings. This condition seems to be true for several other species from tribe Nyctagineae that grows in similar conditions (*e.g., Acleisanthes chenopodioides, Anulocaulis gypsogenus* var. *gypsogenus, Boerhavia torreyana* – pers. obser.). The adaptation to arid environments and drought stress in *Allionia* has been linked also with the association with endophytes – arbuscular mycorrhizal – an adaptation that has been demonstrated for more than 40 species, including *Allionia* (Lugo et al. 2015). Other species of Nyctaginaceae growing in deserts (e.g., *Boerhavia* and *Commicarpus*) have acquired the ability to survive in dry conditions by avoiding desiccation also through the production of tannins, thickened stomata walls and additional collenchyma as supporting tissue (Struwig et al. 2011).

A common characteristic of roots, stems and leaves in *Allionia* is the abundance of calcium oxalate crystals in the form of raphides, as also observed in other Nyctaginaceae (Metcalfe & Chalk 1950; Struwig et al. 2011). Calcium oxalate crystals play different roles in plants such as accumulation of excesses, ionic/osmotic calcium regulation, detoxification/heavy metal tolerance and defence against herbivores (Mauseth 1988; Franceschi & Nakata 2005; Molano-Flores 2001; Struwig et al. 2011). However, this defence system is probably constitutive rather than inducible and not likely associated with soil calcium concentration (Ruiz et al. 2002). Nonetheless, the occurrence of calcium oxalate crystals as a mechanism to sequester calcium in a physiologically unavailable form has been recently investigated on gypsophytes – plants that grow on gypsum (CaSO4·H2O) soils – given that they can extract structural H_2O molecules from gypsum (Merlo et al. 2011; Borer et al. 2012; Palacio et al. 2014a,b; Mota et al. 2017). *Allionia* can grow on gypsum (gypsovag) but is not a gypsophyte (Moore et al. 2014). Curiously, *Anulocaulis gypsogenus var. gypsogenus* and *Acleisanthes lanceolata* are endemic to gypsum and comparatively show less crystals in their vegetative parts compared to *Allionia* (pers. obser.).

In *Allionia,* some stem anatomical features can be associated to their herbaceous habit, including the absence of rays (raylessness), wide fibers with thin walls, delayed or absence of periderm and the reduced amount of secondary growth despite the presence of successive cambia. Most of these characteristics can be linked with specific hydraulic and/or biomechanical demands of herbaceous plants. The absence of rays (raylessness), for instance, might be related to the fact that longitudinal conduction of photosynthates outweighs radial conduction (Carlquist 2012). In addition, given that medullary bundles seem to remain functional even in mature stems (Cunha Neto et al. 2020), their high proportion of vessels for a specific stem section can represent a crucial path for maintenance of its hydraulic function. However, the functional anatomy of herbaceous annuals and plants with medullary bundles remain underexplored, since this topic has been investigated predominantly in large woody plants.

Overall, our results indicate that vegetative anatomy in *Allionia* species do not contribute to species delimitation. However, its general stem structure and

development is unique. While we have revealed different interpretations for the phenomena accounting for the complexity of the stem vascular system in *Allionia* (e.g., polycyclic eustele, medullary bundles, continuous concentric procambium and successive cambia), a broader analysis of the diversity and evolution of the secondary structure within Nyctaginaceae is yet to be performed. Our future investigations will address this and other questions concerning the diversity and evolution of the vascular system for the family and sister lineages.

APPENDIX 1

Information on taxa, collectors, localities, and herbarium vouchers for the analyzed species.

Abronia fragrans **Nutt. ex Hook.,** Douglas 2290 (FLAS), Las Cruces, New Mexico, USA. *Abronia neealleyi* **Standl.,** Douglas 2281, (FLAS) Eddy County, Yeso Hills, New Mexico, USA. *Acleisanthes lanceolata* **(Wooton) R.A. Levin,** Douglas 2277 (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Acleisanthes chenopodioides* **(A. Gray) R.A. Levin,** Douglas 2289, 2293 (FLAS), Las Cruces, New Mexico, USA. *Acleisanthes longiflora* **A. Gray,** Douglas 2279 (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Anulocaulis leiosolenus* **(Torr.) Standl. var.** *leiosolenus*, Douglas 2278 (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Boerhavia linearifolia* **A. Gray,** Douglas 2284 (FLAS), New Mexico, USA. *Boerhavia torreyana* **(S. Watson) Standl.,** Douglas 2294 (FLAS), Las Cruces, New Mexico, USA. *Boerhavia wrightii* **A. Gray,** Douglas 2288 (FLAS), Las Cruces, New Mexico, USA. *Commicarpus scandens* **(L.) Standl.,** Douglas 2291 (FLAS), New Mexico, USA. *Cyphomeris gypsophiloides* **(M. Martens & Galeotti) Standl.,** Douglas 2287 (FLAS), Organ Mountains-Desert Peaks National Monument, Las Cruces, New Mexico, USA. *Mirabilis aggregata* **(Ortega) Cav.,** Pace 728 (MEXU), Ixmiquilpan, Hidalgo, Mexico. *Mirabilis cf. albida* **(Walter) Heimerl.,** Douglas 2286 (FLAS), New Mexico, USA. *Mirabilis jalapa* **L.,** Acevedo-Rodríguez 16480 (US), Veracruz, Mexico. *Mirabilis viscosa* **Cav.,** Pace 727 (MEXU), Ixmiquilpan, Hidalgo, Mexico. *Nyctaginia capitata* **Choisy,** Douglas 2282 (FLAS), New Mexico, USA. *Okenia hypogaea* **Schltdl. & Cham.,** Pace 749 (MEXU, SPF), Veracruz, Mexico.

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Chapter 3

Diversity, distribution, development, and evolution of medullary bundles in Nyctaginaceae*

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Diversity, distribution, development, and evolution of medullary bundles in Nyctaginaceae

PREMISE: Medullary bundles, i.e., vascular units in the pith, have evolved multiple times in vascular plants. However, no study has ever explored their anatomical diversity and evolution within a phylogenetic framework. Here, we investigated the development of the primary vascular system within Nyctaginaceae showing how medullary bundles diversified within the family.

METHODS: Development of 62 species from 25 of the 31 genera of Nyctaginaceae in stem samples was thoroughly studied with light microscopy and micro-computed tomography. Ancestral states were reconstructed using a maximum likelihood approach.

RESULTS: Two subtypes of eusteles were found, the regular eustele, lacking medullary bundles, observed exclusively in representatives of Leucastereae, and the polycyclic eustele, containing medullary bundles, found in all the remaining taxa. Medullary bundles had the same origin and development, but the organization was variable and independent of phyllotaxy. Within the polycyclic eustele, medullary bundles developed first, followed by the formation of a continuous concentric procambium, which forms a ring of vascular bundles enclosing the initially formed medullary bundles. The regular eustele emerged as a synapomorphy of Leucastereae, while the medullary bundles were shown to be a symplesiomorphy for Nyctaginaceae.

CONCLUSIONS: Medullary bundles in Nyctaginaceae developed by a single shared pathway, that involved the departure of vascular traces from lateral organs toward the pith. These medullary bundles were encircled by a continuous concentric procambium that also constituted the polycyclic eustele, which was likely a symplesiomorphy for Nyctaginaceae with one single reversion to the regular eustele.

KEY WORDS: Caryophyllales, evo-devo, Nyctaginaceae, ontogeny, primary growth, stem anatomy, trait evolution, vascular bundles.

Medullary bundles are complete vascular bundles located in the pith and may be arranged in two or more concentric rings or as bundles scattered within the pith in addition to the bundles of the stele ring (de Bary, 1884; Esau, 1967; Ogura, 1972; Beck et al., 1982; Schmid, 1982; Cutter, 1987; Mauseth, 1988; Beck, 2010; Isnard et al., 2012). Such organization of the vascular tissue constitutes the "polycyclic eustele" subtype in the stele classification by Beck et al. (1982) and Schmid (1982). Medullary bundles have also been addressed in the context of "anomalous structures" (de Bary, 1884; Eames and McDaniels, 1925; Metcalfe and Chalk, 1950; Beck, 2010; Yang and Chen, 2017), a concept not followed here, since we consider the so-called anomalous vascular structures to be a variant type of secondary growth (Carlquist, 2001; Angyalossy et al., 2012, 2015). Regardless, medullary bundles are a remarkable feature of vascular plants, which also contributes to their complexity and morphological diversity (Eames and McDaniels, 1925; Beck et al., 1982).

Medullary bundles have evolved multiple times in the history of vascular plants, being present in ferns (e.g., Cyatheaceae and Dennstaedtiaceae [*Pteridium*], Ogura, 1927, 1972; Eames and McDaniels, 1925; Lucansky, 1974), and more frequently in the flowering plants, where they have been recorded in approximately 60 families, including magnoliids (Isnard et al., 2012; Trueba et al., 2015) and eudicots (Wilson, 1924; Lambeth, 1940; Boke, 1941; Holwill, 1950; Metcalfe and Chalk, 1950; Davis, 1961; Esau, 1967; Pant and Bhatnagar, 1975; Raj and Nagar, 1980, 1989; Kirchoff and Fahn, 1984; Mauseth, 1993, 2006; Costea and DeMason, 2001; Schwallier et al., 2017; Kapadane et al., 2019). In some families, this character is found in just a few representatives (e.g., *Nepenthes,* Nepenthaceae; Schwallier et al., 2017) or is restricted to a specific lineage (subfamily Cactoideae, Cactaceae; Mauseth, 1993, 2006; Terrazas and Arias, 2002), while in others it seems to be widespread within the family (e.g., Amaranthaceae, Nyctaginaceae, Piperaceae) (Metcalfe and Chalk, 1950). However, comparative analyses or investigations within a phylogenetic context are still lacking, except for Cactaceae (Mauseth, 1993, 2006).

Nyctaginaceae is a family distributed in tropical and subtropical regions worldwide, currently

encompassing 31 genera and more than 400 species of herbs, subshrubs, lianas, shrubs, and trees (Bittrich and Kühn, 1993; Douglas and Manos, 2007; Douglas and Spellenberg, 2010; Hernández-Ledesma et al., 2015). Medullary bundles are a common feature in Nyctaginaceae, and different studies have aimed to explain their occurrence in relation to the vascularization of the vegetative shoot (Inouye, 1956; Nair and Nair, 1961; Sharma, 1962; Balfour, 1965; Pulawska, 1972; Zamski, 1980). Nevertheless, studies within Nyctaginaceae are restricted to a few genera (i.e., *Boerhavia*, *Bougainvillea*, and *Mirabilis*), and the distribution, anatomy, diversity and evolution of medullary bundles have yet to be fully characterized.

The structure and ontogeny of medullary bundles for most taxa of Nyctaginaceae remain unknown or need re-evaluation, in part because only the adult stems of these plants have been studied in most cases (de Bary, 1884; Metcalf and Chalk, 1950) or the anatomical origin and developmental processes have been interpreted differently. For instance, medullary bundles are reported to arise from cauline bundles, those that originate in the shoot apical meristem but do not form leaf traces (Inouye, 1956; Gibson, 1994), or from vascular strands departing from leaves and lateral appendages (Balfour, 1965; Zamski, 1980). There are also cases in which authors failed to recognize the medullary bundles, naming them either "primary bundles" (Nair and Nair, 1961; Stevenson and Popham, 1973; Mikesell and Popham, 1976) or "leaf traces" (Pant and Mehra, 1963; Gibson, 1994). Moreover, while some authors stated that only the innermost bundles are of primary origin (Inouye, 1956), others have argued that at least some of them are of secondary origin (for example, those situated more peripherally; de Bary, 1884; Sabnis, 1921; Balfour, 1965; Pulawska, 1972).

Here, we conducted a detailed anatomical study of the diversity and evolution of the medullary bundles in Nyctaginaceae using a time-calibrated phylogeny as framework for the analyses. Specifically, we evaluated the occurrence, development, distribution, diversity, and evolution of medullary bundles and how they affect the organization of the eustele in the monophyletic Nyctaginaceae, in which

medullary bundles are widespread. This study characterizes for the first time the complexity of the primary vascular system of Nyctaginaceae and shows how different subtypes of eustele have evolved within the family.

MATERIALS AND METHODS

Taxon sampling and material collection

Stems of 62 species representing 25 of the 31 genera of the family Nyctaginaceae were collected either in natural populations, herbaria, or wood collections. Nyctaginaceae is currently subdivided into seven tribes (Douglas and Spellenberg, 2010): Nyctagineae, Pisonieae, Bougainvilleae, Boldoeae, Colignonieae, Leucastereae, and Caribeaeae. Genera from all tribes were sampled except for the monotypic Caribeaeae (*Caribea litoralis* Alain), known only from the type collection and which may be extinct (Douglas and Spellenberg, 2010). The sampled species, habits, number of specimens studied, and the type of anatomical analysis performed for each species are summarized in Table 1. At least one species from the main lineages of Nyctaginaceae was selected for the

developmental study (Table 1). For these species, we sampled stems from the shoot apex until the fifth internode. In addition, fully developed stems were also analyzed. For that, different samples along the stem and at the base of the plant were obtained in the case of herbs, subshrubs, shrubs, scandent-shrubs, and lianas, while branches up to 1.5–2 cm in diameter were analyzed for trees. Detailed information on the species, localities of collection, and herbaria where vouchers were deposited is included in Appendix 1.

Besides Nyctaginaceae, 11 species as outgroups for the ancestral character state reconstruction were sampled (further information below). The species were distributed across Agdestidaceae, Petiveriaceae, and Phytolaccaceae, which represent three of the four most closely related families to Nyctaginaceae within the phytolaccoid clade (sensu Walker et al., 2018). No information could be obtained for Sarcobataceae, the remaining family in the phytolaccoid clade. For these 11 species, character state information was obtained either from natural populations, wood or herbarium collections or directly from

original published images (see Appendix 1 for information).

Anatomical procedures

For anatomical studies, all specimens collected in the field were immediately fixed in either FAA (formaldehyde–acetic acid–70% ethanol, 1:1:18 v/v) (Berlyn and Miksche, 1976) or 70% isopropyl alcohol for a week, and then stored in 70% ethanol (Johansen, 1940). Subsequently, samples from different developmental stages were either dehydrated in an ethanol series and embedded in a methacrylate resin (Historesin; Leica Microsystems, Heidelberg, Germany), or dehydrated in a butanol series, infiltrated with mixtures of butanol + Paraplast with increasing concentrations of paraplast and embedded in paraplast (Fisher Healthcare, Houston, TX, USA) (Johansen, 1940). Transverse and longitudinal sections were cut using either a rotary (Leica RM2145, Nussloch, Eisfeld, Germany) or a sliding microtome (Leica SM2010R). Sections cut from methacrylate were 5–8 μm thick and stained with 0.05% w/v toluidine blue O in 0.1 M phosphate buffer (pH 4.7; O'Brien and McCully,

1964), while sections cut from Paraplast were 10–12 μm thick and doubled-stained in 1% w/v astra-blue and 1% w/v safranin (Bukatsch, 1972). Samples from herbaria and/or wood collections were rehydrated by boiling the material in a mixture of water and glycerin (Angyalossy et al., 2016). Afterward, small samples were embedded in methacrylate resin and stained with 0.05% toluidine blue. For even larger stem samples, inclusion in polyethylene glycol 1500 was adopted (Barbosa et al., 2010). Permanent steel knives perfectly sharpened with sandpaper were used (Barbosa et al., 2018). The slides were mounted in synthetic resin (Permount; Fisher Scientific, Fair Lawn, NJ, USA). In addition, some samples were dehydrated in ethanol, hand-sectioned, doubled-stained in 1% astra-blue and 1% safranin and mounted in 50% v/v glycerin.

For examining the morphology of vessel elements and fibers, tissue was macerated using Jeffrey's solution (10% aqueous nitric acid + 10% aqueous chromic acid v/v; Johansen 1940). The dissociated material was washed in water, stained with safranin, and the slides were mounted in 50% glycerin.

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The anatomical analyses and photographs were performed using a Leica DMBL light microscope coupled with a digital camera (Leica DFC310, Leica Microsystems, Wetzlar, Germany). The images were edited and, sometimes, auto-aligned to build larger pictures using Adobe (San Jose, CA, USA) Photoshop (v. CS5, 64-bit) or Leica Application Suite (LAS, v. 4.0).

Micro-computed tomography

A fixed sample of *Colignonia glomerata* (less than 2 mm thick) was used for micro-computed tomography (SkyScan 1176, Bruker, Kotich, Belgium). The scanner was adjusted with a 50 kV/500 µA power supply, Al 0.5 mm filter and 18 µm average resolution. After three-dimensional reconstruction, images were captured using CTVox 3D visualization software (v. 3.1.2 64-bit; Micro Photonics,

Allentown, PA, USA). The micro-CT analyses allowed the observation of the stem structure sequentially and nondestructively. The results obtained with this technique were compared with anatomical sections.

Phylogeny and ancestral character state reconstruction

A well-supported genus-level molecular phylogeny of Nyctaginaceae (Douglas and Manos, 2007; Douglas and Spellenberg, 2010) was used as the basis for ancestral character state reconstruction for the different eustele subtypes. Since some species sampled in this study were not sampled in that phylogeny, the tree was edited to include only the species sampled in this work by collapsing them into the least inclusive nodes. After the species that were not sampled in our anatomical analyses were pruned, a tree containing all the taxa analyzed in the present study was obtained, and this tree was used for ancestral character state reconstructions. To establish the relationship within tribe Pisonieae, we followed the most recent classifications by Rossetto et al. (2019). A tree including representatives of four

TABLE 1. Species name for all studied Nyctaginaceae and outgroups, including habit, number of specimens analysed, and type of anatomical analysis (a– development, b– primary growth, c– secondary growth; d–maceration). Species with development studied ('**a**') are in bold. For information on collection site and vouchers see Appendix 1.

Taxon	Habit	No. of specimens analyzed	Anatomical analysis
Nyctagineae			
Abronia fragrans	Herb	1	b–c
Abronia neealleyi	Herb	1	**a–d**
Acleisanthes acutifolia	Herb	1	c
Acleisanthes chenopodioides	Herb	3	**a–d**
Acleisanthes lanceolata	Herb	2	b–c
Acleisanthes longiflora	Subshrub	1	b–c
Allionia choisyi	Herb	1	c
Allionia incarnata	Herb	5	**a–d**
Anulocaulis leiosolenus* var. *leiosolenus	Herb	3	**a–d**
Anulocaulis leiosolenus var. *gypsogenus*	Herb	3	c
Boerhavia diffusa	Subshurb	2	c
Boerhavia hereroensis	Herb	1	b–c
Boerhavia linearifolia	Herb	2	b–c
Boerhavia torreyana	Herb	2	b–c
Boerhavia wrightii	Herb	2	c
Commicarpus scandens	Scendent–shrub	3	**a–d**
Cyphomeris gypsophiloides	Herb	2	**a–d**
Mirabilis aggregata	Herb	1	b–c
Mirabilis* cf. *albida	Herb	2	**a–d**
Mirabilis jalapa	Subshurb	1	**a–d**
Mirabilis viscosa	Herb	1	c
Mirabilis sp.	Herb	1	c
Nyctaginia capitata	Herb	2	**a–d**
Okenia hypogea	Herb	3	**a–d**
Pisonieae			
Grajalesia fasciculata	Tree	2	**a–c**
Guapira linearibracteata	Tree	1	c
Guapira pernambucensis	Scendent–shrub	2	**a–c**
Guapira graciliflora	Tree	1	**a–c**
Guapira laxa	Tree	1	**a–d**
Guapira ligustrifolia	Tree	1	c
Guapira opposita	Tree	1	c
Neea delicatula	Tree	1	c
Neea hermaphrodita	Tree	2	**a–c**
Neea laetevirens Standl.	Tree	1	c
Neea macrophylla	Tree	1	c
Neea ovalifolia	Tree	1	c
Neea psychotrioides	Tree	1	c
Pisonia aculeata	Liana	2	**a–d**
Pisonia zapallo	Tree	1	c
Pisonia sp1.	Tree	1	c
Pisonia sp2.	Tree	1	**a–c**
Pisonia sp3.	Tree	1	c
Pisoniella arborescens	Liana	2	**a–c**
Pisoniella glabrata	Liana	2	**a–d**
Bougainvilleae			
Belemia fucsioides	Liana	2	c
Bougainvillea berberidifolia	Shrub	2	**a–d**
Bougainvillea campanulata	Shrub	2	**a–c**
Bougainvillea modesta	Tree	2	c
Bougainvillea stipitata	Tree	2	b–c
Bougainvillea spectabilis	Scendent–shrub	1	c
Bougainvillea sp1.	Shrub	1	c
Bougainvillea sp2.	Shrub	1	c
Phaeoptilum spinosum	Shrub	1	c
Colignonieae			
Colignonia glomerata	Liana	2	**a–d**
Colignonia rufopilosa	Liana	1	c
Boldoeae			
Cryptocarpus pyriformis	Liana	1	c
Salpianthus arenarius	Shrub	1	c
Salpianthus macrodontus	Shrub	1	c
Salpianthus purpurascens	Shrub	1	**a–d**
Leucastereae			
Andradea floribunda	Tree	2	**a–c**
Leucaster caniflorus	Liana	3	**a–c**
Ramisia brasiliensis	Tree	2	**a–c**
Reichenbachia hirsuta	Shrub	2	**a–d**
Outgroups*			
Agdestidaceae			
Agdestis clematidea	Liana	1	–
Petiveriaceae			
Gallesia integrifolia	Tree	2	**a–c**
Hilleria latifolia	Herb	1	–
Monococcus echinophorus	Shrub	1	–
Petiveria alliacea	Herb	2	–
Trichostigma octandrum	Liana	1	–
Rivina humilis	Herb	2	–
Seguieria americana	Tree	2	–
Seguieria langsdorffii	Shrub	1	–
Phytolaccaceae			
Phytolacca dioica	Tree	2	–

*Based mostly on the literature, except for *Gallesia integrifolia*. See Appendix 1 for details.

sister lineages (i.e., Petiveriaceae, Phytolaccaceae, Sarcobataceae, and Agdestidaceae) was also used to perform reconstruction analyses. For this tree, the relationships established by Walker et al. (2018) were followed. Among these families, Agdestidaceae were monotypic, Sarcobataceae are monogeneric (ca. two species), while Phytolaccaceae have three genera, and Petiveriaceae have nine genera (Stevens, 2001 onward; Hernández-Ledesma et al., 2015).

The ancestral character reconstruction analyses were performed using maximum likelihood

algorithms as implemented in Mesquite (ver. 3.6; Maddison and Maddison 2009).

RESULTS

Primary vascular system: eustele subtypes

In Nyctaginaceae, two subtypes of eustele were observed, the regular eustele (Fig. 1A, B) and the polycyclic eustele (Fig. 1C, D). The regular eustele was characterized by the presence of a discrete ring of collateral bundles delimiting the pith (Fig. 1A), whereas the polycyclic eustele was marked by several medullary bundles within the pith and encircled by a continuous concentric procambium that later
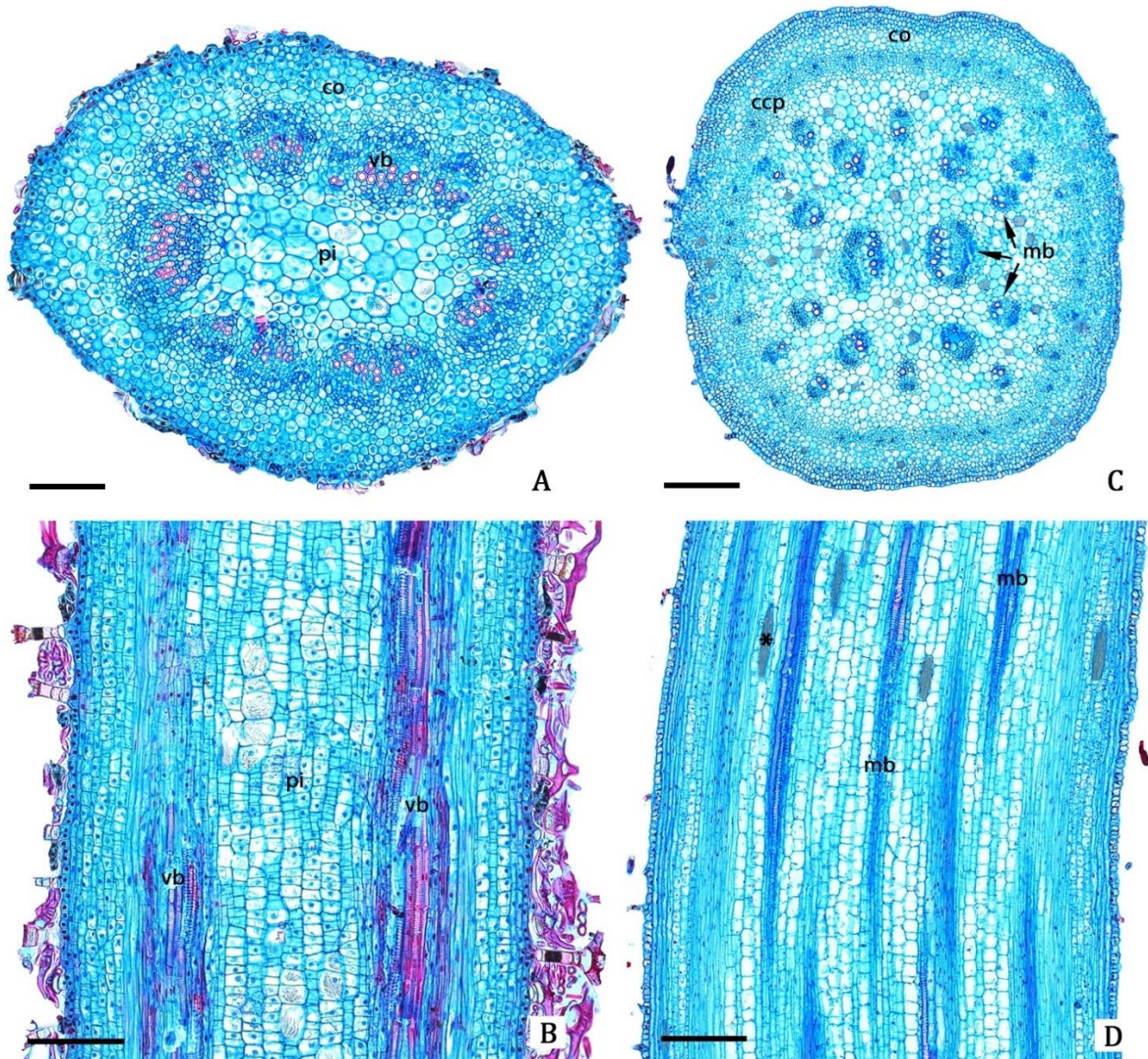
FIGURE 1. Subtypes of eustele in Nyctaginaceae. (A, B), *Reichenbachia hirsuta*, regular eustele, formed by collateral vascular bundles forming a ring that delimits the pith. (C, D) *Pisoniella glabrata*, polycyclic eustele, formed by medullary bundles arranged in rings inside the pith and encircled by a continuous concentric procambium. Asterisk, raphides; co, cortex; ccp, continuous concentric procambium; mb, medullary bundle; pi, pith; vb, vascular bundle. Scale bars: A = 100 μm; B = 150 μm; C, D = 200 μm. Stained with astra blue and safranin.

differentiated into vascular bundles (Fig. 1C). In the following sections, we described the different developmental, structural, and evolutionary aspects of medullary bundles within Nyctaginaceae.

Origin and development of medullary bundles

In Nyctaginaceae, medullary bundles originated from procambium strands that interconnected the stem with lateral organs or appendages, including leaves, branches, and thorns (Fig. 2A–E, 4A–D, 5A). The medullary bundles were the first vascular tissues formed in the polycyclic eustele (Fig. 2A–E, 4C, D), followed by a new, continuous procambial layer that subsequently resulted in vascular bundles (see below *Continuous concentric procambium*). As stem development progressed, additional leaf, bud, and/or thorn traces contributed to the appearance of new medullary bundles externally to the ones that had already established, as seen in *Colignonia glomerata* when comparing early and later formed nodes and internodes (Fig. 2A). The same was observed in *Bougainvillea berberidifolia* (Fig. 5A–C).

At the nodal level, medullary bundles sometimes twisted, underwent anastomoses and/or bifurcations, which eventually resulted in changes in their arrangement, size and disposition (Fig. 2A, 3A, 3B, 5A–C). In *C. glomerata,* numerous anastomoses were observed, forming what is known as a nodal plexus (Fig. 2A). As new leaf, thorn, and bud traces were produced, some of them anastomosed with already present medullary bundles (Fig. 5A–C), so that their number did not increase indefinitely.

Organization and diversity of medullary bundles

During primary growth, at the first internode near the shoot apex, different organizations of medullary bundles were observed in different taxa (Fig. 6A–I). The number of medullary bundles in Nyctaginaceae varied from eight to more than 20, as seen in *Pisoniella glabrata* (Fig. 1C) and *C. glomerata* (Fig. 6F). The vascular bundles showed three general types (Table 2). Type 1 consisted of eight medullary bundles distributed in a pair of three opposite bundles and two larger ones either in the center (Fig. 6A, C) or forming a ring (Fig. 6B, D–E). Type
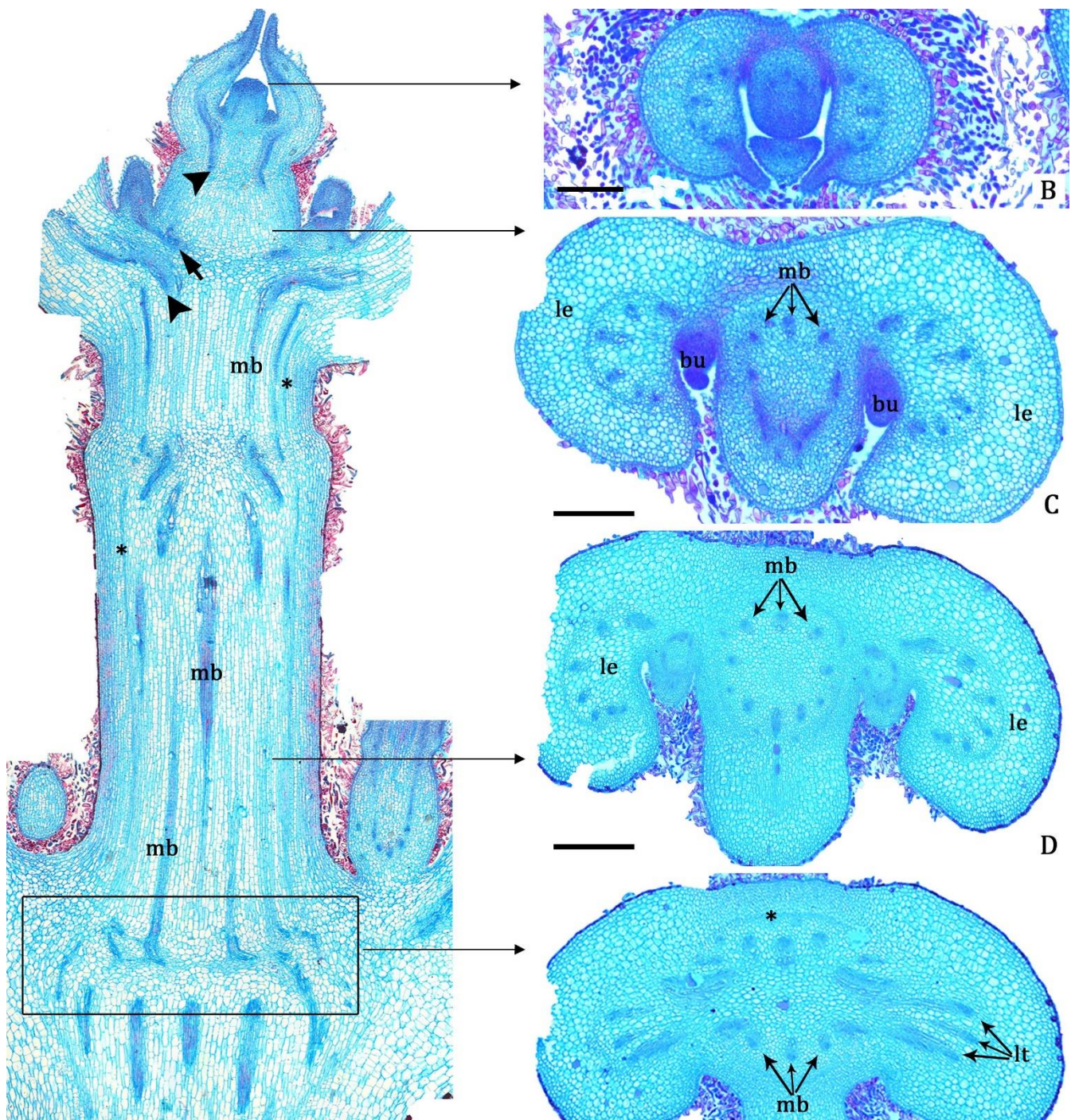
FIGURE 2. Origin and development of medullary bundles in *Colignonia glomerata*. (A) Shoot apex with leaf (arrowheads) and bud (arrow) traces in which medullary bundles anastomose and/or bifurcate. (B–E) Sequence of images from the apex toward developed internodes showing establishment of medullary bundles. Horizontal arrows indicate approximate levels of cross sections. (B) Shoot apical meristem without differentiated medullary bundles. (C–E) Developed medullary bundles in the stem. Notice nodal region in (E) showing three medullary bundles functioning as leaf traces. Asterisk, continuous concentric procambium; bu, axillary bud; mb, medullary bundles; le, leaf; lt, leaf trace. Scale bars: A, C–E = 400 μm; B = 200 μm. Stained with astra blue and safranin.

2 consisted of ≥10 medullary bundles regularly spaced, arranged in two to three concentric rings; the inner ring is
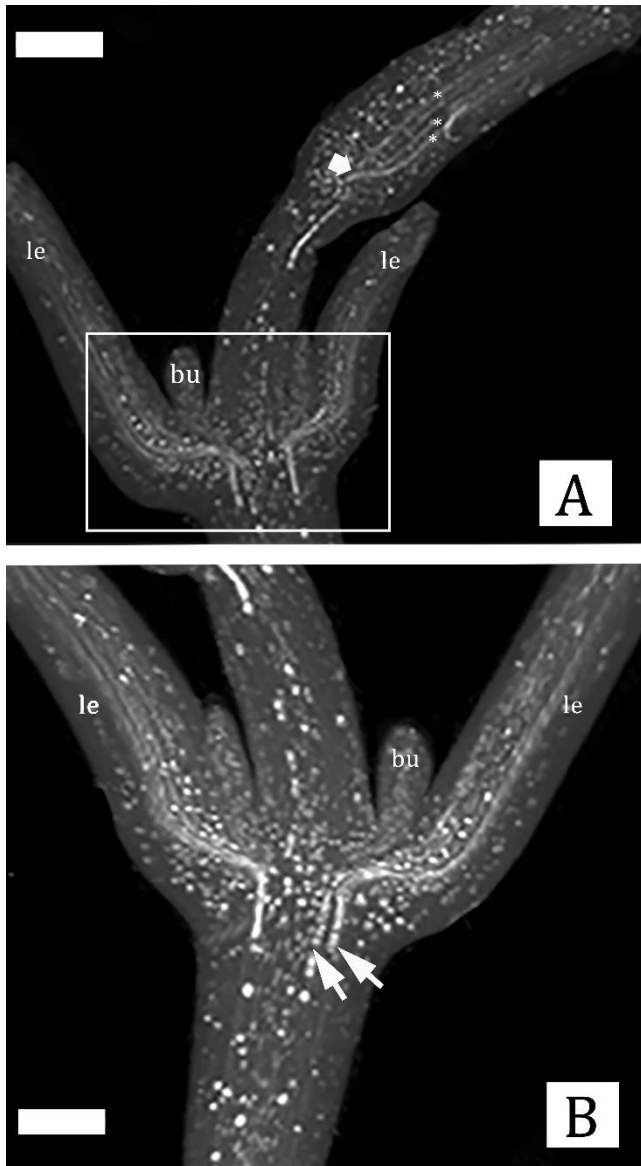
FIGURE 3. Micro-computed tomography images of *Colignonia glomerata*. (A) General view of stem showing trajectory of medullary bundles (asterisks), which anastomose at the nodal region (thick arrow). (B) Close-up of boxed area A in the mirror side, indicating bifurcation of procambial strands (arrows), which establishe as medullary bundles in the pith. bu, axillary bud; le, leaf. Scale bars: 2000 µm.

composed of larger bundles and the outer one constituted of smaller bundles (Fig. 1C, 6F). Type 3 consisted of ≥10 medullary bundles in a nonspecific arrangement (Fig. 6G–I). Species with eight medullary bundles represented the most common pattern (type 1), although small variations in number and arrangement occurred in each type. The organization of medullary bundles was independent from phyllotaxy and the presence or absence of thorns (Table 2). For example, different genera in tribe Nyctagineae such as *Abronia*, *Acleisanthes*, *Boerhavia*, and *Nyctaginia* had the same phyllotaxy (i.e., opposite), but different types of medullary bundles were found among them (Table 2).

The ancestral character state reconstruction (Fig. 12) suggested type 3 as the most likely ancestral character state for the ancestor of all Nyctaginaceae (45% likelihood presence versus 12.5% for type 1, 22.5% for type 2, and 20% for medullary bundles absent). Types 1 and 2 had multiple, parallel evolutions within the family, being present in taxa with different habits and from different

tribes. There was no single character state that defined an entire clade.

In some species, the number and arrangement of medullary bundles were found to be constant throughout the species life span, such as in the two species of *Pisoniella* (Fig. 6E) and some species of *Neea* and *Guapira*.

Pisoniella arborescens typically belonged to type number and arrangement of medullary bundles in stems with secondary growth showed frequent variations derived from anastomoses and/or bifurcations at the nodal regions (Fig. 2A, 5A–C).
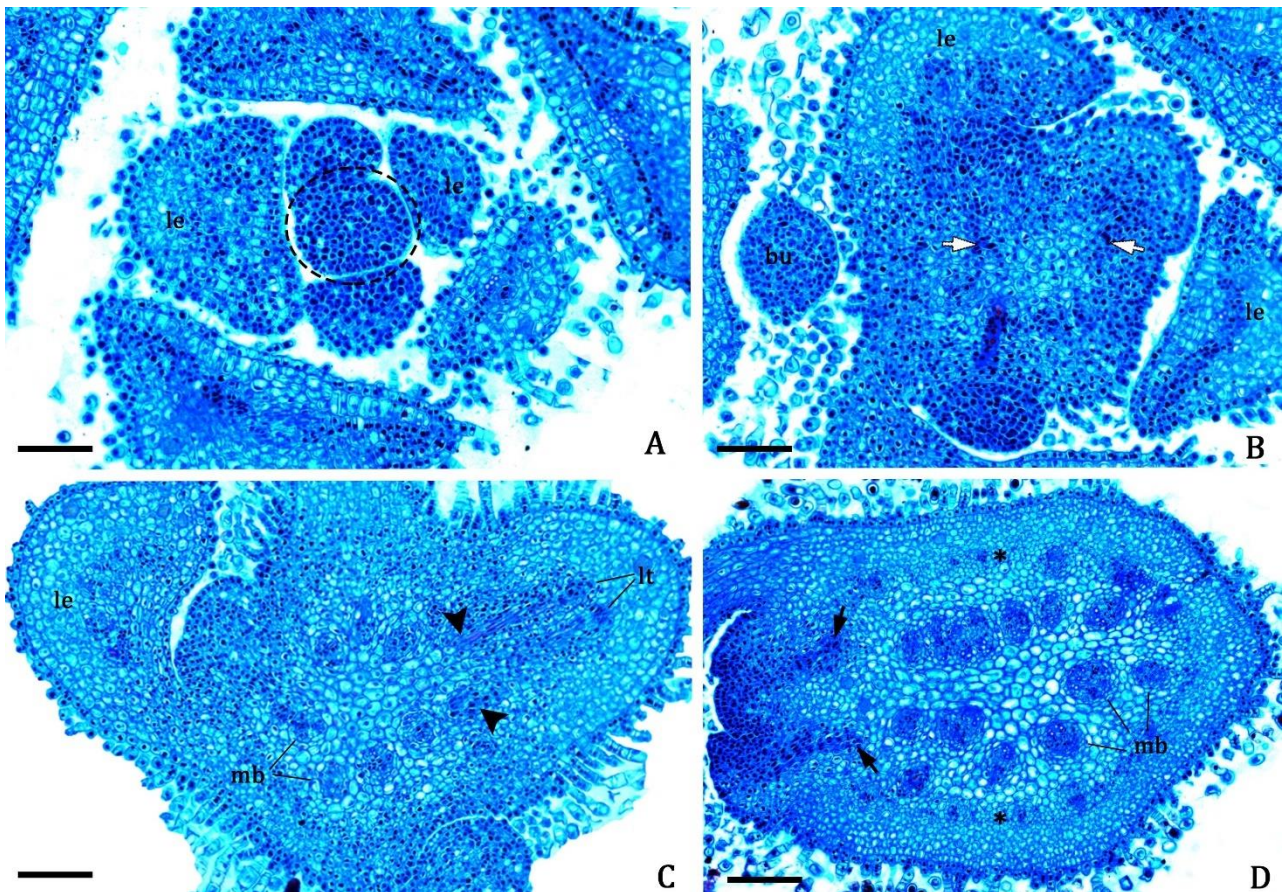
FIGURE 4. Origin and development of medullary bundles in *Bougainvillea berberidifolia*. (A–D) Sequence of images from apex toward more developed internodes showing establishment of medullary bundles from leaves and buds. (A) Shoot apical meristem without differentiated medullary bundles (dashed line). (B) Differentiation of procambial cells (dark stained) from developing leaf traces (white arrows). (C) Differentiated medullary bundles and two future medullary bundles (arrowheads) arising from leaf traces. (D) Greater number of medullary bundles and two new medullary bundles arising from axillary bud (black arrows). Notice the developing continuous concentric procambium (asterisks). bu, axillary bud; le, leaf; lt, leaf trace; mb, medullary bundle. Scale bars: 100 µm. Stained with astra blue and safranin.

Anatomy of medullary bundles

In all taxa studied, the medullary bundles were constituted of phloem to the outside and xylem to the inside, being, therefore, collateral (Fig. 7A, B).

During primary growth, medullary bundles were composed solely of conducting cells and parenchyma (Fig. 8B, C). Vessel elements in the primary xylem had different types of wall
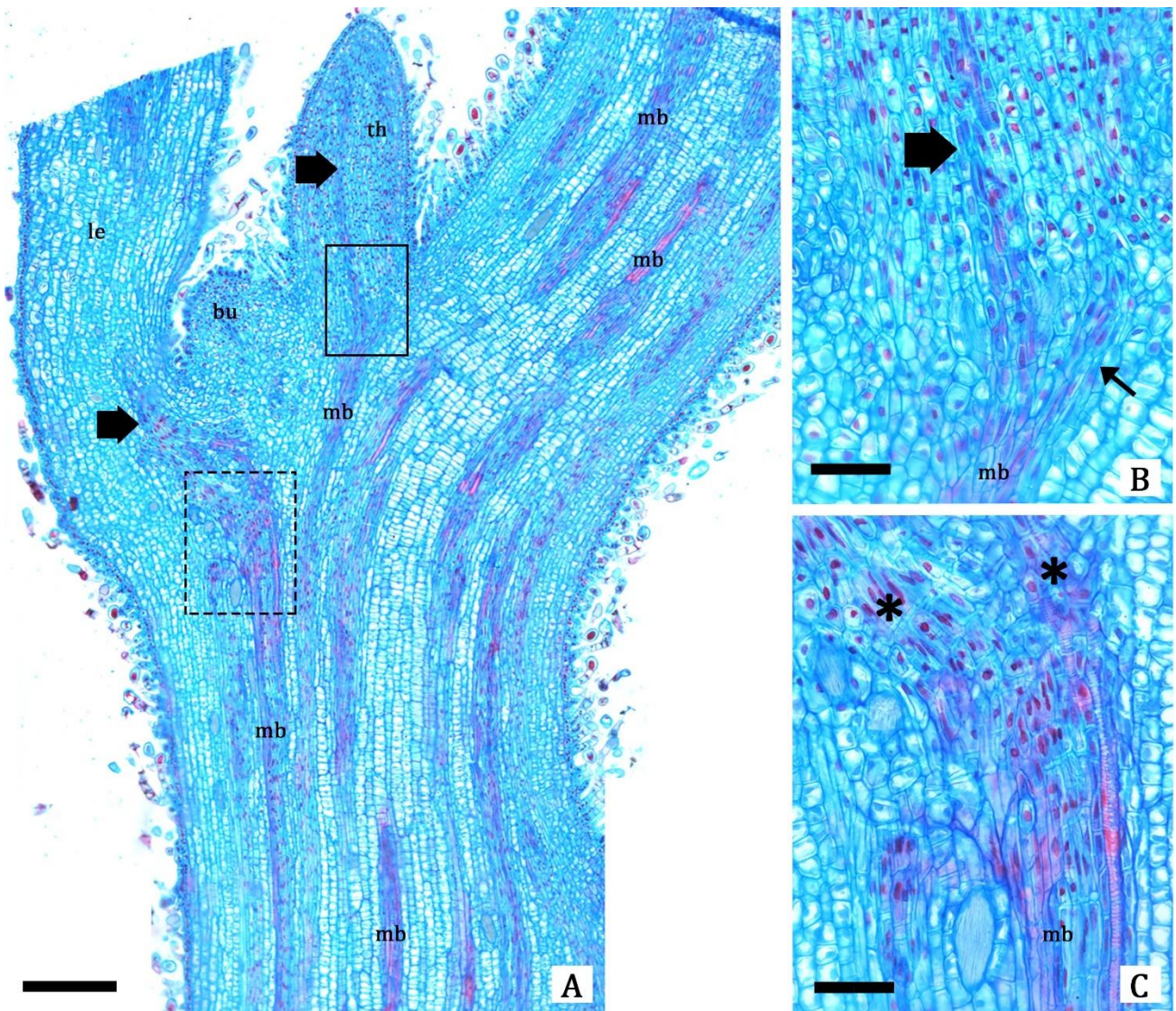
FIGURE 5. Details of the development of medullary bundles in *Bougainvillea berberidifolia.* (A) Overview of stem internodes showing the trajectory of medullary bundles. Notice leaf and thorn traces (thick arrows) which will anastomose with previous established medullary bundles. Box and dashed box indicate close-up in figures (B) and (C), respectively. (B) Detail of thorn trace (thick arrow) diverging towards the pith and fusing with another medullary bundle (thin arrow). (C) Detail of leaf traces (asterisks) diverging towards the pith to form a single medullary bundle. bu, axillary bud; le, leaf; lt, leaf trace; mb, medullary bundle; th, thorn. Scale bars: A = 200 μm; C–D = 50 μm. Stained with astra blue and safranin.

thickening, varying between annular and helical (Fig. 8B, C).

In all species, as stem secondary growth begun, the medullary bundles
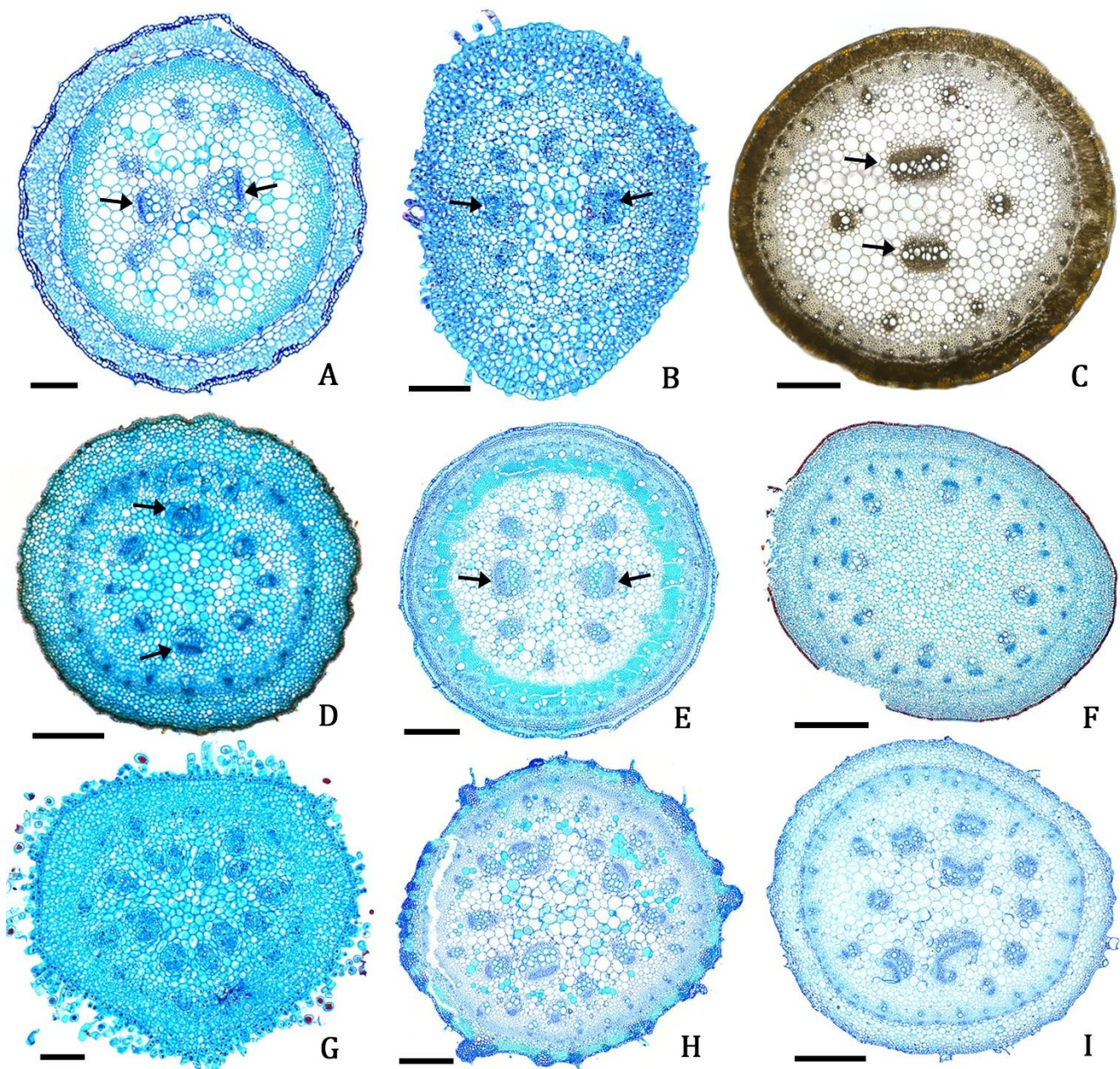
FIGURE 6. Organization and diversity of medullary bundles. (A–E) Type 1, eight medullary bundles organized in a ring. (A) *Acleisanthes chenopodioides.* (B) *Commicarpus scandens.* (C) *Anulocaulis leiosolenus* var. *gypsogenus.* (D) *Guapira laxa.* (E) *Pisoniella arborescens,* illustrating that, for this species, this arrangement is present in primary structure, as well as in stems with secondary growth. (F) Type 2, medullary bundles arranged in two concentric rings, *Colignonia glomerata.* (G–I) Type 3, medullary bundles showing several bundles irregularly distributed. (G) *Bougainvillea berberidifolia.* (H) *Salpianthus purpurascens.* (I) *Nyctaginia capitata.* arrows, larger (central) medullary bundles. Scale bars: A, B, G = 100 µm; C, D, I = 400 µm; E, F, H = 300 µm. A, E, G–I, stained with toluidine blue; B, D, F, stained with astra blue and safranin; C, no stain.

TABLE 2. Information on the types of arrangement of medullary bundles in relation to other morphological characteristics. The arrangement of medullary bundles was inferred from specimens with "a" and/or "b" in Table 1, since this organization may change with secondary growth. When only the genus name is included, then all species collected in that genus were studied; otherwise, the species name was specified.

Arrangement of medullary bundles in primary growth	Tribe	Taxa	Phyllotaxy	Presence/ absence of thorns	Hollow stems	Observations
Type 1 – eight medullary bundles organized in one or two rings; the central ones are larger than the others. **See Fig. 6A–6E.**	Nyctagineae	*Abronia neealleyi, Acleisanthes chenopodioides, Acleisanthes lanceolata, Acleisanthes longiflora, Anulocaulis leiosolenus* var. *leiosolenus, Allionia incarnata, Boerhavia linearifolia, Commicarpus, Cyphomeris, M. albida, Okenia*	Opposite	–	–	Sometimes one bundle was lacking and only seven constituted the ring, as in *Allionia and Cyphomeris*. The arrangement is usually lost in fully developed stems due to new bundles.
	Pisonieae	*Pisoniella arborescens*	Opposite	–	–	There was also one sample with 10 medullary bundles. The arrangement was maintained in developed stems.
		Guapira laxa, Guapira pernambucensis, Neea hermaphrodita	Subopposite	–	–	The arrangement is usually lost in fully developed stems due to new bundles.
Type 2 – ≥ than 10 medullary bundles arranged in two or more rings. Medullary bundles in the inner ring are usually larger. **See Fig. 1C, 6F.**	Nyctagineae	*Mirabilis jalapa, M. aggregata, Mirabilis sp.*	Opposite	–	–	
	Colignonieae	*Colignonia glomerata*	Opposite	–	+	The medullary bundles delimit a large pith, which disintegrate later forming a hollow stem.
		Pisoniella glabrata	Opposite	–	–	The arrangement was maintained in developed stems.
Type 3 – ≥ than 10 medullary bundles in a non-specific arrangement. **See Fig. 6G–I.**	Nyctagineae	*Boerhavia herehoensis, B. torreyana*	Opposite	–	–	
		Mirabilis viscosa		–	–	
		Nyctaginia		–	–	
	Bougainvilleae	*Bougainvillea*	Alternate	+	–	
	Boldoeae	*Salpianthus purpurascens*	Alternate	–	–	
	Pisonieae	*Guapira graciliflora*	Subopposite	–	–	
		Grajalesia fasciculata	Opposite	–	–	
		Pisonia aculeata	Subopposite	+	–	
		Pisonia sp.2	Subopposite	–	–	

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also underwent secondary growth (Fig. 8D–G). The secondary xylem was marked by the presence of pitted vessels, fibers, and sometimes rays (Fig. 8D–G). In mature stems, medullary bundles were maintained, showing vessels with the different types of wall thickenings, varying between annular to pitted (Fig. 8E). As the secondary growth proceeded, cells from the primary phloem were crushed to a greater or lesser extent forming a variable amount of nonconducting, collapsed phloem (Fig. 8D). On the other hand, even medullary bundles on fully developed stems had conducting xylem and phloem, which in some cases showed little crushed phloem, but several living sieve-tube elements,

recognized by the presence of protoplasts in their companion cells (Fig. 8G).

In *Cryptocarpus pyriformis* and some species within tribe Pisonieae (e.g., *Guapira ligustrifolia, Neea ovalifolia*), fibers encircling the phloem and to some extent the xylem were observed, as evident in *G. ligustrifolia* (Fig. 9A). In adult stems of *Phaeoptilum spinosum,* the medullary bundles were surrounded by lignified pith tissue (Fig. 9B). In most species, numerous parenchymatic cells containing starch grains were observed in the pith and/or around the medullary bundles, as seen in *Acleisanthes chenopodioides* (Fig. 9C).
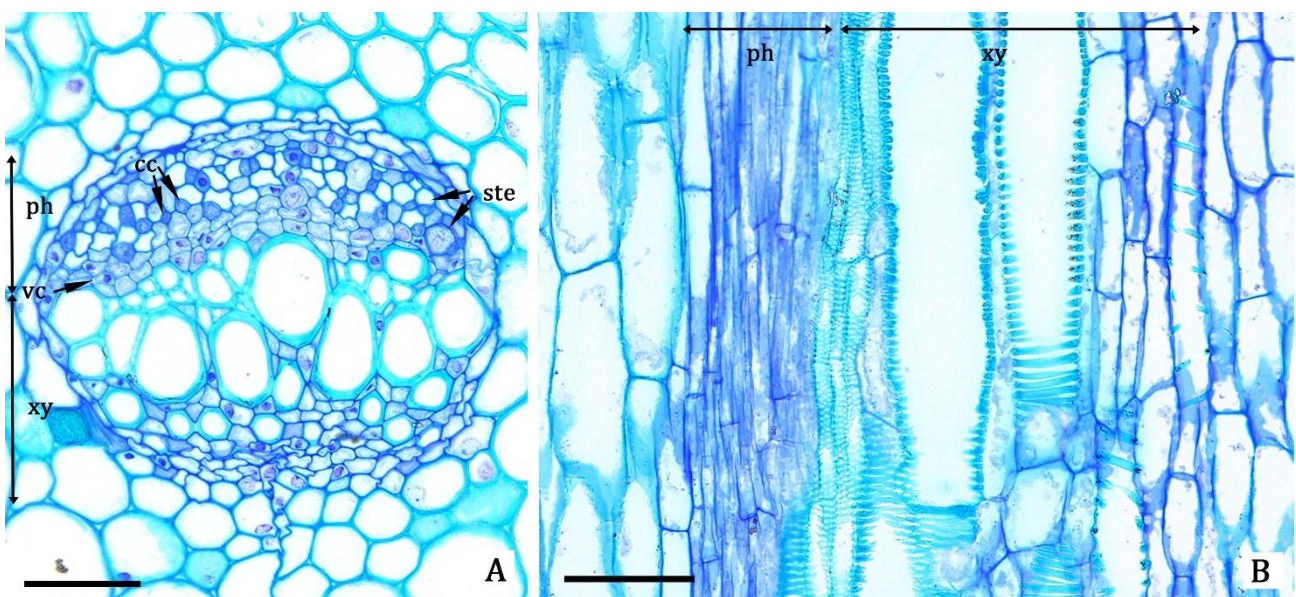
FIGURE 7. Collateral medullary bundles in *Anulocaulis leiosolenus* var. *gypsogenus*. (A) Transverse section. (B) Longitudinal section. cc, companion cell; ph, phloem; ste, sieve-tube element; vc, vascular cambium; xy, xylem. Scale bars: 50 μm. Stained with toluidine blue.

Continuous concentric procambium

In addition to the medullary bundles, the primary vascular system in Nyctaginaceae species with polycyclic eustele had an additional developmental step, with the formation of a hollow continuous concentric procambium (Fig. 1C, 2A, 10A–F). This continuous concentric procambium appeared early in stem development, after the second or third node (Fig. 2A, 10A), immediately after the establishment of the medullary bundles (Fig. 2E, 4D, 10A–D). It was characterized by cells with dense cytoplasm, round or irregular contour and a smaller size, as seen in cross section (Fig. 10C, D). These same cells were elongated or appeared fusiform in longitudinal sections (Fig. 10E, F). Later, these procambial cells underwent several periclinal and anticlinal divisions in some regions (Fig. 10D), resulting in the differentiation of primary xylem and phloem in discrete collateral bundles (Fig. 1A–C), and a ring of parenchymatic cells in the outermost part of the vascular cylinder (Fig. 10E, F). As a result, several vascular bundles had differentiated along the circumference of the continuous concentric procambium establishing a single ring outside the medullary bundles and internally to the starch sheath (Fig. 10A, B).

Unlike the species with polycyclic eusteles, the representatives of tribe Leucastereae had the primary vascular system formed solely by a ring of collateral vascular bundles (Fig. 1A). In these species, the development of the vascular bundles followed a regular pattern in which the vascular bundles delimited the pith, i.e., forming a typical eustele (Fig. 1A).

Ancestral state reconstruction of the eustele subtypes onto the phylogeny of Nyctaginaceae is shown in Fig. 12. Ancestral character state reconstruction of eustele subtypes across Nyctaginaceae suggested that the ancestral state for the family was the polycyclic eustele (78% presence; 22% absence), with one independent loss to the regular eustele in tribe Leucastereae.

Among the outgroups, we found that most representatives of Petiveriaceae had a regular eustele (i.e., *Gallesia*, *Hilleria*, *Monococcus*, *Petiveria*, *Trichostigma*, *Rivina*, and *Seguieria*); no information was
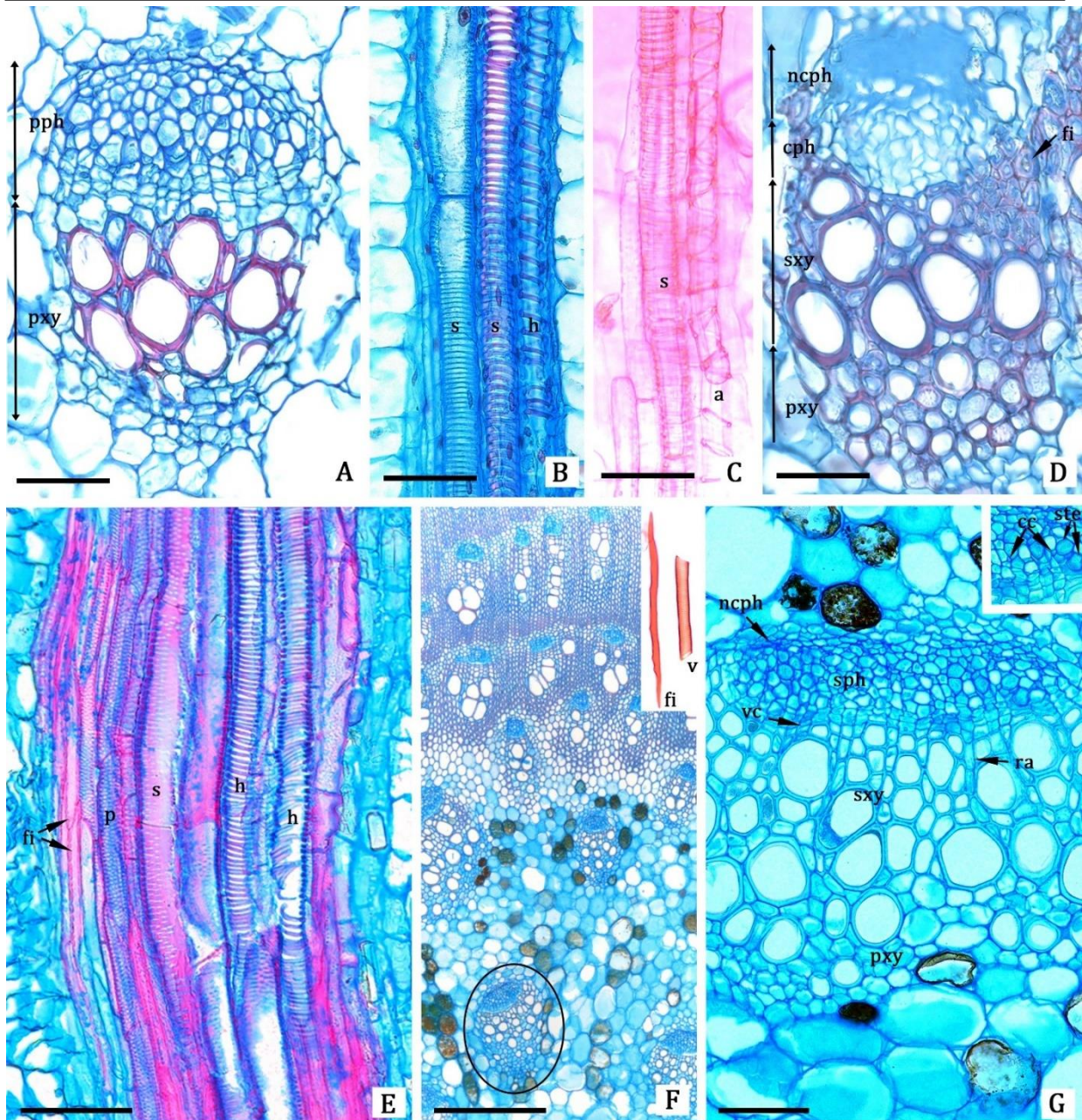
FIGURE 8. Anatomy of medullary bundles. (A, B) *Pisoniella glabrata*, collateral medullary bundles during primary growth. Notice that primary xylem and primary phloem are constituted only of conducting elements and parenchyma. Vessel thickenings are annular (a) or helical (h) in images B and C. (C) *Colignonia glomerata,* macerated medullary bundles from a sample in primary growth. (D, E) *Guapira laxa*, medullary bundles during secondary growth evidenced by secondary xylem with fibers, secondary phloem, and collapsed primary phloem. (D) Transverse section. (E) Longitudinal section. Notice fibers, scalariform (s) and pitted vessels (p). (F–G) *Salpianthus purpurascens*. (F) Adult stem showing the presence of functional medullary bundles (ellipse). Macerated fiber and pitted vessel (inset) from medullary bundles during secondary growth. (G) Detail of medullary bundles in secondary growth, evidenced by the secondary xylem, conducting secondary phloem with functional sieve-tube elements and companion cells (inset), vascular ray, and nonconducting phloem at the periphery. a, annular vessel thickening; cc, companion cell; cph, conducting phloem; fi, fiber; h, helical vessel thickening; mb, medullary bundles; ncph, nonconducting phloem; p, pitted vessel; pc, procambium; pph, primary phloem; pxy, primary xylem; ra, vascular ray; s, scalariform vessel; sph, secondary phloem; ste, sieve-tube element; sxy, secondary xylem; vc, vascular cambium; v, vessel element. Scale bars: A–D = 50 μm; E, G = 100 μm; F = 500 μm. A–F, stained with astra blue and safranin; G, stained with toluidine blue.

obtained for the remaining genera (i.e., *Ledenbergia* and *Schindleria*). In contrast, the representatives of other outgroup families were found to have the polycyclic eustele, as in *Agdestis clematidea* (Agdestidaceae) and *Phytolacca dioica* (Phytolaccaceae). The analysis including Nyctaginaceae and the representatives of the sister families indicate that the ancestor of the phytolaccoid clade also had medullary bundles (81% presence; 19% absence), with one independent loss in the ancestors of Petiveriaceae and Nyctaginaceae (tribe Leucastereae) (Fig. 12). Therefore, the presence of medullary bundles was most likely a symplesiomorphy for Nyctaginaceae.

DISCUSSION

The evolution of different stele types (and subtypes) is one of the crucial aspects in the diversification of vascular plants (Eames and McDaniels, 1925; Esau, 1954; Beck et al., 1982). However, the vascular architecture in plants with medullary bundles has long been considered difficult to characterize (Wilson, 1924; Davis, 1961), which makes this topic either controversial or little understood in

several aspects, from terminology—with the use of different names (see Schmid, 1982; Nair and Nair, 1961; Gibson, 1994)—to its potential adaptive and/or evolutionary significance. In a general sense, additional vascular tissues in roots and stems, have been thought to be adaptations for higher capacity in the storage and translocation of water and photosynthates, being advantageous especially for some plants occurring in harsh environments (Holwill, 1950; Mauseth, 1993; Carlquist, 2001; Hearn, 2009; Ogburn and Edwards, 2013; Males, 2017). However, the functional significance of medullary bundles is still unclear, and explicit physiological studies are needed to address this question.

Origin of medullary bundles and the continuous concentric procambium

Various reports have explored the origin of medullary bundles. Some suggested that the medullary system originates from independent procambial strands in the shoot apical meristem or as cauline bundles (e.g., Apiaceae: Lambeth, 1940; Cactaceae: Boke, 1941; de Bary, 1884; Nyctaginaceae: Inouye, 1956), while

others suggested an origin from regular stelar bundles that branch toward the pith (e.g., Melianthaceae: Metcalf and Chalk, 1950; Phytolaccaceae: Kirchoff and Fahn, 1984), from the rib meristem (e.g., Cactaceae: Boke, 1941; Convolvulaceae: Pant and Bhatgnar, 1975) or even from mature parenchyma pith cells (e.g., Cactaceae: MacDougal, 1926; Cyatheaceae [ferns]: Lucansky, 1974; Convolvulaceae: Kapadane et al., 2019). In addition, medullary bundles are believed to arise by conversion of intraxylary phloem as for the Cucurbitaceae (Worsdell, 1915), Apocynaceae and Gentianaceae (Metcalfe and Chalk, 1950), and Euphorbiaceae (Hayden and Hayden, 1994).

Our findings for Nyctaginaceae did not support any of these statements. The origin of medullary bundles in Nyctaginaceae conforms with the generalization that they arise from procambial traces associated with leaves, buds, thorns, and inflorescences (Nair and Nair, 1961; Sharma, 1962; Stevenson and Popham, 1973; Mikesell and Popham, 1976; Zamski, 1980).

As pointed out by other authors (de Bary, 1884; Inouye, 1956; Pant and Mehra, 1961, 1963) and observed in the present study, medullary bundles may eventually anastomose or bifurcate in the nodes on their trajectory between the lateral organs and the axis. In our study, this pattern was particularly evident in *Colignonia glomerata*, with
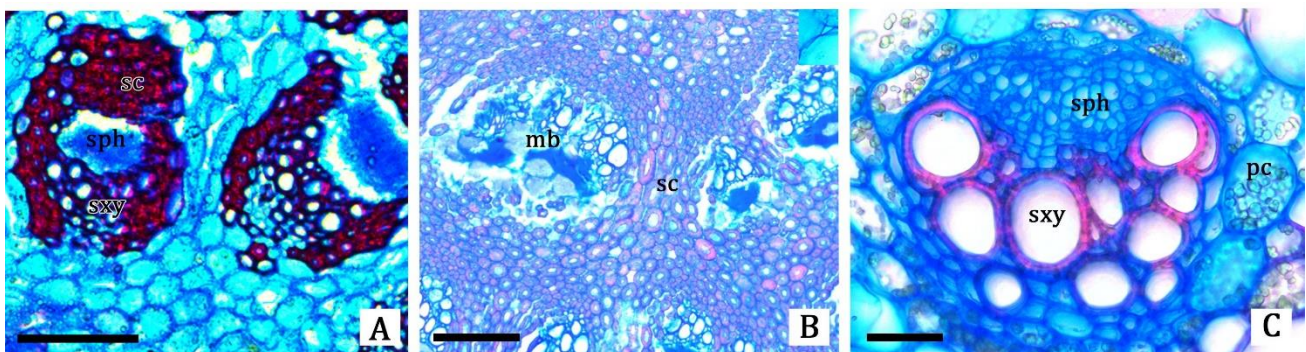
FIGURE 9. Details of medullary bundles in developed stems. (A) *Guapira ligustrifolia*, medullary bundles encircled by a sheath of sclerenchyma. (B) *Phaeoptilum spinosum*, medullary bundles immersed in sclerenchymatic tissue. (C) *Acleisanthes chenopodioides*, medullary bundle encircled by parenchymatic cells containing starch grains. mb, medullary bundle; pc, parenchymatic cell; sc, sclerenchyma; sph, secondary phloem; sxy, secondary xylem. Scale bars: A = 200 µm; B = 250 µm; C = 50 µm. Stained with astra blue and safranin.

the formation of a nodal plexus, comparable to those is described for monocot stems (Tomlinson and Fischer, 2000; Vita et al., 2019). Similarly, instead of "ending blindly in the stem" as mentioned by earlier authors (Lambeth, 1940; Pant and Bhatnagar, 1975), medullary bundles eventually shifted their trajectory and/or anastomosed with other bundles. Given that one or more bundles frequently connect among them forming a single bundle, phenomena as shifting anastomoses or bifurcations of medullary bundles directly interfere in their organization in stem cross sections. Similar phenomena have also been reported for Amaranthaceae and Convolvulaceae, among other families (Wilson, 1924; Pant and Mehra, 1961, 1963; Pant and Bhatnagar, 1975) (further explored later in *Arrangement of medullary bundles and their independence from shoot morphology*).

Our observation and description of the continuous concentric procambium was fundamental for understanding the primary vascular system in Nyctaginaceae. Three main aspects need to be highlighted regarding the presence of the

continuous concentric procambium: (1) it occurs in all species with medullary bundles; (2) as a primary meristem, it produces primary tissues organized in vascular bundles; (3) they constitute part of the primary vascular system; i.e., they are part of the polycyclic eustele.

Previous authors have generally overlooked or given different denominations for this external continuous concentric procambium (de Bary, 1884; Pant and Mehra, 1961, 1963; Sharma, 1962; Balfour, 1965; Zamski, 1980). As a result, different terms have been used to name this meristem and its products, including perimedullary bundles and outer ring (Zamski, 1980), third ring (Inouye, 1956), peripheral or "belated" ring (Pant and Mehra, 1961, 1963), peripheral procambial ring (Sharma, 1962), and "extrafascicular cambium" (de Bary, 1884). Here we demonstrated that the continuous concentric procambium is a peripheral ring of procambial cells that differentiates beneath the innermost cortex layer. The primary origin of these vascular bundles was confirmed due to the presence of vessel elements with annular and helical thickenings (typical of primary xylem, i.e.,
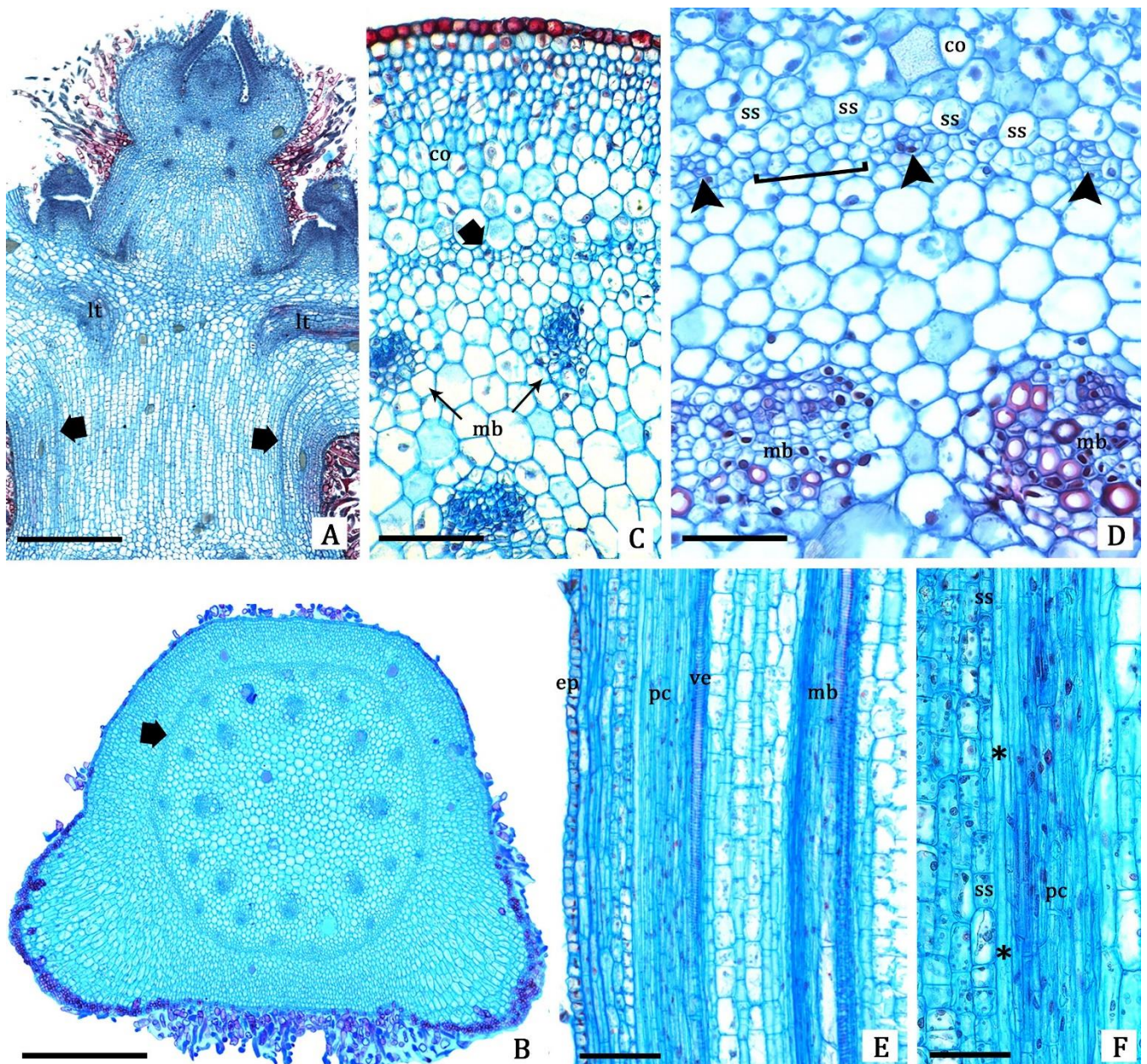
FIGURE 10. Origin and development of continuous concentric procambium. (A–C) *Colignonia glomerata,* view from developing continuous concentric procambium in early developmental stages (arrows). (A) Longitudinal section. (B, C) Transverse sections. (B) General view. (C) Detail of medullary bundles and continuous concentric procambium. (D) *Commicarpus scandens*, continuous concentric procambium cells dividing to form the first vascular elements (arrowhead). (E, F) *Pisoniella glabrata.* (E) Detail of the continuous concentric procambium in a region where a vascular bundle is differentiating. Note the presence of the first differentiated vessel element. (F) Continuous concentric procambium formed by elongated cells that differentiate into vascular bundles and the parenchymatic cells forming the outermost part of the vascular system (asterisks). co, cortex; ep, epidermis; lt, leaf trace; mb, medullary bundle; pc, continuous concentric procambium; ss, starch sheath; ve, vessel element. Scale bars: A, B = 400 μm; C, E = 100 μm; D, F = 50 μm. A, C, D, stained with astra blue and safranin; B, E, F, stained with toluidine blue.

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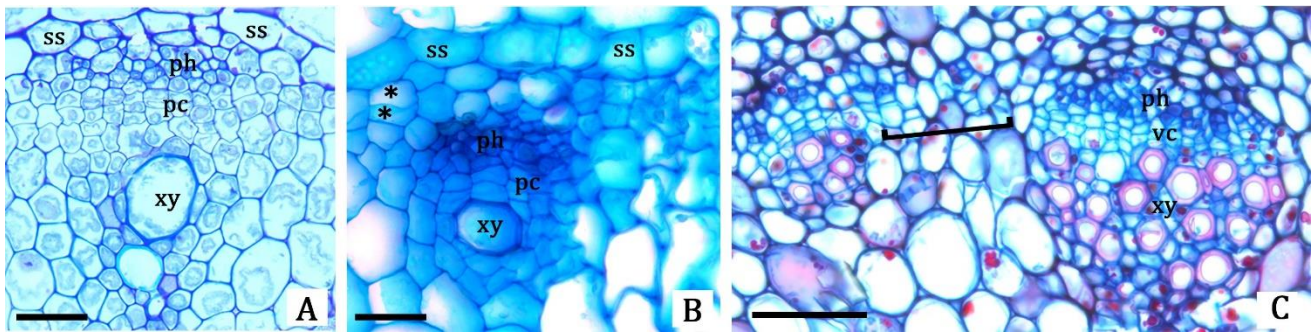
FIGURE 11. Vascular bundles originated from the continuous concentric procambium. (A) *Nyctaginia capitata,* collateral vascular bundle. (B) *Colignonia glomerata*, detail of vascular bundle, outermost cells of vascular system (asterisks) and starch sheath. (C) *Guapira graciliflora*, differentiated vascular bundle evidenced by primary xylem and primary phloem, and interfascicular region (bracket). pc, procambium; ph, phloem; ss, starch sheath; vc, vascular cambium; xy, xylem. Scale bars: A–C = 50 μm. A, stained with toluidine blue; B, C, stained with astra blue and safranin.

protoxylem and metaxylem), as also indicated by Inouye (1956).

The polycyclic eustele in Nyctaginaceae

The results on the origin and development of medullary bundles and the continuous concentric procambium are directly related to the stelar concept. As we have shown, the way that both medullary bundles and the vascular bundles from the continuous concentric procambium are built in most Nyctaginaceae means that they correspond to the primary vascular bundles, which constitute the eustele. Therefore, the concept of polycyclic eustele in Nyctaginaceae should be slightly modified to "medullary bundles arranged within the pith which are surrounded by vascular bundles

particularity of Nyctaginaceae and other Caryophyllales from the phytolaccoid clade.

Because the polycyclic eustele contains several medullary bundles within the pith, this arrangement of the vascular system in eudicots has long been compared to the "atactostele" of the monocots (de Bary, 1884; Wilson, 1924; Pant and Mehra, 1963; Isnard et al., 2012). Although the arrangement of medullary bundles in Nyctaginaceae may be similar in appearance to the stele of monocots, there are differences in origin, development, and structure. Differently from Nyctaginaceae and other plants with medullary bundles, monocots generally possess several bundles seemingly uniformly spaced (i.e.,

ordered; *sensu* Korn, 2016), which may be formed by two types of bundles, the bundles that form leaf-traces and cauline (axial) bundles that are not continuous with the leaves (Tomlinson, 1984; Cattai and Menezes, 2010; Botânico and Angyalossy, 2013; Vita et al., 2019).

Another general idea is that the medullary bundles are comparable to the regular bundles (stele bundles) of eudicots, which, during evolution,

diverged inward at the nodes (Wilson, 1924; Zamski, 1980; Costea and DeMason, 2001). The observations in this study may corroborate this observation, since the medullary bundles in Nyctaginaceae do not form an independent system but rather constitute the primary vascular system, which is formed from leaf and thorn traces. Therefore, analyzing the morphological complexity acquired by the species with polycyclic eustele in
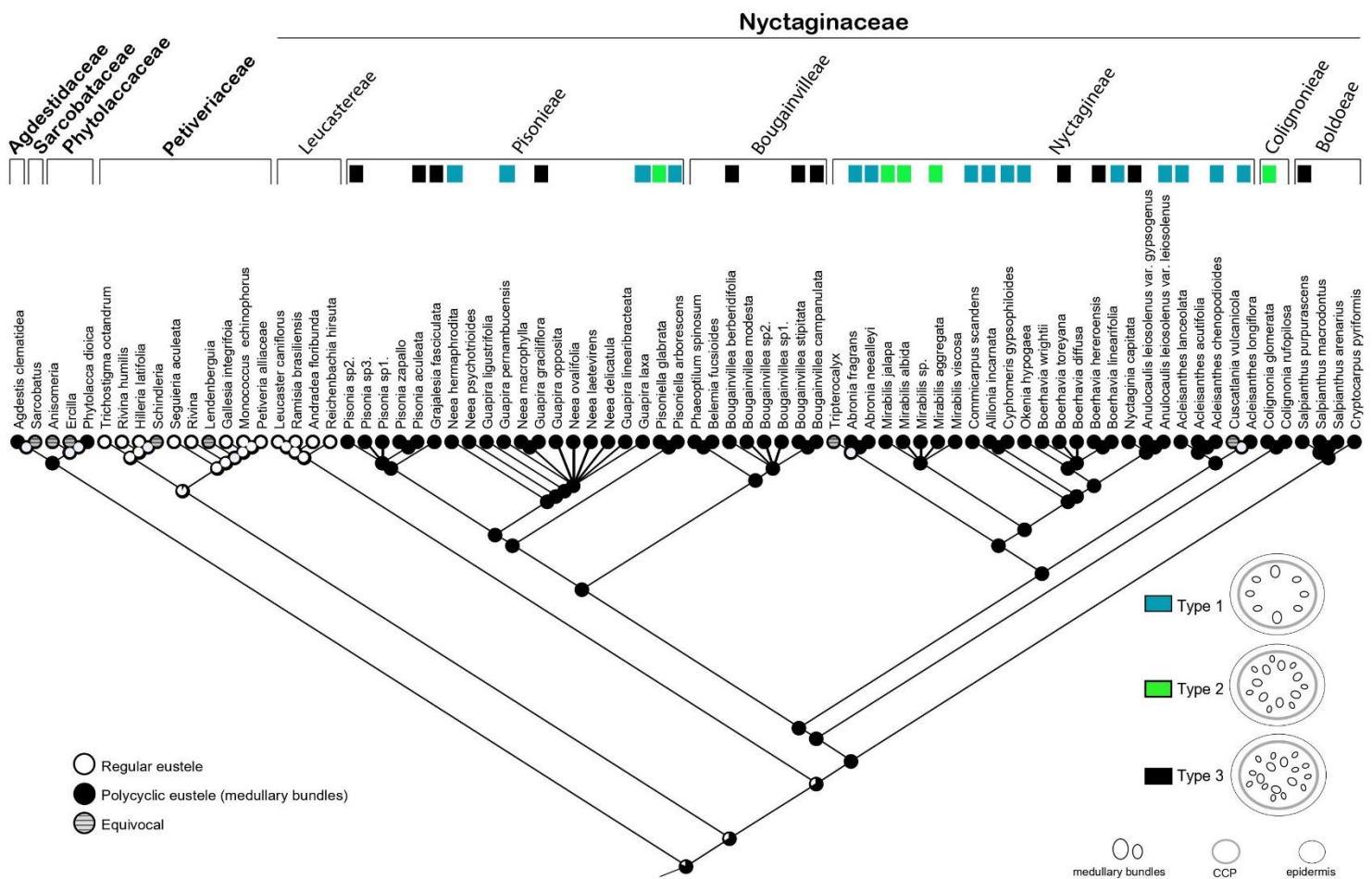
FIGURE 12. Different arrangements of medullary bundles; ancestral character state reconstruction of the presence of medullary bundles in Nyctaginaceae and sister groups. The deepest node inferred 81% probability for presence of medullary bundles (black) versus 19% for absence (white). Nyctaginaceae most recent common ancestor was inferred to have 45% likelihood of being type 3. CCP continuous concentric procambium.

Nyctaginaceae, two main developmental shifts are observed: (1) the establishment of leaf-traces in the pith as medullary bundles and (2) the belated appearance of a continuous concentric procambium forming the ring of vascular bundles that delimits the pith—equivalent to the ring of vascular bundles forming the regular eustele in Leucastereae and eudicots in general.

Arrangement of medullary bundles and their independence from shoot morphology

The presence of medullary bundles is directly related to the formation of lateral organs, since medullary bundles form leaf, branch, and thorn traces. Therefore, different arrangement of medullary bundles might be expected to arise in direct correlation with the external morphology. However, we found the number and arrangement of medullary bundles in Nyctaginaceae to be independent from the external morphology, such as the different phyllotaxies or the presence or absence of thorns (Table 1). Likewise, the study of Hernández-Ledesma et al. (2011) with 24 species of *Mirabilis* found

that the number of medullary bundles varied from 4 to 40 with different organizations, even though all the species exhibit the same phyllotaxy. Similar results are found in other groups, such as for the genus *Piper* (Piperaceae) where seven species of equal phyllotaxy showed a variable number of medullary bundles (Yang and Chen, 2017). Overall, the organization of medullary bundles depends on the proximity of the nodes due to bifurcations or anastomoses that may occur along the medullary bundle's trajectory in the internodes.

An interesting case was found in the genus *Pisoniella.* Investigation of the number and arrangement of medullary bundles showed that the two species of *Pisoniella* have different patterns, despite their similar external morphology. While *P. glabrata* always had around 20 bundles distributed in two to three concentric rings, *P. arborescens* had mostly eight bundles arranged in a single ring. Interestingly, the number and arrangement of medullary bundles in these two species are quite stable and remained constant when comparing different developmental stages, even among young or adult stems. Although *P.*

arborescens and *P. glabrata* have been distinguished as different species by some authors (Fay, 1980; Spellenberg, 2001; Nee, 2004), others have classified the genus as monotypic (Standley, 1911; Bittrich and Kühn, 1993) or consisting of one species divided into two varieties (López and Anton, 2006). As expected, the vegetative and reproductive morphology of these two taxa are very similar, but apparently, they have a disjunct distribution with *P. glabrata* occurring in South America, while *P. arborescens* is restricted to Mexico and Central America. Now, differences in the structure of primary vascular system add additional evidence that these two species should be considered different.

Anatomy of medullary bundles

All medullary bundles in Nyctaginaceae are collateral, as also demonstrated by previous works with the family (Nair and Nair, 1961; Stevenson and Popham, 1973; Mikesell and Popham, 1976). However, other types of medullary bundles have also been reported in eudicots, including amphicribal, inversely oriented, or variations between these patterns (Metcalfe and Chalk, 1950; Davis, 1961).

Later in development, the collateral medullary bundles in Nyctaginaceae increase in size due to the production of secondary tissues (Balfour, 1965; Rajput et al., 2009; this study), as also observed in Apiaceae (Lambeth, 1940), Cactaceae (Mauseth, 1993), and Convolvulaceae (Kapadane et al., 2019). We noticed that the secondary growth in the medullary bundles is synchronous with the secondary growth of the external ring of bundles derived from the continuous concentric procambium. Thus, while the stem increases in diameter, the medullary bundles also continue to produce secondary xylem and secondary phloem to some extent, and their conducting cells maintain function throughout the plant's lifespan. Secondary growth is noticeable due to the presence of a cambium, with the production of fibers, pitted vessels, and the presence of collapsed cells in the phloem, which indicate that new phloem elements have been produced.

Ancestral character state reconstruction of eustele subtypes

The observed differences in distribution of the eustele subtypes is enough to define an entire clade, since

the regular eustele is exclusive to all the representatives of tribe Lecaustereae and is here suggested as an anatomical synapomorphy of this clade. The exclusive occurrence of regular eustele in tribe Leucastereae is quite interesting, given that this tribe is also known as having a set of other morphological characteristics differing from other tribes of Nyctaginaceae, e.g., stellate trichomes, absence of anthocarp (typical fruit of Nyctaginaceae) and bisexual flowers (Bittrich and Kühn, 1993; Douglas and Manos, 2007; Douglas and Spellenberg, 2010; Rossetto et al., 2019). As a result, the occurrence of medullary bundles in the remaining taxa has an important systematic implication at the tribal and generic level within Nyctaginaceae.

The ancestral character state reconstruction for the eustele characters within Nyctaginaceae showed that the ancestors for the family likely had a polycyclic eustele. Consequently, the occurrence of a regular eustele in tribe Leucastereae represents a single evolution (autapomorphy) within the Nyctaginaceae. When Nyctaginaceae is put in a larger phylogenetic context, including their sister groups, i.e.,

Agdestidaceae, Phytolaccaceae, and Sarcobataceae, the sister groups of Nyctaginaceae, which all except Petiveriaceae share the presence of medullary bundles, it becomes evident that the most recent common ancestor of this entire clade is likewise reconstructed as having a polycyclic eustele. Therefore, the presence of regular eustele in tribe Leucastereae and the family Petiveriaceae reconstructs as a reversal (an independent evolution) to the character state ancestral for all lignophytes (the clade including extinct progymnosperms, gymnosperms, and angiosperms, all having a bifacial vascular cambium), which is thought to be a regular eustele, while the presence of a polycyclic eustele in Nyctaginaceae and close-related families likely constitutes a synapomorphy for the phytolaccoid clade and symplesiomorphy for Nyctaginaceae.

Three configurations for the polycyclic eustele were encountered. Our results showed that the ancestor of Nyctaginaceae probably had a polycyclic eustele with more than or 10 medullary bundles in a nonspecific arrangement (type 3), with multiple

evolution of types 1 (8 medullary bundles in symmetrical groups) and 2 (≥ 10 medullary bundles in two to three concentric rings) during the evolutionary history of this character. Considering this reconstructed likelihood, the shifts that led to the appearance of type 2 simply represented a change in the disposition of the medullary bundles resulting in the formation of rings. On the other hand, the evolution of type 1 would entail a reduction in the number of medullary bundles and their organization in groups. The number and organization of medullary bundles is diverse in Nyctaginaceae, but their occurrence is widespread and independent from phyllotaxy, habit, and habitat. Except for the prevalence of type 1 within Nyctagineae, a lineage composed mostly by herbs or subshrubs, no other pattern emerged among the clades.

Medullary bundles have apparently evolved not only in the phytollacoid clade, but also independently in other families within the Caryophyllales, both among the core families, such as Amaranthaceae and Cactaceae and in the noncore Caryophyllales, as in Nepenthaceae

(Metcalfe and Chalk, 1950; Mauseth, 1993; Schwallier et al., 2017). Among these families, the medullary bundles have been investigated in the families Nepenthaceae and Cactaceae. In the first, Schwallier et al. (2017) reported that it is a derived feature, which evolved multiple times independently, although it is present only in eight of the 40 species investigated. For the latter, Mauseth (1993) found that they appear to be relictually absent in the family, originating during early stages of evolution of subfamily Cactoideae.

CONCLUSIONS

The medullary bundles share the same origin and development across Nyctaginaceae, except for representatives of tribe Leucastereae where they are absent. Our observations showed that the number and organization of medullary bundles in stem cross section are unrelated to phyllotaxy, habit, and habitat. Given the presence or absence of medullary bundles, the primary vascular system of Nyctaginaceae can be subdivided in two subtypes of eustele; the polycyclic eustele and the regular eustele. Here, we showed that their absence is inferred as a potential synapomorphy

of tribe Leucastereae. Although we have revealed the character history of medullary bundles for Nyctaginaceae, their evolution across Caryophyllales requires further investigation. Information on the evolution of medullary bundles for the order is of interest given that they are present in several families both in the core and noncore Caryophyllales. Their occurrence in the close relatives of Nyctaginaceae, for instance, resulted in the inference of medullary bundles as a symplesiomorphy for Nyctaginaceae and a synapomorphy for the phytolaccoid clade. Whether the same developmental genetic pathways (deep homology) are responsible for the presence of medullary bundles in other Caryophyllalean families is something to be addressed in future studies.

AUTHOR CONTRIBUTIONS

I.L.C.N. and V.A. conceived the research; I.L.C.N., M.R.P., N.A.D., M.H.N., C.F.C.S., and M.J.M. assisted in plant collection; I.L.-C.-N. performed anatomical procedures, phylogenetic comparative analysis and wrote the manuscript; M.J.M. and N.A.D. provided the phylogenetic tree; I.L.C.N. and V.A. analyzed and interpreted the results; all authors revised, complemented, and corrected the text and gave final approval for publication.

DATA AVAILABILITY

Supplementary data are available online at FigShare (Table S1: Character and character states for ancestral reconstructions of medullary bundles in Nyctaginaceae and related families, https://doi.org/10.6084/m9.figshare.118 15260; Fig. S1: Diversity of medullary bundles in stems of Nyctaginaceae species, https://doi.org/10.6084/m9.figshare.118 15209.v2).

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APPENDIX 1. Species name for all studied Nyctaginaceae (divided by tribes) and the outgroups. The information includes vouchers followed by the abbreviations for herbaria where the samples are deposited, geographical region, and herbaria/wood collections where specimens were obtained. Herbaria: FLAS, Florida Museum of Natural History; HURB, Universidade Federal do Recôncavo da Bahia; MEXU, Universidad Nacional Autónoma de México; MW, Moscow State University; RB, Jardim Botânico do Rio de Janeiro; SPF, Universidade de São Paulo; US, Smithsonian Institution; USZ, Museo de Historia Natural Noel Kempff Mercado, Universidad Autónoma Gabriel René Moreno.

Species name, Collector/collector number (Herbarium), Collection site.

Tribe Nyctagineae

Abronia fragrans **Nutt. ex Hook.,** *Douglas 2290* (FLAS), Las Cruces, New Mexico, USA. *Abronia neealleyi* **Standl.,** *Douglas 2281*, (FLAS) Eddy County, Yeso Hills, New Mexico, USA. *Acleisanthes acutifolia* **Standl.,** US 842034, Coahuila, Mexico. *Acleisanthes chenopodioides* **(A.Gray) R.A.Levin.,** *Douglas 2289, 2293* (FLAS), Las Cruces, New Mexico, USA. *Acleisanthes lanceolata* **(Wooton) R.A.Levin,** *Douglas 2277* (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Acleisanthes longiflora* **A.Gray.,** *Douglas 2279* (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Allionia choisyi* **Standl.,** *US 498327*, New Mexico, USA; *Allionia incarnata* **L.,** *Nee 64124–64126*, (USZ), Parque Nacional Amboró, Pampa Grande, Santa Cruz, Bolivia; *Douglas 2293* (FLAS), Las Cruces, New Mexico, USA. *Anulocaulis leiosolenus* **(Torr.) Standl. var.** *leiosolenus*, *Douglas 2278* (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Anulocaulis leiosolenus var. gypsogenus* **(Waterf.) Spellenb. & Wootten,** *Douglas 2280* (FLAS) Eddy County, Yeso Hills, New

Mexico, USA; *Douglas 2283* (FLAS), Crest of 7 Rivers Hills, New Mexico, USA. **Boerhavia diffusa** L., *Pace 753* (MEXU, US), Veracruz, Mexico. *Boerhavia hereroensis* **Heimerl.,** *Sukhorukov 517* (MW), Namib Desert, Karas Region, Namibia. **Boerhavia linearifolia** A.Gray., *Douglas 2284* (FLAS), New Mexico, USA. **Boerhavia torreyana** (S.Watson) **Standl.,** *Douglas 2294* (FLAS), Las Cruces, New Mexico, USA. **Boerhavia wrightii** A.Gray., *Douglas 2288* (FLAS), Las Cruces, New Mexico, USA. **Commicarpus scandens (L.) Standl.,** *Acevedo-Rodríguez 16250* (US), Tonalá, Oaxaca, Mexico; *Douglas 2291* (FLAS), New Mexico, USA. **Cyphomeris gypsophiloides** (M. **Martens & Galeotti) Standl.,** *Douglas 2287* (FLAS), Organ Mountains-Desert Peaks National Monument, Las Cruces, New Mexico, USA. **Mirabilis** sp., *Pace 730* (MEXU), Ixmiquilpan, Hidalgo, Mexico. **Mirabilis aggregata (Ortega) Cav.,** *Pace 728* (MEXU, US), Ixmiquilpan, Hidalgo, Mexico. **Mirabilis** cf. **albida (Walter) Heimerl.,** *Douglas 2286* (FLAS), New Mexico, USA. **Mirabilis jalapa** L., *Acevedo-Rodríguez 16480* (US), Veracruz, Mexico. **Mirabilis viscosa** Cav., *Pace 727* (MEXU), Ixmiquilpan, Hidalgo, Mexico.

Nyctaginia capitata **Choisy.,** *Douglas 2282* (FLAS), New Mexico, USA. **Okenia hypogea** Schltdl. & Cham., *Pace 749* (MEXU, SPF, US), Veracruz, Mexico.

Tribe Pisonieae

Grajalesia fasciculata (Standl.) **Miranda.,** *Pace 765* (MEXU, SPF, US), Chiapas, Mexico. *Guapira linearibracteata* (Heimerl) **Lundell.,** *USw 29941.,* Belize. *Guapira pernambucensis* (Casar.) **Lundell.,** *Cunha Neto 04-05* (HURB), Alagoinhas, Bahia. **Guapira graciliflora (Mart. ex J.A.Schmidt) Lundell.,** *Cunha Neto 06* (HURB), Alagoinhas, Bahia. **Guapira laxa (Netto) Furlan.,** *Cunha Neto 08* (HURB), Universidade Estadual de Feira de Santana, Feira de Santana, Bahia. **Guapira ligustrifolia (Heimerl) Lundell,** *USw 1886,* San Gabriel Island, República Dominicana; *USw 1982,* Hispaniola Island, República Dominicana. **Guapira opposita (Vell.) Reitz.,** *Cunha Neto 07* (HURB), Alagoinhas, Bahia. **Neea delicatula Standl.,** *Pace 689* (US), Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica. **Neea hermaphrodita S.Moore,** *Nee 64112* (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de

la Sierra, Bolivia. *Neea laetevirens* **Standl.,** *Pace 713, 716* (US), Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica; *USw 16154*, Los Santos, Panamá. *Neea macrophylla* **Britton ex Rusby.,** *USw 40851*, San Martín, Peru. *Neea ovalifolia* **Spruce ex J.A. Schmidt,** *USw 42783*, Régina, French Guiana. *Neea psychotrioides* **Donn. Sm.,** *Pace 763* (MEXU), Estación de Biología Tropical Los Tuxtlas, Veracruz, Mexico; *USw 29939*, Belize. *Pisonia* **sp1.,** *Nee 64108* (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia. *Pisonia* **sp2.,** *Nee 64132* (USZ), Parque Nacional Amboró, Vallegrande, Santa Cruz, Bolivia. *Pisonia* **sp3.,** *Pace 789* (MEXU), Puente Nacional, Veracruz, Mexico. *Pisonia aculeata* **L.,** *Acevedo-Rodríguez 16549* (US), Bonito, Mato Grosso do Sul, Brazil. *Pisonia zapallo* **Griseb.,** *Nee 64110* (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia. *Pisoniella arborescens* **(Lag. & Rodr.) Standl.,** *Pace 738-739* (MEXU, SPF, US), Alfajayucan, Hidalgo, Mexico. *Pisoniella glabrata* **(Heimerl) Standl.,** *Nee 64137, 64151* (USZ), Parque Nacional Amboró, Vallegrande, Santa Cruz, Bolivia.

Tribe Bougainvilleeae

Belemia fucsioides **Pires.,** *Farney 4887, 4888* (RB). *Bougainvillea* **sp1.,** *Nee 64176* (USZ), Rio Grande, Santa Cruz de la Sierra, Bolivia. *Bougainvillea* **sp2.,** *Nee 64182* (USZ), Rio Grande, Santa Cruz de la Sierra, Bolivia. *Bougainvillea berberidifolia* **Heimerl.,** *Nee 64140* (USZ), Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia. *Bougainvillea campanulata* **Heimerl.,** *Acevedo-Rodríguez 16772* (US), Mato Grosso do Sul, Brazil; *Nee 64142* (USZ), Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia. *Bougainvillea modesta* **Heimerl.,** *Nee 64115* (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia. *Bougainvillea stipitata* **Griseb.,** *Nee 64121* (USZ), Parque Nacional Amboró, Samaipata, Santa Cruz, Bolivia. *Bougainvillea spectabilis* **Willd.,** *Rossetto 453* (RB), Estrada Carlos Chagas-Teófilo Otoni, Minas Gerais, Brazil. *Phaeoptilum spinosum* **Radlk.,** *MADw 37340*, Mocamedes, Angola.

Tribe Colignonieae

Colignonia glomerata **Griseb.,** *Nee 64157-64159* (USZ), Parque Nacional Amboró, Samaipata, Santa Cruz,

Bolivia. *Colignonia rufopilosa* **Kuntze,** *Nee 64061* (USZ), Cochabamba, Bolivia.

Tribe Boldoeae

Cryptocarpus pyriformis **Kunth,** *US 2833648*, Galápagos. *Salpianthus arenarius* **Bonpl.,** *US 1893480*, Vallecitos, Mexico. *Salpianthus macrodontus* **Standl.,** *US 2219249*, Sinaloa, Mexico. *Salpianthus purpurascens* **(Cav. ex Lag.) Hook. & Arn.,** *Pace 774* (MEXU, SPF, US), El Cobanal, Chiapas, Mexico.

Tribe Leucastereae

Andradea floribunda **Allemão,** *US 2627753*, Espírito Santo, Brazil; *Rossetto 445* (RB), Linhares, Espírito Santo, Brazil. *Leucaster caniflorus* **(Mart.) Choisy,** *US 2839822*, Jacarepaguá, Rio de Janeiro, Brazil; *Rossetto 447* (RB), Linhares, Espírito Santo, Brazil; *Rossetto 455* (RB), Teófilo Otoni, Minas Gerais, Brazil. *Ramisia brasiliensis* **Oliv.,** *US 2947296*, Nova Venécia, Espírito Santo, Brazil; *Rossetto 448* (RB), Nanuque, Minas Gerais, Brazil. *Reichenbachia hirsuta* **Spreng.,** *Nee 64109* (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia; *Nee 64169* (USZ), Rio Grande, Santa Cruz de la Sierra, Bolivia.

Outgroups

Agdestidaceae: *Agdestis clematidea* **Moc. & Sessé ex DC.,** Carlquist, 1999.

Petiveriaceae: *Gallesia integrifolia* **(Spreng.) Harms,** SPFw 5241; Rossetto 446 (RB), Linhares, Espírito Santo, Brazil; Carlquist, 2000; *Hilleria latifolia* **(Lam.) H.Walter,** Carlquist, 2000; *Monococcus echinophorus* **F.Muell.,** Jansen et al., 2000. *Petiveria alliaceae* **L.,** Carlquist, 2000; Duarte & Lopes, 2005; *Trichostigma octandrum* **(L.) H.Walter,** Carlquist, 2000; *Rivina humilis* **L.,** Carlquist, 2000; Pugialli & Marquete, 1989; *Seguieria americana* **L.,** Rossetto 451 (RB), Nanuque, Minas Gerais, Brazil; Carlquist, 2000; *Seguieria langsdorffii* **Moq.,** Rossetto 452 (RB), Nanuque, Minas Gerais, Brazil.

Phytolaccaceae: *Phytolacca dioica* **L.,** Kirchoff & Fahn, 1984; Carlquist, 2000; *Phytolacca* **sp.,** SPFw 5601.

Chapter 4

A new interpretation of the successive cambia of some Nyctaginaceae as interxylary phloem*

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Premise of research.   The alternative patterns of secondary growth (vascular cambial variants) in stems of Nyctaginaceae are outstanding and have been widely investigated since late 19th century. However, there are controversial interpretations in the literature regarding the existence of either one or two types of cambial variants in this family (successive cambia vs. interxylary phloem). We aim to explore the anatomical diversity of stems in Nyctaginaceae, to document the real nature of the cambial variant present in most species of the family.

Methodology.   We analyzed 60 species, focusing on 18 species from 12 genera, for developmental studies. Anatomical and ontogenetic features were characterized from images produced by standard plant techniques for macro and microscopic analyses.

Pivotal results.   Our analyses reveal that most species of Nyctaginaceae present stems with polycyclic eusteles, which later develop a single cambium that produces secondary xylem and secondary phloem at unequal rates around the stem circumference. This unusual activity results in the absence of a regular cylinder of secondary vascular tissues and in the formation of secondary phloem strands (surrounded by variable amounts of sheathing axial parenchyma) embedded within the secondary xylem. In cross-section, adult stems can exhibit different tissue arrangements (i.e., phloem islands/strands, patches, or concentric bands) that result from differences in rates of production of phloem and associated sheathing axial parenchyma forming the strands. The cambial variant in these stems is described as interxylary phloem, as similarly observed in other eudicot lineages.

Conclusions.   Our examination of the stem development of Nyctaginaceae confirms the presence of interxylary phloem, which has been overlooked in the family as most previous studies have reiterated descriptions of successive cambia as the common cambial variant within the family. These findings emphasize the importance of developmental studies encompassing a representative number of genera to further our understanding of stem macromorphologies and to highlight the complexity and diversity of stem architectures in Nyctaginaceae.

Keywords: cambial variant, Caryophyllales, ontogeny, polycyclic eustele, secondary growth, stem anatomy

Introduction

The prevalence of families with cambial variants in the order Caryophyllales, compared to other angiosperm orders, has been reported since the first treatises on the subject (De Bary, 1884; Schenck, 1893; Pfeiffer, 1926). Cambial variants are reported in at least 19 of the 39 families currently recognized in the Caryophyllales (Gibson, 1994; Carlquist, 2010; Hernández-Ledesma et al., 2015). Until the late 20th century, the majority of papers on the formation of alternative vascular anatomies in this order followed the traditional view (Schenck, 1893; Pfeiffer, 1926) that regarded the main cambial variant in the family as successive cambia, i.e., additional increments of vascular tissue through the formation of new cambia outside of the first cambium. More recently, Carlquist's outstanding contributions to the understanding of vascular anatomies in Caryophyllales (Carlquist, 1991, 1999, 2000, 2001 2003, 2004, 2007, 2010) have concurred with earlier views that successive cambia is the cambial variant occurring in the order. This view has been subsequently shared by other authors (Rajput and

Rao, 1998; Rajput and Marcati 2013; Rajput, 2015; Rajput et al., 2009; 2012; Hernández-Ledesma et al., 2011; Hernández-Ledesma et al., 2015; Myśkow et al., 2019; Zumaya-Mendoza et al., 2019).

Nyctaginaceae is one of the most emblematic families in the core Caryophyllales and the anatomy of the stem and cambial variants have been widely investigated. For the family, de Bary (1884) and Schenk (1893) were some of the first to characterize the presence of successive cambia in stems of some species (e.g., in the genera *Bougainvillea, Mirabilis*). This topic was further investigated by Esau and Cheadle (1969) and Carlquist (2004). The former authors undertook detailed ontogenetic analyses of stems and roots in *Bougainvillea spectabilis*, which demonstrated a pattern of successive cambia originating from the pericycle and producing multiple additional bifacial cambia. Carlquist's study investigated different genera and species (including *B. spectabilis*), interpreting all of them as having successive cambia that originated from an independent meristem arising in the cortex, which Carlquist termed a

master cambium. Despite these interpretations, the pattern of secondary growth in some species of Nyctaginaceae has also been recurrently interpreted as interxylary phloem in a number of studies (Chalk and Chattaway, 1937; Metcalfe and Chalk, 1950; Studholme and Philipson, 1966; Lopes et al., 2008; Sonsin et al., 2014). Nevertheless, most of these studies were either focusing on a single species or did not explore in detail the origin, development, and anatomy of the vascular system of Nyctaginaceae comparing multiple species, where situations intermediate between successive cambia and interxylary phloem have been hiding in some species, especially non-ornamental ones. As a result, given the widespread distribution of cambial variants in Nyctaginaceae and their complex macromorphologies, different names and classifications have been proposed to describe their cambial variants. These different classifications emphasize different structural aspects including the origin, type of the meristem that forms the tissues and the arrangement of cell types in stem cross section (stem topologies) (see Supplemental Material – Table A1).

Successive cambia and interxylary phloem are recognized as two entirely different types of cambial variants, whose correct identification can be hindered by their common (although ontogenetically different) characteristic, i.e., the presence of secondary phloem amid the secondary xylem in stem cross section (Carlquist, 2001, 2013; Angyalossy et al., 2012, 2015). Overall, in the case of successive cambia, new bands of xylem, phloem, and sometimes conjunctive parenchyma are produced by the formation of subsequent cambia (and their products) outside of the original cambium. Species with successive cambia generally initiate their secondary growth forming a regular vascular cylinder of secondary xylem and phloem, and only later additional rings or concentric increments of vascular tissue are produced, the first of which originating either from the cortex, the pericycle, the phloem parenchyma (Angyalossy et al., 2015, Pace et al., 2018) or from procambium remnants (Myśkow et al., 2019). On the other hand, the appearance of phloem within the xylem in species with interxylary phloem results from a single cambium (Carlquist, 2001, 2013;

Angyalossy et al., 2016), and three subtypes of interxylary phloem can be distinguished based on how the phloem cells are produced by the cambium (see the IAWA Bark Committee, Angyalossy et al., 2016). Regardless of the mode of phloem production in stems with this cambial variant, the interxylary phloem generally forms small groups of cells, for which reason it has been referred to as "included phloem" or "phloem islands" (IAWA Committee 1989; van Veenendaal and den Outer, 1993; Lens et al., 2008; Rajput et al., 2009; Carlquist, 2013; Gondaliya and Rajput, 2017). The overall result of these anatomical similarities and differences is that when stems with cambial variants display long or continuous rings of vascular increments in cross section, authors are prone to identify them as successive cambia, while if secondary phloem is observed within the xylem forming small strands they are typically recognized as interxylary phloem. However, this distinction becomes difficult to apply when all gradations between these two extremes are present, which is the case of Nyctaginaceae.

Here, we performed a comparative analysis between species of Nyctaginaceae with a clear-cut aspect of interxylary phloem and intermediate forms, using a broad sampling of the family, to understand the real nature of the cambial variant present in most species of the Nyctaginaceae.

Material and Methods

Plant collection and sampling

We analyzed 60 species of the Nyctaginaceae (Appendix), of which we focused on 18 species from 12 genera distributed in five of the seven tribes to understand if successive cambia or interxylary phloem better explain the cambial variants in these species (Table 1). Most of the specimens were collected by ourselves in natural populations, while some were studied in slides deposited in wood collections (Appendix). Some representatives were used for the ontogenetic study, while well-developed stems of all species/specimens were analyzed to observe the entire range of anatomical variability.

The material collected included different portions of the stems, from the stem apex to the plant base in the case of scandent plants and herbs, and fragments from branches and the main trunk in the case of trees and shrubs. During fieldwork, the specimens were immediately fixed in FAA 70 (formaldehyde–acetic acid–ethanol or 70% isopropanol) and then stored in 70% ethanol (Johansen, 1940).

Anatomical procedures

Samples of the stems in different stages of development were embedded in polyethylene glycol 1500 (Rupp, 1964) and sectioned in transverse, longitudinal radial, and longitudinal tangential planes with a Leica sliding microtome (Nussloch, Eisfeld, Germany), using a Styrofoam resin coat (Barbosa *et al.* 2010). Sections were typically 16–22 μm thick and sectioned with permanent steel knives sharpened with sandpapers (Barbosa *et al.* 2018). Anatomical sections were double stained with Safrablau (Bukatsch, 1972, modified by Kraus and Arduin, 1997) and mounted in Canada balsam to make permanent slides.

To describe the cambium, we embedded small portions of the stem containing the cambium in Historesin (Leica) to observe its activity during primary and secondary growth. Sections, typically 5–10 μm thick, were cut with the rotary microtome, then stained with toluidine blue (O'Brien et al., 1964). The slides were observed and photographed using a light microscope (Leica DMLB).

Results

The transition from primary to secondary growth: from the polycyclic eustele to a single cambium

In primary growth, most species of Nyctaginaceae have a polycyclic eustele, which consists of medullary bundles and a continuous cylindrical procambium (CCP) (Fig. 1A-C). Subsequently, the CCP divides into fascicular and interfascicular sectors, giving rise to a ring of vascular bundles (primary vascular tissue) that delimit the pith (Fig. 1B-C). Circumscribing the vascular tissue, a parenchymatous pericycle that later becomes lignified, and an endodermis (starch sheath or innermost cortical layer) characterized by larger cells sometimes containing starch are usually present (Fig. 2A-B,

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D-F; 3A-E; 4A-C; 5B, D-E). Later, the CCP undergoes structural changes with several periclinal and anticlinal divisions around its entire extent, initiating the transition from primary to secondary growth (Fig. 1D). Thus, the cambium develops in continuity with the CCP, since the continuous ring of fascicular and interfascicular procambium becomes the continuous fascicular plus interfascicular cambium (Fig. 1C-D; 2A-F).

Table 1

List of studied species from Nyctaginaceae used for developmental analyses.

Tribe	Species	Habit	Source / Collector	Location
Nyctagineae				
	Acleisanthes chenopodioides (A.Gray) R.A.Levin.	Herb	Douglas 2289, 2293 (FLAS)	Las Cruces, New Mexico, USA.
	Commicarpus scandens (L.) Standl.	Liana	Acevedo-Rodríguez 16250 (US); Douglas 2291 (FLAS)	Tonalá, Oaxaca, Mexico; New Mexico, USA.
	Cyphomeris gypsophiloides (M. Martens & Galeotti) Standl.	Herb	Douglas 2287 (FLAS);	Organ Mountains-Desert Peaks National Monument, Las Cruces, New Mexico, USA.
	Mirabilis cf. *albida* (Walter) Heimerl.	Herb	Douglas 2286 (FLAS)	New Mexico, USA.
	Mirabilis jalapa L.	Subshrub	Acevedo-Rodríguez 16480 (US)	Veracruz, Mexico.
Pisonieae				
	Grajalesia fasciculata (Standl.) Miranda	Scandent-tree	Pace 765 (MEXU, SPF, US)	Chiapas, Mexico.
	Guapira laxa (Netto) Furlan.	Tree	Cunha Neto 08, 09 (HURB)	Universidade Estadual de Feira de Santana, Feira de Santana, Bahia.

	Guapira pernambucensis (Casar.) Lundell.	Scandent-shrub	Cunha Neto 04, 05 (HURB)	Alagoinhas, Bahia.
	Pisonia aculeata L.	Liana	Acevedo-Rodríguez 16549 (US); Aw 1073	Bonito, Mato Grosso do Sul, Brazil; Soledad, Cuba.
	Pisoniella glabrata (Heimerl) Standl.	Liana / scandent-shrub	Nee 64137, 64151	Parque Nacional Amboró, Vallegrande, Santa Cruz, Bolivia.
	Pisoniella arborescens (Lag. & Rodr.) Standl.	Liana	Pace 738, 739	Hidalgo, Mexico.
	Neea hermaphrodita S.Moore	Shrub	Nee 64112 (USZ)	Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia.
Bougainvilleae				
	Bougainvillea berberidifolia Heimerl.	Shrub	Nee 64140 (USZ)	Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia.
	Bougainvillea campanulata Heimerl.	Shrub	Acevedo-Rodriguez 16772 (US); Nee 64142 (USZ)	Mato Grosso do Sul, Brazil; Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia.
	Bougainvillea spectabilis Willd.	Scandent-Shrub	Rossetto 453 (RB); Aw 24470	Estrada Carlos Chagas-Teófilo Otoni, Minas Gerais, Brazil.
Colignonieae				
	Colignonia glomerata Griseb.	Liana	Nee 64157-64159	Parque Nacional Amboró, Samaipata, Santa Cruz, Bolivia.
	Colignonia rufopilosa Kuntze	Liana	Nee 64061 (USZ)	Cochabamba, Bolivia.
Boldoeae				
	Salpianthus purpurascens (Cav. ex Lag.) Hook. & Arn	Shrub	Pace 774	El Cobanal, Chiapas, Mexico

The transition from primary to secondary growth is also marked by the lignification of several cells in the periphery of the pith (Fig. 2C, E; 3A–B).

In early stages of secondary growth, additional secondary xylem and phloem conducting cells are produced by the fascicular cambium within the vascular bundles, while the interfascicular cambium give rises mostly to secondary xylem fibers internally and phloem parenchyma cells externally (Figs. 1D, 2A–B, E–F). Therefore, at this stage, a single cambium that produces secondary xylem to the inside and secondary phloem to the outside is formed (Fig. 2A). Soon, this cambium initiates an irregular activity (described in detail below) whereby secondary xylem and secondary phloem are produced at unequal rates around the circumference of the stem (Fig. 2C, E–F; 3C–E). Due to this irregular activity of the cambium and because the first secondary conducting cells are formed within the vascular bundles (by the fascicular cambium), the primary and secondary phloem of the bundles, derived from the CCP and the fascicular cambium, respectively, become the first interxylary phloem

immersed within the secondary xylem, near the pith, as seen in stem cross-sections (Fig. 2C, E–F; 3A–B). Therefore, a regular cylinder of secondary xylem and phloem is not formed in these species (Fig. 2B; 3A–B).

A single cambial zone maintains a dynamic irregular activity leading to complex stem anatomies

In the studied Nyctaginaceae species, the cambium maintains an irregular activity due to differential rates and arrangements of its derivatives. To the inner side, the cambium produces mostly xylem fibers, except for the regions where conducting elements (vessels) are formed, which are co-radial with the phloem strands (figs. 2E, 2F, 3C, 3D, 4B–4E). In the secondary phloem, the axial parenchyma is predominant and usually forms a set of radially oriented cells (Fig. 4A–F). These layers of parenchyma vary from 2-10 (Fig. 2E–F; 3A, C–D; 4A–C) to several rows (>10) (Fig. 4E–F; 5A). The conducting cells of the phloem appear as strands (Fig. 4C–D), which are surrounded by the innermost layers of the phloem axial parenchyma, named here "sheathing axial parenchyma" (Fig. 4A–B, D–E; 5A, C). Externally to the
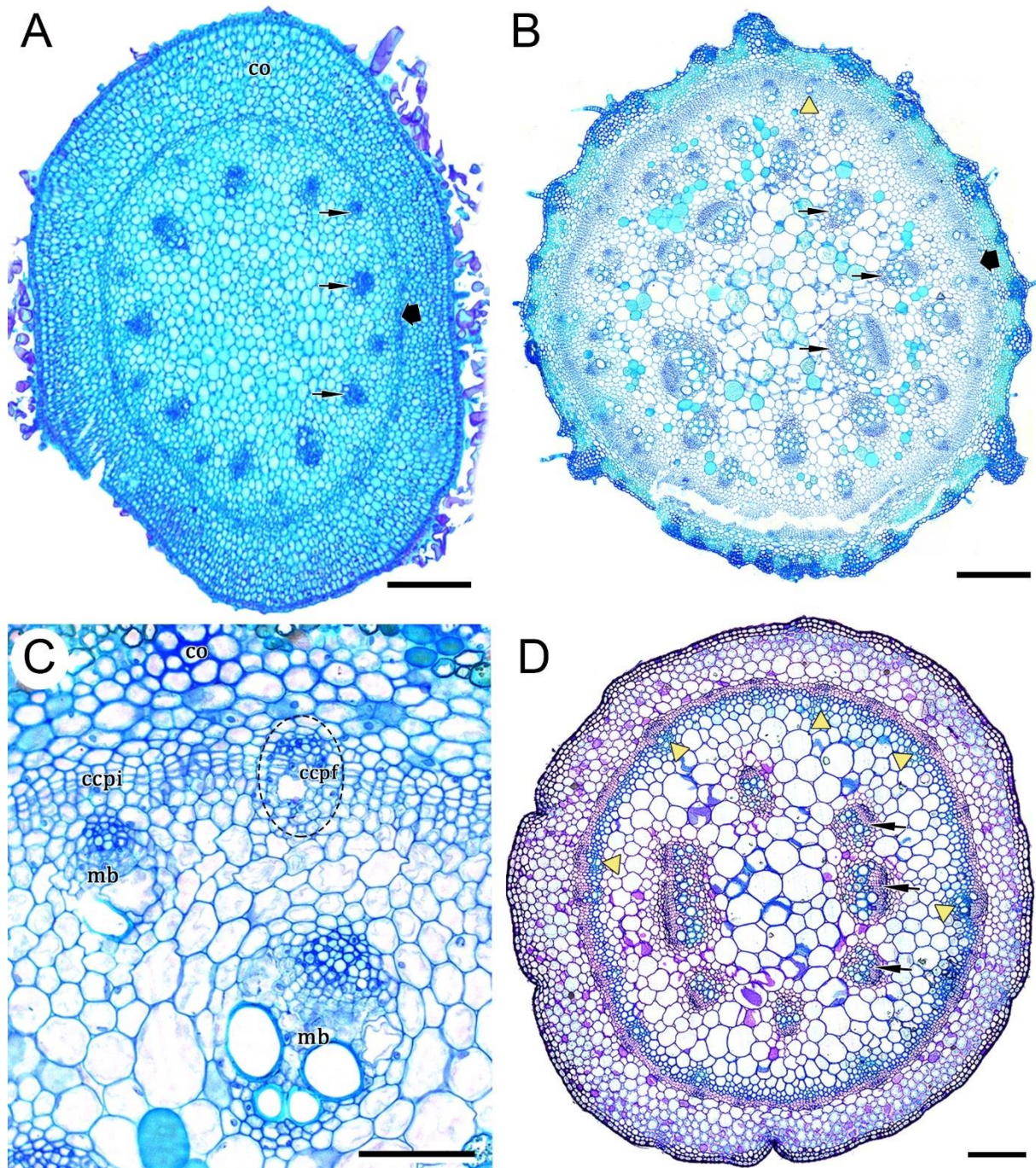
Fig. 1 Overview of young stems showing polycyclic eustele. A, *Colignonia glomerata*. B–C, *Salpianthus purpurascens.* The yellow triangle in D indicates a vascular bundle developed from the CCP, shown in detail in "C" (dashed ellipse). D, *Cyphomeris gypsophiloides,* early secondary growth (see details in Fig. 2). Scale bars = 100 μm (A–B). Abbreviations: co, cortex; ccpf, continuous cylindrical procambium, fascicular; ccpi, continuous cylindrical procambium, interfascicular; mb, medullary bundles; thick arrows, continuous cylindrical procambium (CCP); thin arrows, medullary bundles; yellow triangles, bundles derived from the CCP. Stained with astrablue (A). Stained with toluidine blue (B–D).
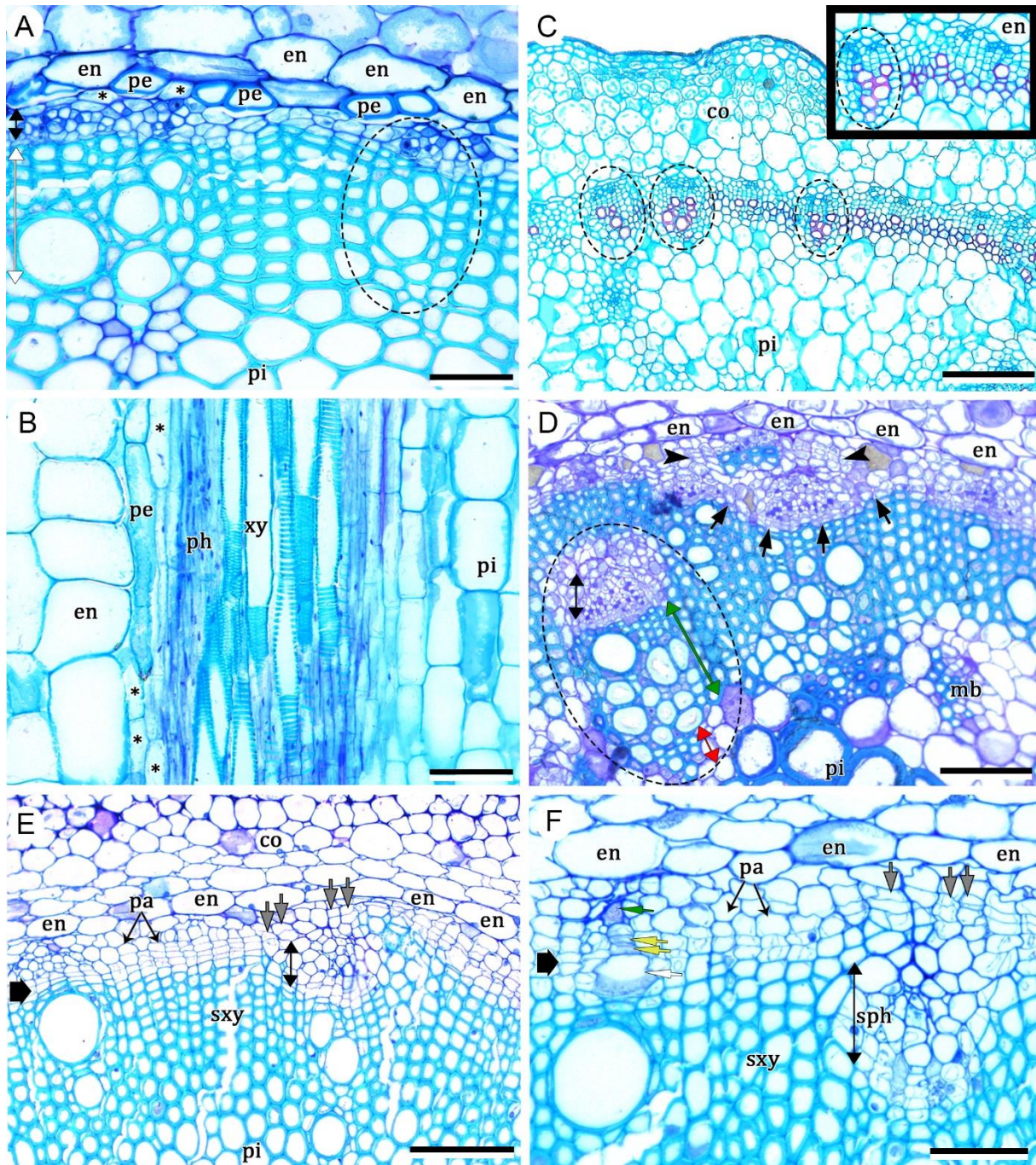
Fig. 2 Details of the onset of secondary growth in young stems. A–B, *Acleisanthes chenopodioides.* Detail of early secondary activity: i) within the bundles by the fascicular cambium and ii) in the interfascicular cambium (centre) producing mostly xylem fibers internally and parenchymatic cells in the secondary phloem, beneath the pericycle. In "A", note the secondary xylem (white double arrow, left) and secondary phloem (black double arrow, left). In "B", note the lignified (pe) and parenchymatous pericycle (asterisks). C–D, *Cyphomeris gypsophiloides.* In "C" and "D", note the production of additional conducting cells within the bundles (ellipse) and, in the interfascicular region, the production of mostly xylem fibers internally and parenchymatic cells in the secondary phloem. In "D", the irregular activity of the cambium has produced xylem fibers delimiting the first phloem strand (black double

arrow) which is constituted of the phloem derived from the bundle of the CCP. Also note the irregularity of the cambium (wavy appearance, arrows) producing xylem fibers and phloem derivatives in different rates, and the developing coalescent cambium (arrowheads) overarching the phloem that will form another phloem strand. E-F, *Mirabilis jalapa.* Detail of the single cambium (thick arrow) producing cell types at differential rates. Note that only phloem parenchyma remains at the periphery, while sieve-tube elements and companion cells are exclusive to the strands. In "E", note phloem parenchyma undergoing cell division (grey arrows) to form the coalescent cambium that will overarch the early produced phloem. In "F", note the presence of cambial cells between the xylem inside (vessel element), and phloem outside (sieve-tube element), evidencing the cambium bifacial activity. These phloem conducting cells will be later overarched by a new coalescent cambium. Scale bars = 100 μm (A-E); 50 μm (F). Abbreviations: Arrow (green), sieve-tube element; arrow (yellow), cambial initial; arrow (white), vessel element; co, cortex; double arrow (green), secondary xylem; double arrow (red), primary xylem; ellipse, vascular bundle from the CCP with additional secondary cells produced by the fascicular cambium; en, endodermis; mb, medullary bundles; pa, phloem parenchyma; pe, lignified pericycle; ph, phloem; pi, pith; sph, secondary phloem; sxy, secondary xylem; xy, xylem. Stained with toluidine blue (A, C-F). Stained with safrablau (B).

phloem, parenchymatous and lignified pericyclic cells may also be observed (Fig. 2A-B).

As secondary growth progresses, from time to time the cambium reduces the production of xylem and increases the production of phloem forming discrete concavities around the cambial girth (Fig. 2D, 3A, D-E; 4A, C-F). Later, the resulting phloem strands are included by the formation of an arc of coalescent cambium external to these concavities (Fig. 3E; 4C, E). The coalescent cambium is formed in continuity with the rest of the cambial layer (i.e., the cambium sectors that have grown in the regular mode, in terms of types and rates of secondary tissues produced). The coalescent cambium differentiates from the inner layers of axial phloem parenchyma located external to the sheathing axial parenchyma that enclose the phloem strands (Fig. 2E-F; 3E; 4C, E; 5E). The coalescent cambium produces xylem and phloem in the usual polarity and overarches the developing strand of phloem, enclosing it within the secondary xylem (Fig. 4D; 5D-E). Through the activity of the coalescent cambium mostly xylem
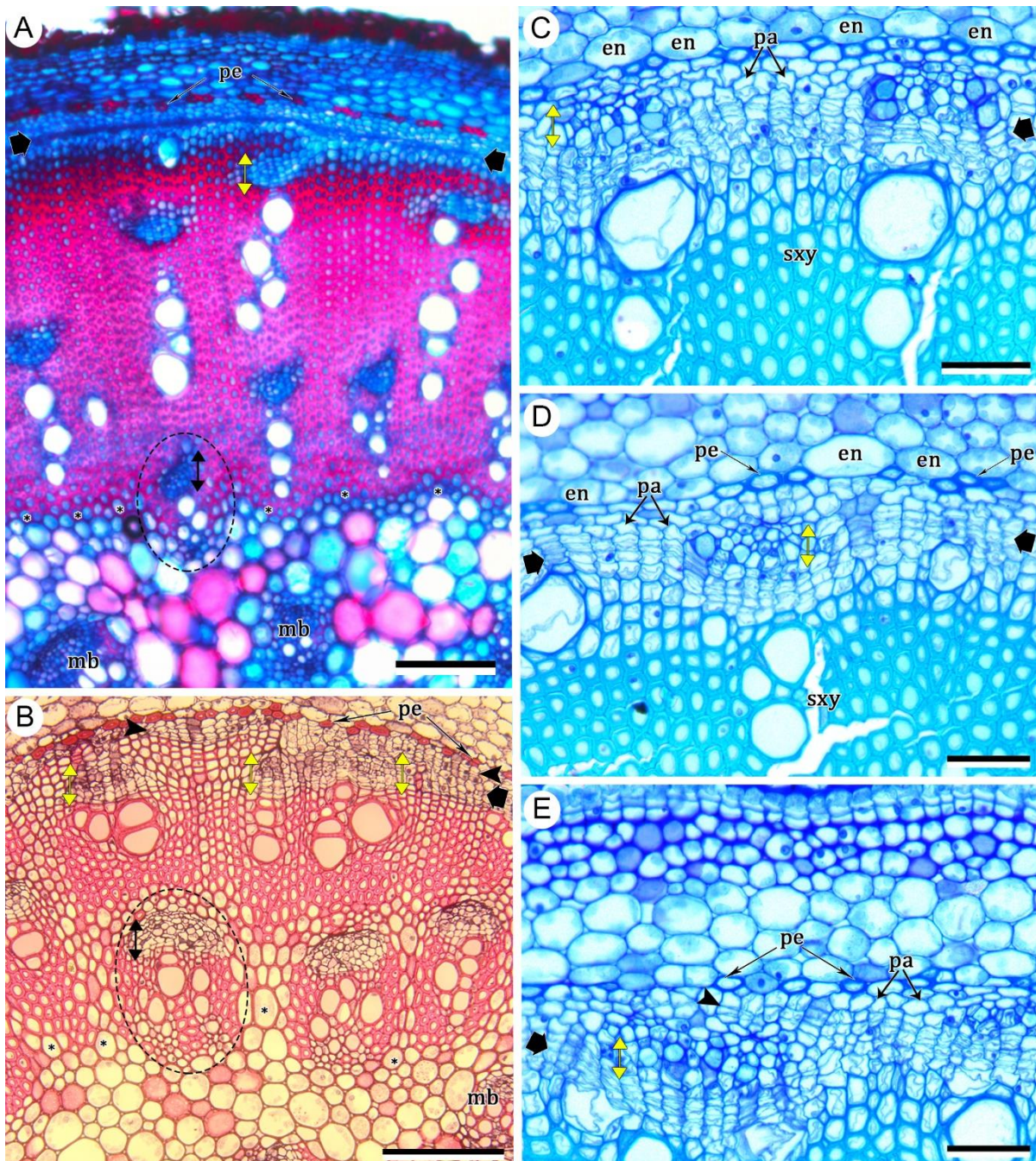
Fig. 3 Cross view of stems in early secondary growth in young stems. A, *Pisoniella glabrata.* B, *Bougainvillea spectabilis.* In both "A" and "B", note the absence of a regular cylinder and the presence of phloem strands within the secondary xylem. Note also lignified cells from periphery of the pith (asterisks) and that the first interxylary phloem (black double arrow) was formed from the primary phloem of the vascular bundles (originated from the CCP) along with some secondary phloem produced by the fascicular cambium within the bundles; new phloem strands (yellow double arrow) are produced by the coalescent cambium (arrowheads) and the irregular activity of the main cambium (thick arrow). C–E, *Pisoniella arborescens.* Note the irregularity (wavy appearance) of the cambium (thick arrows), which produces cell

types at differential rates, producing less secondary xylem, and more secondary phloem (yellow double arrow; left in "C" and "E", centre in "D"). Also note that the developing coalescent cambium (arrowhead) will overarch the phloem strand (yellow double arrow), which maintains part of the main cambium (thick arrow). Scale bars = 100 μm (A-E). Abbreviations: arrowheads, coalescent cambium; en, endodermis; mb, medullary bundles; pa, phloem parenchyma; pe, lignified pericycle; sc, sclerified parenchyma; sxy, secondary xylem. (A) Stained with safrablau, (B) stained with safranine, (C-E) Stained with toluidine blue.

fibers are produced internally, while only phloem parenchyma are formed externally (Fig. 4D). By this whole process, all regions with conducting cells of the secondary phloem with their original cambial segment are embedded within the secondary xylem (Fig. 4D, F; 5B-F; 6A-H). Thus, in these species, the conducting phloem is restricted to the strands immersed within the secondary xylem, and the secondary phloem external to the single cambium is composed only by non-conductive phloem cells, i.e., phloem parenchyma (Fig. 4A-C, E; 5A-F). The cambium enclosed within each strand is functional for some time, and both conducting and non-conducting phloem can be distinguished (Fig. 4A, F).

Despite the irregular activity of the cambium forming phloem strands throughout the stem and over the entire life span of the plant (see images of the cambium in mature stems, Fig.

4A-F), the secondary xylem and phloem produced by this cambial activity also exhibit features associated with typical cambial growth. This is demonstrated by features such as the formation of rays (Fig. 4A, E; 5A-D; 7A-B), which are continuous between the xylem and phloem in the external portion of the phloem (Fig. 5A-B);the alternation of thick-walled and thin-walled fibers (Fig. 4F); and the formation of growth rings formed either by thick-walled fibers or axial parenchyma in lines (Fig. 7A-B, D). In some species, some axial phloem parenchyma cells become sclerified (Fig 4F; 5A-B, D), while other species exhibit conspicuous ray dilatation in the secondary phloem and sclerification of axial and radial cells of the non-conducting secondary phloem (Fig. 5A).
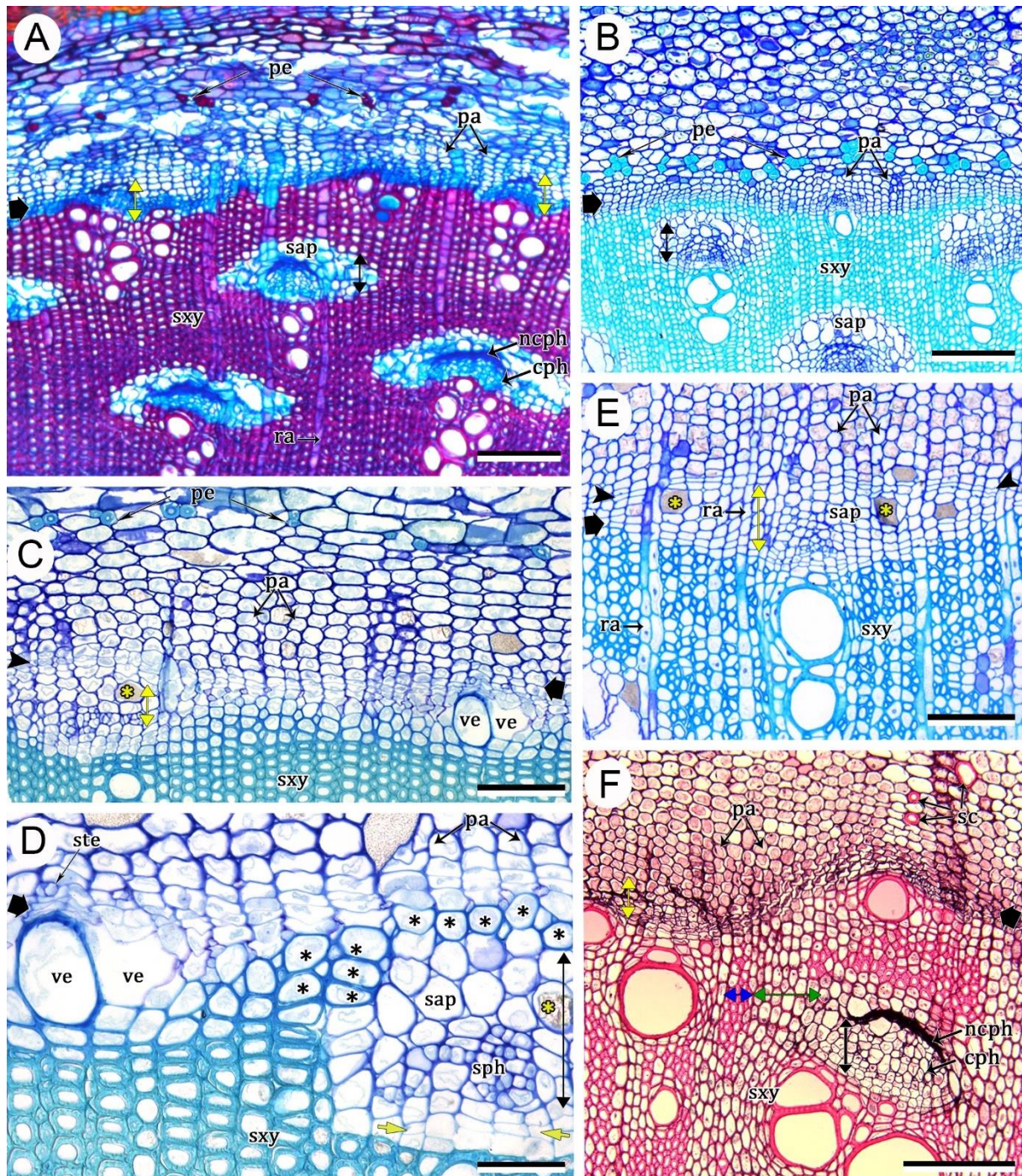
Fig. 4 Details of cambium in mature stems. A, *Neea hermaphrodita.* Note the lignified pericycle delimiting the vascular system and the irregular cambium (thick arrow) producing new phloem strands (yellow double arrows). Notice also developed phloem strands (black double arrows) which are formed by conducting and non-conducting cells in the centre and surrounded by sheathing axial parenchyma, also originated from the cambium. B, *Grajalesia fasciculata.* Detail of cambium (thick arrow) and phloem strands (black double arrows). C-D, *Guapira pernambucensis.* C, Detail of cambium (thick arrows) and the coalescent cambium (arrowhead) overarching the developing phloem strands (yellow double arrows). Note that, in the centre, only

xylem fibers are produced internally, and parenchymatic cells to the outside. Vessels and sieve-tube elements are formed to the left and to the right (see detail in D). D, In the left, vessels and sieve-tube elements are produced by the cambium. In the right, we observe a phloem strand (black double arrow) with included cambium (yellow arrows), sieve-tube elements with their companion cells in the centre and sheathing axial parenchyma surrounding them. As the coalescent cambium becomes active, new xylem fibers (asterisks) are produced and enclose the phloem strand. E, *Pisonia aculeata,* detail of cambium (thick arrow) and coalescent cambium (arrowheads) overarching the future phloem strand. F, *Bougainvillea spectabilis.* Detail of irregular cambium producing phloem strands (yellow double arrow). Notice that both thick-walled xylem fibers (green double arrow) and thin-walled xylem cells (blue double arrow) are both produced by the same cambial initials – they arise in a succession in radial files. Scale bars = 250 μm (A); 200 μm (B, E); 100 μm (C, F); 50 μm (D). Abbreviations: arrow (green), sieve-tube element; asterisks (yellow), crystals; co, cortex; cph, conducting phloem; pa, phloem parenchyma; pe, lignified pericycle; ncph, non-conducting phloem; pa, phloem parenchyma; ra, vascular ray; sap, sheathing axial parenchyma; sc, sclerified parenchyma; ste, sieve-tube element; sxy, secondary xylem; ve, vessel element. (A) Stained with safrablau, (B-E) Stained with toluidine blue, (F) Stained with safranine.

Different arrangements of interxylary phloem occur in Nyctaginaceae

The type of secondary growth resulting from the developmental sequence described above indicates that the stems can be interpreted as having interxylary phloem. In Nyctaginaceae, the ontogenetic pathway generating this pattern gives rise to several distinct macromorphologies (Fig. 6A-H; 7A-C). The different arrangements are distinguished in cross-sections: Type 1, strands of phloem and sheathing axial parenchyma confined to small islands (Fig. 6A-B; 7A); Type 2, strands of phloem with the sheathing axial parenchyma forming a patchy arrangement due to tangential confluences of the sheathing axial parenchyma connecting two or more islands (Fig. 6C-D; 7B); and Type 3, long concentric tangential bands of sheathing axial parenchyma with small phloem strands within them (Fig. 6E-H; 7C). The three different arrangements result from variation in the extent of the coalescent cambium: the longer the coalescent cambium is, the longer are the islands or bands of sheathing axial parenchyma produced. It is observed that in species with type 3
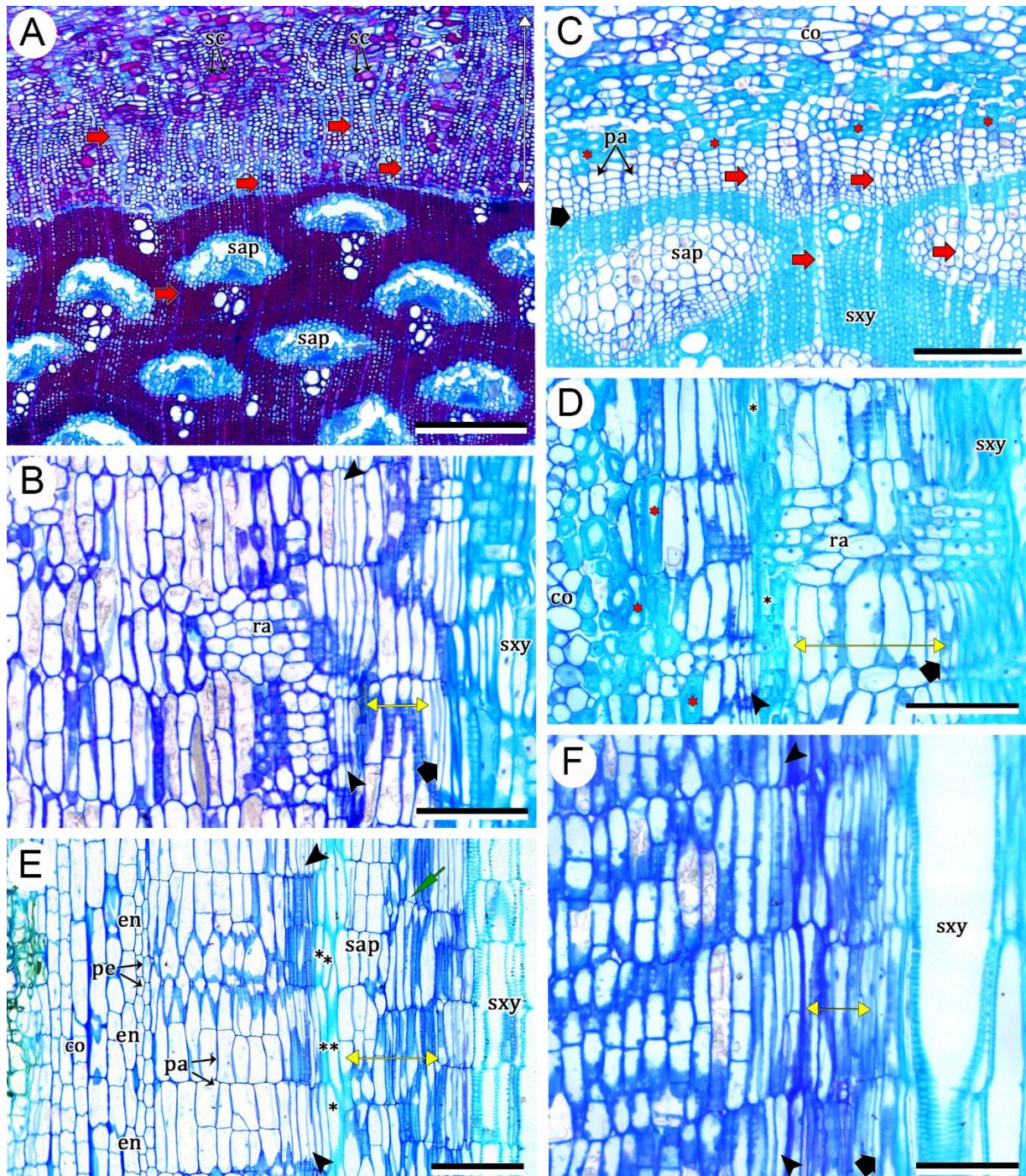
Fig. 5 Details of cambium and secondary tissues in mature stems. (A–B) cross sections; (C–F) longitudinal radial sections. A, *Pisonia ligustrifolia.* Notice the continuity of the vascular rays between the xylem and phloem, and ray dilatation (to the left) and sclerified parenchyma, common features of the developed non–conducting phloem (white double arrow). B, *Pisonia aculeata,* notice the cambium (thick arrow) and the coalescent cambium (arrowheads) enclosing the phloem strand (yellow double arrow). C–D. *Guapira laxa.* Vascular rays crossing the xylem, the phloem and reaching the lignified pericycle. Note that some phloem parenchyma become sclerified (red asterisks), intermixing with the lignified pericycle, and the

xylem fibers (asterisks) originated from the coalescent cambium (arrowheads) which encloses the phloem strand (yellow double arrow). E, *Colignonia glomerata*. Secondary xylem (sxy), phloem strand (yellow double arrow) and fibers (asterisks) derived from the coalescent cambium (arrowheads). F, *Bougainvillea campanulate*. Note the secondary xylem (sxy), the developing phloem strand (yellow double arrow), and the coalescent cambium (arrowheads). Scale bars = 200 µm (A, C, E); 100 µm (B, D, F). Abbreviations: arrow (black, thick), single cambium; arrow (green), sieve-tube element; arrow (red), vascular ray; arrowhead (black), coalescent cambium; asterisk (black), xylem fibers; asterisks (red), sclerified parenchyma; co, cortex; en, endodermis; pa, phloem parenchyma; pe, pericycle; ra, vascular ray; sap, sheathing axial parenchyma; sc, sclerified parenchyma; sxy, secondary xylem. (A) Stained with safrablau, (B-F) Stained with toluidine blue.

arrangement, the concentric aspect is given by the continuity of confluent sheathing axial parenchyma, while the sieve-tube elements and associated cells are still concentrated in small segments of these bands, in positions opposite the xylem vessels (Fig. 6E-F; 7C, E). In some cases, the rays are usually wider, and may form a networking arrangement with the tangential sheathing axial parenchyma (Fig. 6D, F, H; 7C-E).

Some taxa are characterized by starting with one anatomical arrangement, like members of *Bougainvillea* that at the beginning have type 1 anatomy (discrete phloem islands), which is followed in ontogeny by a type 2 anatomy (patches of phloem and sheathing axial parenchyma), and eventually mature to a typical type 3 anatomy (Fig. 7C). In terms of adult forms, some genera have predominantly one single arrangement (e.g., *Bougainvillea, Colignonia* – both with type *3*), while other genera have species with different adult arrangements. For instance, *Guapira, Neea,* and *Pisonia* show predominantly phloem islands (type 1), but some species, such as *Guapira pernambucensis* and *Pisonia obtusata*, also have phloem in patches (type 2) (see Table 2). The genus *Pisoniella,* with only two recognized species, is interesting because each species has a different arrangement: *P. glabrata* has type 1 anatomy (phloem islands), while *P. arborescens* exhibits type 3 anatomy (concentric bands) (Table 2). More generally, the species with interxylary phloem are noteworthy for possessing conducting elements of both xylem and phloem grouped along narrow

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Table 2

Characteristics and distribution of the different arrangements of interxylary phloem in Nyctaginaceae.

Arrangements	Taxa	Observations
Type 1 – Phloem islands: small strands of secondary phloem embedded surrounded by sheathing axial parenchyma.	*Acleisanthes, Anulocaulis, Boerhavia, Commicarpus, Cryptocarpus, Grajalesia, Guapira, Neea, Pisonia, Pisoniella glabrata, Mirabilis*	The genera *Abronia* and *Nyctaginia* belong probably to this category, but they are herbs with few secondary growths and with very slender stems.
Type 2 – Patches: longer tangential strands, usually forming confluences between two or more phloem islands.	*Commicarpus, Guapira, Neea, Pisonia, Pisoniella glabrata*	In *Guapira, Neea* and *Pisonia,* some species may show patches in later secondary growth and/or in scandent-shrubs, as observed in *G. pernambucensis.*
Type 3 – Continuous concentric: long tangential bands of sheathing axial parenchyma with secondary phloem embedded in it.	*Bougainvillea, Colignonia, Cyphomeris, Phaeoptilum, Pisoniella arborescens, Salpianthus.*	*Belemia* may fall into this category since it is a member of the Bougainvilleae tribe. However, the samples from this genus were obtained from the herbarium and were too young.
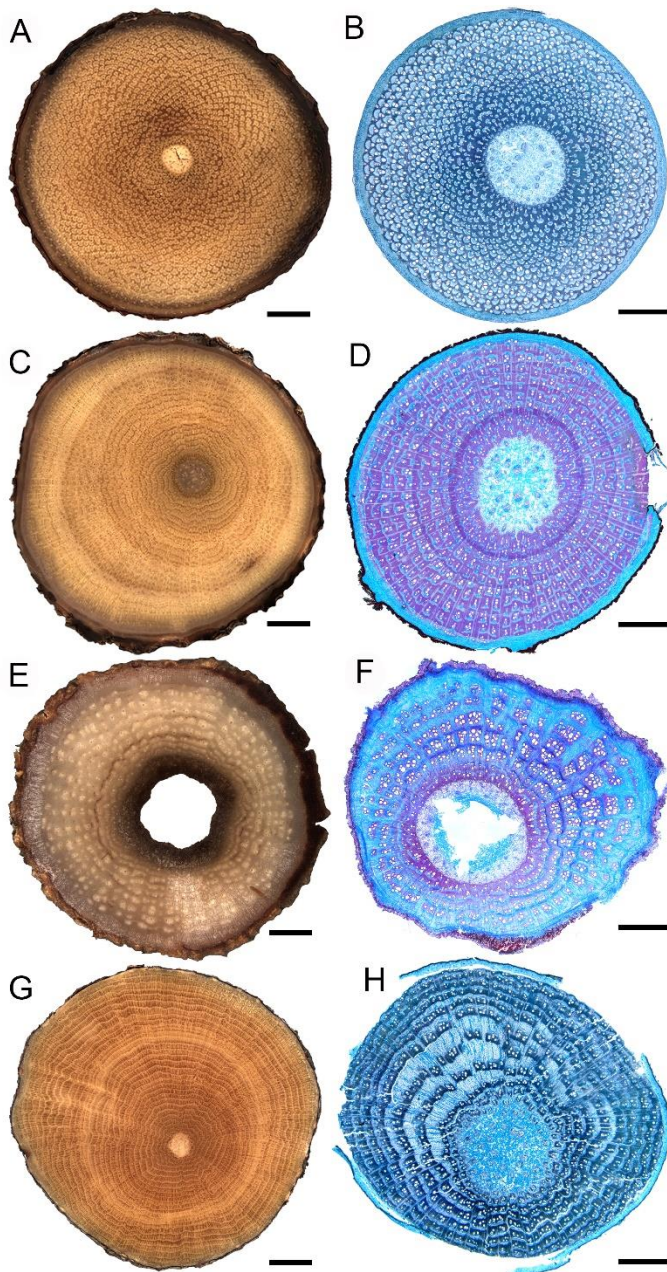
Fig. 6 Diversity of stem vascular architectures of Nyctaginaceae species with interxylary phloem. (A, C, E and G) Macroscopic view. (B, D, F and H) Microscopic view. A, *Neea hermaphrodita*. B, *Pisonia aculeata*. C–D, *Pisoniella glabrata*. E, *Colignonia glomerata*. F, *Colignonia rufopilosa*. G, *Bougainvillea berberidifolia*. H, *Bougainvillea campanulata.* Scale bars: A, C, E, G = 5mm; B, D, F, H = 2mm.

segments of the cambium, which produce a cluster-like arrangement in the overall patterning of the secondary tissues (Fig. 4A–B).

However, the fibrous tissue around these clusters of conducting cells is also xylem, produced by the cambium, as demonstrated by the formation of growth rings delimited by radially narrow fibers (Fig. 7A) and also marginal lines of axial parenchyma (Fig. 7B, D).

Discussion

Interxylary phloem in angiosperms

Interxylary phloem, i.e., strands of phloem embedded in the secondary xylem, is a type of cambial variant observed in more than 10 angiosperms families distributed mostly within the rosids and asterids, such as Acanthaceae, Apocynaceae, Combretaceae, Icacinaceae, Loganiaceae, Thymeleaceae (Chalk and Chattaway, 1937; van Veenendaal and den Outer, 1993; Carlquist, 2001, 2013; Lens et al., 2008; Patil and Rajput, 2008; Gondaliya and Rajput, 2017; Angyalossy et al., 2012, 2015; Luo et al., 2020). In most of these families, interxylary phloem seems to be present in only a few genera or species (e.g., *Strychnos*

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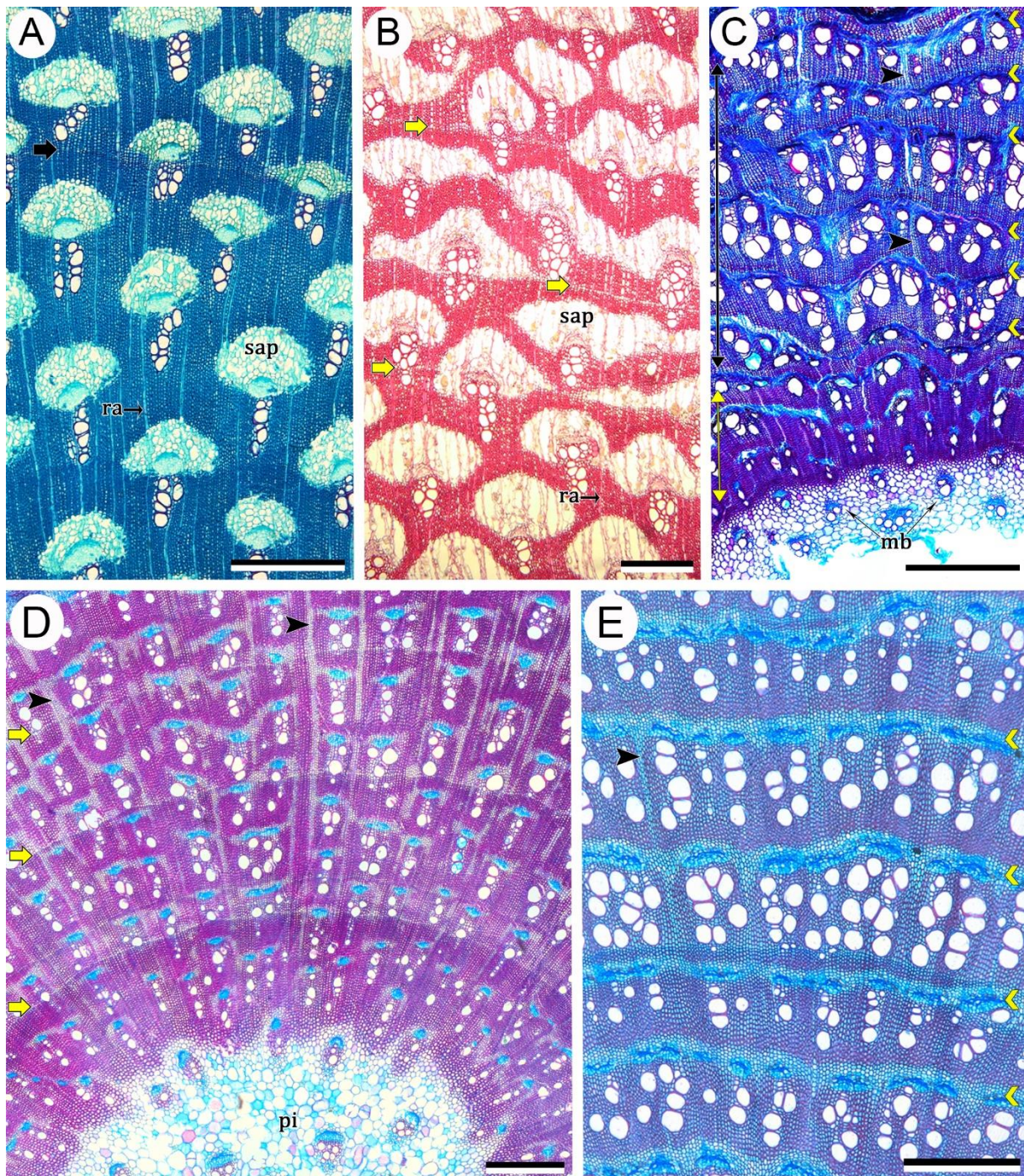
Fig. 7 Cross section of mature stems showing different arrangements of interxylary phloem. A, *Guapira laxa* (type 1, phloem islands). B, *Pisonia obtusata* (type 2, patches formed by longer phloem strands). C, *Colignonia rufopilosa* (type 3, continuous concentric); note that at the beginning of secondary growth small phloem strands are formed (type 1 – yellow double arrow) and later, various tangential bands are produced (type 3 – black double arrow). D, *Pisoniella glabrata* (type 2, patches), note the connection between neighbouring phloem strands (blue cells) through the larger abundance of sheathing axial parenchyma (tangential whiteish tissue). E, *Pisoniella arborescens* (type 3, tangential bands). Darker blue cells represent the phloem

conducting cells, while the lighter cells are sheathing axial parenchyma. Scale bars = 500 μm (A-E). Abbreviations: arrow (black), growth rings delimited by thick fibers; arrow (yellow), growth rings delimited by parenchyma in lines; arrowhead, vascular rays; mb, medullary bundles; pi, pith; pointer (yellow), tangential bands of phloem and sheathing axial parenchyma; sap, sheathing axial parenchyma. (A, C-F) Stained with safrablau, (B) Stained with safranine.

— Loganiaceae, some *Combretum* — Combretaceae, *Erisma* — Vochysiaceae), while some taxa have regular mode of vascular cambial growth (Carlquist, 2013). In addition, in some families, interxylary phloem is not the only cambial variant to occur, but other types might also be present including successive cambia (e.g., Acanthaceae, Icacinaceae) (Lens et al., 2008; Angyalossy et al., 2012; Carlquist, 2013).

The formation of interxylary phloem does not follow the same ontogeny in all families. To date, at least three main types have been characterized. One of these is the *Strychnos* type, produced by segments of vascular cambium that temporarily form less secondary xylem and more secondary phloem, generating depressions or concavities that later are overarched by a flanking cambium that encloses the phloem and starts producing vascular tissue in the usual way, xylem to the inside and phloem to

the outside. In particular, this pattern creates interxylary phloem in which the phloem strands is maintained with a portion of the cambium (van Veenendaal and den Outer, 1993; Rajput et al., 2010). The second type is the *Thunbergia* type, in which small segments of the cambium produce secondary phloem (instead of xylem) to the inside for a short period of time, and then resume formation of xylem, embedding phloem islands within the wood. In this type of interxylary phloem, no cambium remains inside the phloem islands. Similar development has been observed in *Combretum* (van Veenendaal and den Outer, 1993) and *Aquilaria* (Thymelaeaceae – Luo et al., 2018, 2020). The third type of interxylary phloem is that seen in the *Ixanthus* type, formed by redifferentiation of axial parenchyma into strands of phloem. This pattern also occurs in other taxa of Gentianaceae (e.g., *Orphium*), as well as in the families

Onagraceae (e.g., *Pseudolopezia*) and Convolvulaceae (e.g., *Turbina*) (Carlquist, 2013).

The terminology applied to interxylary phloem, sometimes called "included phloem", has undergone critical reexamination as a result of improved understanding of anatomical and developmental diversity of cambial variants. Currently, the term interxylary phloem is preferred instead of "included phloem" because in the past some studies used the latter term to describe stems with similar macromorphologies that originated from different ontogenies (Carlquist, 2013). For example, the presence of "included phloem" was used to define groups within Icacinaceae, but it has been demonstrated by Lens et al. (2008) that both successive cambia and interxylary phloem can occur in the family, and that some species of *Sarcostigma* were inaccurately identified as having successive cambia instead of interxylary phloem. Here, we showed that a similar case seems to have occurred for Nyctaginaceae.

The interxylary phloem of

Nyctaginaceae

In Nyctaginaceae, just as in other families, interxylary phloem results from a single cambium, which has an unequal activity switching on and off in different sectors to generate interxylary phloem. These phloem strands are formed in such a way that a portion of the original cambium is maintained, similar to what is observed in *Strychnos* (Loganiaceae), as reported in several studies (Scott and Brebner, 1889; van Veenendaal and den Outer, 1993; Rajput et al., 2010; Moya et al., 2018). However, the dynamics of the cambium in Nyctaginaceae species generate phloem strands consisting of conducting cells associated with the sheathing axial parenchyma (remaining phloem parenchyma cells) in several amounts and arrangements, which makes this type of interxylary phloem novel and unique.

The formation of interxylary phloem in Nyctaginaceae seems to be intrinsically related with the type of eustele, since virtually most of the species with polycyclic eustele (but not all, e.g., *Allionia* – Cunha Neto et al. 2020*b*) have this type of cambial variant. As a rule, the ontogeny of stems with this cambial variant in Nyctaginaceae involves three steps

that deviate from the typical developmental pattern found in plants with regular mode of vascular cambial growth: i) the cambium is formed in a continuum with a continuous cylindrical procambium (CCP); ii) the activity of the cambium is regular for a short period of time and soon becomes irregular leading to the absence of a complete regular cylinder in the stem; iii) the unusual dynamics of the cambium lead to the production of phloem strands embedded within the secondary xylem throughout the stem body.

In Nyctaginaceae, the formation of this cambial variant, which generates phloem strands, is based on modifications at the cellular level, especially the pattern and/or rate of cell division and differentiation of cambium initials and its derivatives, in different sectors of the cambium. There is evidence that the meristematic activity of the vascular cambium is regulated by genetic mechanisms that can independently control distinct developmental modules that consist of distinct sets of processes, such as periclinal divisions, the control of radial polarity or the independent differentiation of xylem and phloem (Du

and Groover, 2010; Etchells et al., 2013; Bossinger and Spokevicius, 2018; Tomescu and Groover, 2019). The interplay of such independent modules regulating these major biological processes might explain the asynchronous development of vascular tissues resulting in disparate morphologies, as is the case of interxylary phloem in Nyctaginaceae. Future transcriptomic studies of the vascular meristems in Nyctaginaceae can further our understanding of the molecular developmental mechanisms underlying procambial identity in the continuous cylindrical procambium, the functioning of the cambium as a bifacial meristem, and its activity in distinct sectors that produce xylem and phloem at unequal rates.

The ontogeny of interxylary phloem as described here is a unique developmental trajectory likely present not only in Nyctaginaceae, but also in related families. In some Amaranthaceae, for example, we note stems (e.g., *Celosia argentea,* Myśkow et al., 2019) that seem to have the same polycyclic eustele with medullary bundles and a continuous cylindrical procambium ("continuous concentric procambium" of Cunha Neto et al.,

2020*a*), while in secondary growth some species also initiate vascular cambial without a regular cylinder of cambium, but forming phloem strands with various arrangements (patches, concentric) in mature stems (e.g., *Simmondsia chinensis,* Schweingruber 2011; *Hebanthe eriantha,* Rajput and Marcati 2013; *Iresine spp.,* Zumaya-Mendoza et al., 2019; *Camissoa altissima,* pers. Observ.). In the case of *Hebanthe eriantha* (Rajput and Marcati, 2013), the stem has phloem islands in the inner region and radially longer phloem strands as they reach the periphery. This observation indicates that even stage-dependent polymorphism in the mode of vascular cambial growth similar to that documented here in some Nyctaginaceae, where two types of arrangements occur in the same stem, is present in other Caryophyllales. However, more studies are needed to confirm these hypotheses. Although most taxa with interxylary phloem in other families have phloem islands (e.g., *Combretum, Strychnos*), concentric arrangement seems to not be limited to Nyctaginaceae or Caryophyllales (see Carlquist, 2013). In their study of Icacinaceae, Lens et al.

(2008) mentioned species of *Pleurisanthes flava* with interxylary phloem that is more or less arranged in rings (not illustrated by those authors), while *Sarcostigma,* another genus with this cambial variant, is described with scattered phloem islands. In Malpighiaceae, the genus *Dicella* has interxylary phloem (Chodat and Vischer 1917), and in *Dicella macrocarpa* this interxylary phloem forms patches that are also surrounded by different amounts of axial parenchyma (Pace, 2015).

The abundance and distribution of axial parenchyma associated with the phloem strands in Nyctaginaceae is remarkable. In previous studies that classified these species as having successive cambia, this tissue surrounding the conducting elements of each vascular increment was termed "conjunctive tissue", whether it was parenchymatous or fibrous (Rajput and Rao, 1998; Carlquist, 2007, 2010; Hernández-Ledesma et al., 2011). Instead of "conjunctive parenchyma", we use the term "sheathing axial parenchyma" for these large parenchyma cells surrounding the phloem, because in the literature the term "conjunctive tissue" has been

used originally for products of the secondary thickening meristem in the monocots (Cheadle 1937; Diggle and DeMason 1983), as well as in the context of successive cambia (Carlquist, 2007, 2010). More importantly, it is reported that in most species with successive cambia the conjunctive tissue originates from the pericycle (master cambium of Carlquist, 2007 or procambium remnants of Myśkow et al., 2019), while the sheathing axial parenchyma of Nyctaginaceae originate directly from the cambium. Within the Nyctaginaceae, in species with type 2 and type 3 arrangement, neighbouring strands can be connected by lateral or radial confluences of sheathing axial parenchyma contributing to the different arrangements as seen in cross-section. These radial connections have been named 'radial plates of conjunctive tissue', 'raylike radial sheets of conjunctive parenchyma' (Metcalfe and Chalk, 1950), and 'radial panels of conjunctive parenchyma' (Esau and Cheadle, 1969). Given the multiple physiological functions of parenchymatic tissue, the relative amounts and distribution of the sheathing axial and radial parenchyma

in species with interxylary phloem is a key feature that still needs experimental investigation. The physiological impact of the conducting phloem confined within the secondary xylem in an apparent regular arrangement is another aspect that deserves to be fully investigated in these taxa.

The classification of the cambial variant: why interxylary phloem and not successive cambia

Some cambial variants are readily identifiable based on topological aspects, as in the case of phloem wedges (common in Bignoniaceae) or axial xylem and phloem elements in plates (common in Aristolochiaceae, Piperaceae). However, the distinction between successive cambia and interxylary phloem has been problematic in several cases (Lens et al., 2008; Carlquist, 2013). For Nyctaginaceae, most of the pioneering works described successive cambia as the cambial variant occurring in the family (e.g., Schenck, 1893; Pfeiffer, 1926), with several subsequent studies reaching the same interpretation (Supplemental Material – Table A1). However, other

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authors have also suggested the presence of interxylary phloem (Chalk and Chattaway, 1937; Lopes et al., 2008; Sonsin et al., 2014), although most of them did not document in detail the development of the stems, using instead only the mature stem anatomy. In addition, the majority of these studies examined only a few species from more common genera, such as *Guapira, Neea*, and *Pisonia*, whose timbers can be found sometimes in the market; these are taxa with remarkably similar anatomy, showing in most cases small phloem strands (phloem islands, i.e., our type 1). Chalk and Chattaway (1937) described the interxylary phloem in Nyctaginaceae as "strands of included phloem isolated, wider tangentially than radially", without any further developmental information. Although these authors recognized that tangential bands could also be formed in some taxa (e.g., *Pisonia*), other genera with concentric arrangement (e.g., *Bougainvillea, Colignonia*) were classified as having successive cambia, in which is inconsistent with our findings. For Studholme and Philipson (1966), who studied the anatomy of a single species of *Pisonia,* the stem diverges from a

regular mode of vascular cambial growth by "the differentiation of longitudinal strands of phloem within the zone of meristematic cells". This idea of a single cambium or a meristematic zone that moves outwards and produce phloem "within" it instead of the outside had been defended by some authors and neglected by others (Supplemental Material – Table A1). Our results also show that the original cambium maintains its activity throughout, producing phloem outwards and xylem inwards and being, therefore, a bifacial cambium. Sectors of this cambium remain with the phloem strands and their activity is maintained for some time (and non-conducting, collapsed phloem is often seen in some species), providing additional evidence for this bifacial cambial activity.

The presence of a single vascular cambium is the main aspect that distinguishes interxylary phloem from the phloem produced by successive cambia (Carlquist, 2001, 2013; Angyalossy et al., 2015). Therefore, interpretations proposing successive cambia for most species of Nyctaginaceae are not supported, since multiple cambia are not observed.

Stems with true successive cambia generally initiate secondary growth with a single cambium producing a complete cylinder of secondary xylem and secondary phloem, and only after some period of such regular secondary growth, the first additional cambium is formed outside of the initial cambium and independent of it. These ontogenetic steps characterize the formation of successive cambia in distantly related lineages including gymnosperms (e.g., Gnetales, *Gnetum*; Carlquist and Robinson, 1995; Carlquist, 1996; Carlquist 2012) and several eudicot families (e.g., Convolvulaceae, Terrazas et al., 2011; Fabaceae, Dias-Leme et al., 2020; Menispermaceae, Tamaio et al., 2009; Sapindaceae, Cunha Neto et al., 2018; Vitaceae, Pace et al., 2018), as well as in some Nyctaginaceae not covered here, as we will present in a future paper (Cunha Neto et al., in prep.). In contrast, in species with interxylary phloem like those we have presented here, secondary growth starts with a variant cambium, and continues with production of secondary phloem and secondary xylem at differential rates throughout its girth. This developmental mode prevents the

stem from forming a typical cylinder of secondary vascular tissues, and phloem strands immersed within the secondary xylem are formed starting in the early stages of secondary growth. In addition, the coalescent cambium overarching the phloem strands is usually continuous with the original cambium. To the best of our knowledge, the development of interxylary phloem with these characteristics has not been reported previously. Why the vascular cambium has this differential activity throughout its girth is still unknown, but given the regularity in the distribution of the phloem strands within the xylem, a robust, canalized regulatory mechanism is expected to exist. Such a mechanism explaining the distribution of phloem strands could involve interactions similar to those of the Turing-like mechanism in the stochastic reaction-diffusion model proposed by Hearn (2019).

Systematic and phylogenetic implications of interxylary phloem in Nyctaginaceae

The observation of interxylary phloem in Nyctaginaceae represents a new piece of information with phylogenetic importance, since it

indicates the evolution of this feature within the large and diverse order Caryophyllales. The presence of interxylary phloem in Nyctaginaceae is also significant at the family level. We encountered this cambial variant in at least 16 genera of Nyctaginaceae, distributed in five out of the seven tribes. Undoubtedly, this is the most widespread type of cambial variant in the family. At the genus level we found an interesting example of how different vascular arrangements can be useful as a taxonomic feature to distinguish related species. By investigating the two species of the genus *Pisoniella*, we found that they have different arrangements of interxylary phloem, with *P. glabrata* showing phloem islands (type 1 and/or 2), whereas *P. arborescens* has stems with concentric arrangement of interxylary phloem (type 3). Possibly related to this difference in vascular cambial growth, we showed in an earlier study that these two species also differ strikingly in their architecture of their primary vascular system, since *P. glabrata* have a polycyclic eustele characterized by concentric rings of medullary bundles (more than 20 bundles), while *P.*

arborescens has only one ring with eight bundles (Cunha Neto et al. 2020*a*).

Conclusions

Our examination of the stem development of Nyctaginaceae confirms the presence of interxylary phloem in the family. This type of cambial variant has been overlooked in Nyctaginaceae as most previous studies have reiterated descriptions of successive cambia as the common pattern of secondary growth within the family. Here we not only confirm the presence of this unusual ontogeny, but also demonstrate that these species combine an uncommon set of features (e.g., polycyclic eustele, absence of regular cambial cylinder, conducting phloem confined within the secondary xylem) that clearly differentiates them from the regular mode of vascular cambial growth of most eudicots and from stems with successive cambia. In addition, our results show that within this same type of cambial variant, there are different stem architectures resulting from similar ontogenetic pathways. These findings emphasize the importance of developmental studies in furthering our understanding of stem macromorphologies, and

highlight the complexity and diversity of stem architectures in Nyctaginaceae. Broader future studies including species of other lineages are planned to shed light onto the distribution and the evolution of development of all cambial variants found in Nyctaginaceae (Cunha Neto et al., in prep.).

Supplemental Material

Supplemental material is available online through Dryad at {https://doi.org/10.5061/dryad.w9ghx3f n1} and consists of the following: Table A1: Historical review of terminology applied to cambial variants in Nyctaginaceae.

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Appendix

List of species examined and site of collection/wood collection where specimens were obtained for all studied Nyctaginaceae (organized by tribes).

Herbaria: A, Harvard University Herbaria; FLAS, Florida Museum of Natural History; HURB, Universidade Federal do Recôncavo da Bahia; MEXU, Universidad Nacional Autónoma de México; MW, Moscow State University; RB, Jardim Botânico do Rio de Janeiro; SPF, Universidade de São Paulo; US, Smithsonian Institution; USZ, Museo de Historia Natural Noel Kempff Mercado, Universidad Autónoma Gabriel René Moreno.

Species name, Collector/collector number (Herbarium), Collection site.

Tribe Nyctagineae

Abronia fragrans **Nutt. ex Hook.**, Douglas 2290 (FLAS), Las Cruces, New Mexico, USA. *Abronia neealleyi* **Standl.**, Douglas 2281, (FLAS) Eddy County, Yeso Hills, New Mexico, USA. *Acleisanthes chenopodioides* (A.Gray) **R.A.Levin.**, Douglas 2289, 2293 (FLAS), Las Cruces, New Mexico, USA.

Acleisanthes lanceolata (Wooton) **R.A.Levin**, Douglas 2277 (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Acleisanthes longiflora* **A.Gray.**, Douglas 2279 (FLAS), Malone Mountains, Sierra Blanca, Texas, USA. *Allionia choisyi* **Standl.**, US 498327, New Mexico, USA; *Allionia incarnata* **L.**, Nee 64124–64126, (USZ), Parque Nacional Amboró, Pampa Grande, Santa Cruz, Bolivia; Douglas 2293 (FLAS), Las Cruces, New Mexico, USA. *Anulocaulis leiosolenus* **var.** *gypsogenus* (Waterf.) **Spellenb. & Wootten**, Douglas 2280 (FLAS) Eddy County, Yeso Hills, New Mexico, USA; Douglas 2283 (FLAS), Crest of 7 Rivers Hills, New Mexico, USA. *Boerhavia diffusa* **L.**, Pace 753 (MEXU, US), Veracruz, Mexico. *Boerhavia linearifolia* **A.Gray.**, Douglas 2284 (FLAS), New Mexico, USA. *Boerhavia torreyana* (S. Watson) **Standl.**, Douglas 2294 (FLAS), Las Cruces, New Mexico, USA. *Boerhavia wrightii* **A.Gray.**, Douglas 2288 (FLAS), Las Cruces, New Mexico, USA. *Commicarpus scandens* (L.) **Standl.**, Acevedo-Rodríguez 16250 (US), Tonalá, Oaxaca, Mexico; Douglas 2291 (FLAS), New Mexico, USA. *Cyphomeris gypsophiloides* (M. **Martens & Galeotti) Standl.**, Douglas

2287 (FLAS), Organ Mountains-Desert Peaks National Monument, Las Cruces, New Mexico, USA. *Mirabilis cf. albida* **(Walter) Heimerl.**, Douglas 2286 (FLAS), New Mexico, USA. *Mirabilis jalapa* **L.**, Acevedo-Rodríguez 16480 (US), Veracruz, Mexico. *Nyctaginia capitata* **Choisy.**, Douglas 2282 (FLAS), New Mexico, USA. *Okenia hypogea* **Schltdl. & Cham.**, Pace 749 (MEXU, SPF, US), Veracruz, Mexico

Tribe Pisonieae

Grajalesia fasciculata **(Standl.) Miranda.**, Pace 765 (MEXU, SPF, US), Chiapas, Mexico. *Guapira bracei* Britton, USw 23138, Monroe, Florida, USA. *Guapira cuspidata* **(Heimerl) Lundell,** USw 35378, Mérida, Venezuela. *Guapira linearibracteata* **(Heimerl) Lundell.**, USw 29941, Belize. *Guapira pernambucensis* **(Casar.) Lundell.**, Cunha Neto 04-05 (HURB), Alagoinhas, Bahia, Brazil. *Guapira graciliflora* **(Mart. ex J.A.Schmidt) Lundell.**, Cunha Neto 06 (HURB), Alagoinhas, Bahia, Brazil. *Guapira laxa* **(Netto) Furlan.**, Cunha Neto 08 (HURB), Universidade Estadual de Feira de Santana, Feira de Santana, Bahia, Brazil. *Guapira ligustrifolia* **(Heimerl) Lundell**, USw 1886, San Gabriel Island,

Dominican Republic; USw 1982, Hispaniola Island, Dominican Republic. *Guapira longifolia* **(Heimerl) Little,** Hw 29374. *Neea cauliflora* **Heimerl in Engl. & Prantl**, USw 9134, Acre, Brazil. *Neea delicatula* **Standl.**, Pace 689 (US), Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica. *Neea hermaphrodita* **S.Moore**, Nee 64112 (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia. *Neea laetevirens* **Standl.**, Pace 713, 716 (US), Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica; USw 16154, Los Santos, Panama. *Neea macrophylla* **Britton ex Rusby.**, USw 40851, San Martín, Peru. *Neea ovalifolia* **Spruce ex J.A. Schmidt**, USw 42783, Régina, French Guiana. *Neea psychotrioides* **Donn. Sm.**, Pace 763 (MEXU), Estación de Biología Tropical Los Tuxtlas, Veracruz, Mexico; USw 29939, Belize. *Pisonia aculeata* **L.**, Acevedo-Rodríguez 16549 (US), Bonito, Mato Grosso do Sul, Brazil; Aw 1037. *Pisonia brevipetiolata* **(Heimerl) Urb.**, USw 1793, Fond Parisien, Etang Saumatre, Haiti. *Pisonia fragrans* **Dum.-Cours.**, USw 35502, Republica Dominicana. *Pisonia ligustrifolia* **Heimerl,** USw 1886, San Gabriel Island, Dominican

Republic. *Pisonia obtusata* **Jacq.,** Aw8322. *Pisonia rotundata* **Griseb.,** USw 21834, Monroe, Florida, USA; Aw 8323. *Pisoniella arborescens* **(Lag. & Rodr.) Standl.,** Pace 738-739 (MEXU, SPF, US), Alfajayucan, Hidalgo, Mexico. *Pisoniella glabrata* **(Heimerl) Standl.,** Nee 64137, 64151 (USZ), Parque Nacional Amboró, Vallegrande, Santa Cruz, Bolivia.

Tribe Bougainvilleeae

Belemia fucsioides **Pires.,** Farney 4887, 4888 (RB). *Bougainvillea sp1.,* Nee 64176 (USZ), Rio Grande, Santa Cruz de la Sierra, Bolivia. *Bougainvillea sp2.,* Nee 64182 (USZ), Rio Grande, Santa Cruz de la Sierra, Bolivia. *Bougainvillea berberidifolia* **Heimerl.,** Nee 64140 (USZ), Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia. *Bougainvillea campanulata Heimerl.,* Acevedo-Rodríguez 16772 (US), Mato Grosso do Sul, Brazil; Nee 64142 (USZ), Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia. *Bougainvillea modesta* **Heimerl.,** Nee 64115 (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia. *Bougainvillea stipitata* **Griseb.,** Nee 64121 (USZ), Parque Nacional Amboró,

Samaipata, Santa Cruz, Bolivia. *Bougainvillea spectabilis* **Willd.,** Rossetto 453 (RB), Estrada Carlos Chagas-Teófilo Otoni, Minas Gerais, Brazil. *Phaeoptilum spinosum* **Radlk.,** MADw 37340, Mocamedes, Angola.

Tribe Colignonieae

Colignonia glomerata **Griseb.,** Nee 64157-64159 (USZ), Parque Nacional Amboró, Samaipata, Santa Cruz, Bolivia. *Colignonia rufopilosa* **Kuntze,** Nee 64061 (USZ), Cochabamba, Bolivia.

Tribe Boldoeae

Cryptocarpus pyriformis **Kunth,** US 2833648, Galapagos. *Salpianthus macrodontus* **Standl.,** US 2219249, Sinaloa, Mexico. *Salpianthus purpurascens* **(Cav. Ex ag.) Hook. & Arn.,** Pace 774 (MEXU, SPF, US), El Cobanal, Chiapas, Mexico.

Tribe Leucastereae

Andradea floribunda **Allemão,** US 2627753, Espírito Santo, Brazil; Rossetto 445 (RB), Linhares, Espírito Santo, Brazil. *Leucaster caniflorus* **(Mart.) Choisy,** US 2839822, Jacarepaguá, Rio de Janeiro, Brazil; Rossetto 447 (RB), Linhares, Espírito Santo, Brazil; Rossetto 455 (RB),

Teófilo Otoni, Minas Gerais, Brazil. *Ramisia brasiliensis* Oliv., US 2947296, Nova Venécia, Espírito Santo, Brazil; Rossetto 448 (RB), Nanuque, Minas Gerais, Brazil. *Reichenbachia hirsuta* **Spreng.,** Nee 64109 (USZ), Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia; *Nee 64169* (USZ), Rio Grande, Santa Cruz de la Sierra, Bolivia.

Table A1. Historical review of terminology applied to cambial variants in Nyctaginaceae.

Reference	Cambial variant	Origin of the cambial variant	Name of the meristem of origin	Products of the meristem	Taxa
De Bary 1884	Secondary thickening(= successive cambia)	Outer margins of the phloem	Extra-fascicular cambium	Vascular bundles to the inner side	*Mirabilis jalapa*
Schenck 1893	Successive cambia	Pericycle	Cambium	Concentric rings of xylem, separated by conjunctive tissue with phloem strands within it	*Bougainvillea spectabilis; Pisonia aculeata;*
Solereder 1908	Anomalous structure (=successive cambia)	Pericycle	Cambium	Secondary collateral bundles and conjunctive tissue to the inner side	*Boerhavia plumbaginea; B. arborea; Bougainvillea spectabilis; Cryptocarpus sp.; Leucaster caniflorus; Mirabilis jalapa; Neea sp.; Phaeoptilum sp.; Pisonia fragrans; P. hirtella; P. nigricans;*
Pfeiffer 1926	Successive cambia (*corpus lignosum circumvallatum*)	Pericycle	Cambium	Rings of xylem, conjunctive tissue and phloem strands	*Boerhavia arborea; Bougainvillea; Cryptocarpus; Leucaster; Mirabilis; Neea; Phaeoptilum; Pisonia; Rockia.*

Maheshwari 1930	Successive cambia	Underneath the cortex	Meristematic zone (cambium)	Xylem and conjunctive tissue to the inner side; Phloem *within* the zone.	*Boerhavia diffusa;*
Chalk and Chattaway 1937	Successive cambia	Pericycle or cortex	–	Xylem internally; phloem externallly	*Bougainvillea spectabilis; Colignonia scandens;*
	Interxylary phloem (*Corpus lignosum foraminatum*)	Single vascular cambium	Cambium	Included strands of phloem inside the xylem;	*Guapira broadwayana; Guapira discolor; Neea amplifolia; N. laetevirens; N. pittieri; N. psychotrioides; N. urophylla; Pisonia aculeata; P. albida; P. linguistifolia; P; macranthocarpa; P. nishimurae; P. obtusa; P. sandwicensis; P. subcordata; Neea amplifolia; N. laetevirens; N. pittieri; N. psychotrioides; N. urophylla;*
Metcalfe and Chalk 1950	Anomalous secondary thickening	Single vascular cambium	Secondary meristem	Secondary bundles	*Boerhavia verticillata; Bougainvillea spectabilis; Colignonia scandens; Leucaster*

	(succession of rings)				*caniflorus; Mirabilis sp.; Neea sp.; Phaeoptilum sp.; Pisonia sp.; Reichenbachia sp.; Guapira sp.; Neea sp.; Pisonia sp.*
	Phloem in islands				
Balfour and Philipson 1962	Intraxylary phloem	Meristematic ring	Extra-fascicular cambium	–	*Bougainvillea spectabilis*
Philipson and Ward 1965	Anomalous secondary growth (=successive cambia)	External to primary bundles	Unidirectional cambium	Xylem internally; Phloem *within* the meristematic zone	*Boerhavia, Mirabilis*
Esau and Cheadle 1969	Successive cambia	The parenchyma beneath the perivascular fibres	Meristematic tissue (cambium)	Xylem and conjunctive tissue to the inside, phloem and conjunctive tissue to the outside	*Bougainvillea spectabilis*
Balfour 1965	No especial name is given	Cortex	Meristematic zone (cambium)	Xylem internally; Phloem *within* the meristematic zone	*B. spectabilis; Pisonia brunonianum*
Studholme and Philipson 1966	Included phloem	Cortex	Meristematic zone; cambium	Xylem to the inside and parenchyma to the outside. Strands of phloem differentiate within the meristematic zone.	*Pisonia brunonianum*

Pulawska 1973	No especial name is given	Procambium of peripheral bundles, and outside them	Parenchymo-vascular cambium	Secondary parenchyma to the outside and secondary vascular bundles and conjunctive tissue inside	*Bougainvillea glabra*
Stevenson and Popham 1973	Anomalous secondary thickening	Pericycle	"Primary thickening meristem"	Complete bundles to the inside	*Bougainvillea spectabilis*
Phillips 1976	Successive cambia	Pericycle/parenchyma outside the phloem	Cambium	Conjunctive tissue, phloem and xylem, forming small collateral vascular bundles	*Allionia incarnata; A. choisyi*
Mikesell and Popham 1976	*Corpus lignosum foraminatum* from Pfeiffer's	Pericycle	"Primary thickening meristem"	Conjunctive tissue to the inside and outside; vascular tissue to the inside.	*Mirabilis jalapa*
Zamski 1980	Anomalous secondary thickening	Outside the vascular bundles	"Primary thickening meristem" (vascular cambium)	Conjunctive tissue and complete bundles to the inside	*Bougainvillea glabra*
Rajput and Rao 1998	Successive rings	Outermost phloem cells	-	Xylem internally; phloem externally	*Boerhavia diffusa; B. rependa; B. verticillata*
Carlquist 2004	Successive cambia	Cortex	Lateral meristem	Conjunctive tissue, vascular cambium (and its products) and rays to the inside, and	*Abronia latifolia; Bougainvillea spectabilis; Guapira discolor; G. guianensis;*

				"secondary cortex" to the outside.	*G. cuspidata; Mirabilis jalapa; Neea macrophylla; P. brunonianum; P. rotundata;*
Carlquist 2007, 2010	Successive cambia	Cortex	Master cambium	Conjunctive tissue, vascular cambium (and its products) and rays to the inside, and "secondary cortex" to the outside.	*Abronia latifolia; Bougainvillea spectabilis; Guapira discolor; G. guianensis; G. cuspidata; Mirabilis jalapa; Neea macrophylla; P. brunonianum; P. rotundata;* and species from other families.
Rajput et al. 2009	Successive cambia	Cortex	Master cambium	Conjunctive tissue centripetally and centrifugally; vascular cambium and its products to the inside	*Mirabilis jalapa*
Hernández–Ledesma et al. 2011	Successive cambia	Cortex	Master cambium	New cambia with xylem internally and phloem externally	Several species of *Mirabilis*
Schweingruber 2011	Successive cambia	–	–	Vascular bundles	*Abronia fragrans; A. latifolia; Bougainvillea spectabilis; B. spinosa; Commicarpus boissieri.*

Lopes et al. 2008	Interxylary phloem	–	Cambium	–	*Pisonia aculeata*
Sonsin et al. 2014	Interxylary phloem	–	–	–	*Guapira noxia*
Cunha Neto et al. 2020	Successive cambia	Pericycle	Meristematic zone	A new cambium is formed from the meristematic zone and forms variant xylem internally and variant phloem externally; conjunctive tissue is formed between new increments of vascular tissue.	*Allionia choisyi, Allionia incarnata.*

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Chapter 5

From procambium to cambium and cambial variants: complex developments shape the diverse stem vascular systems of Nyctaginaceae*

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From procambium to cambium and cambial variants: complex developments shape the diverse stem vascular systems of Nyctaginaceae

Abstract

Background: The presence of alternative patterns of secondary growth in stems of Nyctaginaceae has been known since a long time. Still, the interpretation of types of cambial variants are controversial. The knowledge of the diversity of stem anatomies in Nyctaginaceae, which is also diverse in habits, offers the opportunity not only to investigate the evolution of complex developments, but also how these anatomies shifted within these habits and if the acquisition of cambial variants impacted the diversification of the family.

Methods: We integrated developmental data within a phylogenetic framework to investigate the diversity and evolution of stem anatomy in Nyctaginaceae using phylogenetic comparative methods. We examined whether anatomical shifts correspond with species diversification rate shifts in the family.

Results: Two types of cambial variants, interxylary phloem and successive cambia, were observed in Nyctaginaceae, which result from four ontogenies. These ontogenetic trajectories develop from two primary structures (regular or polycyclic eustele) yet, they contain shared developmental stages which generate stem morphologies with deconstructed boundaries of morphological categories (continuum morphology). Unlike our previous hypotheses, interxylary phloem is reconstructed as the ancestral character for the family, with three ontogenies characterized as successive cambia evolving in few taxa. Cambial variants are not contingent in habits, and their transitions do not increase species diversification.

Conclusions: These findings suggests that multiple evolutionary mechanisms rather than additional developmental stages generate the transitions between these two patterns. Therefore, it also helps us to understand how the changes in the ontogeny of vascular systems across the phylogeny and show how these features play a role in the evolution of stem architectures.

Keywords: anatomy, Caryophyllales, continuum morphology, developmental processes, evolution, ontogeny, vascular tissue, species diversification.

Background

In the context of evolutionary developmental biology and phylogenetic research, morphology and anatomy have played a major role in our challenge to unravel the complexity and diversity of organisms [1,2]. Thus, one of the fundamental pillars of this discipline remains to investigate how modifications in developmental programmes of organisms contributes to the diversity of phenotypes encountered in nature [1,3–5]. The evolution of plant diversity is achieved by modifications in developmental processes (e.g., heterochrony, heterotopy, homeosis, evolutionary novelties) [6–8]. In stem development, previous studies have demonstrated different developmental programmes interacting in the evolution of various anatomical architectures, which evidenced also anatomical shifts that likely triggered their diverse forms, coped with possible hydraulic and biomechanical functions so critical to plant survival not only different pathways in the formation of vascular anatomies [9–12]. Therefore, by studying the alterations in developmental trajectories in stem formation, we are studying the mode of how natural selection has acted in the evolution of nature's most complex stem architectures.

Regular secondary growth – a single bifacial cambium producing wood and bark – is believed to have first appeared in the ancestor of progymnosperms, gymnosperms, and angiosperms [13–15]. Within this large and diverse lineage, known as the lignophytes, alternatives to this regular growth are not uncommon [16–19]. Several modifications on the regular growth include a single cambium with differential activity and/or multiple cambia [18,20–22]. These alternative patterns of secondary growth produce diverse and complex stems architectures, also known as cambial variants [20,21]. Many types of cambial variants are found in lineages containing lianas, although they occur also in plants with distinct growth forms, such as trees, shrubs and herbs [22,23]. While the development of cambial variants has been largely investigated in recent years, the evolutionary history underlying the formation of these complex patterns is still little understood. Integrative studies using stem anatomical data within a phylogenetic context illustrating how these disparate macromorphologies evolved from regular anatomies are reported in a few groups [11,12], and evidence they derive from different developmental mechanisms. Therefore, much has yet to be performed to understand the total realm of changes in

developmental trajectories that can contribute to the major complexity and diversity of the vascular system of plants in distant phylogenetic lineages.

Nyctaginaceae is a family of c. 400 species of broad distribution across the Americas, Africa and Indo-Pacific, which grows in a wide range of habits from arid deserts to tropical rain forests [24,25]. Their species can be prostrate herbs, shrubs, scandent delicate plants, bulky lianas, large trees and suffrutescent species [24,25]. Regardless of growth forms, the stem vascular anatomy of most Nyctaginaceae is remarkable for their polycyclic eustele [26] and cambial variants that appear under secondary growth [27–29]. Most other plant families only have cambial variants in certain clades or their lianescent taxa (Malpighiaceae [30]; Sapindaceae [12,31]), although they are also found in self-supporting plants deriving from lianescent ancestors (Bignoniaceae [11]; Convolvulaceae [32]). According to Gianoli [33,34], the evolution of climbers substantially increased the species richness of climbing clades if compared to their non-climbing sister groups. However, since many clades containing climbing plants (mostly lianas) are also characterized for showing cambial variants, we ask whether it is the shifts in habit or the appearance of cambial variants that is associated with species richness within these lineages. Given that Nyctaginaceae have a wide diversity of habits and because all lineages present cambial variants, the family is the perfect model to test these hypotheses.

Distinct types of cambial variants have been reported for Nyctaginaceae and more recently new approaches to their types have been proposed, demonstrating that taxa initially described as having successive cambia have interxylary phloem instead ([35] – Chapter 4). As for the origin, both the pericycle [36, 37, 38] and a meristem derived from the cortex (i.e., the master cambium [39]) has been suggested as the place where the cambial variants arise [29,40,41]. Either way, the understanding of anatomical and developmental diversity of the vascular system in Nyctaginaceae has been limited given the absence of ontogenetic studies in a broader taxonomic scale. In the family, few genera and species have been investigated in previous studies, which include generally only the ornamental taxa, such as *Bougainvillea* and *Mirabilis* [28,29,40,41]. Given the diversity of vascular anatomies, in this study we aim to investigate the developmental processes causing the evolution of disparate vascular

architectures, which is widespread in the family and likely independent of habits and habitats.

Here, we performed a comparative stem developmental analyses in the context of a well-supported phylogenetic hypothesis to understand how developmental processes evolved over time and shaped the diversity of stem architectures in Nyctaginaceae. Among the remarkable findings of this evo-devo study we highlight: i) the anatomical changes underlying the evolution of four ontogenetic trajectories in stem development; ii) the anatomical, developmental and evolutionary lability of vascular meristems, especially the vascular cambium; and iii) the significance of developmental mechanisms for evolutionary diversity of stem anatomical architectures. In addition, we evaluated whether species diversification rate has changed in Nyctaginaceae to understand the potential impact that climber habit, in the multiple instances it evolved, have had on the diversification of the family.

Materials and methods

Taxon sampling and anatomical analysis

This study represents the broadest taxonomic sampling for stem anatomical studies in Nyctaginaceae to date. Stem samples of 54 species (~74 specimens) from 25 genera were collected, representing all major clades within the family, based on the most recent phylogenies for the group [24,25,42,43]. Specimens were obtained mostly from field collections in different countries in both North and South America (Supplementary Data – Table S1). Additional samples were obtained from dried stems from either herbarium vouchers or wood collections (Supplementary Data – Table S1).

Samples from living plants were harvested at different heights of the stem to ensure that different developmental stages would not be missed. For herbs, complete stems were collected; for shrubs, lianas and scandent-shrubs, samples were obtained at the base of the plant and at least three different heights towards the shoot apex. For trees, we collected trunk samples at breast height (1.30m) and at different heights of selected branches. See Supplementary Data – Table S1 for information on stem diameter for each specimen.

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For the ontogenetic analyses, 27 species belonging to 22 genera of Nyctaginaceae were selected to account for all the variation both in terms of their phylogenetic distribution and anatomical patterns (Fig. 1; Supplementary Data – Table S1). For these species, sections were taken from different internodes beginning at the shoot apex until reaching the fully developed stem. For the remaining species, analyses of adult stems (the most developed stem available, from fully grown plants) were carried out to ensure the cambial variant types studied previously in detail were consistent.

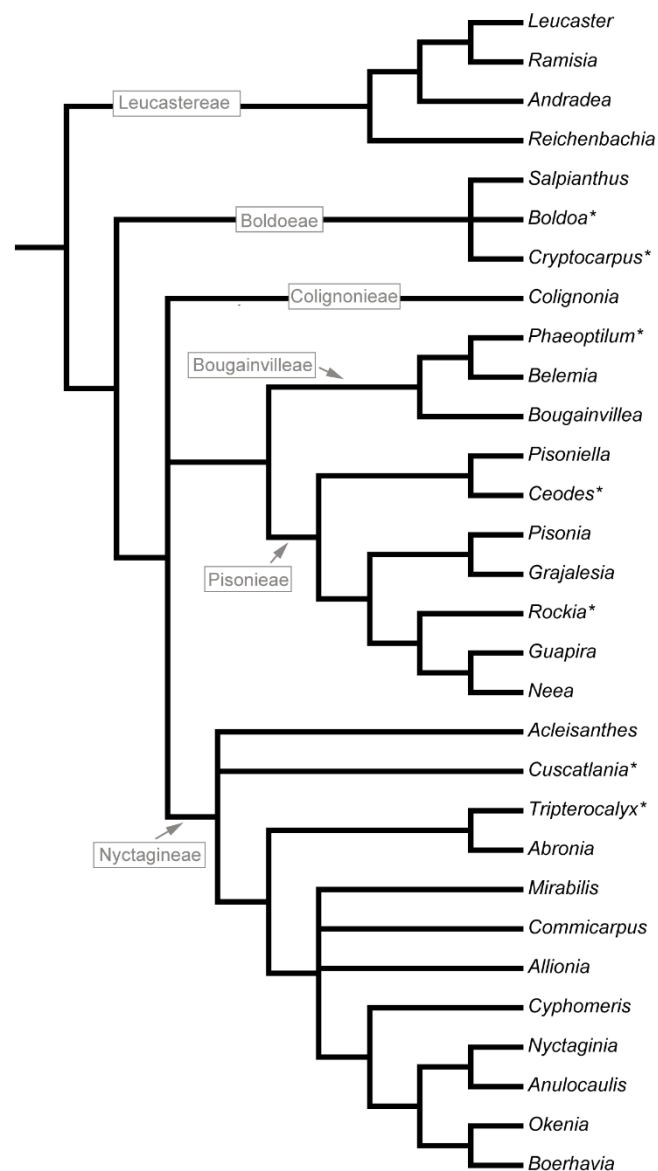**Fig. 1** Phylogenetic relationship of major Nyctaginaceae lineages based on the most recent data (Douglas & Manos, 2007; Douglas & Spellenberg, 2010; Hernández–Ledesma et al., 2015; Rossetto et al., 2019). In the present study, mature stems of all genera in the tree were analyzed (except for *Tripterocalyx, Cuscatlania* and *Boldoa*), and ontogenetic studies were carried out for all genera except for the genera indicated with asterisk.

During field work the samples were fixed in FAA 50 (10% formalin, 5%acetic acid, 50% ethanol) for one week and then transferred to a solution of 70% ethanol [44]. Anatomical sections were obtained following two different procedures: i) young and small stems samples were dehydrated in an ethanol series, embedded in Historesin (Leica Mycrosystem, Wetzlar, Germany), sectioned in a rotary microtome (Leica RM2145, Nussloch, Eisfeld, Germany), and stained in 0.05% toluidine blue in glacial acetic buffer at pH 4.7 [45]; ii) adult and large samples were softened in 10% ethylenediamine for up to 2–5 days [46], gradually embedded in polyethylene glycol 1500, sectioned with the help of polystyrene (foam) resins applied upon the stem blocks before sectioning in a sliding microtome with a permanent steel knife sharpened with different grids of sandpapers (Leica SM2010R, Nussloch, Eisfeld, Germany) [47,48] and double stained in 1% astra blue and 1% safranine [49]. Sections were mounted with coverslip in Canada Balsam or Entellan® synthetic resin (Merck KGaA, Darmstadt, Germany) to make permanent slides.

Phylogenetic framework, ancestral state reconstructions

For the ancestral state reconstructions of ontogenetic pathways, we used the same phylogenetic tree and optimization applied by Cunha Neto et al. [26 – chapter 3], under the Mesquite version 3.5 (Maddison and Maddison 2019).

Diversification analysis

Divergence times.

To estimate the age of Nyctaginaceae, we conducted a Bayesian inference with BEAST v.2.6.5 [51], using two secondary calibrations derived from a thorough study of the divergence times of the angiosperm families [52]. We applied a uniform prior distribution to calibrate the root of the tree corresponding to the stem age of a group comprising Gisekiaceae and Nyctaginaceae, where the maximum value of the distribution was 83.6 Ma (Million years ago), and the minimum value was 52 Ma. We also applied a uniform prior distribution to calibrate the crown node of Nyctaginaceae, with a maximum value of 47.59 Ma and minimum value of 18.12. In BEAUti, we assigned a molecular substitution model as GTR + Γ, using empirical base frequencies, molecular clock set as uncorrelated with rates obtained from a log-normal

distribution (UCLN; [53]), and a birth-death tree prior. We ran two independent analyses, each with 400 million generations, sampling parameters every 10,000 generations. We corroborated the correct mixing of the Markovian chains in Tracer v.1.6 [54], where the Effective Sample Size was equal or higher than 200 for all the parameters. We obtained the Maximum Clade Credibility (MCC) tree with TreeAnnotator v.2.6.5 (beast2.org/treeannotator).

Diversification rate estimation

Using a time-calibrated phylogeny, we evaluated whether there have been changes or shifts in the diversification rate through time and among lineages. The diversification rate corresponds to the net number of species/lineages generated per time unit (speciation) considering the extinction [55,56]. For this, we implemented a Bayesian analysis of macroevolutionary mixtures (BAMM v.2.5.0; [57]). BAMM estimates diversification and morphological rate shifts under a compound Poisson process through time and among lineages, using reversible-jump Markov chain Monte Carlo (rjMCMC) samplers to evaluate models that vary in the number of shifts proposed [57]. We selected a set of priors calculated in the R package BAMMtools [58] for the speciation and extinction initial values. We specified a proportion of taxon sampling to consider the missing species of Nyctaginaceae and outgroups. We ran the analysis for 100 million generations. We evaluated the convergence of chains and with the package coda [59] we corroborated that the ESS of the MCMC was 200 or above.

Results

Four ontogenetic pathways link procambium to cambium and cambial variants

In Nyctaginaceae, the stems may present two types of eustele, the regular or the polycyclic (with medullary bundles) (Fig. 2). The vascular system is also characterized by a distinguishable pericycle that can be uni- or multiseriate and which is divided into a portion of lignified cells and other that remains parenchymatous (Fig. 2; 4a–d; 9b, d). In mature stem, two types of cambial variants can be recognized, i.e., successive cambia and interxylary phloem, which derive from four different ontogenies (Fig. 2). Interestingly, representatives of all lineages of the family present

DEVELOPMENTAL PATHWAYS

ADULT STEM
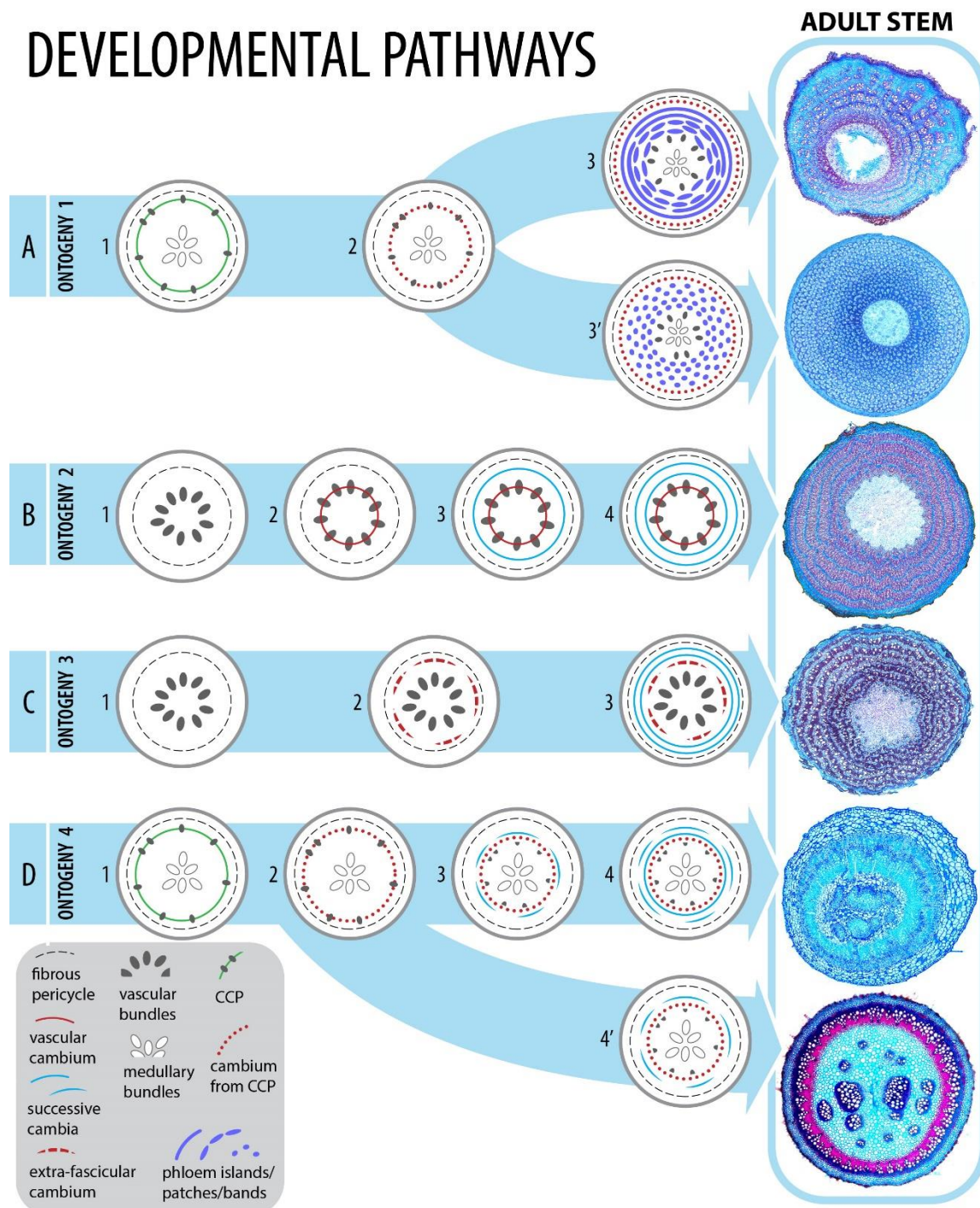
Fig. 2 Diversity of stem ontogenies in Nyctaginaceae, illustrating developmental steps from eustele types to cambial variants. The developmental pathways were divided into steps (illustrated on the left). On the right, microscopic images represent mature stems. Representations of cambium include the cambium itself and its products. **a.** Ontogeny 1 (interxylary phloem): polycyclic eustele, cambium derived from the CCP, phloem strands with different arrangements, illustrated by *Colignonia glomerata* (upper) forming bands and *Pisonia aculeata* (lower) forming phloem islands. **b.** Ontogeny 2 (successive cambia): regular eustele, extra–fascicular cambium derived from the pericycle, additional successive bands or rings; *Leucaster caniflorus.* **c.** Ontogeny 3 (successive cambia): regular eustele, regular cambium, new cambium formed *de novo* from the pericycle, additional successive bands or rings; *Reichenbachia hirsuta.* **d.** Ontogeny 4 (successive cambia): polycyclic eustele, regular cambium derived from the CCP, new cambium formed *de novo* from the pericycle, additional small bands or rings of successive cambia; *Allionia incarnata* (upper) and *Okenia hypogaea* (lower). Drawing: Marcelo Kubo.

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variant vascular anatomies during secondary growth. Below we detail each of these ontogenies.

Ontogeny 1 **(summarized from Cunha Neto et al., 2021 – chapter 4) – Steps: i. Polycyclic eustele, ii. cambial zone derived from the cylindrical continuous procambium (CCP), iii. formation of phloem strands, iv. interxylary phloem (Fig. 2a; 3a-e).**

This developmental pathway begins with the establishment of a polycyclic eustele – medullary bundles + cylindrical concentric procambium (Fig. 2a and 3a). After vascular bundles are formed from the CCP, a cambial zone is established from the procambium of the bundles (Fig. 3b). At maturity, the cambial zone presents an irregular activity leading to the formation of secondary xylem and phloem derivatives at different rates along the stem circumference, which results in the formation of phloem strands (Fig. 3c). At some points, segments of the cambial zone slightly reduce the production of xylem derivatives concomitantly with the increased formation of phloem derivatives outwards (Fig. 3c). Subsequently, these phloem patches are overarched by cambial segments formed in continuity with the single cambium and originated by differentiation of the axial phloem parenchyma (Fig. 3c); this cambium produces secondary xylem inwards and phloem outwards enclosing the islands of phloem, constituted mainly by conducting cells and axial parenchyma cells which here we denominate sheathing axial parenchyma (Fig. 3c, d). This process occurs repeatedly in the cambial zone and, as a result, many phloem strands are formed with the original cambial segment embedded within the secondary xylem (Fig. 3e).

The cambial variant described above characterizes in several aspects an interxylary phloem pattern. In Nyctaginaceae, this developmental pathway produces disparate stem architectures ranging from well-defined phloem islands with less sheathing axial parenchyma to long concentric bands of phloem and much sheathing axial parenchyma diffused tangentially (Fig. 2a). Intermediates between these two types also occur, forming phloem strands produced by confluences or patches (chapter 4).

Ontogeny 1 is the most common type within Nyctaginaceae, occurring in genera and species of various growth habits and from five out of the seven tribes, i.e.,
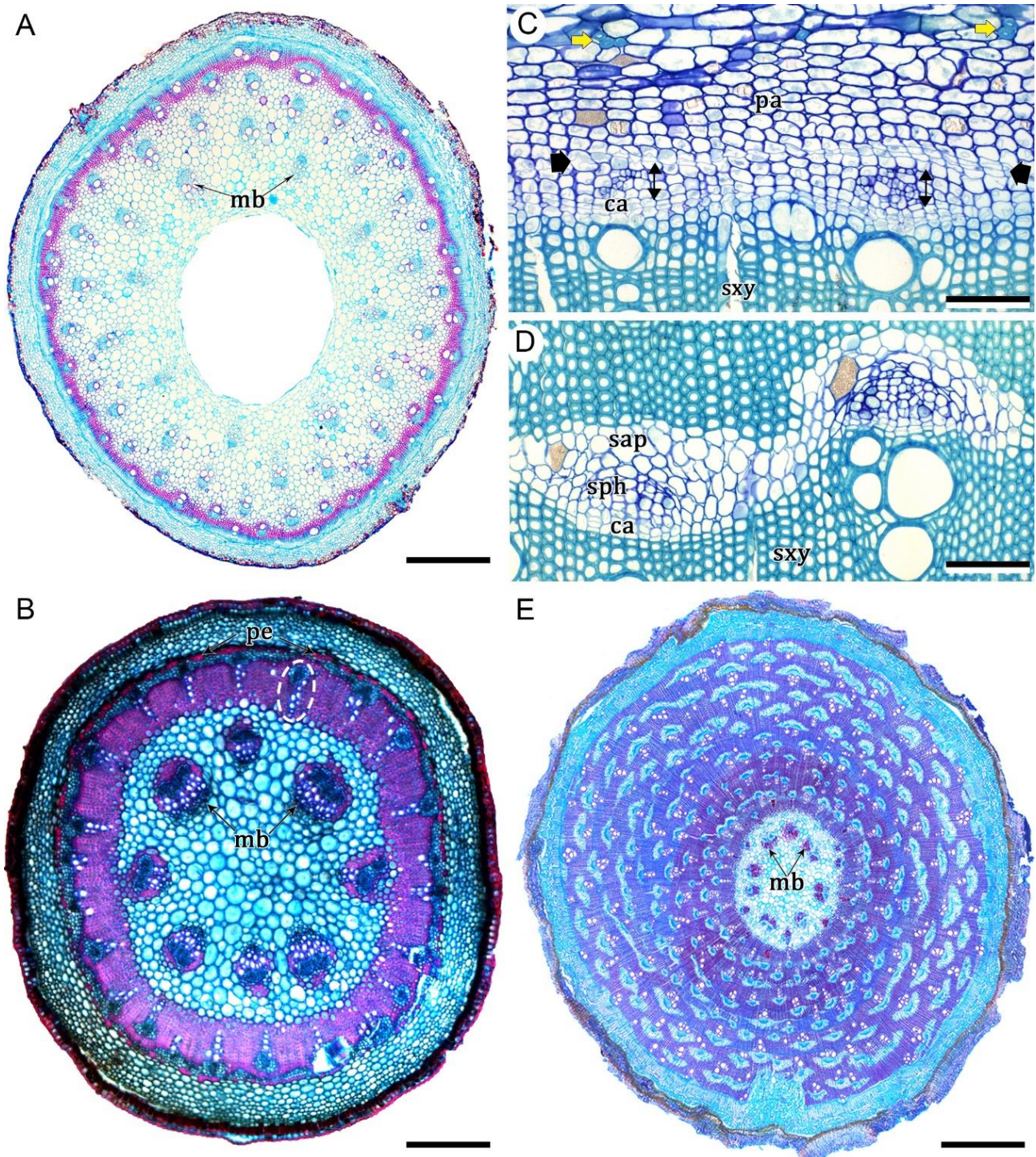
Fig. 3 Development of interxylary phloem in stems of *Colignonia glomerata* and *Guapira pernambucensis* (ontogeny 1). **a.** *Colignonia glomerata,* young stem showing polycyclic eustele and the transition from primary to secondary growth. **b-e.** *Guapira pernambucensis.* **b.** Stem in transition from the activity of the CCP to the cambium; note the vascular bundles (ellipse) formed by the CCP, whose phloem will be the first phloem island. **c** Irregular activity of the cambium which results in phloem islands (double arrows) after the development of the coalescent cambium (thick arrows). Note that the coalescent cambium is originated from the axial phloem parenchyma. Yellow arrows indicate pericyclic fibers. **d.** Two phloem islands which are formed by secondary phloem and sheathing axial parenchyma. **e.** Mature stem with phloem islands and some patches. Scale bars: 200 μm (a–c); 100 μm (d–e). Abbreviations: ca, cambium; mb, medullary bundles; pa, axial phloem parenchyma; pe, pericycle; sap, sheathing axial parenchyma; sph, secondary phloem; sxy, secondary xylem. (a–b, e) Stained with astra blue and safranin. (c–d) Stained with toluidine blue.

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Boldoeae, Bougainvillea, Colignonieae, Nyctagineae and Pisonieae.

Ontogeny 2 – Steps: i. Regular eustele, ii. regular cambium, iii. installation of variant cambium, iv. successive cambia (Fig. 2b; 4a-d; 5a-f).

This ontogeny initiates with the formation of a regular eustele characterizing the primary vascular system (Fig. 4a-b). This genus lacks medullary bundles. Later, a regular cambium develops from the fascicular and interfascicular cambium and starts to produce secondary tissues in the usual way, i.e., secondary xylem centripetally and secondary phloem centrifugally (Fig. 4a-c; 5a-d). Initially, the interfascicular cambium may produce mostly phloem axial parenchyma to the outside and produce xylem fibres, vessel and ray to the inside (Fig. 4c-d; 5c-d). After some period of regular growth, a new meristematic zone arises through subsequent divisions of pericyclic parenchyma cells outside of the primary phloem (Fig. 5a-c). Then, a new cambium (variant cambium) differentiates in the middle of the meristematic zone (Fig. 5b), whereas some inner layers of cells differentiate into conjunctive tissue (Fig. 5e). This variant cambium produces variant xylem towards the inside and variant phloem towards the outside (Fig. 4c; 5c-d). Subsequently, by the same mechanism of the first variant cambium, new cambia arise successively in centrifugal, concentric order, each originating from the outer derivatives of the preceding meristematic zone (Fig. 5e).

The cambial variant presented here is characterized as successive cambia and give rise to a stem architecture formed by concentric continuous increments of variant xylem and phloem (Fig. 2b). In mature stems, the tangential conjunctive tissue forms a network arrangement with narrow and wide vascular rays (Fig. 5e). The sieve-tube elements and their companion cells form mostly strands surrounded by the tangential conjunctive parenchyma, located at an opposite pole of the radially arranged vessel elements (Fig. 5e-f).

Ontogeny 2 was observed exclusively in the shrubby species of genus *Reichenbachia* (tribe Leucastereae).
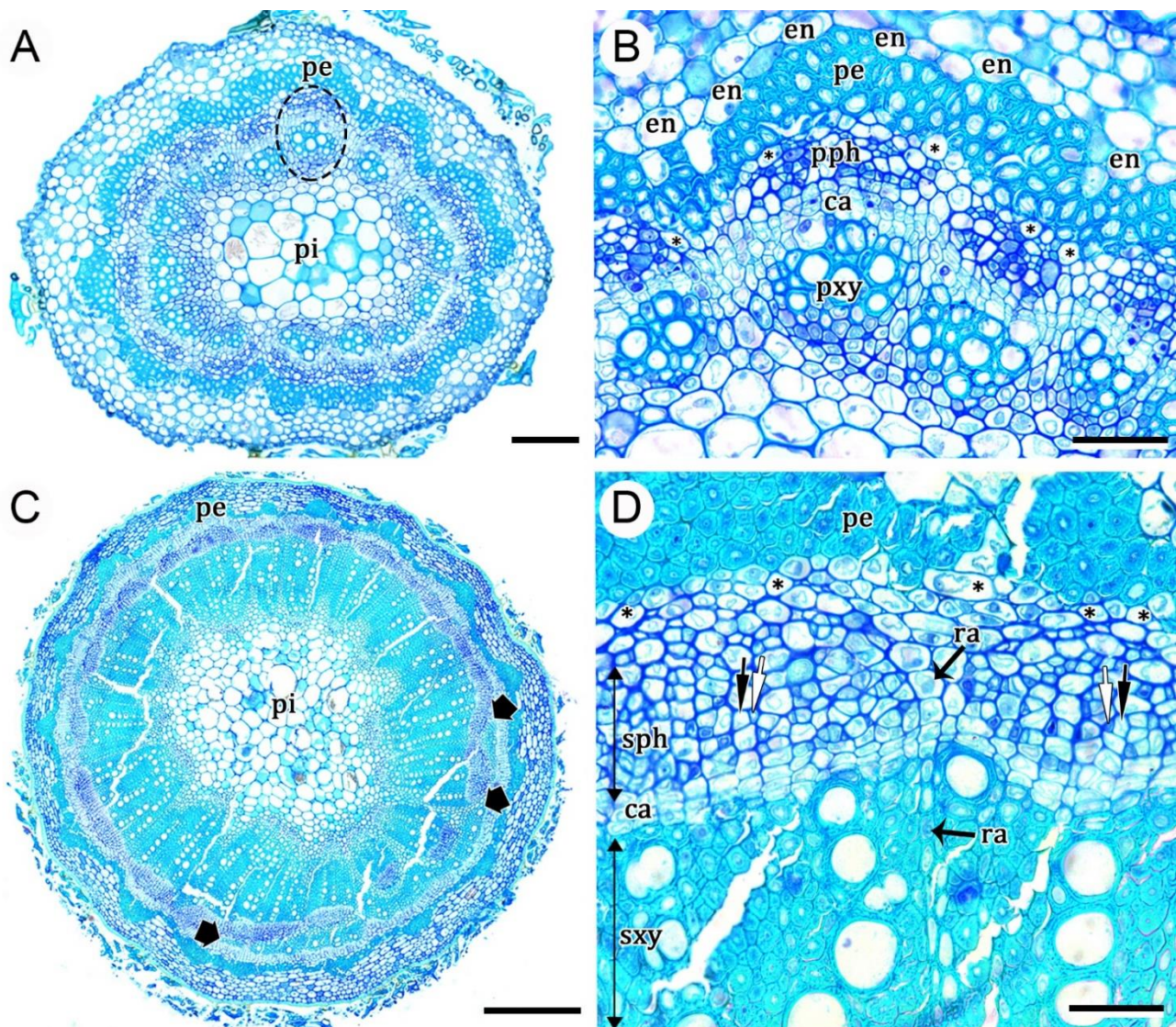
Fig. 4 Stem development in *Reichenbachia hirsuta* (ontogeny 2). **a.** Young stem showing early secondary growth derived from a regular eustele; the dashed ellipse indicates the position of a vascular bundle. **b.** Detail of regular cambium and its derivatives, primary vascular tissues, fibrous pericycle and parenchymatous pericyclic cells (asterisks). **C.** Stem during regular secondary growth. Note the appearance of the first new cambium and its derivatives (thick arrow); see details in next figures (**8 a-d**). **d**. Detail of previous image showing regular cambium producing secondary xylem and secondary phloem. Scale bars: 50 μm (a-c); 400 μm (d). Abbreviations: Arrow (black), sieve-tube element; Arrow (white), companion cell; Asterisks, pericyclic parenchyma cells; ca, regular cambium; en, endodermis; mz, meristematic zone; pe, pericyclic fibres; pi, pith; pph, primary phloem; pxy, primary xylem; ra, ray; sph, secondary phloem; sxy, secondary xylem. (a-d) Stained with toluidine blue.

Ontogeny 3 – **Steps: i. Regular eustele, ii. installation of variant cambium (extra-fascicular cambium), iii. successive cambia (Fig. 2C; 6A-D; 7A-B; 8A-C).**

This pattern differs from ontogeny 2 (see description above) for not forming a regular cambium, even though their primary vascular system is similarly characterized by a regular eustele (Fig. 6A). Similarly to species in Ontogeny 2, the genera under this

ontogeny lack medullary bundles. Instead of forming a regular cambium, the first cambium is already the first variant cambium, differentiated from a meristematic zone formed by divisions of the pericyclic parenchyma cells located between the primary phloem and the fibrous pericycle (Fig. 6b-d). This variant cambium differentiates externally to the vascular bundles (i.e., extra-fascicular cambium), giving rise to secondary xylem produced internally and secondary phloem formed to the outside (Fig. 7a-b). Other additional cambia are formed outwards from remaining cells of the previous meristematic zone, whereas some parenchyma cells constitute the tangential conjunctive tissue between the two increments of vascular tissue. In some cases, the first new vascular tissues are initially formed in relatively small tangential segments (patches) (Fig. 6d), while the following cambia are displayed in more continuous and concentric rings (Fig. 7a-b). However, vascular increments forming confluent bands can also be observed in later developmental stages (Fig. 8c), evidencing that the activity of the new cambia do not always form complete concentric rings. Depending on the organization of the conjunctive tissue, the rings of successive cambia seems can appear forming a wavy appearance (Fig. 8A-B), or more or less concentric rings (Fig. 2c; 8c). Despite that, the sieve-tube elements and their companion cells are formed always at the opposite side of the vessel elements, while conjunctive parenchyma form an intricate network with the vascular rays (Fig. 8b-c).

Ontogeny 3 was observed in the genera *Andradea, Leucaster* and *Ramisia,* which are composed of trees or lianas and all belonging to tribe Leucastereae.

Ontogeny 4 – Steps: i. Polycyclic eustele, ii. cambial zone derived from the cylindrical continuous procambium (CCP), iii. installation of variant cambium, iv. successive cambia (Fig. 2d; 9a-b; 10a-e).

This ontogeny differs from ontogeny 2 for having a polycyclic eustele in the primary vascular system, constituted of medullary bundles and the cylindrical continuous procambium (CCP) (Fig. 9a). The CCP is constituted by the fascicular procambium that originates the vascular bundles and the interfascicular procambium that gives rise to fibres (Fig. 9b). Subsequently, a vascular cambium develops from the procambium and interfascicular procambium; this cambium produces regular secondary xylem and phloem in the usual way, but this activity is maintained for a relatively short
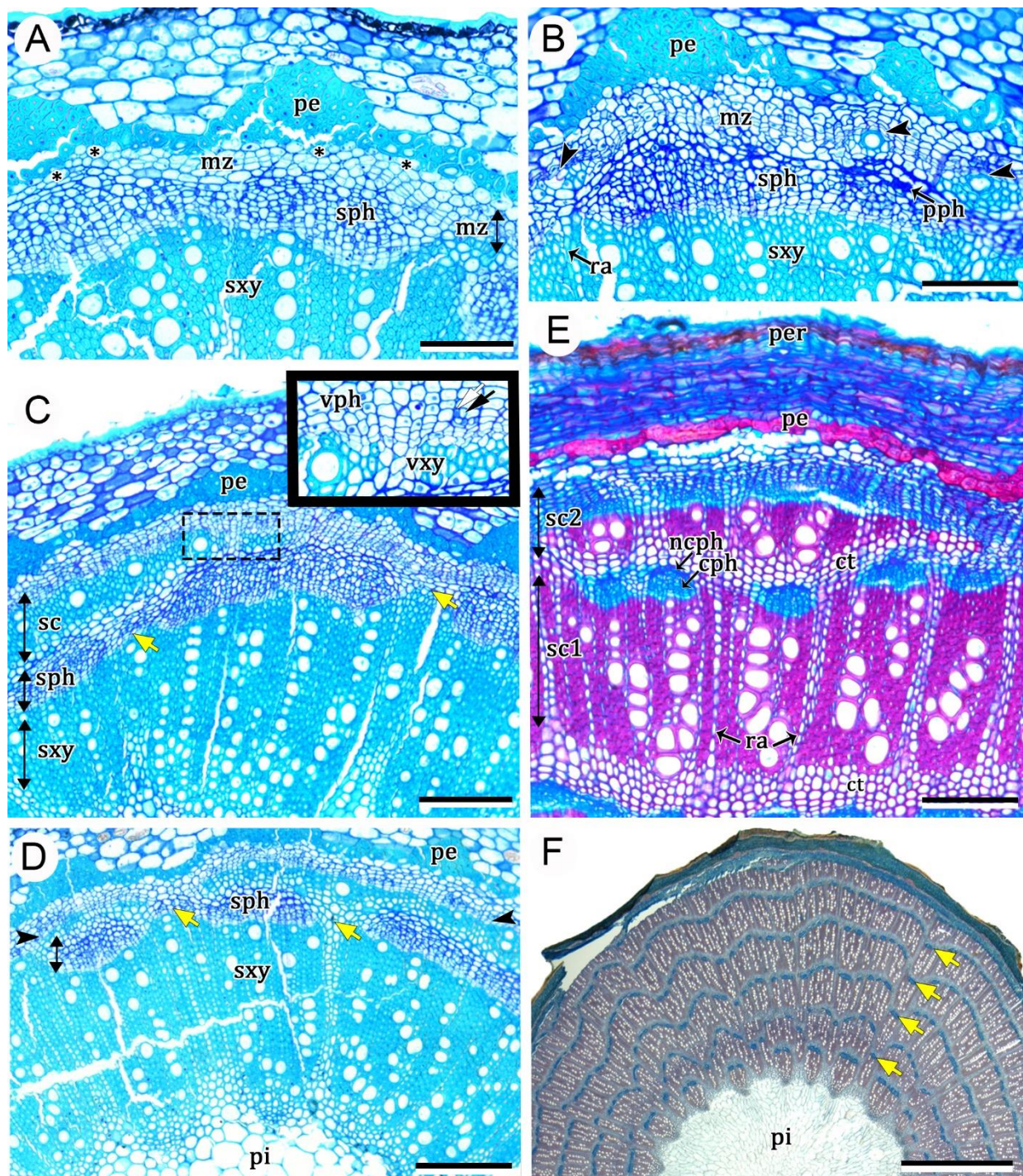
Fig. 5 Development of successive cambia in stems of *Reichenbachia hirsuta* (ontogeny 2). **a.** Meristematic zone; asterisks indicate pericyclic cells that remain parenchymatous. **b.** Development of the first new cambium within the meristematic zone. **c.** First ring of successive cambia with their variant xylem and variant phloem (sc). Dashed rectangle indicates the inset. **d.** Initially, the interfascicular cambium (yellow arrow) produces mostly phloem axial parenchyma to the outside; the vascular tissue may be produced at unequal rates which contribute to irregular bands of vascular tissue (double arrow). **e.** Detail of developed stem showing two rings of successive cambia with conjunctive tissue between them. **f.** Cross view of adult stem showing several rings of vascular tissue; note the irregular regions resulted from differential activity at some regions of the new cambia (yellow arrows). Scale bars: 100 µm (a–c); 200 µm (d–e); 500 µm (f). Abbreviations: Arrow (black), sieve-tube element; Arrow (white), companion cell; Arrowhead, variant cambium; cph, conducting phloem; ct, conjunctive tissue; ncph, non-conducting phloem; mz, meristematic zone; pe, pericyclic fibres; per, periderm; pph, primary phloem (crushed); ra, ray; sc, sc1 e sc2, increments of successive cambia; sph, secondary phloem; sxy, secondary xylem; vph, variant phloem; vxy, variant xylem. (A–D) Stained with toluidine blue. (D–E) Stained with astra blue and safranin.

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period of time (Fig. 10a-b). Later, divisions of the pericyclic parenchyma cells (Fig. 10b) form a meristematic zone, more conspicuous in *Allionia* than in *Okenia*. New segments of cambia develop within the meristematic zone producing new vascular increments composed of variant secondary xylem and phloem in the usual polarity (Fig. 10c-e). This pattern of secondary growth is characterized as a successive cambia system.

Ontogeny 4 was found only in two small genera from tribe Nyctagineae, which are constituted of herbaceous species, i.e., *Allionia* and *Okenia.* Although successive cambia establish in an early period of their lifespan, these species show relatively little amount of secondary tissues even in the most developed stems, as seen in *Allionia incarnata* (Fig. 2d, 10e) and *Okenia hypogae* (Fig. 2D, 9C). In *Allionia,* one or two complete rings of vascular increments are possible (see chapter 2), whereas in *Okenia hypogeae* only small cambial segments of first order were seen in the most developed stems (Fig. 2D; 10a).

Character mapping and phylogenetic reconstruction

To assess the evolution of stem development in Nyctaginaceae, the ontogenies were mapped onto the current phylogeny of the family. Each of the four ontogenetic pathways were delimited as character states. The phylogenetic analysis showed that ontogeny 1 (polycyclic eustele + interxylary phloem) is the most common and was reconstructed as the ancestral state (79% presence), with a few secondary losses (Fig. 11, e.g., Leucastereae, Nyctagineae). Ontogeny 4 (polycyclic eustele + successive cambia) evolved at least twice, being found in genera belonging to crown clades (i.e., Nyctagineae) that includes the majority of the herbaceous species,
whereas ontogeny 2 (regular eustele + regular cambium + successive cambia) and ontogeny 3 (regular eustele + extra-fascicular cambium + successive cambia) evolved each only once, occurring in genera of tribe Leucastereae (the clade sister to the rest of the family) which is formed by shrubs, trees and/or lianas.

Successive cambia and interxylary phloem occur in species with distinct habits (Fig. 11, 12), which has an ambiguous ancestral reconstruction for the ancestral node of the family (Fig. 12).
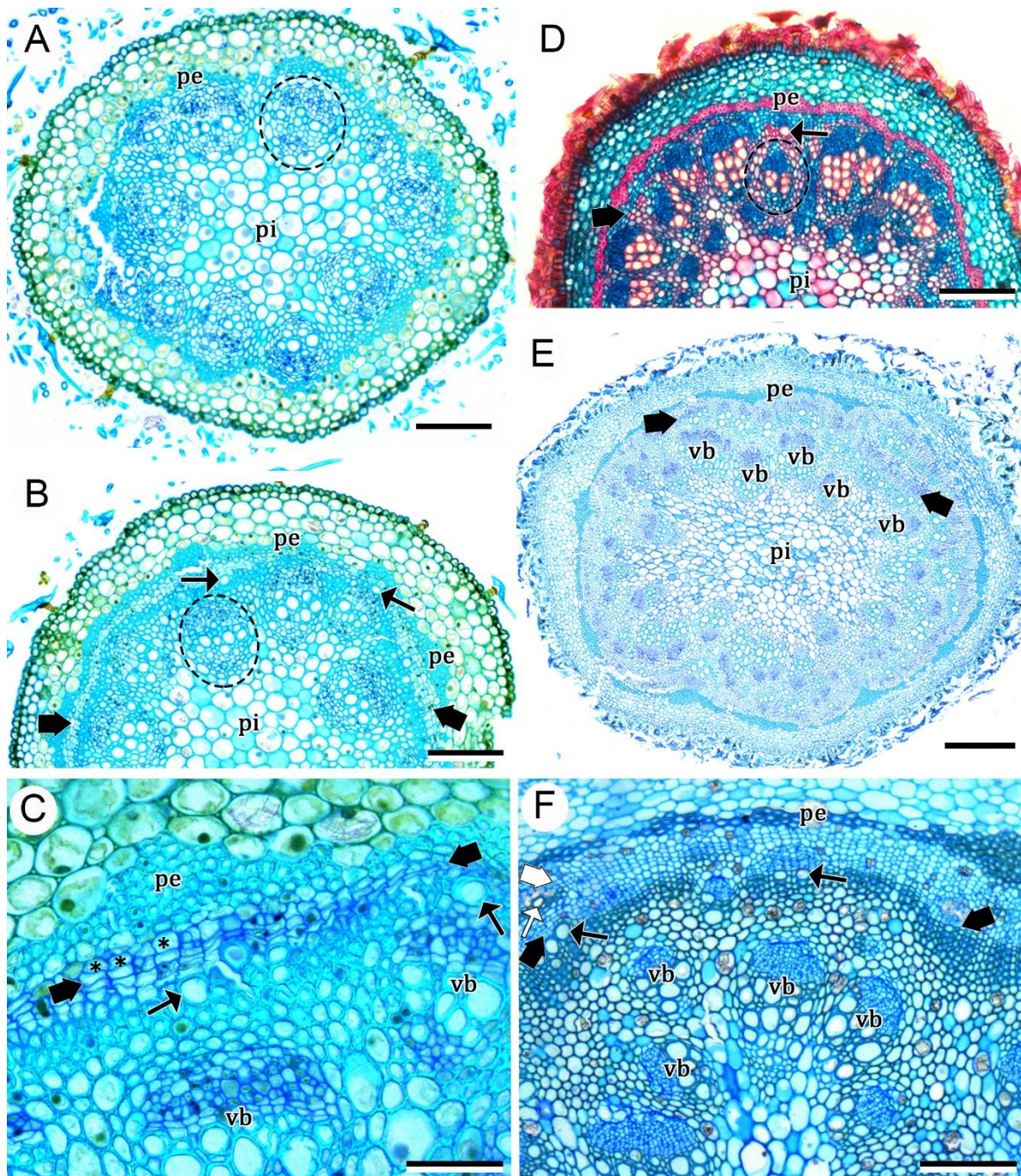
Fig. 6 Development of successive cambia in stems of *Andradea floribunda, Leucaster caniflorus* and *Ramisia brasiliensis* (ontogeny 3). **a–c.** *Leucaster caniflorus.* **a.** Young stem showing vascular bundles (dashed ellipse) forming a regular eustele. **b.** Initiation of secondary growth through the formation of the extra-fascicular cambium (thick black arrows) and first formed vessels of the secondary xylem (thin black arrows). **c.** Detail of the extra-fascicular cambium (thick black arrows) and first formed vessels (thin black arrows); note the pericycle parenchyma cells (asterisks), and the extra-fascicular cambium (variant) **d–f.** *Andradea floribunda.* **d** and **e.** Note the extra-fascicular cambium and their vascular products (thick black arrows) with the first formed vessels (thin black arrows), externally to the vascular bundles (dashed ellipse in **d**). **f.** Detail of the extra-fascicular cambium (thick black arrows) and first formed vessels of the secondary xylem (thin black arrows) and the subsequent ring of successive cambia and their products (thick white arrows), with the first formed vessels of the successive cambia (thin white arrows). Scale bars: 100 µm (a-b, d); 50 µm (c); 400 µm (e); 200 µm (f). Abbreviations: pe, pericyclic fibres; pi, pith; vb, vascular bundles of regular eustele. (a–d, e-f) Stained with toluidine blue. (c) Stained with astra blue and safranin.

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Divergence times and diversification rate estimation

We estimated the age of divergence of Nyctaginaceae and close relatives with BEAST2 (Fig. 13). We obtained the Maximum Clade Credibility tree (Fig. 13), which shows the mean age for the main nodes in the phylogeny. Table 1 shows the mean crown age estimates for the major clades and its associated credibility interval represented by the 95 % Highest Posterior Density (HPD). Nyctaginaceae probably diverged from its sister group (stem age), Phytolaccaceae, 48 Ma (HPD: 38.97–55.56 Ma) and diversified (crown age) 42.3 Ma (HPD: 32.82–47.59 Ma), both events occurring at the Middle Eocene.

Results from the diversification rate estimation are observed in the phylorates plot of the Maximum *a posteriori* probability (MAP) configuration tree shown in Fig. 14, where the estimated rate at each segment of the branches is the mean of the marginal posterior density of the diversification rate. The posterior probability included a set of changes in the diversification rate (i.e., speciation minus extinction), where two shifts (green circles in Fig. 14) in diversification rate are strongly supported. One diversification increase derived from a rise in the speciation rate alone in the clade Pisonieae-Bougainvilleae-Nyctagineae, and another diversification increase derived from rises in both speciation and extinction rates in bulk of *Commicarpus*.

Discussion

The evolution of cambial variants in Nyctaginaceae may represent an example of continuum morphology

Although successive cambia and interxylary phloem have been recognized in the literature as two types of cambial variants, their occurrence in Nyctaginaceae indicates that there is a blurry boundary delimitation between these two patterns. Here we emphasize that the diversity of these two cambial variants in Nyctaginaceae is represented by distinct intermediate forms and seen as an example of continuum morphology in two levels ([60,61] – also known as fuzzy morphology or Fuzzy Arberian Morphology [62]). First, plants with ontogeny 1 that are characterized with interxylary phloem may present phloem strands immersed within the xylem with different arrangements (i.e., phloem islands, patches or bands). These arrangements result
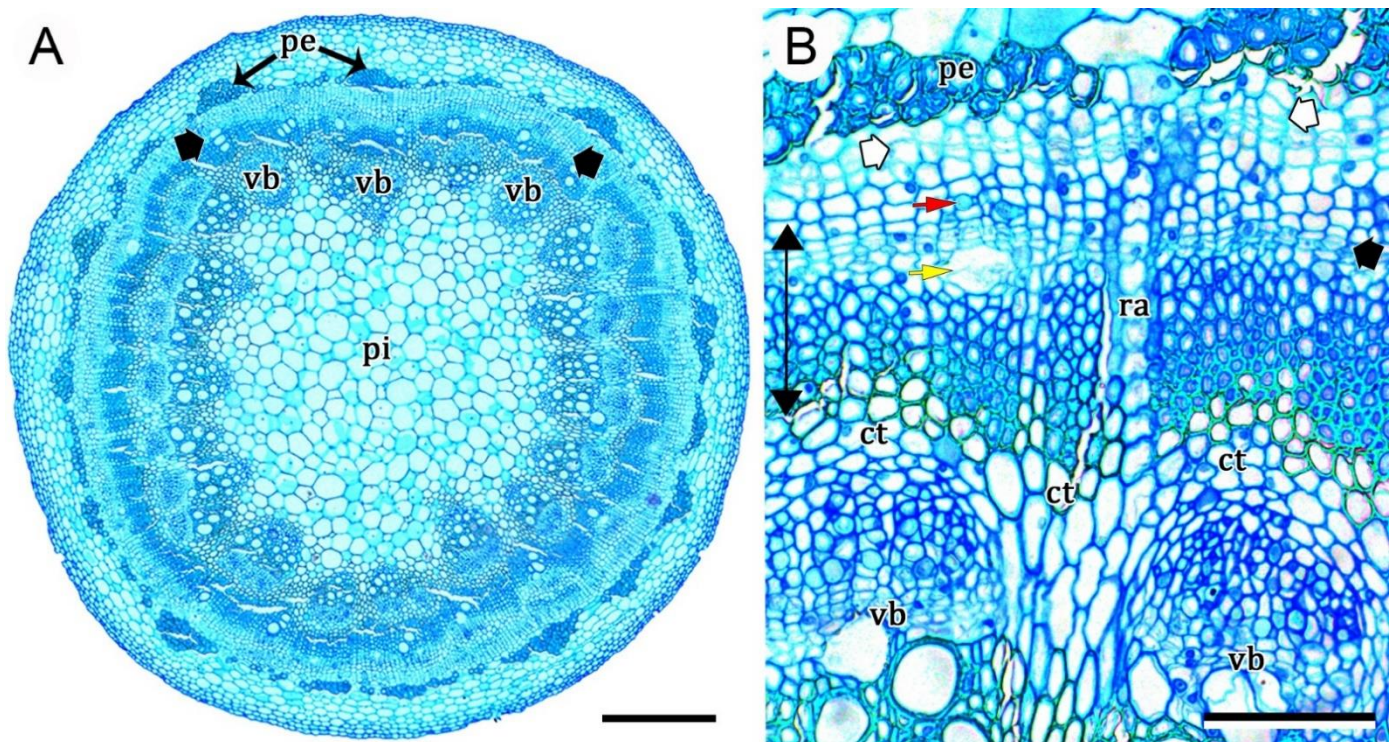
Fig. 7 Details of the extra-fascicular cambium and first successive cambia in *Ramisia brasiliensis* (ontogeny 3). **a.** General view of the stem during the establishment of the extra-fascicular cambium (arrows) outside the vascular bundles of the eustele. **b.** Secondary vascular tissue (double arrow) originated from the extra-fascicular cambium (black arrow). The yellow arrow indicates a vessel element, and the red arrow indicates a sieve-tube element and its companion cell. Note the developing meristematic zone (white arrows), still with little layers of cells, and that will give rise to the subsequent ring of successive cambia; also note the conjunctive parenchyma cells between the vascular bundles and the first ring of successive cambia. Scale bars: 400 μm (a); 100 μm (b). Abbreviations: ct, conjunctive tissue; pe, pericyclic fibres; pi, pith; ra, ray; vb, vascular bundle of the eustele. (a, b) Stained with toluidine blue.

from different extensions of the coalescent cambium which is formed in continuity with the single cambium and encloses the phloem strands. Given that in plants with bands the stem initiates forming phloem islands followed by patches and then bands, the development of this ontogeny itself indicate the existence of a continuum between the different stem macromorphologies. Second, the ontogenies characterized with successive cambia shows that the independent cambia arise and form usually long tangential bands of vascular tissues similar to the phloem strands in bands of some species with interxylary phloem (the type of cambial variant present in the ancestor of the family). In this sense, with the use of fuzzy morphology, we can propose that the successive cambia arise from interxylary phloem due to the larger extension of
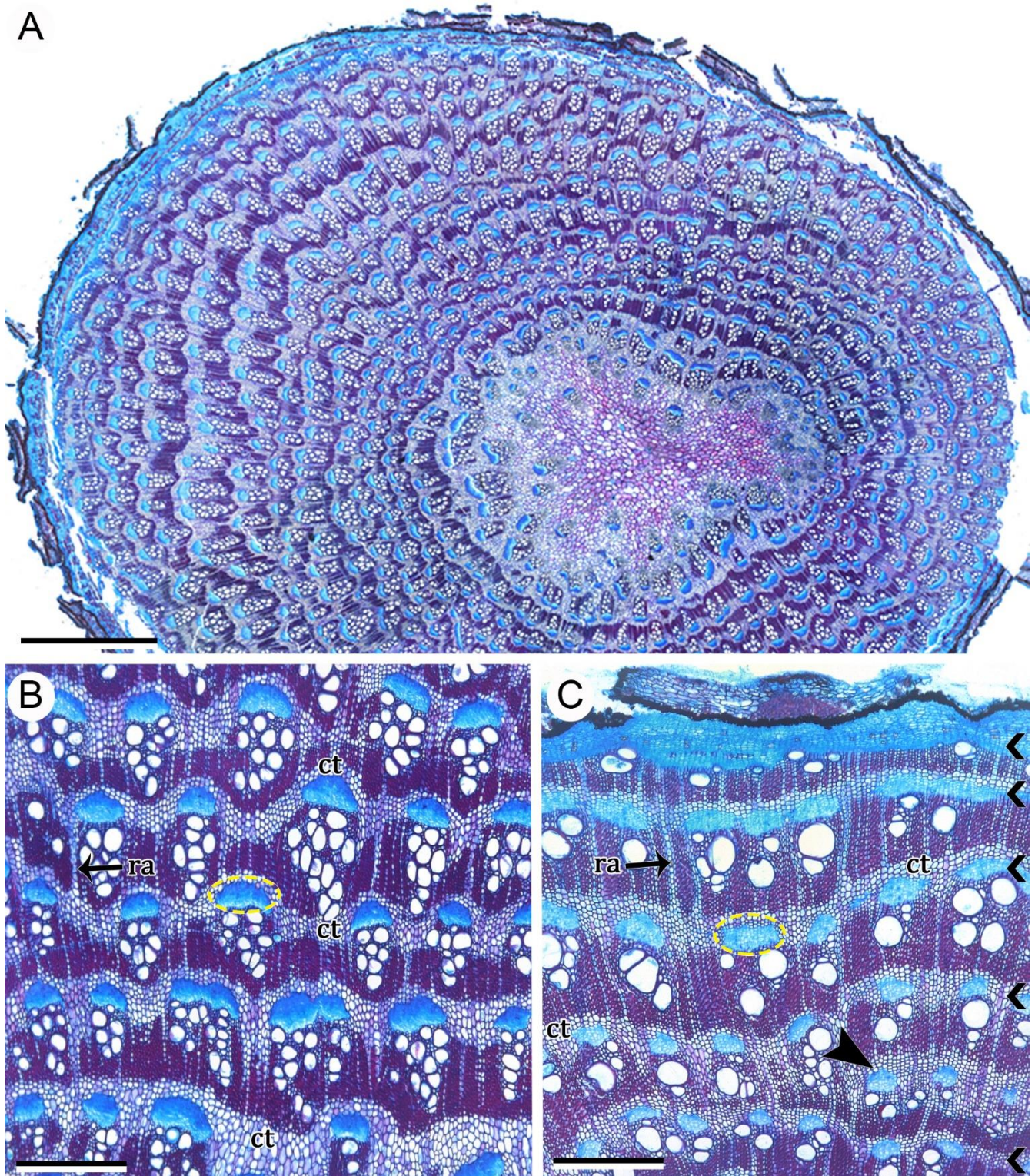
Fig. 8 Cross view of developed stems with successive cambia (ontogeny 3). **a** and **b**. *Andradea floribunda*. **c**. *Leucaster caniflorus*. **a**. Adult stem showing several rings of successive cambia. **b**. Detail of the successive cambia and their vascular products; the phloem form small strands (yellow ellipse) and are composed mainly by sieve-tube elements and their companion cells bordered by the conjunctive tissue; note the wavy appearance of the new increments caused by the conjunctive tissue. **c**. Rings of successive cambia in more or less regular concentric arrangement (pointers); the arrowhead indicates an incomplete segment between the other upper and lower rings; note the phloem forming small strands (yellow ellipse). Scale bars: 2000 μm (a); 500 μm (a-b). Abbreviations: ct, conjunctive tissue; pe, pericyclic fibres; pi, pith; ra, ray. (a-c) Stained with astra blue and safranin.

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coalescent cambium until it is so large that in appearance it can be considered an independent additional cambium.

The fuzzy worldview has been used to distinct morphological systems especially in organ identity [63–66]. Here for the first time the diversity of cambial variants is interpreted under the concept of continuum morphology. These observations for Nyctaginaceae enriches our understanding of these complex vascular morphologies because it gives us the notion of how ontogenies changed across evolutionary time producing intermediate forms that at some point can be distinguished as discrete categories.

The origins and developments determining the cambial variants in Nyctaginaceae

Different developmental pathways accounts for the disparate secondary vascular architectures observed in Nyctaginaceae. The recognition of interxylary phloem along with the occurrence of successive cambia is based primarily on their differences in development, i.e., single vs. multiple cambia [35 - chapter 4], but these developmental pathways result in architectonically similar anatomies and to a certain level can be considered to integrate into intermediate forms.

Here we identified that to the four ontogenies, all events share an origin to their secondary growth internally to the pericyclic fibres. These findings contradict that the cambial variants in Nyctaginaceae are formed from a meristem arising in the cortex as previously suggested [29,39–41,67]. Instead, all cambial variants originated from procambial-derived   cells (i.e., pericycle or cambium), as demonstrated by other authors [37,38 - chapter 2]. These findings confirm that multiple origins (e.g., primary phloem, secondary phloem, cortex - [32,68–71]) and developmental trajectories, as we will see below, can lead to the formation of successive cambia, unlike the idea of a universal phenomenon for the formation of this cambial variant across different plant groups [39,67,72]. Indeed, successive cambia can be established from different stem regions, such as primary phloem [68], secondary phloem [69], pericycle [32,70], or cortex [71]. Nevertheless, except for the cortex and secondary phloem, in all other cases the origin of the new cambium can be considered developmentally linked with a pre-existing vascular meristem because both pericycle and primary phloem are produced by the procambium. A similar developmental parallel can be established for
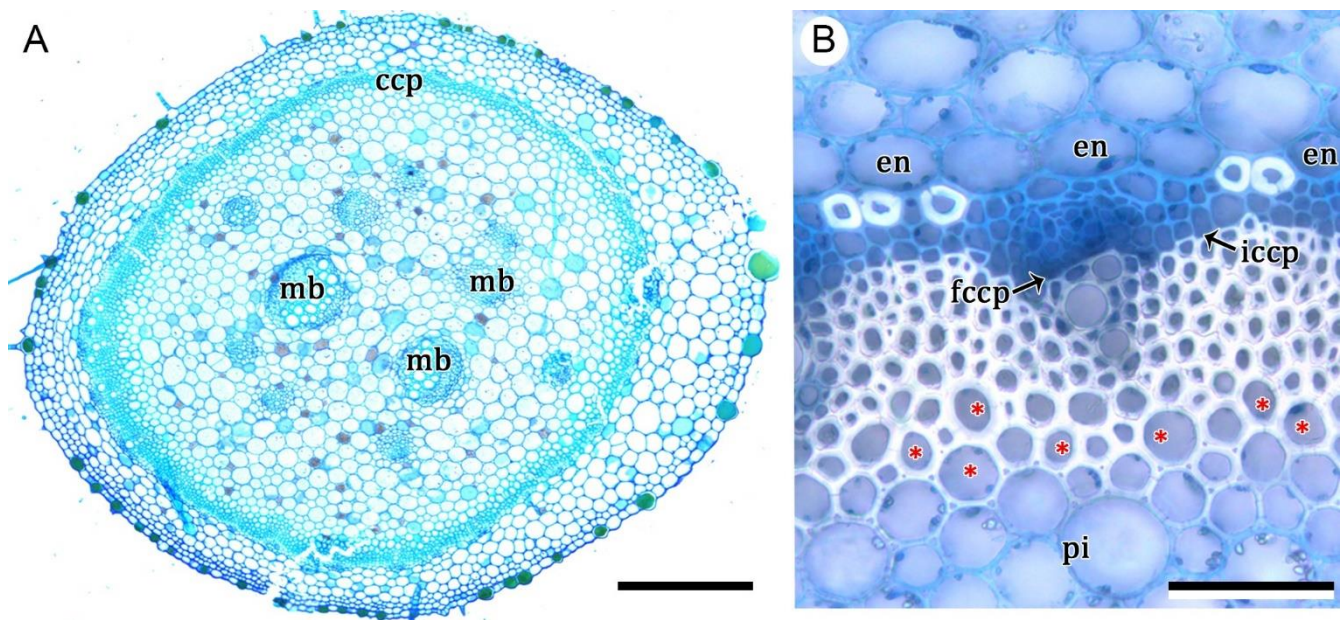
Fig. 9 Cross sections of *Okenia hypogaea* (ontogeny 4) in primary growth. **a.** Note the polycyclic eustele with medullary bundle and cylindrical concentric procambium. **b.** Detail of fascicular procambium (fccp) forming the vascular bundles and interfascicular procambium (iccp) forming mostly xylem fibres; note also pericyclic fibres (large fluorescent cells beneath the endodermis) and lignification of peripheral pith cells (red asterisks). Scale bars: 200 μm (a); 100 μm (b). Abbreviations: ccp, cylindrical concentric procambium; en, endodermis; fccp, fascicular CCP; iccp, interfascicular CCP; mb, medullary bundles; pe, pericyclic fibres; pi, pith. (a) Stained with toluidine blue; (b) Stained with aniline blue.

the origin of interxylary phloem and successive cambia in Nyctaginaceae because both the cambium giving rise to phloem strands within the secondary xylem (interxylary phloem) and the meristematic zone producing a *de novo* cambium (successive cambia) may be traced back to the procambium at some point in stem development. Therefore, successive cambia and interxylary are evolutionarily linked in Nyctaginaceae.

Although the cambial variants in Nyctaginaceae present similar origins at the cell lineage level (i.e., procambium-derived cells), the eustele types and subsequent events in their development are diverse, leading to four distinct ontogenies. In a previous work we showed that the origin and development of interxylary phloem in Nyctaginaceae is similar to that shown in other groups, except for the fact that the single cambium is originated from the cylindrical concentric procambium (CCP), which is part of the polycyclic eustele, that   also   includes   the medullary bundles [35 - chapter 4]. Although ontogenies 2, 3 and 4 are characterized as successive cambia at maturity, they are built upon three different step-wise developmental

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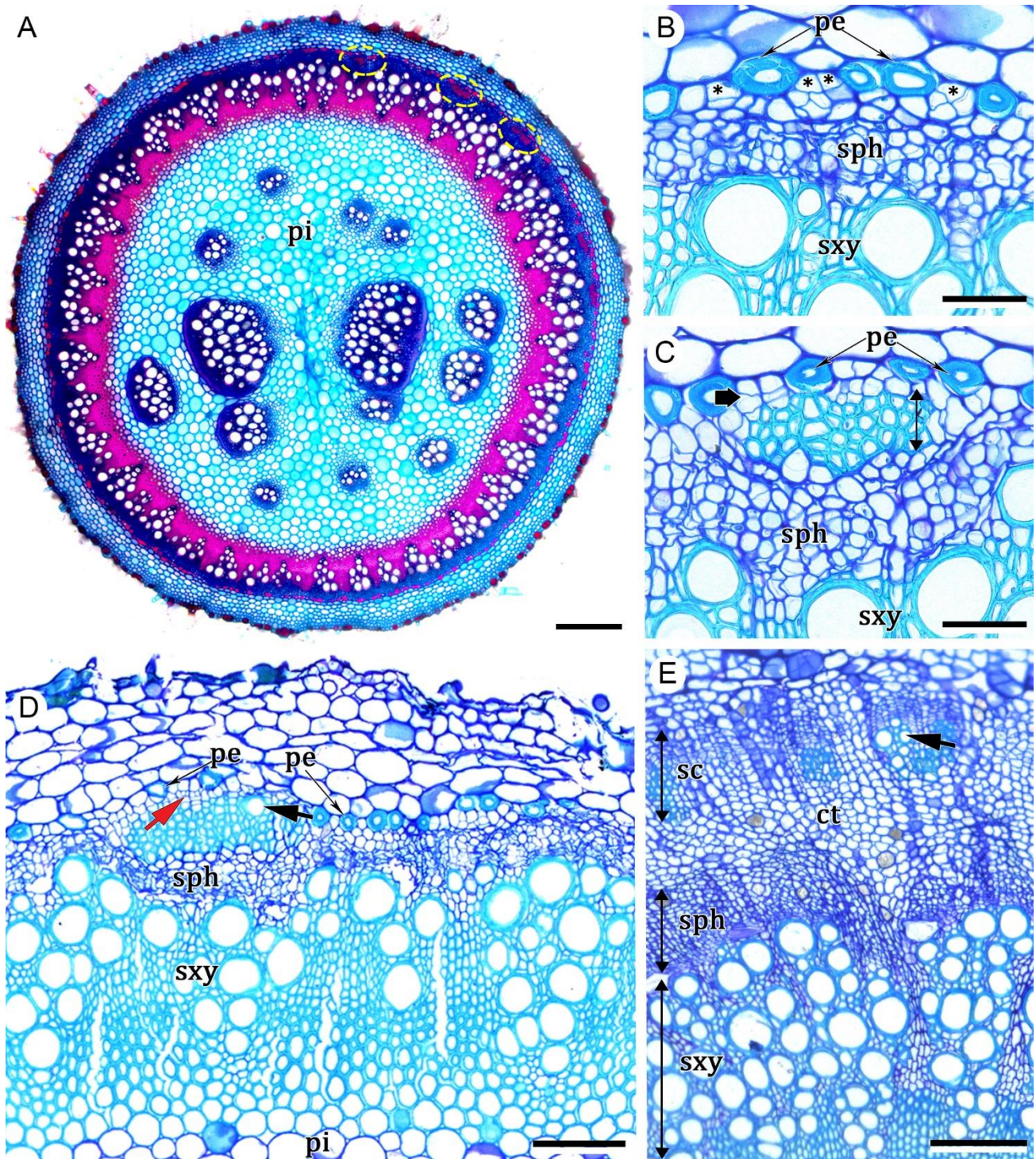
Fig. 10 Development of successive cambia in stems of *Okenia hypogaea* and *Allionia incarnata* (ontogeny 4). **a-d.** *Okenia hypogaea.* **e.** *Allionia incarnata.* **a.** Stem with cambial variant. Dashed ellipses indicate the successive cambia; see detail in figures 10d; note the medullary bundles in the pith. **b.** Regular xylem and regular phloem. **c.** Developing variant cambium (thick arrow) and variant xylem formed mostly by fibres (double arrow). **d.** Developing arc of successive cambia showing variant xylem with first formed vessel (black arrow) and secondary phloem with sieve-tube elements (red arrow). **e.** Regular xylem and regular phloem separated from the first ring of successive cambia by conjunctive tissue. Black arrow indicates vessel from the variant xylem. Scale bars: 400 μm (a); 50 μm (b, c); 100 μm (d, e). Abbreviations: mb, medullary bundles; pe, pericyclic fibres; ct, conjunctive tissue; pe, pericyclic fibers; pi, pith; sph, regular secondary phloem; sxy, regular secondary xylem. (a) Stained with astra blue and safranin. (b-e) Stained with toluidine blue.
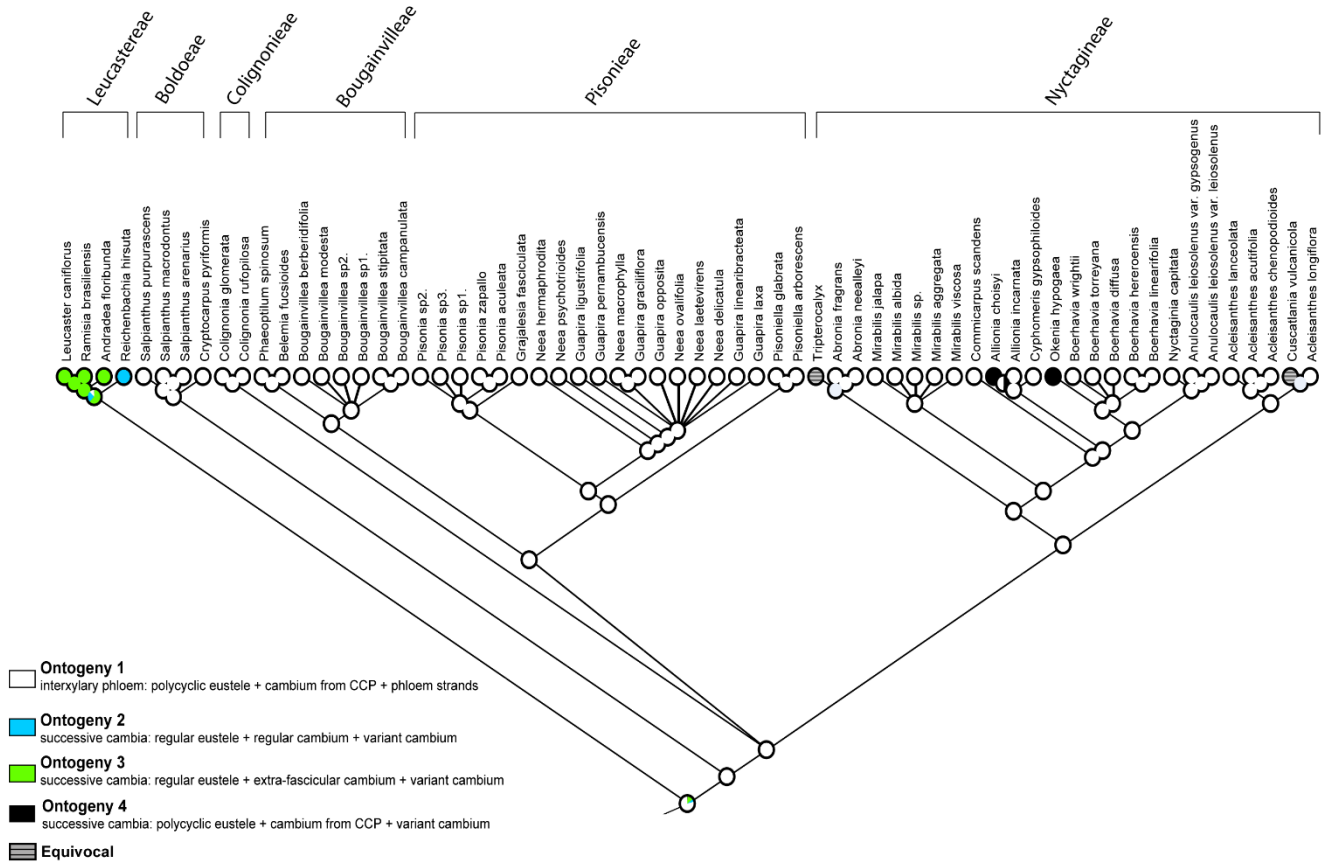
Fig. 11 Maximum likelihood reconstruction of ontogenetic pathways mapped with Mesquite on a phylogenetic tree of Nyctaginaceae based on Cunha Neto et al. (2020).
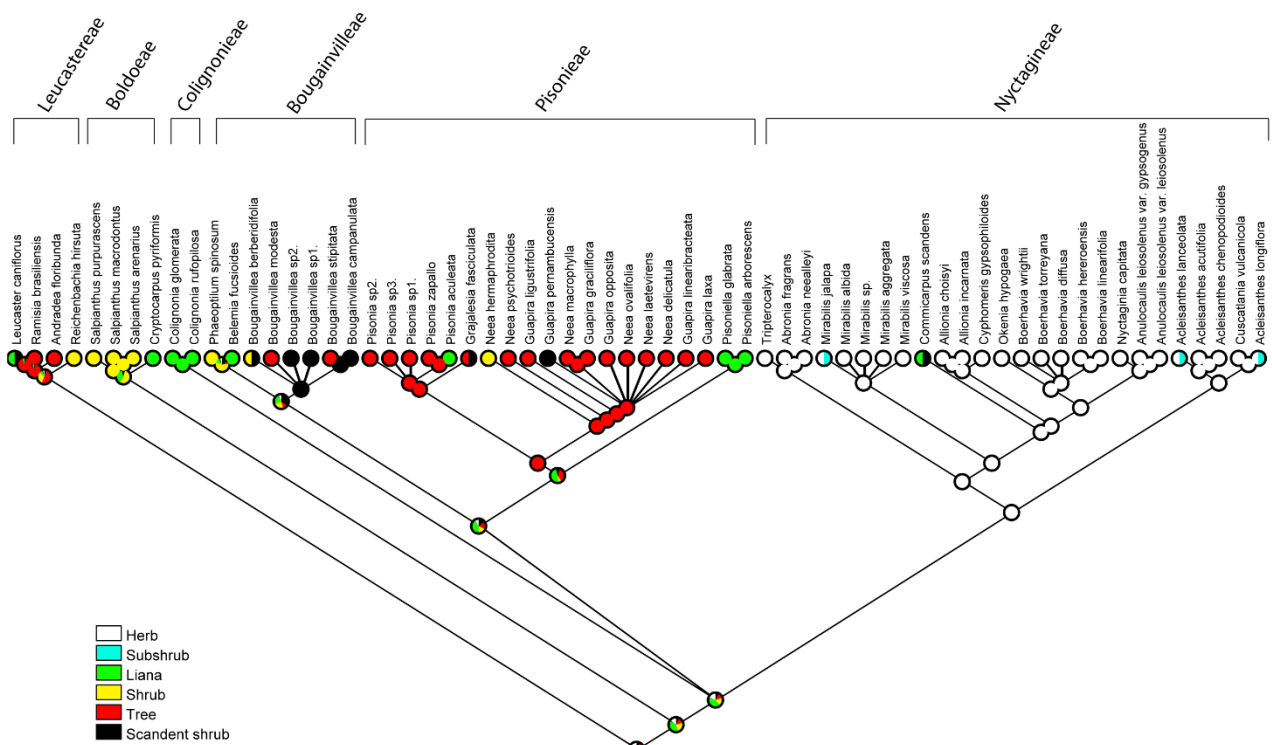
Fig. 12 Maximum likelihood reconstruction of habits mapped with Mesquite on a phylogenetic tree of Nyctaginaceae based on Cunha Neto et al. (2020).

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pathways. The developmental steps in the formation of successive cambia in *Reichenbachia* (ontogeny 2) is the same as described in most families with this cambial variant, i.e., a new cambium is formed *de novo* (mostly but not always) from the pericycle in stems with regular eustele; this is the case of species both in the gymnosperms (e.g., *Gnetum, Cycas* – [39]) and angiosperms from several families (e.g., Menispermaceae [73]; Convolvulaceae [32]; Sapindaceae [70]). On the other hand, successive cambia as described in ontogenies 3 and 4 differ from the taxa mentioned above because they either do not produce a regular cambium, forming an extra-fascicular cambium (ontogeny 3) or because the stem begins with a polycyclic eustele and the first cambium is derived from the CCP instead of a regular cambium (ontogeny 4). It is important to highlight that the appearance of the extra-fascicular cambium, which is independent from the primary vasculature, corroborates the potential of perivascular tissues (i.e., the pericycle) to produce new meristems, as it is observed in species with successive cambia following ontogeny 2 or 4. In addition, except for ontogeny 1, all other developmental pathways in Nyctaginaceae include a *de novo* formation of new cambia.

Besides the presence of distinct developmental pathways, Nyctaginaceae stands out for having all extant lineages characterized by some type of variant anatomy (see discussion on 'evolution of development' below). Regular vascular growth with eustele and a regular cambium forming xylem centripetally and phloem centrifugally as observed in most eudicots is observed only during the initial developmental stages of *Reichenbachia* (ontogeny 1), which later develops successive cambia. Species with ontogeny 4 also have a short period of secondary vascular tissues being produced by a bifacial cambium in the central cylinder, but it is developed from a polycyclic eustele [38 - chapter 2, this study].

Our observations on the origin and development of vascular meristems in Nyctaginaceae as presented here and in previous studies [26 - chapter 3, 35 - chapter 4, 38 - chapter 2] challenge a number of interpretations raised in previous investigations: (1) Cambial variants in Nyctaginaceae arise in the cortex [29,39,67] – we showed that the origin of cambial variants is from the pericycle (which is procambium-derived) ([38 - chapter 2, this study). (2) Successive cambia are the only type of cambial variant in Nyctaginaceae [29,39,58,68] – two types of cambial variants
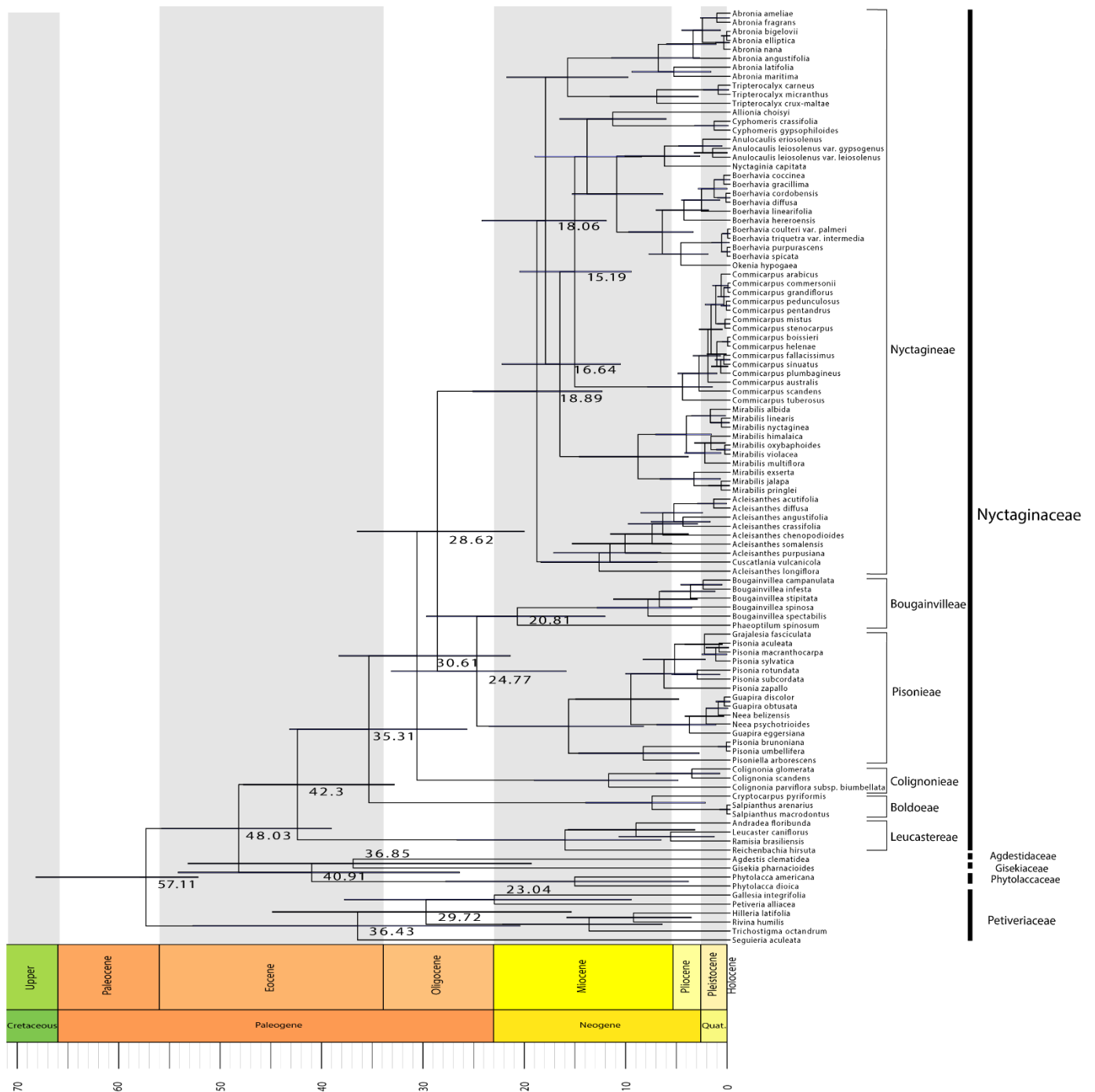
Fig. 13 Maximum clade credibility tree (MCC) with divergence time estimates for Nyctaginaceae and related families. Numbers correspond to mean age estimates (in million years). Bars indicate age confidence intervals from a 95% Highest posterior density (HPD).

Table 1 Divergence-time estimation of mean crown ages in Million years (Ma). Highest posterior density interval (95% HPD).

Family	Crown age (Ma)	95 % HPD
Nyctaginaceae	42.3	32.82–47.59
Phytolaccaceae	40.91	26.45–53.95

occur in Nyctaginaceae, interxylary phloem and successive cambia (35 - chapter 4, this study). (3) The existence of an 'extra-fascicular cambium' forming vascular bundles [27] – the extra-fascicular cambium produces secondary vascular tissues, observed exclusively in ontogeny 3 [this study]. (4) The presence of an unidirectional cambium forming both secondary xylem and secondary phloem to the inside [75] – instead of an unidirectional cambium we found that a single (bidirectional) cambium acts in the formation of interxylary phloem [35 - chapter 4, this study]. (5) The presence of a "Primary Thickening Meristem" similar to the monocots [76–78] – the presence of medullary bundles was probably the main reason for this interpretation, and we have demonstrated that these vascular structures arise from a cylindrical continuous procambium, different from what is observed in the monocots [26 - chapter 3, 38 - chapter 2].

In addition, because the vessel elements and sieve-tube elements are usually restricted to short tangential areas resembling discrete "vascular bundles", authors have used different terminologies to describe the secondary vascular tissues in stems of Nyctaginaceae, such as "secondary medullary bundles" [79], "vascular strands" [80], "vascular bundles" [28,81] or "secondary bundles" [68].

The evolutionary history of the diverse cambial variants in Nyctaginaceae

The phylogenetic distribution presented here demonstrates that the ancestor of Nyctaginaceae already had cambial variant, with interxylary phloem (ontogeny 1) reconstructed as the most likely character state for the ancestral node of the family. This observation is remarkable because it indicates that Nyctaginaceae is one of the few examples where the cambial variants are present in all members, and is likely shared with its sister group, the phytolaccoid clade, being therefore plesiomorphic
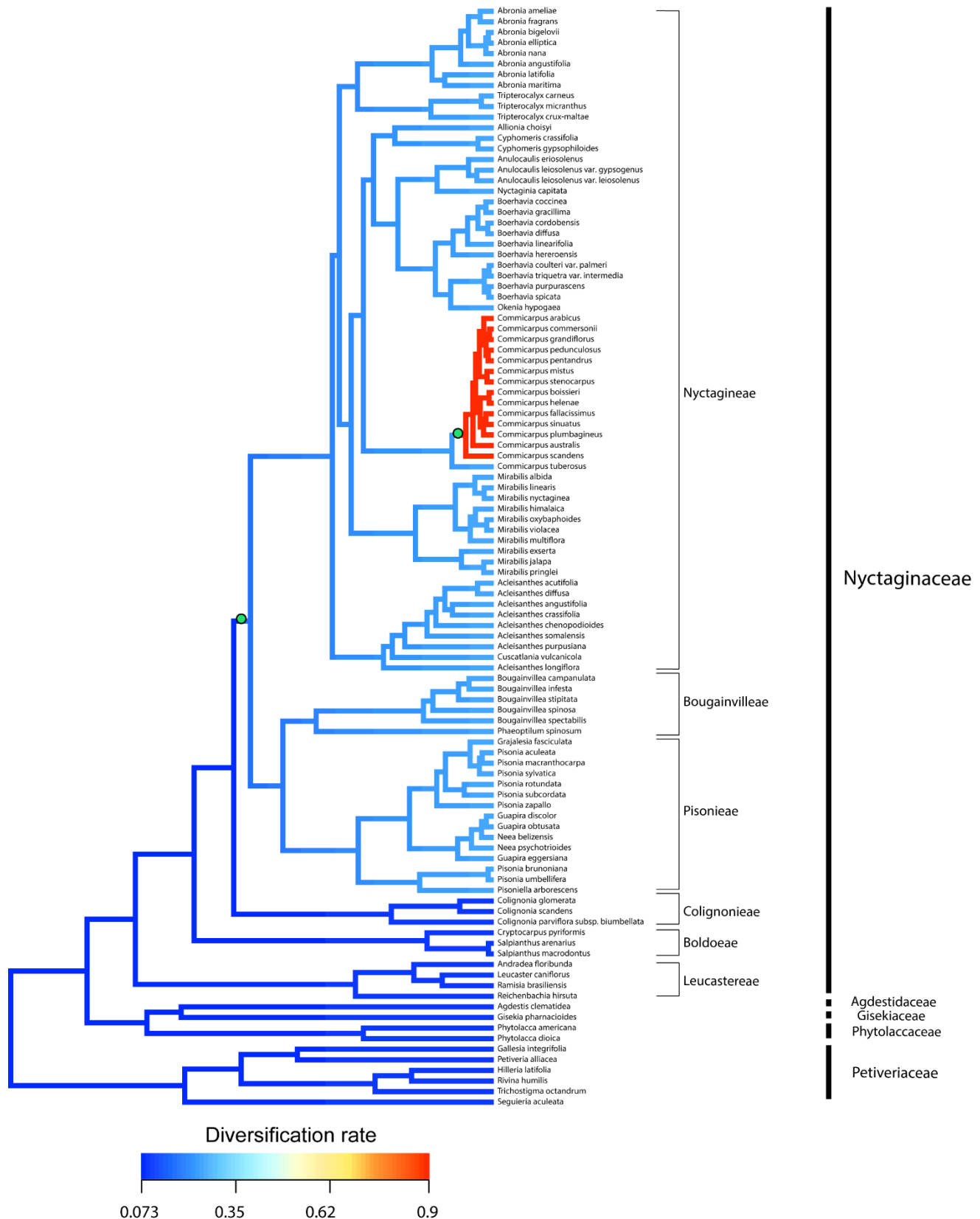
Fig. 14 Net diversification rate dynamics in Nyctaginaceae and most-closely related families estimated by BAMM. Branch color reflects the mean of the marginal posterior density of net diversification rates for each segment of the branches, with rates increasing from blue to red. Two green circles indicate the most probable rate shift configuration found using BAMM.

for Nyctaginaceae. In most families with cambial variants they appear only in one group, mostly in clades containing lianas or descending from lianas (e.g., Bignoniaceae, Convolvulaceae) [11,12,31,82]. Contrary to expectations, interxylary phloem (ontogeny 1) is also the most common type of cambial variant in the family, occurring in five out of seven tribes, inclusive in those most studied genera such as *Bougainvillea, Boerhavia, Mirabilis* and *Pisonia* that used to be classified as having successive cambia (reviewed by [35 – chapter 4].

The ontogenies are not evolutionary labile since each of them appeared only once, except for ontogeny 4 that evolved twice. The evolution of ontogenies 2 and 3 in members of Leucastereae is interesting because the tribe has other morphological (e.g., type of trichomes, pollen and fruit – [42,76,77] and vascular anatomical characters (e.g., type of stele, 26 – chapter 3) that are exclusive or unusual if compared to other lineages of the family. Curiously, from all stem ontogenies of Nyctaginaceae, the pattern observed exclusively in *Reichenbachia* (ontogeny 2) is the only taxon following the commonly described development for successive cambia, i.e., regular cylinder + successive cambia [29,39,67].

Ontogeny 4 is the only type with more than one evolution with two transitions in tribe Nyctagineae. The evolution of successive cambia in *Allionia* and *Okenia* is noteworthy for the fact that they are both small herbs with limited secondary growth in the regular cylinder, but the successive cambia still develop in some way. However, this might not be surprising since the presence of cambial variants in other herbs (annuals or perennials) is reported in many sister-related families across the Caryophyllales [67,85]. This observation suggests that if given time to grow, most herbaceous plants in this lineage can form new additional rings of successive cambia or as presented here, forming new phloem islands/patches/bands as in species with interxylary phloem. Nevertheless, the occurrence and diversity of variant anatomies in Nyctaginaceae seem to be not contingent on specific habits, since both cambial variants occurs in species with all the range of growth forms in the family.

The development and evolution of distinct patterns of cambial variants in Nyctaginaceae is remarkable because successive cambia has been reiterated as the only cambial variant in the family [29,41,67,85] and considered one of the notorious morphological convergent adaptations for the group along with other biological

features (e.g., betalain pigmentation [86,87]; floral morphology [88–90]; ecophysiological adaptations [91]). Although we observed multiple ontogenetic pathways resulting in distinct cambial variants in Nyctaginaceae, similar patterns may also be present in other caryophyllalean families (35 - chapter 4). Comparably to other traits that seem to represent apomorphic tendencies (e.g., floral morphology [88–90]), the presence of cambial variants likely share developmental and genetic programs (deep homology) triggering the recurrent evolution of this morphological feature in multiple Caryophyllales lineages.

Evolution of development: how different ontogenies generate similar stem macromorphologies

Because ontogeny is a linear process and given that primary and secondary vascular tissue may have intrinsic developmental relations, the investigation of the diversity and evolution of vascular anatomies in Nyctaginaceae needs to include the products of procambium, cambium and cambial variants to thoroughly comprehend the anatomical and developmental shifts in stem ontogeny. Here the integration between ontogeny and phylogeny showed that adult stems with distinct cambial variants evolved from different eustele types. Therefore, the organization of the primary vascular system is not a prerequisite for the evolution of patterns of secondary growth in Nyctaginaceae, achieved through distinct ontogenetic pathways. This scenario diverges from the evolution of secondary growth patterns in stems of Bignoniaceae [11] or *Paullinia* from the Sapindaceae [12] where cambial variants trace back to stems with regular growth in previous stem developmental stages.

For the evolution of interxylary phloem in the ancestor of Nyctaginaceae, several developmental modifications were needed in stem development of the putative ancestor of inner nodes of the phylogeny of the Caryophyllales, which likely had a regular anatomy. Considering that some of the anatomical changes include: i) the decrease in the formation of xylem derivatives at specific locations, ii) the increased phloem production at the same location and iii) the development of the flanking cambium. Similarly, considering that the ancestor of Nyctaginaceae had interxylary phloem (ontogeny 1), the appearance of each new ontogeny requires a set of transformations in the developmental pathway of its ancestor. First, in the case of

ontogeny 3, the main steps include the loss of the regular cambium and the appearance of the extra-fascicular cambium, which is probably regulated by the same mechanism leading to the evolution of the new cambium from the pericycle in ontogeny 2, that result in the formation of successive cambia. The difference from ontogeny 2 to ontogeny 3 is that the later regained the regular cambium and form regular xylem and phloem for some period. For the evolution of ontogeny 4, the evolution of new cambia from the pericycle as in ontogeny 2 and 3 is also observed, but this time the primary vascular system is characterized by the polycyclic eustele, while the main cambium produces regular tissue for a shorter period of time compared to ontogeny 2. Surprisingly, at maturity, the stem macromorphology of all ontogenies in Nyctaginaceae are alike, especially the ones with successive cambia (e.g., *Reichenbachia* – ontogeny 2) or interxylary phloem forming bands (e.g., *Bougainvillea, Colignonia* – ontogeny 1). Species with interxylary phloem forming only phloem islands are more easily distinguished in stem topology, although the development follow virtually the same steps in species with patches/bands, except for the length of the coalescent cambium.

Evolutionary mechanisms are hard to be interpreted for the evolution of secondary vascular patterns in Nyctaginaceae due to multiple and complex developmental transitions. Here, three processes are inferred to generate the stem diversity found in the family: homoeosis, heterochrony, and heterotopy. (1) The formation of interxylary phloem (ontogeny 1) in relation to a putative ancestor with regular anatomy seems likely to represent a case of homoeosis, since the unusual activity of the cambium leads to the presence of phloem strands in the place of secondary xylem. Similar cases of homoeosis in woody plants has been hypothesized for example in species with parenchymatized xylem, that is, in cases where non-lignified parenchyma occur where fibers, vessels and lignified axial parenchyma would be present (e.g., lianas, succulents) [10, 11]. (2) In the evolution of ontogeny 3 from ontogeny 1, the extra-fascicular cambium appeared, and this suggests a case of heterotopy since the first vascular cambium arises in a different position from that present in the ancestor. In addition, the development of ontogeny 3 is based on the earlier onset of formation of the cambial variant by suppressing one of the ontogenetic stages (i.e., formation of a regular cambium), therefore, it may also

illustrate for the first time a case of predisplacement, a form of peramorphosis (heterochrony). In wood anatomy, most cases of heterochrony suggest the occurrence of prolonged juvenile characteristics into adult forms (paedomorphosis) [10,92,93], and a case of peramorphosis (hypermorphosis – evolution by developmental additions) is also suggested for the origin of successive cambia in *Paullinia,* Sapindaceae [12]. In addition, because ontogeny 3 evolved from ontogeny 1, this transition also requires modifications in the primary vascular system which indicates developmental changes that are regulated by an independent developmental module [94]. Thus, modularity may also be a source for anatomical diversity in this group. The evolution of ontogeny 2 from ontogeny 3 implicates in the appearance of a regular cambium. This transition indicates that a partial regression to the state of the ancestor of the family occurred in this lineage, if we consider that the regular cambium occurs in the same position of the single cambium generating interxylary phloem. Similarly, the evolution of ontogeny 4 requires a reversion from the cambium with unusual activity to the regular cambium, and then a new cambium is formed constituting the successive cambia, which suggests an additional developmental event.

In the challenge to understand developmental changes during evolution, one can notice that evolutionary changes such as heterochrony and heterotopy are likely to involve fewer processes than other changes such as homeosis or evolutionary novelty [95]. These set of developmental trajectories may be under complex gene regulation, given that multiple cellular and tissue processes are involved in the formation of each cambial variant [35 – chapter 4]. In addition, it is likely that the formation of the primary vascular system function as a module independent of the establishment of secondary growth since different secondary architectures can evolve from similar pre-vascular conditions in the primary stem.

Sheathing axial parenchyma vs. conjunctive tissue: origin, classification, and functional significance.

The term conjunctive tissue has been applied predominantly in the context of cambial variants, particularly for successive cambia [18,22,29,39,68], but also in cases of interxylary phloem (=included phloem, [96]). In the context of cambial variants,

conjunctive tissue is described as the parenchymatous or fibrous tissue between vascular increments (rings) derived from the meristematic zones that produce the new cambium in the successive cambia system. This interpretation has been maintained for cases of successive cambia in Nyctaginaceae [38 – chapter 2; this study]. However, to describe the parenchymatous tissue bordering the conducting cells of phloem strands in species with interxylary phloem a different name (i.e., sheathing axial parenchyma) has been applied because this tissue originates from the phloem axial parenchyma formed by the main cambium [35 - chapter 4] while conjunctive tissue is formed from remaining cells of the meristematic zone that originates the variant cambium [38 – chapter,2,61,67]. As indicated in relation to the origin of the cambial variants, the sheathing axial parenchyma and conjunctive tissue as products of these two systems are also regarded as developmentally linked.

The spatial distribution of conjunctive tissue and sheathing axial parenchyma is one of the main aspects resulting in the diversity of stem macromophologies in Nyctaginaceae. Given their structural and organization similarities, these tissues are likely to develop the same functions indicated to wood axial parenchyma (e.g., storage, involvement in mechanical strength, defence against pathogens and in hydraulic maintenance – [87,88,89]). In addition, the abundant parenchyma present in some plants with cambial variants may also represent adaptive advantages for increased flexibility, mechanical strength and injury repair as suggested to climbing plants [20-22,68]. Other functions have also been attributed to species with successive cambia (e.g., salt sequestration, xylem and phloem three-dimensional network – [68]). Curiously, successive cambia and interxylary phloem, are two types of cambial variants that can be now observed in species with distinct habits, from herbs to lianas and large trees [32,68,90,this study]. In any case, the capacity of producing multiple cambia or secondary phloem within the secondary xylem in such intricate organization might represent a beneficial physiological alternative to the typical regular growth of woody plants [29,39,86], given the multiple evolution of these cambial variants across angiosperms in both scandent and self-supporting plants. Experimental work is still needed to substantiate these hypotheses.

The impact of transitions in habits, habitats and cambial variants in the diversification of Nyctaginaceae

The diversification of Nyctaginaceae was most probably in the Middle Eocene (~48 Ma), when most of the extant angiosperm families were already established forming the contemporaneous tropical biomes [52]. Other estimates for the split between Nyctaginaceae and close families assumes an interval lying between 13 and 33 Ma [101], as inferred for the divergence from Aizoaceae + Phytolaccaceae (e.g., 26 Myr, [102]).

Speciation/diversification rate increased in Nyctaginaceae 28.62 Ma, at the time of emergence of a group comprising the Bougainvilleeae and Pisonieae ("B&P") clade + the Nyctagineae ("NAX") clade [42], and has been maintained since then. There is not an apparent unique characteristic for this group that could explain its increase in diversification. However, different hypotheses have been pointed out for the high number of species in each tribe individually. For instance, a remarkable radiation of genera from the NAX clade occurred in deserts of North America, and they are associated with multiple evolutions of cleistogamy and edaphic endemism to grow on gypsum soils (Douglas & Manos, 2007); the B&P clade stands out by having most of the neotropical and large, woody species of the family, which include both the *Guapira/Neea/Pisonia* trees, as well as the shrubby–scandent or tree species of *Bougainvillea* [42]. In addition, the evolution of fleshy anthocarps (the fruits of Nyctaginaceae) in the *Guapira/Neea* lineage and the likely appearance of endozoochory, seems to be one of the possible explanations for the rapid radiation of taxa of this lineage [42,43].

Commicarpus has experienced a high turnover of species, where many species have been generated but also went extinct, as observed by the rise in both speciation and extinction rate that ultimately involve an increase in the diversification rate. This genus is one of several lineages of Caryophyllales where a diversification rate shift has been detected, indicating a very recent and rapid radiation [103]. In some other caryophyllalean lineages, genome duplications (polyploidy species) were associated with diversification shifts, which was not identified in *Commicarpus* in that study sampling. Within the NAX clade, *Commicarpus* stands out for having few American species and being mostly diverse in Africa, with several species showing restricted

distributions (endemics) in tropical regions, some of them also growing on gypsum or limestone [42,104,105].

Our results also suggest that there was no increase in diversification in the lineages containing lianas (e.g., *Colignonia*), therefore, contrary to previous hypotheses [33,34]. This observation is noteworthy because it indicates for the first time with an explicit analysis that higher speciation rates in the evolution of lianas seem not to hold at least when the whole group has a cambial variant, as it is the case of Nyctaginaceae. Therefore, the question on whether cambial variants or the lianescent habit is what is likely boosting the diversification of lianas in the studies of Gianoli [33,34] remains to be answered. Future analyses that directly estimate the contribution of morphological characters on the diversification dynamics will provide new insights on the proportional role that habit, or other functional traits have on these rate diversifications.

Conclusion

By comparing the stem developments in all main lineages of Nyctaginaceae, we discovered that the mature vascular architectures range from typical successive cambia to interxylary phloem, following four disparate ontogenies. These ontogenies share developmental stages and thus may contain intermediate forms between the typical state of these two cambial variants. This way, the stem diversity in Nyctaginaceae, which is driven by developmental changes triggered by heterochronic, heterotopic and homeotic processes, may represent a strong case of continuum morphology. Nyctaginaceae is also one of the first groups to show cambial variants in all members of the family, whose ancestor was reconstructed as having interxylary phloem instead of the most endorsed type, successive cambia. These cambial variants are built upon two dissimilar primary vascular organizations, the regular or polycyclic eustele, suggesting that distinct developmental modules are present in the stem ontogeny of these plants. We also presented that high species richness in Nyctaginaceae has probably not been driven by transitions in habits or cambial variants, which indicates that other functional traits may have been more important in their diversification. The complex and diverse developmental pathways shown by Nyctaginaceae may be present in close-related families and be of important

phylogenetic significance within Caryophyllales, given the likely potential for convergent evolution in this group. Further investigations in the evolution of development of other caryophyllalean families remain essential in our desire for a better interpretation of the morphological evolution in this important lineage. In addition, understanding the genetic regulatory network underlying stem development in Nyctaginaceae seems to be the next step, since it will be easier to identify the role of genes once it is investigated in plants where the developmental and evolutionary patterns are further comprehended.

Supplementary information
Additional file 1: Table S1: List of studied species.

Acknowledgements

Funding

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Supplementary Information – Table S1

Specimen information for stem anatomical analyses of Nyctaginaceae. Names in bold were used for ontogenetic analysis.

Species name, Collector/collector number (Herbarium), Locality/Collection source, Stem diameter.

Taxa	Collector, collector number (Herbarium)[1]	Locality	Habit	Stem diameter (mm)[2,3]
Nyctagineae				
***Abronia fragrans* Nutt. Ex Hook.**	Douglas 2290 (FLAS)	Las Cruces, New Mexico, USA	Herb	4
Abronia neealleyi Standl.	Douglas 2281 (FLAS)	Eddy County, Yeso Hills, New Mexico, USA	Herb	2.5
Acleisanthes acutifolia Standl.	Purpus 4753 (US 842034)*	Coahuila, Mexico	Herb	2
***Acleisanthes chenopodioides* (A.Gray) R.A.Levin.**	Douglas 2289, 2293 (FLAS)	Las Cruces, New Mexico, USA	Herb	3.5
Acleisanthes lanceolata (Wooton) R.A.Levin	Douglas 2277 (FLAS)	Malone Mountains, Sierra Blanca, Texas, USA	Herb	3.5
Acleisanthes longiflora A.Gray.	Douglas 2279 (FLAS)	Malone Mountains, Sierra Blanca, Texas, USA	Herb	2
***Allionia incarnata* L.**	Nee 64124-64126 (USZ)	Parque Nacional Amboró, Pampa Grande, Santa Cruz, Bolivia	Herb	5

Allionia choisyi Standl.		Mesilla Valley, New Mexico, USA	Herb	4
Anulocaulis leiosolenus (Torr.) Standl. *Var. leiosolenus*	Douglas 2278 (FLAS)	Malone Mountains, Sierra Blanca, Texas, USA	Herb	12
Boerhavia diffusa L.	Pace 753 (MEXU, US)	Veracruz, Mexico	Herb	8
Boerhavia wrightii A.Gray.	Douglas 2288 (FLAS)	Las Cruces, New Mexico, USA	Herb	1.5
Commicarpus scandens (L.) Standl.	Acevedo-Rodríguez 16250 (US); Douglas 2291 (FLAS)	Tonalá, Oaxaca, Mexico; New Mexico, USA	Scandent-shrub	11.5
Cyphomeris gypsophiloides (M. Martens & Galeotti) Standl.	Douglas 2287 (FLAS)	Organ Mountains-Desert Peaks National Monument, Las Cruces, New Mexico, USA	Herb	16
Mirabilis aggregata (Ortega) Cav.	Pace 728 (MEXU, US)	Ixmiquilpan, Hidalgo, Mexico	Herb	12
Mirabilis cf. albida (Walter) Heimerl.	Douglas 2286 (FLAS)	New Mexico, USA	Herb	4
Mirabilis jalapa L.	Acevedo-Rodríguez 16480 (US)	Veracruz, Mexico		11
Nyctaginia capitata Choisy.	Douglas 2282 (FLAS)	New Mexico, USA	Herb	5
Okenia hypogea Schltdl. & Cham.	Pace 749 (MEXU, SPF, US)	Veracruz, Mexico	Herb	5
Pisonieae				
Grajalesia fasciculata (Standl.) Miranda.	Pace 765 (MEXU, SPF, US)	Chiapas, Mexico	Scandent-tree	12; trunk
Guapira bracei Britton	Scott 386 (USw 23138)*	Florida, United States of America	Tree	Trunk

Species	Voucher	Locality	Habit	Diameter
Guapira pernambucensis (Casar.) Lundell.	Cunha Neto 04–05 (HURB)	Alagoinhas, Bahia	Scandent-shrub	23
Guapira graciliflora (Mart. Ex J.A.Schmidt) Lundell.	Cunha Neto 06 (HURB)	Alagoinhas, Bahia	Tree	22
Guapira laxa (Netto) Furlan.	Cunha Neto 08–09 (HURB)	Universidade Estadual de Feira de Santana, Feira de Santana, Bahia	Tree	15; trunk
Guapira linearibracteata	Gentle 481 (USw 29941)*	Belize	Tree	trunk
Neea amplifolia	Pace 720 (US)	Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica	Tree	12; trunk
Neea delicatula Standl.	Pace 689 (US)	Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica	Tree	15
Neea hermafrodita S. Moore	Nee 64112-64113 (USZ)	Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia	Tree	11.5
Neea laetevirens Standl.	Pace 713, 716 (US); Stern 196 (USw 16154)*	Reserva Biológica La Selva, Sarapiquí, Heredia, Costa Rica; Los Santos, Panamá.	Tree	15; trunk
Neea ovalifolia Spruce ex J.A. Schmidt	Feuillet 10218 (USw 42783)*	Régina, French Guiana	Tree	18.5

Neea psychotrioides Donn. Sm.	Pace 763 (MEXU)	Estación de Biología Tropical Los Tuxtlas, Veracruz, Mexico.	Tree	9
Pisonia aculeata L.	Acevedo-Rodríguez 16549 (US)	Bonito, Mato Grosso do Sul, Brazil	Liana	23.5
Pisonia fragrans Dum.Cours.	Stern, 2456 (USw 35502)*	Dominican Republic	Tree	Trunk
Pisonia ligustrifolia Heimerl	Abbott 2251 (USw 1982)*	Hispaniola Island, Dominican Republic	Tree	21
Pisonia zapallo Griseb.	Nee 64110 (USZ)	Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia	Tree	10
Pisoniella arborescens (Lag. & Rodr.) Standl.	Pace 738-739 (MEXU, SPF, US)	Alfajayucan, Hidalgo, Mexico	Liana	15.5
Pisoniella glabrata (Heimerl) Standl.	Nee 64137, 64151 (USZ)	Parque Nacional Amboró, Vallegrande, Santa Cruz, Bolivia	Scandent-shrub, liana	11
Bougainvilleeae				
Belemia fucsioides Pires.	Farney 4887, 4888 (RB); Cunha Neto 16, 17	Espírito Santo, Brazil	Liana	5
Bougainvillea berberidifolia Heimerl.	Nee 64140 (USZ)	Parque Nacional Amboró, Comarapa, Santa Cruz, Bolivia	Shrub	18
Bougainvillea campanulata Heimerl.	Acevedo-Rodríguez 16772 (US)	Mato Grosso do Sul, Brazil; Nee 64142 (USZ), Parque	Shrub	22

		Nacional Amboró, Comarapa, Santa Cruz, Bolivia		
Bougainvillea modesta Heimerl.	Nee 64115 (USZ)	Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia	Tree	20
Bougainvillea stipitata Griseb.	Nee 64121 (USZ)	Parque Nacional Amboró, Samaipata, Santa Cruz, Bolivia	Tree	19
Bougainvillea spectabilis Willd.	Rossetto 453 (RB)	Estrada Carlos Chagas–Teófilo Otoni, Minas Gerais, Brazil	Shrub	14
Phaeoptilum spinosum Radlk.	Dechamps 1213 (MADw 37340)*	Mocamedes, Angola	Shrub	20
Colignonieae				
Colignonia glomerata Griseb.	Nee 64157–64159 (USZ)	Parque Nacional Amboró, Samaipata, Santa Cruz, Bolivia	Liana	17
Colignonia rufopilosa Kuntze.	Nee 64061 (USZ)	Cochabamba, Bolivia	Liana	11
Boldoeae				
Cryptocarpus pyriformis Kunth	Fosberg 44705 (US 2833648)*	Galápagos	Liana	5.5
Salpianthus macrodontus Standl.	Annetta 3251 (US 2219249)*	Sinaloa, Mexico	Herb	6

Salpianthus purpurascens (Cav. Ex Lag.) Hook. & Arn.	Pace 774 (MEXU, SPF, US)	El Cobanal, Chiapas, Mexico	Shrub	20
Salpianthus arenarius Humb. & Bonpl.	Hinton 10215 (US 1893480)*	Vallecitos, Guerrero, Mexico	Shrub	3
Leucastereae				
Andradea floribunda Allemão	Rossetto 445 (RB); Serviço Florestal 152 (SPFw 5033)*	Linhares, Espírito Santo, Brazil; Distrito Federal, Brazil.	Tree	21; trunk
Leucaster caniflorus (Mart.) Choisy	Rossetto 447, 455 (RB)	Linhares, Espírito Santo, Brazil; Teófilo Otoni, Minas Gerais, Brazil	Liana	19
Ramisia brasiliensis Oliv.	Rossetto 448 (RB); Serviço Florestal 2614 (SPFw 5035)*	Nanuque, Minas Gerais, Brazil; Minas Gerais, Brazil.	Tree	18; trunk
Reichenbachia hirsuta Spreng.	Nee 64109, 64169 (USZ)	Living Collection Jardín Botánico Municipal de Santa Cruz de la Sierra, Santa Cruz de la Sierra, Bolivia; Rio Grande, Santa Cruz de la Sierra, Bolivia	Shrub	13
Reichenbachia colombiana Stand.	Dugand 979 (SJRw 32385)*	Colombia	Shrub	35

[1] In this list, specimens with asterisk (*) were obtained in the herbarium or wood collection indicated in parenthesis; the collection number is also indicated.

[2] Largest stem sample of adult specimens analyzed for the anatomical study.

[3] The indication "trunk" means that a sample was obtained from the main stem at ca. 1.30 cm height.

Collections acronyms: FLAS, Florida Museum of Natural History; HURB, Universidade Federal do Recôncavo da Bahia; MADw (Forest Products Laboratory, Madison, WI, USA); MEXU, Universidad Nacional Autónoma de México; RB, Jardim Botânico do Rio de Janeiro; SJRw, Samuel J. Record (acquired by the Forest Products Laboratory, Madison, WI, USA) SPF and SPFw, Universidade de São Paulo; US, Smithsonian Institution; USZ, Museo de Historia Natural Noel Kempff Mercado, Universidad Autónoma Gabriel René Moreno.

Final Remarks

In this study, we used an **integrative approach** including mostly **development** and **phylogenetic comparative methods** to understand the **diversity** and **evolution** of the **vascular system** in Nyctaginaceae, a predominantly **neotropical family**. The development of this study increased our knowledge on different aspects of stem anatomy of the main lineages of Nyctaginaceae, except for Caribeaeae, which is a monotypic group, from Cuba, and likely extinct. Explanation of the most important conclusions of each chapter are summarized below.

In the first chapter, we revealed the **anatomy** and **histochemistry** of a poorly known **secretory structure** in the genus *Anulocaulis,* which seem to be present also in other genera (e.g., *Boerhavia, Cyphomeris*). We showed that the secretory structure is a group of elongated epidermal cells (or unicellular glandular trichomes) which secrete a complex exudate. From the second chapter onward, we focused on the diversity of the vascular system, which proved to be more **diverse** and **complex** than previously thought. Chapter two was dedicated to a case study using the genus *Allionia,* with only two species with unresolved **taxonomy**. In this study, we discovered that the two species have conserved vegetative anatomy and that even though being small **herbs**, the vascular system possesses interesting characteristics such as **polycyclic eustele** and **cambial variants**, characterized as **successive cambia**. In addition, we demonstrated through a detailed **ontogenetic study** that the **origin** of this cambial variant which originate in the **pericycle** contradicts the vast literature stating that a meristem derived from the **cortex** generate the successive cambia system.

From chapter three onwards, our approach changed to a broader taxonomic scale using **comparative morphological analyses** under a phylogenetic context. In chapter three we investigated the diversity and evolution of the vascular system in Nyctaginaceae and related families and showed that two subtypes of **eustele** occur in this lineage, the **regular eustele** and the **polycyclic eustele**, which consists of **medullary bundles** and a **cylindrical continuous procambium** (CCP). The polycyclic eustele develops as **vascular traces** of lateral organs and the medullary bundles may appear in various number (from eight to ~30 bundles) and arrangements (ordered or non-ordered). This type was reconstructed as the **ancestral character** for the family,

and as a **symplesiomorphy** for the clade phytolaccoid of the Caryophyllales, that includes families such as Phytolaccaceae and Petiveriaceae.

In chapter four, we developed the largest comparative study of **stem anatomy** in Nyctaginaceae to begin to understand the complexity and diversity of the secondary vascular system, which is marked by the presence of **cambial variants**. Nonetheless, the types and developments of these patterns is much more intricate and complex than previously reported. For this reason, this paper was dedicated to unravelling the development of the most common type of cambial variant in the family, which is not successive cambia as stated since late nineteenth century. Because the gross morphology of the stems is achieved through the activity of a **single cambium**, the secondary growth occurring in representatives of most tribes of the family is better described as **interxylary phloem** – characterized mostly by the presence of phloem strands immersed within the secondary xylem. Despite sharing this character, the anatomy in these plants is diverse, ranging from stems with small phloem islands, to others forming patches and long bands of phloem recovered by the sheathing axial parenchyma interrupting the secondary xylem.

In chapter five, we expand our analyses of the development of cambial variants to all lineages of the family. We discovered that two patterns are present in the family, interxylary phloem and successive cambia, following four different **developmental pathways.** Unlike our expectations, interxylary phloem is the most common pattern and was reconstructed as **ancestral** in the family. Interxylary phloem occur in most lineages of Nyctaginaceae, being absent likely only in **Leucastereae –** an almost exclusive Brazilian tribe – distinguished also by being the only lineage with regular eustele instead of polycyclic eustele (chapter two). From this pattern, successive cambia evolved three times in the family, each following a different ontogeny. This **complexity** is because the ontogenies depart from different eustele subtypes and due to the appearance of unusual features such as the **extra–fascicular cambium**, as well as the well–known development of successive cambia with a *de novo* formation of a new meristem in the **pericycle**. For example, successive cambia may be derived from both regular eustele or polycyclic eustele, whereas interxylary phloem is always derived from polycyclic eustele. Other important findings from this study include the observation that regular secondary growth is found only during the initial growth of

Reichenbachia, which later develop successive cambia. All other Nyctaginaceae present variations in their development through the formation of polycyclic eustele + interxylary phloem (known for 19 genera in all tribes, except Leucastereae and Caribeaeae), regular eustele + successive cambia (4 genera from Leucastereae) or polycyclic eustele + successive cambia (2 genera from Nyctagineae). This observation is important because makes Nyctaginaceae one of the few families with cambial variants in all representatives.

In the evolution of the developments of cambial variants in Nyctaginaceae, **heterochrony**, **heterotopy** and **modularity** are the main **developmental processes** accounting for the increased morphological diversity in the group. In addition, we showed that the only way to understand the evolution of successive cambia from interxylary phloem is using the **fuzzy worldview**, since that there are intermediate forms between each category and between these two morphological types. This is the first time that cambial variants are discussed under the **continuum morphology** concept and that one variant can originate another. Also exciting were the results obtained with the **diversification analyses**, which showed that being a liana did not increase species diversification compared to their non-climbing sister groups. This result opens new avenues to investigate the role of cambial variants in lianescent groups, since their presence instead of the climbing habit might be the key innovation promoting **species diversification**.

Considering the anatomical diversity found in the vascular system of Nyctaginaceae, we must, therefore, recognize that the generally accepted definitions of **procambium** and **cambium** cannot be employed strictly. As we could observe from Nyctaginaceae, the primary vascular **meristem**, the **procambium**, appears in different configurations, and the development of the primary and secondary growth merges, gradually and continuously, following diverse **ontogenetic pathways** that result in **disparate morphologies** (e.g., eustele subtypes, cambial variants). These observations are remarkable because they were unnoticed for quite some time and because they show unique anatomies compared to the patterns found in most **eudicots**. Here, again, we realize that in biology there is almost not a rule not including exceptions.

In summary, anatomical and ontogenetic studies under a phylogenetic approach are essential to our understanding of the developmental processes that generate disparate morphologies.

Future research directions – To continue to expand our understanding of the anatomical diversity of the vascular system in Nyctaginaceae, an investigation of the diversity and evolution of wood and bark characters would be a great benefit. Efforts to summarize this information has already begun using our large taxonomic sampling and slide collection used in this thesis. Other research ideas deriving from this project and under progress include: i) a study of the diversity and evolution of medullary bundles within Caryophyllales; in this study we aim at integrating the anatomical data with biomechanical and/or hydraulic experiments to explore the adaptive function of medullary bundles; ii) an anatomical comparative study of vegetative organs of the emblematic genus *Belemia,* including an updated taxonomic treatment for the group.

In addition, given that now we understand better the cambial variants in Nyctaginaceae in terms of anatomy, using a model plant from this group to investigate the molecular basis underlying these patterns would put an enhanced perspective into the evolution of its diversity. Investigations on molecular genetics of plant development have become increasingly common in recent years, yet much of these plant evo-devo studies have concentrated on the flower and the leaf. Nevertheless, to understand morphological evolution, these concepts and research need to be developed across all biological systems of land plants. Therefore, investigating the developmental genetics in groups with such intriguing morphologies as it is the Nyctaginaceae and perhaps other families of the Caryophyllales would bring more light to our knowledge on the aspects of wood, bark and cambial variants development and evolution.

Resumo

Nyctaginaceae, a família da primavera, tem ampla ocorrência nos Neotrópicos, e com espécies com uma notável variedade de hábitos e padrões anatômicos vasculares em seus caules. Por exemplo, os caules dessas plantas têm sido descritos com feixe medulares e variações cambiais desde o século 19, sendo na maioria das vezes interpretadas como tendo câmbios sucessivos e em algumas poucas vezes como floema interxilemático. Todavia, pouco se sabe sobre a real diversidade, origem, desenvolvimento e evolução dessas anatomias vasculares e ainda menos conhecida é sua distribuição filogenética e se elas impactaram a diversificação dos clados dentro da família. Portanto, o presente estudo teve como objetivo compreender a diversidade anatômica, desenvolvimento, distribuição e evolução dos diferentes padrões anatômicos vasculares em Nyctaginaceae. Amostras de caules de mais de 90 espécies distribuídas em 26 dos 34 gêneros conhecidos, e pertencentes a seis das setes tribos da família foram investigadas. Nossos resultados apontam que a diversidade e complexidade do sistema vascular caulinar em Nyctaginaceae é maior do que previamente imaginado. Há dois subtipos de eustelo na família, regular e policíclico (que inclui feixes medulares e um procâmbio contínuo), e dois tipos de variações cambiais, câmbios sucessivos e floema interxilemático. Nós vimos que caules com feixes medulares e floema interxilemático representam a condição mais comum e ancestral para a família. Entretanto, a evolução de variações cambiais não é contingente em relação ao sistema vascular primário, e nem aos hábitos. Vimos também que Nyctaginaceae se destaca como uma das poucas famílias onde representantes de todas as linhagens apresentam variações cambiais. Finalmente, a diversidade dessas anatomias complexas é discutida sob o conceito de morfologia contínua, dado que a evolução desses padrões em Nyctaginaceae ocorreu com transições intermediárias entre as diferentes categorias. O impacto dos hábitos, habitats e variações cambiais na diversificação da família também são discutidos. Este trabalho demonstra que estudos ontogenéticos realizados em um contexto filogenético continuam sendo um excelente método para desvendar a enorme diversidade morfológica encontrada nos organismos, sua evolução e seus impactos na diversificação das espécies.

Abstract

Nyctaginaceae, the four o'clock family, has a broad occurrence in the Neotropics, containing species with an outstanding diversity of habits and stem vascular anatomical patterns. For instance, the stems in Nyctaginaceae have been described with medullary bundles and cambial variants since the 19th century, being the majority of times interpreted as having successive cambia, and a few times with interxylary phloem. However, little is known about the real diversity, origin, development and evolution of these vascular anatomies, but even less understood is its phylogenetic distribution and if they impacted the diversification of clades within the family. For that, stem samples from more than 90 species distributed in 26 from the 34 genera currently recognized and belonging to six out of the seven tribes of the family were investigated. Our results indicate that the diversity and complexity of the stem vascular system in Nyctaginaceae is larger than previously anticipated. There are two subtypes of eustele in the family, regular and polycyclic (which includes medullary bundles and a continuous procambium), and two types of cambial variants, successive cambiums and interxylary phloem. We have seen that, stems with medullary bundles and interxylary phloem represent the most common and ancestral condition for the family. However, the evolution of cambial variants is not contingent on the primary vascular system, nor on habits. We have also seen that Nyctaginaceae stands out as one of the few families where representatives of all lineages have cambial variants. Finally, the diversity of such complex anatomies is discussed under the concept of continuum morphology, given that the evolution of these patterns in Nyctaginaceae occurred with transitions of intermediate forms between the different categories. The impacts of habits, habitats and cambial variants in the diversification of the family are also discussed. Thus, this work demonstrates that ontogenetic studies carried out in a phylogenetic context continue to be an excellent method to unravel the immense morphological diversity observed in organisms, their evolution and their impact on species diversification.

Extra files

Extra file I – First page of the published articles with results from this project

Chapter 1

WHAT ARE THE "STICKY RINGS" ON STEMS OF *ANULOCAULIS* AND RELATED TAXA (NYCTAGINACEAE) FROM ARID REGIONS?

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ABSTRACT

Anulocaulis, commonly known as "ringstem," is a small, unusual genus restricted to the Chihuahuan, Sonoran, and Mojave deserts of North America. Here we combined light microscopy and histochemical tests to characterize for the first time the "sticky structures" (here called secretory rings) found on the stem internodes of *Anulocaulis.* The secretory rings were shown to be groups of epidermal cells, or unicellular glandular trichomes, which largely differ from their neighboring cells both in structure and histochemistry. The cells start to differentiate in early stages of stem development. They begin as regular epidermal cells, but later their anticlinal and external tangential walls start to enlarge. At maturity the cells become remarkably elongated, even balloon-like, with dense cytoplasmic content. Although the secretory rings have been reported as "mucilaginous structures" based on morphological observations, preliminary histochemical analyses showed that its exudate is complex, including a mixture of mucilage, proteins, and phenolic compounds. Future investigations are needed to compare the anatomy of the secretory rings within related genera of Nyctaginaceae and characterize the chemical components of their exudate more specifically to search for potential homologies and adaptive functions of these structures.

RESUMEN

Anulocaulis, comúnmente conocido como "ringstem," es un género pequeño que se encuentra restringido a los desiertos de Chihuahua, Sonora y Mojave de América del Norte. En este estudio, usamos microscopía óptica y pruebas histoquímicas para caracterizar por primera vez las "sticky structures" (aquí denominadas "secretory rings") que se encuentran en los entrenudos del tallo de *Anulocaulis.* Se demostró que los anillos secretores son grupos de células epidérmicas, o tricomas glandulares unicelulares, que se diferencian en gran medida de sus células vecinas tanto en estructura como en histoquímica. Las células comienzan a diferenciarse en las primeras etapas del desarrollo del tallo. Comienzan como células epidérmicas normales, pero luego sus paredes anticlinal y tangencial externa comienzan a agrandarse. En la madurez, las células se vuelven notablemente alargadas, incluso como globos, con un contenido citoplasmático denso. Aunque los anillos secretores han sido descritos como "estructuras mucilaginosas" basadas en observaciones morfológicas, los análisis histoquímicos preliminares mostraron que su exudado es complejo, incluyendo una mezcla de mucílago, proteínas y compuestos fenólicos. Son necesarias investigaciones futuras que permitan estudiar comparativamente la anatomía de los anillos secretores en los géneros de Nyctaginaceae y así mismo caracterizar los componentes químicos de su exudado más específicamente para buscar posibles homologías y funciones adaptativas de estas estructuras.

KEY WORDS: Anatomy, Caryophyllales, Nyctagineae, secretory structures, Chihuahuan Desert, glandular trichomes

INTRODUCTION

Anulocaulis is a small genus of perennial herbs with just five species. It is included in tribe Nyctagineae, which is the largest and most diverse tribe in the family Nyctaginaceae (Douglas & Spellenberg 2010). The genus is endemic to arid regions of North America (e.g., Chihuahuan, Sonoran, and Mojave Deserts) and is distributed from northern Mexico to southeastern California in the United States of America (Spellenberg 1993; Douglas & Spellenberg 2010). *Anulocaulis* may be divided into two groups based on fruit morphology (Spellenberg 1993). The first encompasses *A. annulatus, A. hintoniorum,* and *A. eriosolenus,* which have smooth anthocarps. *Anulocaulis annulatus* is restricted to low, hot elevations near Death Valley in the Mojave Desert, while *A. hintoniorum and A. eriosolenus* are restricted to the Chihuahuan Desert in Mexico and Texas. The second group comprises *A. leiosolenus* (considered to have four varieties, Spellenberg 1993) and *A. reflexus.* These are characterized by their variously winged and wrinkled anthocarps and are primarily gypsum endemic plants

Chapter 2

ANATOMY OF VEGETATIVE ORGANS IN *ALLIONIA* (NYCTAGINACEAE), WITH EMPHASIS ON THE VASCULAR SYSTEM

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ABSTRACT

Allionia is a small genus within the tribe Nyctagineae (Nyctaginaceae) which has a controversial, infrageneric delimitation. Here, we investigated the two known species of *Allionia* in order to characterize the anatomy of leaves, stems and roots, with further notes on vascular system development. Additionally, the present study aimed to broaden our knowledge of stem vascular diversity and to survey for anatomical features with diagnostic value in distinguishing *A. choisyi* from *A. incarnata*. Leaf anatomy of other Nyctagineae taxa was also analysed. Anatomical and ontogenetic observations from the vegetative organs in *Allionia* revealed no diagnostic features to distinguish the two species. We illustrated the occurrence of Kranz anatomy, which in Nyctaginaceae is only known in *Allionia, Boerhavia,* and *Okenia*. The stem primary vascular system was unusual in showing a polycyclic eustele (medullary bundles + continuous concentric procambium). Likewise, mature stems and roots show vascular cambial variants (successive cambia) that arise from the pericycle. The anatomy and histochemistry of multicellular glandular trichomes observed in aerial organs were presented. Raphids were seen in all organs. Although no strong xerophytic features were observed in *Allionia*, several characteristics can be associated with their arid habitats. Our findings on the vascular system of *Allionia* showed the two species to be much the same and reinforced earlier findings that the stem anatomy of Nyctaginaceae is complex and intriguing.

RESUMEN

Allionia es un género pequeño dentro de la tribu Nyctagineae (Nyctaginaceae) con delimitación infragenérica controversial. Analizamos las características anatómicas de hojas, tallos y raíces de las dos especies conocidas de *Allionia* e incluimos comentarios sobre el desarrollo del sistema vascular. El presente estudio pretende, examinar características diagnósticas entre *A. choisyi* y *A. incarnata* y de esta forma ampliar el conocimiento sobre la diversidad vascular del tallo. Adicionalmente, analizamos la anatomía foliar de otros taxa de Nyctagineae. Las observaciones anatómicas y ontogenéticas de los órganos vegetativos en *Allionia* no mostraron características diagnósticas que permitieran diferenciaran entre las dos especies. La anatomía Kranz para Nyctaginaceae, restringida únicamente a *Allionia, Boerhavia* y *Okenia* fue ilustrada. Presentamos la anatomía e histoquímica de tricomas glandulares multicelulares observados en órganos aéreos. El sistema vascular primario del tallo era incomum al mostrar un eustele policíclico (haces medulares + procambio concéntrico continuo). Así mismo, tallos y raíces maduras mostraron variantes cambiales vasculares (cambios sucesivos) que surgen en el periciclo. Todos los órganos presentaron rafidios. No fueron observadas características xerofíticas en *Allionia*, sin embargo, varias características pueden estar relacionadas con ambientes áridos. Estos hallazgos esclarecen y corroboran la complejidad anatómica de las especies de Nyctaginaceae, y muestran la intrigante diversidad de patrones anatómicos caulinares.

KEY WORDS: *Allionia choisyi, Allionia incarnata,* Caryophyllales, cambial variants, Nyctagineae, ontogeny

INTRODUCTION

Nyctaginaceae have about 30 genera and 400 species which include trees, shrubs, subshrubs, lianas and herbs (Douglas & Manos 2007; Douglas & Spellenberg 2010; Hernández-Ledesma et al. 2015). The species are distributed mostly in the tropics and subtropics of the New World, except for some genera that occur in the Old World (e.g., *Boerhavia, Commicarpus, Pisonia, Phaeoptilum,* and *Mirabilis*) (Hernández-Ledesma et al. 2015). In the most recent classification, the family has been divided into 7 tribes: Nyctagineae, Boldoeae, Leucastereae, Bougainvilleeae, Pisonieae, Colignonieae, and Caribeeae (Douglas & Spellenberg 2010).

Allionia L. belongs to tribe Nyctagineae and comprises species of annual or perennial herbs with procumbent, decumbent or prostrate stems (Fig. 1). Two species are recognized *A. choisyi* Standl. and *A. incarnata* L. which are very similar morphologically, differing only in some fruit characteristics (e.g., number of lateral expansions, length of glands) (Spellenberg 2003), which makes the delimitation of infrageneric categories

Chapter 3

American Journal of Botany — BOTANICAL SOCIETY OF AMERICA

RESEARCH ARTICLE

Diversity, distribution, development, and evolution of medullary bundles in Nyctaginaceae

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PREMISE: Medullary bundles, i.e., vascular units in the pith, have evolved multiple times in vascular plants. However, no study has ever explored their anatomical diversity and evolution within a phylogenetic framework. Here, we investigated the development of the primary vascular system within Nyctaginaceae showing how medullary bundles diversified within the family.

METHODS: Development of 62 species from 25 of the 31 genera of Nyctaginaceae in stem samples was thoroughly studied with light microscopy and micro-computed tomography. Ancestral states were reconstructed using a maximum likelihood approach.

RESULTS: Two subtypes of eusteles were found, the regular eustele, lacking medullary bundles, observed exclusively in representatives of Leucastereae, and the polycyclic eustele, containing medullary bundles, found in all the remaining taxa. Medullary bundles had the same origin and development, but the organization was variable and independent of phyllotaxy. Within the polycyclic eustele, medullary bundles developed first, followed by the formation of a continuous concentric procambium, which forms a ring of vascular bundles enclosing the initially formed medullary bundles. The regular eustele emerged as a synapomorphy of Leucastereae, while the medullary bundles were shown to be a symplesiomorphy for Nyctaginaceae.

CONCLUSIONS: Medullary bundles in Nyctaginaceae developed by a single shared pathway, that involved the departure of vascular traces from lateral organs toward the pith. These medullary bundles were encircled by a continuous concentric procambium that also constituted the polycyclic eustele, which was likely a symplesiomorphy for Nyctaginaceae with one single reversion to the regular eustele.

KEY WORDS Caryophyllales; evo-devo; Nyctaginaceae; ontogeny; primary growth; stem anatomy; trait evolution; vascular bundles.

Medullary bundles are complete vascular bundles located in the pith and may be arranged in two or more concentric rings or as bundles scattered within the pith in addition to the bundles of the stele ring (de Bary, 1884; Esau, 1967; Ogura, 1972; Beck et al., 1982; Schmid, 1982; Mauseth, 1988; Beck, 2010; Isnard et al., 2012). Such organization of the vascular tissue constitutes the "polycyclic eustele" subtype in the stele classification by Beck et al. (1982) and Schmid (1982). Medullary bundles have also been addressed in the context of "anomalous structures" (de Bary, 1884; Eames and MacDaniels, 1925; Metcalfe and Chalk, 1950; Beck, 2010; Yang and Chen, 2017), a concept not followed here, since we consider the so-called anomalous vascular structures to be a variant type of secondary growth (Carlquist, 2001; Angyalossy et al., 2012, 2015). Regardless, medullary bundles are a remarkable feature of vascular plants, which also

contributes to their complexity and morphological diversity (Eames and MacDaniels, 1925; Beck et al., 1982).

Medullary bundles have evolved multiple times in the history of vascular plants, being present in ferns (e.g., Cyatheaceae and Dennstaedtiaceae [*Pteridium*], Ogura, 1927, 1972; Eames and MacDaniels, 1925; Lucansky, 1974), and more frequently in the flowering plants, where they have been recorded in approximately 60 families, including magnoliids (Isnard et al., 2012; Trueba et al., 2015) and eudicots (Wilson, 1924; Lambeth, 1940; Boke, 1941; Holwill, 1950; Metcalfe and Chalk, 1950; Davis, 1961; Esau, 1967; Pant and Bhatnagar, 1975; Raj and Nagar, 1980, 1989; Kirchoff and Fahn, 1984; Mauseth, 1993, 2006; Costea and DeMason, 2001; Schwallier et al., 2017; Kapadane et al., 2019). In some families, this character is found in just a few representatives (e.g., *Nepenthes*,

Chapter 4

A NEW INTERPRETATION OF THE SUCCESSIVE CAMBIA OF SOME NYCTAGINACEAE AS INTERXYLARY PHLOEM

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Premise of research. The alternative patterns of secondary growth (vascular cambial variants) in stems of Nyctaginaceae are outstanding and have been widely investigated since the late nineteenth century. However, there are controversial interpretations in the literature regarding the existence of either one or two types of cambial variants in this family (successive cambia vs. interxylary phloem). We aim to explore the anatomical diversity of stems in Nyctaginaceae to document the real nature of the cambial variant present in most species of the family.

Methodology. We analyzed 60 species, focusing on 18 species from 12 genera, for developmental studies. Anatomical and ontogenetic features were characterized from images produced by standard plant techniques for macro- and microscopic analyses.

Pivotal results. Our analyses reveal that most species of Nyctaginaceae present stems with polycyclic eusteles, which later develop a single cambium that produces secondary xylem and secondary phloem at unequal rates around the stem circumference. This unusual activity results in the absence of a regular cylinder of secondary vascular tissues and in the formation of secondary phloem strands (surrounded by variable amounts of sheathing axial parenchyma) embedded within the secondary xylem. In cross section, adult stems can exhibit different tissue arrangements (i.e., phloem islands/strands, patches, or concentric bands) that result from differences in rates of production of phloem and associated sheathing axial parenchyma forming the strands. The cambial variant in these stems is described as interxylary phloem, as similarly observed in other eudicot lineages.

Conclusions. Our examination of the stem development of Nyctaginaceae confirms the presence of interxylary phloem, which has been overlooked in the family as most previous studies have reiterated descriptions of successive cambia as the common cambial variant within the family. These findings emphasize the importance of developmental studies encompassing a representative number of genera to further our understanding of stem macro-morphologies and to highlight the complexity and diversity of stem architectures in Nyctaginaceae.

Keywords: cambial variant, Caryophyllales, ontogeny, polycyclic eustele, secondary growth, stem anatomy.

Online enhancement: supplemental table.

Introduction

The prevalence of families with cambial variants in the order Caryophyllales, compared with other angiosperm orders, has been reported since the first treatises on the subject (de Bary 1884; Schenck 1893; Pfeiffer 1926). Cambial variants are reported in at least 19 of the 39 families currently recognized in the Caryophyllales (Gibson 1994; Carlquist 2010; Hernández-

Ledesma et al. 2015). Until the late twentieth century, the majority of papers on the formation of alternative vascular anatomies in this order followed the traditional view (Schenck 1893; Pfeiffer 1926) that regarded the main cambial variant in the family as successive cambia (i.e., additional increments of vascular tissue through the formation of new cambia outside the first cambium). More recently, Carlquist's outstanding contributions to the understanding of vascular anatomies in Caryophyllales (Carlquist 1991, 1999, 2000, 2001, 2003, 2004, 2007, 2010) have concurred with earlier views that successive cambia is the cambial variant occurring in the order. This view has been subsequently shared by other authors (Rajput and Rao 1998; Rajput et al. 2009, 2012; Hernández-Ledesma et al. 2011, 2015; Rajput and Marcati 2013; Rajput 2015; Myśkow et al. 2019; Zumaya-Mendoza et al. 2019).

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Extra file II – List of works presented in meetings with results from this project

1. CUNHA NETO, I.L., PACE, M.R., ANGYALOSSY, V. When what seems successive cambia in Nyctaginaceae (Caryophyllales) is actually a new type of interxylary phloem. Botanical Society of America Conference – Botany 2021, Online, USA. 2021. (Oral-video).

2. CUNHA NETO, I.L., Anatomia do caule em plantas vasculares: o crescimento secundário nas espermatófitas. II Webnário Botânico da Faculdade de Educação, Ciências e Letras de Igatu (FECLI), da Universidade Estadual do Ceará, May 2021. Brazil (Oral-online).

3. CUNHA NETO, I.L., PACE, M.R., DOUGLAS, N.A., NEE, M.H., SÁ, C.F.C., MOORE, M.J., ANGYALOSSY, V. Stele diversity and evolution in Nyctaginaceae: medullary bundles across the family. Botanical Society of America Conference – Botany 2020, Online, USA. 2020. (Oral-video).

4. CUNHA NETO, I.L., DOUGLAS, N.A., ANGYALOSSY, V. "First report on the structure and histochemistry of the "sticky rings" in stems of Anulocaulis (Nyctaginaceae)". Botanical Society of America Conference – Botany 2019, Tucson, AZ, USA. 2019. (Poster).

5. CUNHA NETO, I.L. "A developmental and evolutionary perspective on the vascular system of Nyctaginaceae". Caryophyllales 2018 Conference, Universidade Nacional Autónoma de México, México City, México. 2018. (Oral).

6. CUNHA NETO, I. L. "Stem anatomy and development as subsidy to vascular plants systematics". IX Winter Botany Course, August 2019, University of São Paulo, SP, Brazil.

7. CUNHA NETO, I. L. "Diversity and evolution of the vascular system in Nyctaginaceae". 69º Congresso Nacional de Botânica (69º CNBot), July 2018, Cuiabá, MS, Brazil.

Extra file III – Awards obtained during the Ph.D.

- 2018 – Cuatrecasas Travel Award, National Museum of Natural History, Smithsonian Institution, Washington D.C., USA.

- 2020 – I.W. Bailey Award 2020, IAWA Journal/ Brill Publishers – awarded by joint candidature with Dr. Joyce G. Chery (Cornell University).

Sobre o autor

Israel L. da Cunha Neto nasceu em Santo Antônio de Jesus, Bahia, Brasil, em 04 de fevereiro de 1991. O interesse pelo mundo natural surgiu principalmente pela vida no interior. Em 2008, ingressou na Universidade Federal do Recôncavo da Bahia (UFRB) para cursar Agronomia. Já no terceiro semestre, se encantou pela botânica de um modo tão profundo que recusou outras oportunidades, até conseguir uma chance no Laboratório de Anatomia Vegetal e Histoquímica, sob orientação do Dr. Fabiano Machado Martins, em 2009. Durante a graduação desenvolveu vários estudos em anatomia e histoquímica de plantas cultivadas e não cultivadas, até conhecer o mundo da anatomia de madeira, lianas e variações cambiais. Entre 2013 e 2014, realizou intercâmbio na Corvinus University of Budapest, Hungria, onde também desenvolveu pesquisa em anatomia vegetal. Em 2014, concluiu o bacharelado com prêmio de aluno destaque daquele ano.

Depois de se graduar, mudou-se para o Rio de Janeiro, onde iniciou o mestrado em botânica no glorioso Museu Nacional da Universidade Federal do Rio de Janeiro (UFRJ), em março de 2015. Neste período realizou pesquisa com foco na anatomia do sistema vascular de lianas da família Sapindaceae, desenvolvendo atividades no Jardim Botânico do Rio de Janeiro (JBRJ) sob supervisão da Dr. Neusa Tamaio. Completou o mestrado em fevereiro de 2017 e, posteriormente, Israel iniciou o doutorado na Universidade de São Paulo (USP) em junho do mesmo ano. Desta vez, sua pesquisa é dedicada ao sistema vascular em plantas da família Nyctaginaceae, sob supervisão da Dr. Veronica Angyalossy. Para este estudo, Israel realizou diversas expedições de coleta no Brasil e no exterior (Bolívia, Estados Unidos, México), além de desenvolver parte dos trabalhos no Museu de História Natural do Smithsonian Institution (Washington, D.C, EUA). Os resultados desta pesquisa já foram apresentados em conferências no Brasil (2018), México (2018) e EUA (2020, 2021) (remotamente).

A lista de publicações do autor pode ser encontrada em seu currículo nas plataformas online Lattes / Orcid / Google Acadêmico / ResearchGate.

About the author

Israel L. da Cunha Neto was born on the 4th of February 1991 in Santo Antônio de Jesus, Bahia, Brazil. Interest in the natural world came from life in the countryside. He joined the Federal University of Recôncavo da Bahia in 2008 to pursue a bachelor's degree in Agronomy. In the third semester, he was fascinated by botany in such a profound way that he refused several internship opportunities, until he was accepted to work at the Laboratory of Plant Anatomy and Histochemistry, under the direction of Dr. Fabiano Machado Martins, from October 2009. During graduation he developed several studies in anatomy and histochemistry of cultivated and non-cultivated plants, until he developed an interest in wood anatomy, lianas and cambial variants. In the meantime, he did an exchange program at Corvinus University of Budapest, Hungary, between 2013 and 2014, where he also developed research in plant anatomy. In 2014, he completed his bachelor's degree obtaining the Outstanding Student Award.

After graduating, he moved to Rio de Janeiro, where he began his master's degree in botany at the celebrated National Museum of the Federal University of Rio de Janeiro (UFRJ), in March 2015. During this period, he conducted research focusing on the anatomy of the vascular system of lianas from the Sapindaceae family, developing activities at the Rio de Janeiro Botanical Garden under the supervision of Dr. Neusa Tamaio. He completed his master's degree in February 2017. Subsequently, Israel began his doctorate at the University of São Paulo in June of the same year. This time, his research is dedicated to the vascular system in plants of the Nyctaginaceae (Four O'clock Family), under the supervision of Dr. Veronica Angyalossy. For this study, Israel carried out several fieldtrips in Brazil and abroad (Bolivia, United States, Mexico), in addition to developing part of the studies at the National Museum of Natural History of the Smithsonian Institution (Washington, DC, USA). The results of this research have already been presented at conferences in Brazil (2018), Mexico (2018) and the USA (2020, 2021) (remotely).

The author's list of publications can be found in his curriculum in online platforms such as Lattes / Orcid / Google Scholar / ResearchGate.

Cover

Micro– (colored) and macro– (brownish) photographs of stems of Nyctaginaceae in different developmental stages.

Cover: *Acleisanthes chenopodioides; Pisonia aculeata; Pisoniella glabrata; Reichenbachia hirsuta; Colignonia glomerata.*

Back cover: *Reichenbachia hirsuta; Bougainvillea berberidifolia; Leucaster caniflorus; Commicarpus scandens; Pisoniella glabrata.*