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Desenvolsimento, atividade secretora e composição química da resina dos ductos de Kielmeyera appariciana Saddi (Calophyllaceae).

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Sumário

RESUMO GERAL

Calophyllaceae é um importante representante do Cerrado brasileiro, apresentando espécies endêmicas e raras. A família apresenta distribuição pantropical e compreende 13 gêneros e 460 espécies com destaque para o gênero *Kielmeyera* devido ao número de espécies, distribuição geográfica e importância econômica. As espécies de Calophyllaceae são especialmente caracterizadas pela presença de ductos secretores, mas não existe nenhuma informação sobre o seu modo de formação e atividade secretora. Esse trabalho visa analisar o desenvolvimento, a estrutura, atividade secretora e composição da secreção dos ductos do sistema caulinar de *Kielmeyera appariciana*. Nossos resultados demonstraram que a espécie possui ductos primários no córtex e medula e ductos secundários no floema. Ambos os ductos são semelhantes, sendo compostos por um epitélio unisseriado circundado por uma bainha e com lume formado por esquizogenia. Contudo, os ductos diferem quanto à composição do exudato. As análises químicas, complementadas pela localização histoquímica, demonstraram que os ductos primários produzem resina, enquanto os secundários produzem goma, sendo este o primeiro relato da ocorrência de dois tipos de ductos em um mesmo órgão. A análise do modo de formação dos ductos resiníferos também comprovou que estes são esquizógenos, apresentando atividade pectinase entre as células centrais da roseta, soltando-as umas das outras. Em seguida, as células afastam-se por crescimento polarizado, mediadas por uma reorganização do citoesqueleto. Com a diferenciação do epitélio, as células iniciam a atividade secretora, percebida pelo acúmulo de secreção no citoplasma. De maneira assincrônica, as células entram em processo de morte celular mediadas pela liberação de espécies reativas de oxigênio, resultando em um acúmulo de substâncias fortemente coradas no citoplasma, degradação de plastídeos e mitocôndrias, condensação nuclear e ruptura do tonoplasto. Atividade celulase foi detectada nas paredes destas células, especialmente na região voltada para o lume do ducto, rompendo a célula e liberando o exudato em seu interior. A participação do citoesqueleto na formação do ducto esquizógeno e a secreção holócrina mediado por processo de morte celular programada são descritos pela primeira vez para ductos secretores.

GENERAL ABSTRACT

Calophyllaceae are important representatives of the Brazilian Cerrado, presenting endemic and rare species. The family has a pantropical distribution and comprises 13 genera and 460 species in which *Kielmeyera* stands out due to the number of species, geographical distribution and economic importance. The species of Calophyllaceae are especially characterized by the presence of secretory ducts but there is no information on their mode of formation and secretory activity. This work aims to analyze the development, structure, secretory activity and composition of the secretion of the ducts of the *Kielmeyera appariciana*. Our results showed that the species has primary ducts in the cortex and pith and secondary ducts in the phloem. Both ducts are similar, being composed of a uniseriate epithelium surrounded by a sheath and with a lumen formed by schizogeny. However, the ducts differ in relation to the composition of the exudate. The chemical analyses, complemented by the histochemical localization, showed that the primary ducts produce resin, while the secondary ones produce gum. This is the first report of the occurrence of two types of ducts in the same organ. The analysis of the formation of the resin ducts also proved that they are schizogenous in which pectinase digests the middle lamella between the central cells of the rosette, releasing them from each other. Then, the cells split away by polarized growth, mediated by a reorganization of the cytoskeleton. With the differentiation of the epithelium, the cells initiate the secretory activity, identified by the accumulation of secretion in the cytoplasm. Asynchronously, epithelium cells start to die mediated by the release of reactive oxygen species, resulting in an accumulation of strongly colored substances in the cytoplasm, degradation of plastids and mitochondria, nuclear condensation and rupture of the tonoplast. Cellulase activity was detected in the walls of these cells, especially in the region facing the duct lumen, breaking the cell and releasing the exudate inwards. The role of the cytoskeleton in the formation of a schizogenous duct and the holocrine secretion mediated by programmed cell death are described for the first time for secretory ducts.

GENERAL INTRODUCTION

Calophyllaceae – a taxonomic perspective

Calophyllaceae have pantropical distribution and comprise 13 genera and 460 species (Stevens 2012), with ethnobotanical and economic importance, such as guanandi (*Calophyllum brasiliense* Cambess), which offers good quality wood and is considered the first hardwood from Brazil (Souza & Lorenzi 2012), and camaçari (Caraipa densifolia), which has anti-inflammatory, antioxidant and anti-tumor properties, being used to treat skin diseases (Silveira 2010).

The first work published by the Angiosperm Phylogeny Group (APG 1998) included Hypericaceae and Calophyllaceae into Clusiaceae in the order Malpighiales. In APG II (2003), Hypericaceae were elevated to family and in APG III (2009) and APG IV (2016), the subfamily Kielmeyeroideae (Stevens 2007) were split off from Clusiaceae and elevated to Calophyllaceae. The recognition of Calophyllaceae as a distinct family from Clusiaceae was confirmed by phylogenetic studies (Ruhfel et al. 2011) that showed that if Calophyllaceae were included in Clusiaceae, the families Bonnetiaceae, Hypericaceae and Podostemaceae should also be included to remain the monophyly of the group (Souza & Lorenzi 2012).

In general, Calophyllaceae are especially characterized by the presence of several secretory structures in all members of the family. These secretory structures are usually ducts (Rizzini 1971; Rizzini & Mors 1976; Cronquist 1981; Mabberley 1987). Secretory ducts can be located in all plant organs, mainly in the vascular region (Solereder 1908, Metcalfe Chalk 1950), associated to the bundles, but they can also occur in the parenchyma, as described in leaves of *Kielmeyera coriacea* and *K. grandiflora* (Trad et al. 2012).

Secretory ducts

Secretory ducts are glands composed of an epithelium of secretory cells that delimit an elongated lumen in which the secretion is released and stored. This exudate may have a variable chemical nature. In the case of Calophyllaceae, secretory ducts are often referred to as gum producers (Cronquist 1981; Mabberley 1987) but latex production (Rizzini 1971; Rizzini & Mors 1976) has also been reported as a translucent, white to yellowish , orange or red secretion (Santos et al. 2015).

Duct development

In relation to formation, secretory ducts may be lysigenous, schizogenous or schizolysigenous. The lysigenous process is when there is autolysis of the initial cells of the duct for the formation of the lumen. Schizogenous formation involves the separation of the initial cells of the duct through digestion of the middle lamella and polarized expansion, creating the lumen. The lysigenous and schizogenous processes can also be combined for the formation of the lumen, in a process called schizolysigenous, where the formation of the duct begins with the autolysis of one or more cells and then, the intercellular space between initial cells expands forming the lumen (Fahn 1979; Turner 1999).

It is important to note that the different types of formation of secretory structures such as ducts have caused doubts and controversies since the first published works in an attempt to elucidate this subject. Currently, there is still a lot of disagreement as to its possible origin, as was registered for Rutaceae, having been described as schizogenous by Solereder (1908), lysigenous or schizolysigenous by Engler (1931) and schizogenous or lysigenous by Metcalfe & Chalk (1950). Turner et al. (1998) investigated *Citrus* and found that the thin walls of the secretory tissue are very sensitive to the osmotic potential of fixatives. While the cells of neighboring tissues are bounded by more rigid walls and usually appear intact, the cells of the secretory epithelium are swelled and collapsed, misinterpreting the *Citrus* glands as having

lysigenous development. Similar work has been carried out to verify the type of development of secretory cavities in *Eucalyptus* species that, at first, was described as lysigenous but studies with more appropriate techniques have shown that the development of secretory cavities of this genus is schizogenous (Carr & Carr 1970).

There are different ways to identify the origin of secretory ducts. Bennici and Tani (2004) clearly demonstrated the schizogenous origin of secretory cavities using developmental analyses based in anatomical and ultrastructural data. In *Pilocarpus*, the secretory cavities would be schizogenous according to Marquete (1981) and schizolysigenous for Spegazzini et al. (2002). This divergence is due to the analysis made only in adult structures, without an ontogenetic study.

Ultrastructural analysis is a useful method for studying the formation of ducts. In *Copaifera trapezifolia*, the separation of epithelial cells at the beginning of development was observed for the schizogenous formation of the lumen. This process was confirmed by ultrastructural analyses which detect dissolution of the middle lamella but no degeneration of epithelial cells (Milani 2009). Turner (1999) reported that fixing artifacts can be misinterpreted as cell lysis and recommends cautious investigations in the study of the development of secretory ducts and cavities. In another study with *C. langsdorffi*, Rodrigues (2008) reports a schizogenous process for the formation of the secretory cavity through light microscopy. However, transmission electron microscopy analysis also showed the occurrence of lysigeny, confirmed by the immunocytochemical test - TUNEL, which indicated programmed cell death of the initial cells.

Studies of the cytoskeleton also contribute to the elucidation of secretory ducts and cavities formation. The cytoskeleton is formed by a set of protein filaments with a structural and mechanical function that ensure eukaryotic cells the ability to maintain their internal structure, their conformation and the ability to change the internal organization of their

components, essential to the growth process, division and adaptation to the environment (Alberts et al. 2004). The immunolabeling of the cytoskeleton can indicate the process of formation of any secretory structure, including the ducts.

Structural changes during the secretory process can culminate in the death of some duct initial cells. This death occurs as a result of a highly organized process called programmed cell death (Gunawardena et al. 2007), which is part of the normal development of plants. From ultrastructural studies of the resin ducts in *Protium heptaphyllum*, changes in the cell wall and dissolution of the middle lamella of the initial cells were observed, as well as irregularly contoured nuclei and mitochondria with signs of programmed cell death. These observations led Palermo et al. (2018) to suggest the schizolysigenous development of the secretory ducts in this species. Other methods of detection of programmed cell death can be used, such as the identification of DNA fragmentation through immunocytochemical tests, which can indicate the moment when epithelial cells are digested to originate the spaces that characterize the secretory ducts (Farradás et al. 2014).

Secretion mode

The secretion mode is a complex phenomenon of separation or isolation of certain substances from the protoplast, which may include a process of synthesis, accumulation in certain intracellular compartments as well as extracellular release or elimination into nearby internal spaces or, otherwise, outside the surface of the plant (Machado 2005). According to Fahn (1979), the secretion can be released by a holocrine or merocrine mechanism. When substances are released from secretory cells as a result of their disintegration, the secretion is named holocrine. In the case of substances that are eliminated from the cell without cell lysis, the secretion is named merocrine. Merocrine release can be divided in two subtypes: eccrine, when secretion passes freely through the plasma membrane as a result of a concentration gradient or by an active process; or granulocrine, when the secreted substance is released

through vesicles that fuse to the plasma membrane, transferring their content out of the protoplast.

For a more accurate and reliable analysis of the secretion mode, ultrastructural analyses are the most suitable, as they provide information at the subcellular level. Thus, we can verify the organelles involved in the process, changes in the cell wall and other characteristics associated with the secretory process. Ultrastructural analyses of the secretory ducts in Anacardiaceae revealed the presence of plastids with osmiophilic inclusions in the epithelial cells of *Tapirira guianensis* and a great prominence of the rough endoplasmic reticulum and free ribosomes in the epithelium of *Spondias dulcis* and *T. guianenses*, suggesting their role in the lipid-secreting activity (Lacchia & Carmelllo-Guerreiro 2009). The authors also suggest the secretion release by eccrine mechanism due to the presence of electron-dense material dispersed in the peripheral cytoplasm and in the periplasmic space.

Kielmeyera

Within Calophyllaceae, *Kielmeyera* stands out in the Brazilian flora, containing about 50 species, most of them in the Cerrado. Some species are frequent and well-distributed, such as *K. coriacea* Mart. & Zucc. and *K. rubriflora* Cambess, while the vast majority have a more restricted occurrence (Jorge 2014). Some species found in the Brazilian Cerrado have been used by the population for the treatment of various diseases, such as schistosomiasis, leishmaniasis, malaria, infection by bacteria and fungi, among others (Pinheiro et al. 2003).

There are many chemical studies of the exudate and extracts of the leaves, fruits and barks of *Kielmeyera* species, with the isolation and identification of secondary metabolites, as well as the analysis of volatile constituents (Pinto et al. 1987; Gramacho 1997; Cortez et al. 1998). Studies carried out by Caddah (2009), Caddah et al. (2012) and Trad (2012) brought

great knowledge about the anatomy of leaves of several species of the genus, especially *K. coriacea*, although they did not provide a detailed description of the secretory structures.

Despite the studies previously mentioned, most aspects of the secretory structures of the genus remain unclear, such as the anatomy, ultrastructure, ontogeny, secretory mechanisms of synthesis and release of exudate, the chemical nature of the exudate and their possible applications in the systematics and taxonomy of the group. In this way, the investigation of ducts from *Kielmeyera* and their implication in the mode of synthesis and release of secretion becomes relevant for a better understanding of these glands in the genus and in Calophyllaceae.

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Chapter 1

Two origins, two functions: the discovery of distinct secretory ducts formed during the primary and secondary growth in *Kielmeyera*

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Background and Aims. Secretory ducts are reported for more than 50 families of vascular plants among primary and secondary tissues. A priori, all ducts of a plant are of the same type regardless their origin and only slight variations in the concentration of their compounds were reported for few species. However, two types of secretion were observed in primary and secondary tissues of *Kielmeyera appariciana*, leading us to investigate the structure and development of its ducts, as well the secretion composition and the possible influence of duct origins on their metabolism.

Methods. Shoot apices and stems in secondary growth of *K. appariciana* were collected and processed according to the usual techniques of plant anatomy for structural and developmental analyses. Histochemical tests were also performed in fresh samples to histolocalize the main classes of metabolites, additionally to the chemical analyses of the exudate from leaves and stems in secondary growth in HPLC.

Key Results. *Kielmeyera appariciana* has primary ducts in the cortex and pith and secondary ducts in the phloem. Both ducts are composed of uniseriate epithelium surrounded by a sheath and a lumen formed by schizogenous process. Despite their similar structure and formation mode, the ducts produce secretions of two types according to the chemical analyses corroborated by histolocalization of the secretory products. The primary ducts produce resin, while the secondary ducts produce gum.

Conclusions. This is the first report of two types of ducts in the same plant. The distinct origin of ducts from ground meristem in primary shoots and vascular cambium in secondary tissues might be related to the metabolic alteration which likely led to suppression of the biosynthetic pathway of terpenoids and phenolics in the secondary ducts. The functional and evolutionary implications of this innovation are discussed in our study and may be related to the diversification of *Kielmeyera* and Calophyllaceae in tropical environments.

Keywords: secretory ducts, origin, metabolism, evolution, structure, secretion composition.

INTRODUCTION

Secretory ducts are internal glands composed of an epithelium formed by secretory cells which release the exudate in an elongated intercellular space named lumen (Fahn, 1979). They occur in 54 families of vascular plants, with prevalence of resin ducts in 48 families (Ciccarelli et al., 2001; Prado and Demarco, 2018) and six families with mucilage ducts: Welwitschiaceae, Chloranthaceae, Combretaceae, Malvaceae, Neuradaceae and Vochysiaceae (Sykes, 1911; Swamy, 1953; Alverson et al., 1998; Sajo and Rudall, 2002; Tilney, 2002). Secretory ducts are particularly common in families as Anacardiaceae, Asteraceae, Burseraceae, Calophyllaceae, Clusiaceae, Salicaceae, and some Fabaceae and Malvaceae (Metcalfe and Chalk, 1950; Fahn, 1979; Langenheim, 2003; Castro and Demarco, 2008; Lacchia and Carmello-Guerreiro, 2009; Thadeo et al., 2014; Prado and Demarco, 2018; Garcia et al., 2020).

Ducts may be originated from ground meristem, procambium and/or cambium (Fahn, 1979) and vary from slightly elongated structures, as found in some Asteraceae, Malvaceae and Salicaceae (Lersten and Curtis, 1986; Thadeo et al., 2014; Garcia et al., 2020), to extremely long and continuous ducts within all over the plant as observed in conifers, Anacardiaceae and Burseraceae (Garcia et al., 2020). Nevertheless, distinct secretory ducts within an organ in each species produce the same type of secretion in all plants, regardless their origin (Fahn, 1979; Rodrigues and Machado, 2009; Prado and Demarco, 2018).

The secretion of the ducts has a variable composition in the species but they can be generically grouped into three types: resin, mucilage and gum (Fahn, 1979; Langenheim, 2003; Prado and Demarco, 2018) and each type of secretion is usually conservative within the families (Metcalfe and Chalk, 1950; Prado and Demarco, 2018), being often used as diagnostic character (Bayer and Kubitzki, 2003). Few families have distinct genera producing different secretions in their respective ducts, such as Anacardiaceae with resin ducts in almost

all genera and gum ducts in *Lannea*, *Operculicarya* and *Rhodosphaera* (Venkaiah and Shah, 1984; Pell et al., 2011). Considering the resin ducts, only small variations in the secretion composition have already been observed comparing ducts of vegetative and reproductive organs in the same species, inferred from a different color of the exudate in each organ or based on the ultrastructure of the epithelial cells (Joel and Fahn, 1980a, b). Different types of ducts occurring side by side have never been reported until now. However, our field observations remarkably showed two very distinct types of secretion being exuded by leaves and stems in secondary growth in *Kielmeyera appariciana* Saddi.

Kielmeyera is one of the largest genera of Calophyllaceae, comprising 50 species which leaf and bark extracts are used in folk medicine such as anti-inflammatory, antioxidant, antibacterial and antifungal. Recently, biological assays have confirmed the efficacy of *Kielmeyera* extracts even against carcinogenic cell strains (Alves et al., 2000; Cortez et al., 2002; Jorge, 2014; Pinheiro et al., 2003). The main secretion found in the genus is resin which is produced by secretory ducts located in all organs, mainly adjacent to the vascular system (Metcalfe and Chalk, 1950).

Secretory ducts occur in all species of Calophyllaceae and there are divergences on respect of their secretion, previously referred to as resin, gum or latex (Metcalfe and Chalk, 1950; Rizzini 1971; Rizzini and Mors, 1976; Cronquist, 1981; Mabberley, 1987). These divergences raise doubts about the type of duct present in the family and about its possible diversity. Additionally, there are still many doubts in the interpretation of the anatomy of these secretory structures as well as the chemical nature of the secreted compounds.

Therefore, we selected *Kielmeyera appariciana* as a model to investigate the structure of its ducts occurring in primary shoots and stems in secondary growth, the chemical nature of their exudate, as well as the origin of these ducts and the possible influence of their origin on their metabolism.

MATERIAL AND METHODS

Plant material

Samples of *Kielmeyera appariciana* Saddi were collected in the campus of the Universidade de São Paulo in São Paulo/SP (Brazil) and the voucher was deposited in the herbarium SPF (USP; Costa, E.R. 1).

Histological analysis

Several primary shoots with leaves at different developmental stages (leaf primordium, developing leaves, and mature leaves) and fragments of stems in secondary growth (more than 1 cm of diameter) were collected and fixed in Karnovsky's solution for 24h at 4°C for the structural analyses. After fixation, shoot apices and stem portions in secondary growth were isolated, dehydrated through a tertiary butyl alcohol series (Johansen, 1940), embedded in Paraplast (Leica Microsystems Inc., Heidelberg, Germany), and serial sectioned at 10 μm thickness on a Leica RM2145 rotary microtome. Longitudinal and transverse sections were stained with astra blue and safranin O (Gerlach, 1984) and the slides were mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Fresh shoot apices and stems in secondary growth were also free-hand sectioned for histochemical analyses of the secretion. The following histochemical tests were applied: Sudan black B and Sudan IV (Pearse, 1985) in bright field and neutral red under blue light (Kirk, 1970) for lipids, Nile blue (Cain, 1947) in bright field and under blue light for neutral and acidic lipids, Nadi reagent (David and Carde, 1964) for terpenoids, copper acetate and rubeanic acid (Ganter and Jollés, 1969, 1970) for fatty acids, ferric chloride (Johansen, 1940) and potassium dichromate (Gabe, 1968) for phenolic compounds, vanillin and hydrochloric acid (Mace and Howell, 1974; Gardner, 1975) for tannins, Dragendorff's reagent (Svendsen and Verpoorte, 1983) and Wagner's reagent (Furr and Mahlberg, 1981) for alkaloids; periodic

acid – Schiff reaction (PAS) (Jensen, 1962) for polysaccharides, ruthenium red (Gregory and Baas, 1989) and Alcian blue (Pearse, 1985) for acidic mucilage, tannic acid and ferric chloride (Pizzolato, 1977) for mucilage, and Coomassie blue and aniline blue black (Fisher, 1968) for proteins. The autofluorescence of the secretion was also analyzed under UV and blue light. All tests and their respective control procedures were carried out according to Demarco (2017).

Observations and photographs were performed using a Leica DMLB light microscope equipped with a HBO 100W mercury vapor lamp and a blue light filter block (excitation filter BP 420-490, dichromatic mirror RKP 510, suppression filter LP 515) and UV filter block (excitation filter BP340-380, dichromatic mirror RKP400, suppression filter LP425).

Chemical analysis

For chemical analysis, duct exudates from mature leaves and from stems in secondary growth (more than 3 cm of diameter) were collected in becker, first extracted with acetone and analyzed. High performance liquid chromatography (HPLC) analyses were carried out using solvents A (H_2O + 0.1% acetic acid) and B (acetonitrile HPLC grade, J. T. Baker®). HPLC-DAD analyses were performed on an Agilent 1260 chromatograph equipped with a 60 mm flow cell and photodiode array detector. Zorbax Eclipse plus reverse phase C_{18} (4.6 x 150 mm) containing 3.5 μm particle diameter was used as the stationary phase. All analyses were carried out on a mobile phase flow rate of 1.0 $mL.min^{-1}$, 45°C of temperature and 3 μL of sample injection at 2 $mg.mL^{-1}$ of concentration. The chromatographic run method used consisted of: 10-25% B in 10 min, followed by 25-50% B in 20 min, and 50-100% B in 20 min, maintaining 100% B for an additional 10 min, in a total of 60 min. HPLC-MS (high performance liquid chromatography coupled to mass spectrometry) analyses were performed on a Shimadzu chromatograph coupled to a MAXIS 3G - Bruker Daltonics® Q-TOF mass

spectrometer with capillary 4500V, nebulizer at 27 psi in a positive mode, allowing to assign the *m/z* values of the peaks observed in the chromatogram obtained by HPLC-DAD.

RESULTS

Kielmeyera appariciana has primary and secondary ducts throughout the shoot system (Fig. 1-3), which form an extensive secretory network that protect all aerial parts of the plant against herbivory.

Primary ducts

The primary ducts are widely distributed in the cortex and pith (Fig. 1A). They are axially elongated and vary in diameter and length (Fig. 1D-F). The ducts occurring in the outer cortex are very narrow compared to the ducts located closer to the phloem (Fig. 1A; B; E and G). On the other hand, the medullary ducts are always wide and are mainly distributed close to the vascular system (Fig. 1A and Fig. 1F). Several ducts merge laterally and some of them even bifurcate, especially in the nodes, where they are continuous between stem and leaf. These merged ducts may or may not split out again (Fig. 1 C-D; Fig. 3F-G).

Each duct is composed of uniseriate secretory epithelium constituted by thin-walled cells with dense cytoplasm and prominent nucleus (Fig. 2E). Additionally, the duct has a sheath that varies from uni- to biseriate and contains phenolic compounds (Fig. 1E-F; Fig. 2E).

Ontogenetic analyses of the shoot apices of *K. appariciana* revealed that the primary ducts are formed just below the shoot apical meristem, after the differentiation of the procambium in the cortical region (Fig. 1B). The medullary ducts are formed soon after the origin of the cortical ducts. Primary ducts originate from a single cell of the ground meristem that has thin walls, dense cytoplasm and prominent nucleus (Fig. 2A). Successive divisions of this initial cell form a rosette of undifferentiated cells that remain in constant division (Fig. 1C; Fig. 2B). The rosette cells actually are arranged in an elongated strand (Fig. 1C), which

start to differentiate into two distinct regions. The central cells will compose the duct epithelium while the peripheral cells will give rise to the phenolic sheath (Fig. 2C).

During the differentiation of the epithelium, a small aperture in the central region of the rosette is formed by separation of cells (Fig. 2C-D). Later, this aperture expands schizogenously giving rise to the lumen of the duct (Fig. 2D-G). Concomitantly, epithelial cells begin to produce secretion which is released into the expanding lumen. At this secretory phase, the epithelial cells are slightly elongated inwards, sinuously outlined (Fig. 2D-F), having cytoplasm filled with secretion. At the final stage of development, mature ducts stop producing secretion and the epithelium becomes flattened (Fig. 2G).

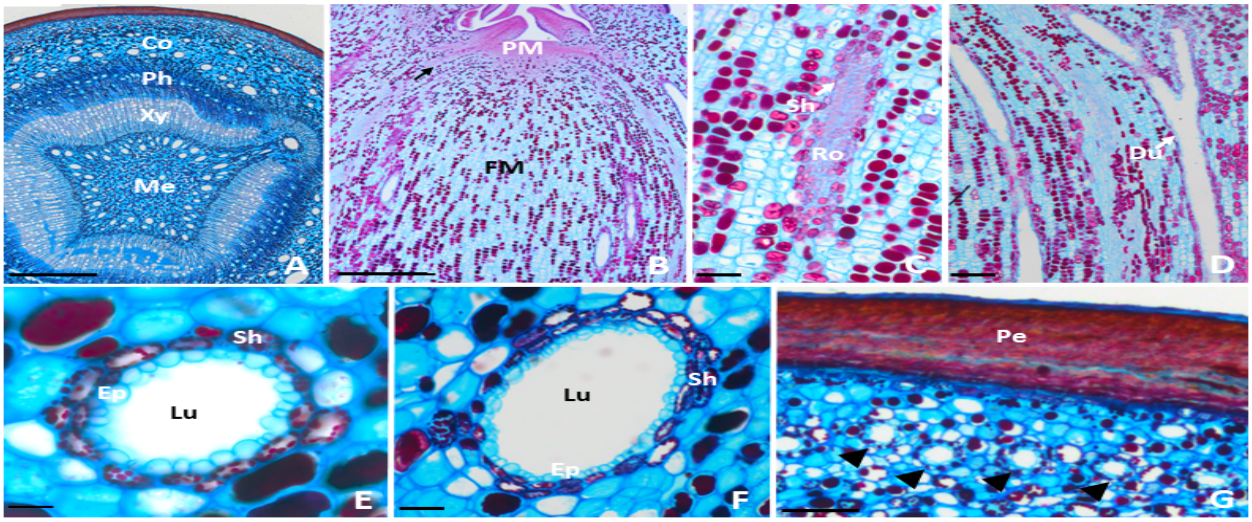

Fig. 1: Distribution and structure of *Kielmeyera appariciana* primary ducts. A: Distribution of primary ducts. B-D: Longitudinal sections of the cauline apex. Note the highlighted procambium cord with the black thin arrow. D: Process of blending and fork of ducts. E-F: Primary ducts that occur in the cortex and medulla with phenolic sheath. G: Highlight for the reduced diameter of the primary ducts that occur in the outermost region of the cortex (Black thick arrow). (Co: Cortex; XSh: Sheath; Ep: Epithelium; Lu: Lumen; FM: Fundamental meristem; Pe: Periderm, PM: Promeristem, Ro: Rosette).

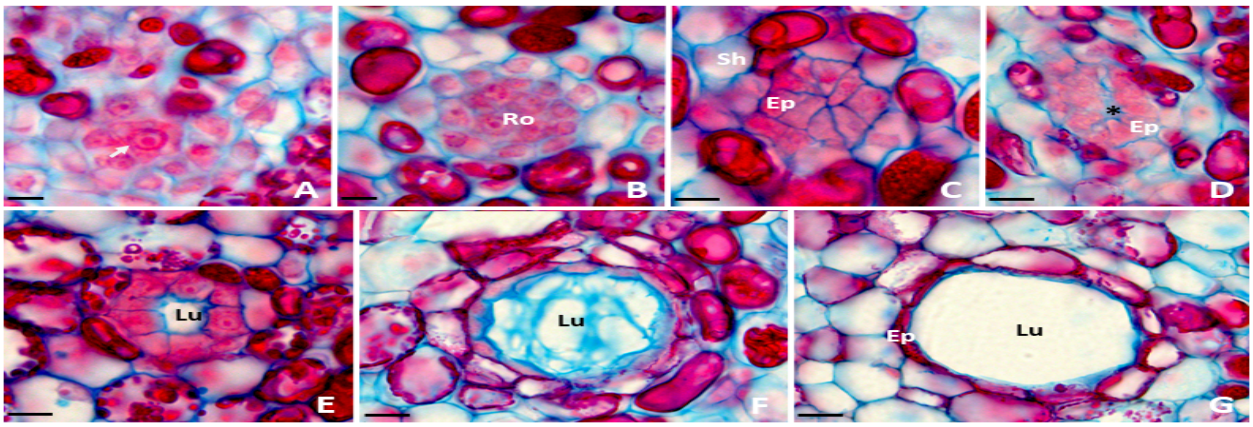

Fig. 2: Development of the primary ducts of *Kielmeyera appariciana*. A-G: Stages of formation of primary ducts. A highlight for the initial cell (thin white arrow). Note (*) the beginning of the lumen formation. F: Note the lumen full of secretion. (Sh: Sheath, Ep: Epithelium, Lu: Lumen, Ro: Rosette).

Secondary ducts

Secondary ducts occur in the secondary phloem (Fig. 3A). They are narrower than the primary ones (Fig. 3A-D) and larger diameters are only observed when two or more adjacent ducts merge laterally (Fig. 3F-G). These ducts are located in axial parenchyma bands with a stratified arrangement (Fig. 3B). Structurally, secondary ducts are similar to the primary ones, having uniseriate epithelium surrounded by a sheath but this latter is parenchymatic (Fig. 3D).

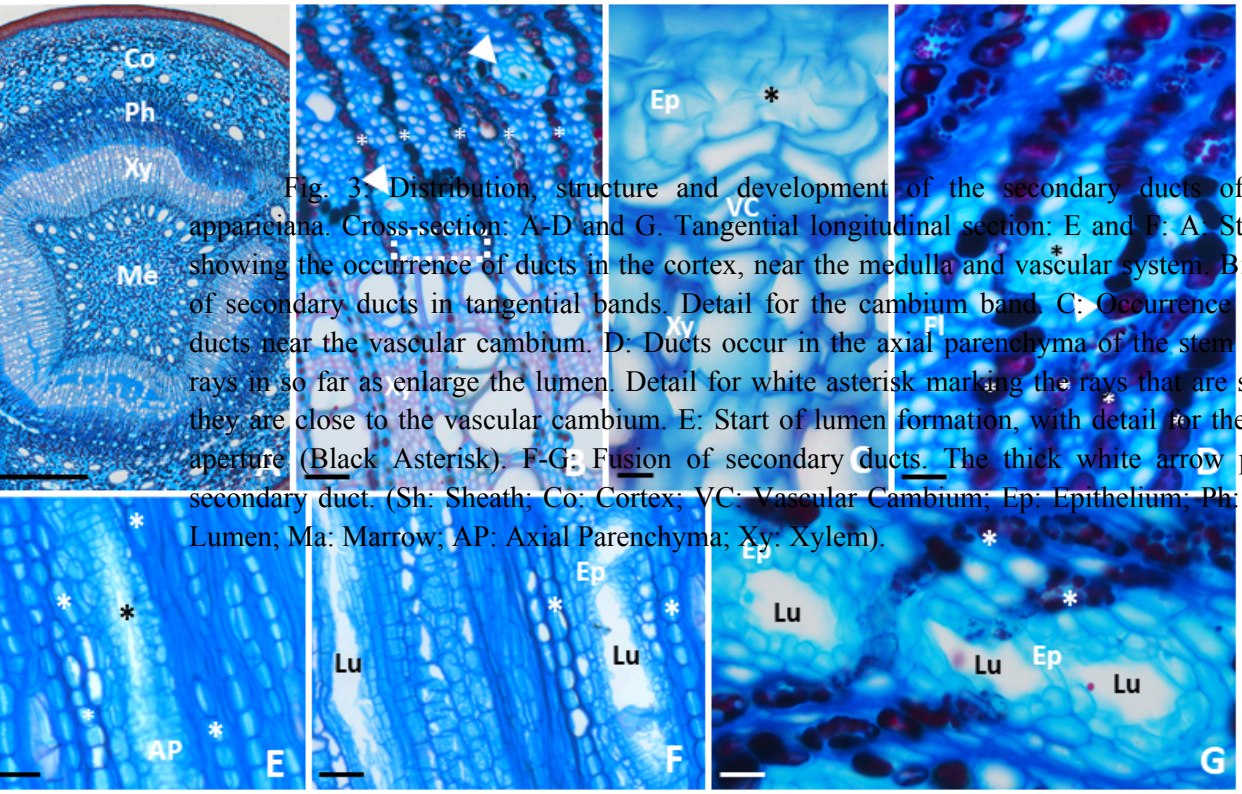

Fig. 3: Distribution, structure and development of the secondary ducts of Kielmeyera appariciana. Cross-section: A-D and G. Tangential longitudinal section: E and F: A. Stem overview showing the occurrence of ducts in the cortex, near the medulla and vascular system. B: Distribution of secondary ducts in tangential bands. Detail for the cambium band. C: Occurrence of secondary ducts near the vascular cambium. D: Ducts occur in the axial parenchyma of the stem and push the rays in so far as enlarge the lumen. Detail for white asterisk marking the rays that are straight when they are close to the vascular cambium. E: Start of lumen formation, with detail for the small initial aperture (Black Asterisk). F-G: Fusion of secondary ducts. The thick white arrow points to the secondary duct. (Sh: Sheath; Co: Cortex; VC: Vascular Cambium; Ep: Epithelium; Ph: Phloem; Lu: Lumen; Ma: Marrow; AP: Axial Parenchyma; Xy: Xylem).

The secondary ducts originate from the vascular cambium (Fig. 3B-C). Ducts have a late origin in the secondary phloem and are only formed after the production of a set of phloem cells during the initial activity of the cambium. Fusiform cells divide intensely and form groups of cells (rosettes) with longitudinally elongated arrangement, similar to the development of the primary ducts (Fig. 3E). The lumen of the secondary ducts is also formed by separation of the rosette cells (Fig. 3E).

There is no radial duct in *K. appariciana* and when the ducts are formed in the direction of the rays, it is observed that the expansion of the lumen affects the path of the ray that becomes sinuous at this point (Fig. 3B) and touch the duct (Fig. 3 D and G).

Secretion composition

The differences between the primary and secondary ducts of *K. appariciana* are not restricted to the origin. Field observations showed that the secretion exuded from both the ducts is initially translucent and viscous, however, shortly after exposure of this exudate to air, a polymerization of the secretion is observed. The exudation of primary ducts in developing leaves and stems tends to solidify and harden, while the secretion exuded by

secondary ducts in stems in secondary growth polymerizes and acquires a gelatinous consistency. Histochemically, the secretions are very distinct. Primary ducts produce resin composed of lipids, including terpenoids (Fig. 4A-F), phenolic compounds (Fig. G-H), polysaccharides (Fig. 4 I-K) and proteins (Fig. 4L), while secondary ducts secrete only gum constituted of polysaccharides (Fig. 5A-B) and proteins (Fig. 5C-D).

Chemical analysis

Chemical analysis also confirmed the different composition of the secretion from ducts with distinct origin. Both secretions were analysed through HPLC-DAD as shown in Fig. 6. From the overlapping of chromatograms obtained from leaves and stems in secondary growth exudates of *K. appariciana*, it is possible to verify only in the leaves exudate the presence of various phenolic compounds. Even when injecting the sample from stems in secondary growth in a higher concentration these compounds were not detected. The phenolic compounds were revealed by their characteristic UV spectrum (Zhang et al., 2013) and the main peaks observed in the chromatogram showed very similar UV spectrum. Through HPLC-MS analysis the seven major peaks observed showed the following *m/z* values: 1. Retention time (R_t): 33.41 min., *m/z* 359.1497; 2. R_t: 37.01 min., *m/z* 373.1643; 3. R_t: 39.05 min., *m/z* 387.1805; 4. R_t: 40.55 min., *m/z* 359.1505; 5. R_t: 43.08 min., *m/z* 343.1560; 6. R_t: 44.71 min., *m/z* 373.1656; 7. R_t: 46.44 min., *m/z* 357.1710. Spectral library search available in the GNPS website (Global Natural Products Social Network: gnps.ucsd.edu) did not indicate correspondence with any known compound.

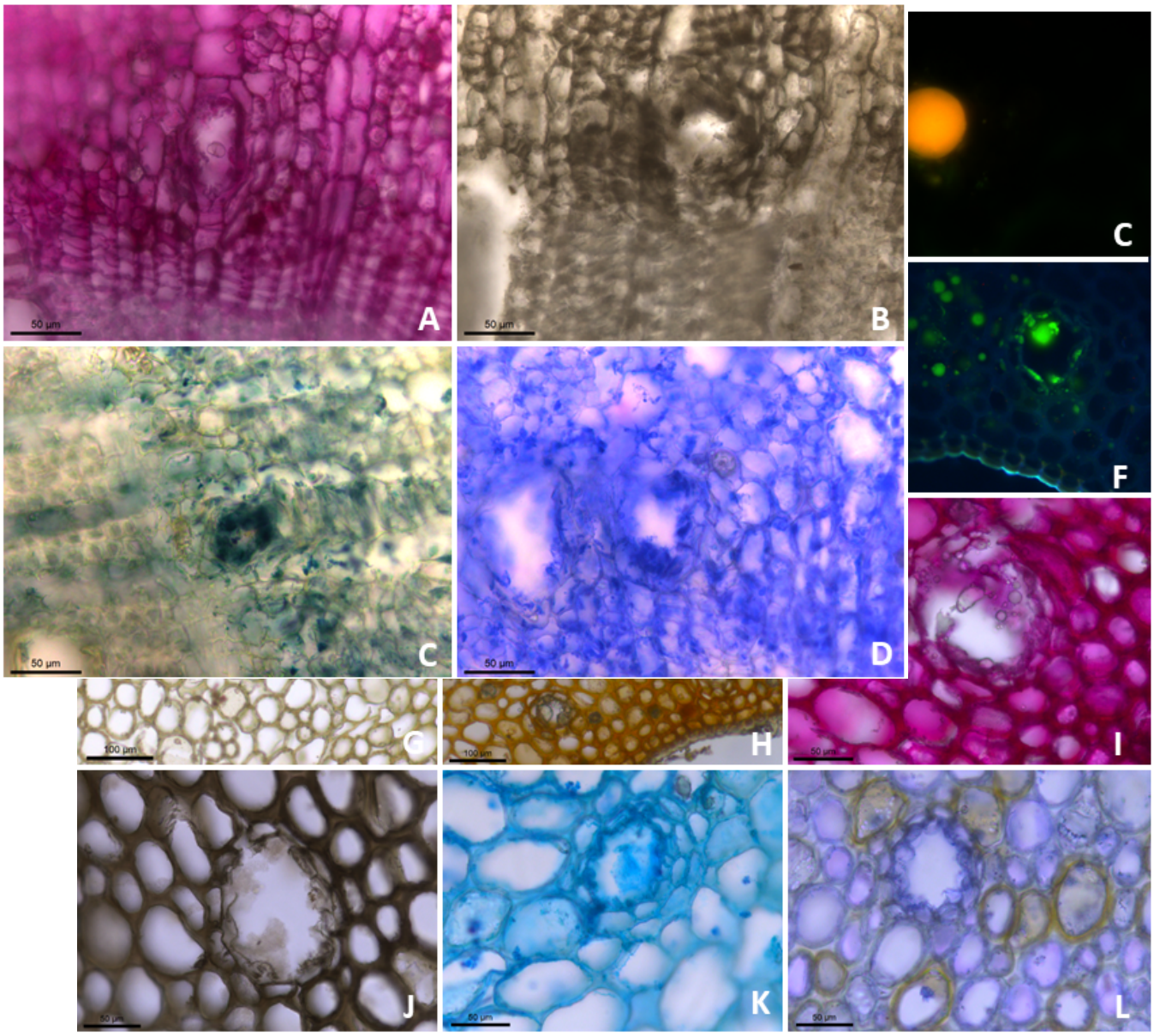

Fig. 4: Histochemical analysis of *Kielmeyera appariciana* primary ducts secretion. A-B: Detected lipids with Sudan black and Nile blue in the bright field; C-D: Identified lipids with Nile blue (C) and neutral red (D) under blue light; E. identified terpenoids with NADI reagent. F Autofluorescence of secretion. G-H: Phenolic compounds detected using ferric chloride (G) and potassium dichromate (H). I-J: Mucilage identified with ruthenium red (I), tannic acid and ferric chloride (J) and Alcian blue (K). L: Proteins detected with Coomassie blue.

Fig. 5: Histochemical analysis of the secretion of the secondary ducts of *Kielmeyera appariciana.* A-B: Mucilage detected by ruthenium red (A) and tannic acid and ferric chloride (B). C -D: Proteins identified by starch black B (C) and Coomassie blue (D).

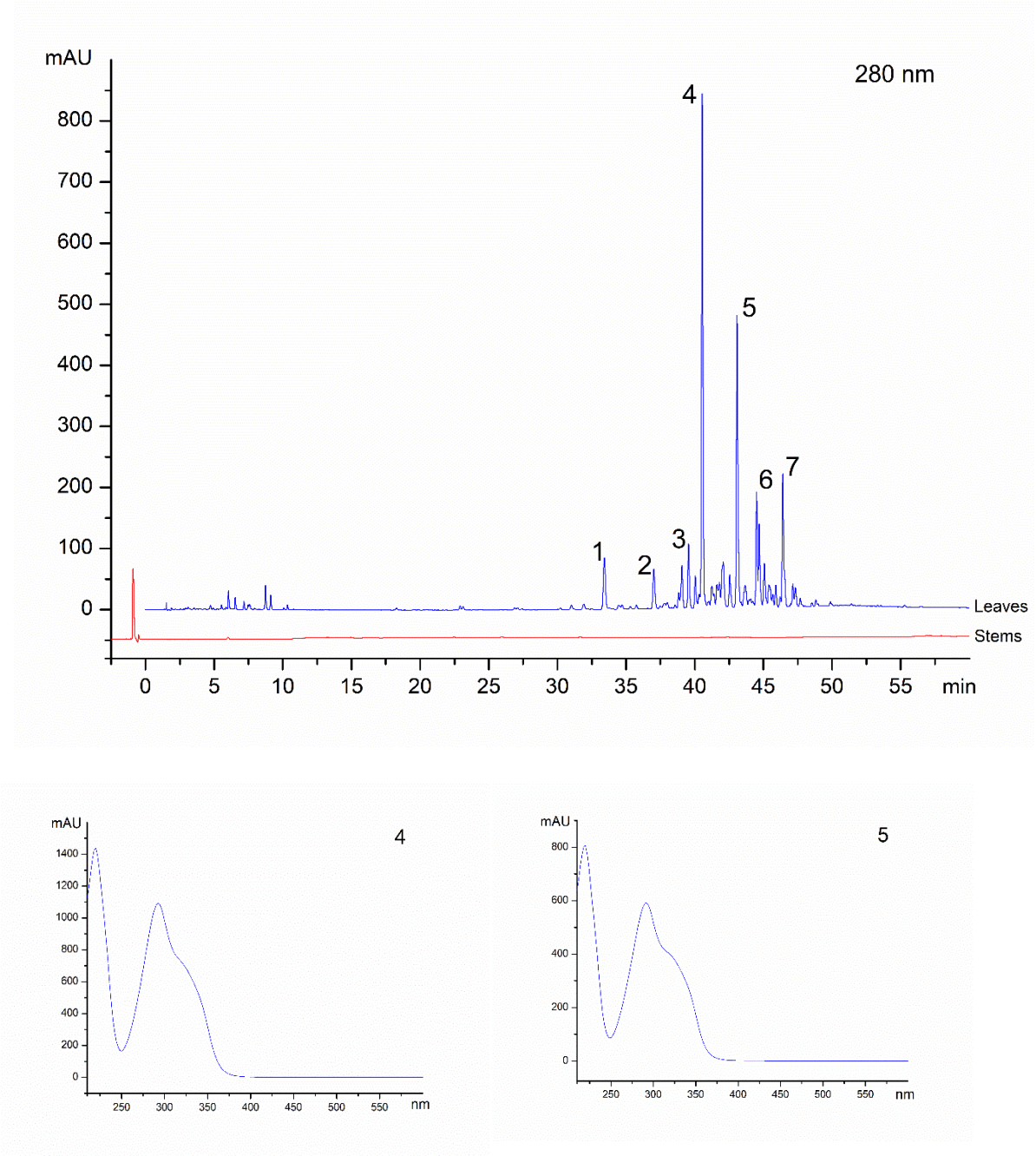

Figure 6. HPLC-DAD analyses of exudated secretion of *K. appariciana* leaves and stems. HPLC-MS analysis indicated: 1. Retention time (R_t): 33.41 min., *m/z* 359.1497; 2. R_t: 37.01 min., *m/z* 373.1643; 3. R_t: 39.05 min., *m/z* 387.1805; 4. R_t: 40.55 min., *m/z* 359.1550; 5. R_t: 43.08 min., *m/z* 343.1560; 6. R_t: 44.71 min., *m/z* 373,1656; 7. R_t: 46.44 min., *m/z* 357.1710.

DISCUSSION

Our study demonstrated for the first time the existence of two types of secretory ducts within a plant. *Kielmeyera appariciana* has resin ducts in the primary stem and leaves, whilst gum ducts in the secondary phloem of the stem.

The majority of plants that have secretory ducts, have these ducts in the primary and secondary regions of the plant body, since most ducts occur in the vascular system (Metcalfe and Chalk, 1950). Considering the 54 families containing secretory ducts, 40 have them in primary and secondary vascular systems (Metcalfe and Chalk, 1950). Ducts occur in five families of Malpighiales – Calophyllaceae, Clusiaceae, Humiriaceae, Hypericaceae and Salicaceae – having fundamental primary ducts and secondary vascular ducts in Calophyllaceae, cortex, medulla and phloem in Clusiaceae, secondary phloem, cortex and medulla in Hypericaceae and for Humiriaceae and Salicaceae having ducts in primary tissues as cortex and medulla (Metcalfe and Chalk, 1950; Ciccarelli et al., 2001; Prado and Demarco, 2018). The occurrence of secretory structures only in primary tissues is common for some types of glands (Fahn, 1979) but the occurrence of one type of secretory structure only in secondary tissues is extremely rare and has only been reported for laticifers of Hippocastanoideae (Sapindaceae; Medina et al,. 2020). The occurrence of glands in the secondary vascular system (i.e. originated by cambium) is expected when the same type of gland also occurs in the primary vascular system (i.e. originated by procambium). Thus, the observation of fundamental primary ducts followed by secondary phloem ducts, as noted in *Kielmeyera*, is not common. Few genera have this type of duct distribution in different tissue systems when comparing primary and secondary regions of the plant body, as observed in *Pinus*, which has primary ducts in the cortex and secondary ducts in the xylem.

Distribution within the plant

The ducts of *K. appariciana* form a network across the entire shoot system in the cortex, pith and secondary phloem. This wide distribution constitutes an efficient defensive system against herbivory, since any region of the plant that be injured will cause the release of the internal secretion to outside. A similar distribution of the primary ducts along the axial system of the stem was also reported to *Parthenium argentatum* (Joseph et al., 1988), *Commiphora wightii* (Arn.) Bhandari (Bhatt, 1987) and *Lannea coromandelica* (Houtt.) Merr. (Venkaiah and Shah, 1984). These ducts may have varied arrangements, such as vertical, horizontal or irregular orientation, and be continuous or discontinuous, branched or unbranched, according to Venkaiah and Shah (1984). Ducts of *Kielmeyera appariciana* form a system of continuous branched tubes which fuse apically and laterally, contributing to a significant expansion of the duct in length and width.

Secondary ducts are located in axial parenchyma and are also referred to as axial ducts by authors such as Kibblewhite and Thompson (1973) and Sato and Ishida (1982) in *Pinus*. These ducts occur within the axial parenchyma bands of the secondary phloem, which may be related to growth layers, as occurs in the wood of *Copaifera langsdorffii* (Marcati et al., 2001). Radial ducts are common in some families as Pinaceae and Anacardiaceae but they are absent in *Kielmeyera* which ray is displaced when a duct is formed in its direction (Kibblewhite and Thompson, 1973; Marcati et al., 2001 and Sato and Ishida, 1982). The occurrence of ducts that are closely linked to the parenchyma rays is common. Wiedenhoeft and Miller (2002) identified the same relation between ducts and rays and warned that even if ray cells pass very close to both sides of the duct, they should not be considered part of the duct.

Duct diversity

Histologically, the ducts are very similar to each other and their diversity is related to their mode of formation of the lumen or to the type of secretion produced. The mode of

formation may be of three types: schizogenous, lysigenous or schizo-lysigenous (Carr and Carr, 1970; Fahn, 1979; Turner et al., 1998; Turner, 1999). The schizogenous mode is when the lumen is formed exclusively by cell separation, as observed in the primary and secondary ducts of *K. appariciana*. On the other hand, the lysigenous mode is when the lumen is formed by programmed cell death of one or more cells of the rosette. Finally, the schizo-lysigenous mode is when both processes occur for the formation of the lumen (Fahn, 1979). The formation mode of ducts varies from species to species (Venning, 1948) and may also vary in different regions of the same organ (Venkaiah and Shah, 1984). However, even when the same secretory structure has different origins in a plant, the nature of secretion is very similar, as reported by several authors (Solereder, 1908; Fahn, 1979; Joel and Fahn, 1980a, b, c; Nair et al., 1983; Lacchia and Carmello-Guerreiro, 2009; Royo et al., 2015).

In relation to the diversity of exudates, ducts can produce resin, mucilage or gum (Fahn, 1979; Langenheim, 2003; Garcia et al., 2020), with a great diversity of composition for the secretions classified as resin (broad sense) which is always mostly lipophilic (terpenic or rarely phenolic; Prado and Demarco, 2018). The wide distribution of resin ducts in vascular plants is directly related to the type of environment in which the groups of resinous plants have evolved, such as tropical environments where the rate of herbivory is higher (Langenheim, 2003; Prado and Demarco, 2018 and references therein) and may explain the chemical diversity found in some groups.

Secretion and metabolism

Our results showed that the differences between the primary and secondary ducts of *K. appariciana* are not restricted only to their origin from ground meristem or cambium. Resin is only produced in cortex and pith, which is mainly composed of terpenes and phenolics but also contains polysaccharides and proteins. Conversely, gum is produced in secondary

phloem, where the production of lipophilic compounds has likely been suppressed. Our chemical analysis has confirmed the different composition of the secretions produced by each duct.

Some factors may be involved in this unusual metabolic alteration. Our hypothesis is that the expression of some genes related to production of terpenoids, such as the terpenoid synthase (TPS) genes, and phenolics, such as phenylalanine ammonia lyase (PAL) genes. TPS is a superfamily of genes conserved in gymnosperms and angiosperms which is likely derived from a single ancestor (Trapp and Croteau, 2001; Cheng et al., 2007). Accordingly, phenolic acids in plants are primarily derived from the phenylpropanoid biosynthetic pathway with the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (PAL; Ma et al., 2016). Changes on TPS and PAL gene sequences or on their gene expression may be related to the origin of two types of ducts in *Kielmeyera appariciana* and this might be an initial hypothesis to be investigated in future studies.

The regulation of plant terpenoid biosynthesis is generally related to spatial and temporal aspects and developmental regulation has already been reported in the production of some terpenoids (Aharoni et al., 2003; Dudareva et al., 2003; Lu et al., 2002; Cheng et al., 2007). In addition, changes in gene regulation that alter terpene quantities are linked with functional shifts according to Theis and Lerdau (2003) and might have conferred adaptive advantages to *Kielmeyera*.

Function

Functionally, the occurrence of two types of secretory ducts in the same plant may represent a specialization of the secretory system of the plant in relation to its ontogenetic stage. While primary resin ducts protect leaves and stem against herbivores during early development of the shoot system and secondary gum ducts abounding polysaccharides assist in the retention and/or translocation of water from the xylem into the phloem (Fahn, 1979;

Gibson and Nobel, 1986; Meyberg, 1998). In general, the marked combination of phenolic compounds and polysaccharides in different regions of the plant provides advantages for the plant as a whole because it is admittedly capable of adsorbing water, acts in protection against herbivory and water economy (Mollenhauer, 1967). The presence of phenolics in primary shoots indicates that the species invests in the protection of its photosynthetic organs against herbivore attacks, since the most predated plant organ is usually the leaf (Aoyama and Labinas, 2012).

Evolutionary implications

The evolutionary emergence of two types of ducts in *K. appariciana* represents an apomorphic character of *Kielmeyera* which may be related to genus diversification and should be searched in other species to evaluate its occurrence in the clade. Secretory ducts have evolved at least three times independently in Malpighiales, occurring in Calophyllaceae, Clusiaceae, Humiriaceae, Hypericaceae and Salicaceae. Secretory ducts have evolved once in the clusioid clade with two reversals in Bonnetiaceae and Podostemaceae and two other emergences in Humiriaceae and Salicaceae in the parietal clade. The formation mode of these ducts are quite distinct in each clade. In the clusioid clade, as observed in *Kielmeyera*, ducts are formed by a strand of meristematic cells, identified as a rosette in transverse sections, as described for most families but ducts of Humiriaceae and Salicaceae are formed by coalescence of cavities originating various transitional shapes between cavities and ducts (Fernandes et al. 2018), as recently described for Malvaceae (Garcia et al., 2020 and references therein).

Despite this being the first report of two types of ducts distinguished by origin and secretory metabolism in the same plant, further studies are needed and lead us to new questions about the relation between the origin and the secretory activity in plant glands, especially in secretory ducts.

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CHAPTER 2

Development and holocrine secretion of resin ducts in *Kielmeyera appariciana* (Calophyllaceae)

Abstract

The mode of formation and release of secretion are complex processes that occur in secretory ducts and have great divergence in the literature. The use of modern techniques for the detection of hydrolytic enzymes, cytoskeleton arrangement and indicators of programmed cell death may help to clarify the processes involved during the ontogeny of this gland. Our study aimed to analyze subcellular changes during schizogenous formation, secretion production and release into the lumen in resin ducts of *Kielmeyera appariciana*. Our results demonstrate the participation of pectinase by loosening the central cells of the rosette, which split subsequently away each other through polarized growth mediated by a rearrangement of the microtubules. The resin is synthesized mainly in plastids and smooth endoplasmic reticulum, being observed inside vesicles and small vacuoles. The secretion release is holocrine and occurs through programmed cell death related to the release of reactive oxygen species, causing the darkening of the cytoplasm, chromatin condensation, vacuole rupture, plastid and mitochondria degeneration. Cellulase activity was identified prior to the rupture of the cell wall, releasing secretion into the lumen of the duct. The participation of the cytoskeleton was observed for the first time during schizogeny of ducts, as well as programmed cell death as part of the process of the releasing holocrine secretion. This type of secretion release may be an innovation in *Kielmeyera*, since it has not been observed in ducts of any other plant so far.

INTRODUCTION

Secretory ducts are glands constituted of an epithelium of secretory cells that delimit an elongated lumen in which the exudate is stored. This exudate has a variable chemical nature and is produced and released into the lumen through various processes (Fahn 1979). During the development of ducts in plants, two distinct processes are involved in their formation and secretion, although these processes are often confused due to the secretory phase usually beginning before the duct reaches its final dimensions (Prado & Demarco 2018).

The formation mode refers to the structural development of the duct, which may involve separation of cells (schizogeny), programmed cell death (lysigeny) or both processes (schizolisygeny; Fahn 1979; Prado & Demarco 2018). On the other hand, the secretion mode (or secretion release mode) is the mechanism by which the compounds produced by secretory cells are transferred to outside of protoplast (Paiva 2016). This process usually occurs without cell disruption and is named merocrine secretion. In this mechanism, the secretion can leave the protoplast crossing the plasma membrane by diffusion (eccrine secretion) or by secretion packaged in vesicles and/or vacuoles which fuse to the plasma membrane and release the secretion (granulocrine secretion; Fahn 1979). Once in the periplasmic space, the secretion can pass through the cell wall by diffusion or be pushed through the active pressure of the protoplast, depending on the composition and viscosity of the compounds (Paiva 2016). The merocrine secretion occurs in most secretory ducts but a second type of mechanism is also reported for many ducts: the holocrine secretion. In this release process, part of the cell or the entire cell breaks, releasing the secretion (Fahn 1979). This process is relatively common in secretory ducts of some plants and has also been reported in other types of gland, such as nectaries and salt glands (Fahn 1979; Gaffal et al. 2007; Vesprini et al. 2008; Bosabalidis 2012).

Cell lysis to secretion release may be related to a process of programmed cell death (PCD) of the secretory cell (Nick 2011). This process is common and occurs in several stages of plant development, being related to various tissue functions during leaf formation, root cap and anther development, ovule fertilization, fruit maturation, seed formation and germination, and the well-known tracheary element differentiation (Pennell & Lamb 1997; Escamez & Tuominen 2017). PCD is usually identified by some events as dark cytoplasm and condensed nuclear chromatin, followed by cell wall loosening, tonoplast and plasma membrane rupture resulting in cell death (Paiva & Machado 2007) It is not yet known all the signaling mechanisms that are behind the cell death process but it is known that the process of producing reactive oxygen species is involved in the activation of this process (Jacobson 1996; Pennell & Lamb 1997; Lam et al. 1999).

All these events culminate in the lysis of the secretory cell, releasing the secretion. The secretory compounds at times have medicinal properties and the secretion is used in folk medicine. Several species of Calophyllaceae produce secretions with anti-inflammatory, antioxidant, antibacterial and antifungal properties, and microbiological tests have confirmed the efficacy of some compounds against cancer cell lineages (Alves et al, 2000; Cortez et al, 2002; Jorge, 2014; Mesquita et al, 2011; Pinheiro et al, 2003; Silveira 2010). Among these medicinal species, we find *Kielmeyera appariciana* which stands out for presenting resin ducts in the primary shoot and gum ducts in the bark (Costa et al. 2020).

Resin ducts of *K. appariciana* have been described as schizogenous but there are no data on cellular changes related to the separation of cells or to the process of secretion release into the lumen. Our knowledge on the action of enzymes and cytoskeleton in gland formation and secretion release is still incipient and have been carried out in ducts, glandular trichomes and laticifers of few species (Tozin & Rodrigues 2016, 2019; Marinho & Teixeira 2019). In view of the scarcity of data and so many questions to be answered, the objective of this work

is to analyze the development of the resin ducts of *K. appariciana* and the processes involved in the synthesis and release of the secretion.

MATERIAL AND METHODS

Samples of *Kielmeyera appariciana* Saddi were collected in the campus of the Universidade de São Paulo in São Paulo/SP (Brazil) and the voucher was deposited in the herbarium SPF (USP; Costa, E.R. 1).

Development of ducts

Shoot apices were were fixed in Karnovsky's solution for 24 h at 4°C and postfixed in 1% osmium tetroxide. Then, the material was dehydrated through a graded ethyl series, embedded in LR White and serial sectioned at 0.5 μm thickness on a Leica Ultracut UCT (Leica Microsystems Inc., Heidelberg, Germany). Longitudinal and transverse semithin sections were stained with toluidine blue and observed in a Leica DMLB light microscope.

Ultrastructure

The same material embedded in LR White were sectioned with 80 nm and the ultrathin sections were stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963) with subsequent observation in a Zeiss EM900 transmission electron microscope.

Pectinase and cellulase activities

Shoot apices were fixed in Karnovsky's solution for 2 h (Karnovsky 1965) and then exhaustively washed in the same buffer of the fixative. Part of the material was incubated in 0.5% pectin solution in 0.1 M sodium acetate buffer and other part was incubated in 0.5% cellulose solution in 0.1 M sodium acetate buffer for 20 min at room temperature. Later, the materials were transferred to Benedict's reagent at 80°C for 10 min and then washed in 0.1 M sodium phosphate buffer. After these treatments, both samples were processed for

transmission eléctron microscopy as usual. The same procedure was applied to the control samples excluding the incubation step in pectin and cellulose.

Programmed cell death (PCD)

For detection of oxygen free radicals/ hydrogen peroxide, free-hand sections of shoot apices were treated with DCFH-DA (2',7'-dichlorofluorescein diacetate) for 10 minutes and washed in deionized water (Bass et al. 1983). The sections were analyzed under a Leica DMLB fluorescence microscope equipped with a blue light filter block (excitation filter BP 420-490, dichromatic mirror RKP 510, suppression filter LP 515).

For DNA labeling, samples fixed in Karnovsky's solution were free-hand sectioned and stained with DAPI (4', 6-diamidino-2-phenylindole; 1 $mg.mL^{-1}$) in phosphate buffered saline (PBS) and 1% Triton X-100 (Sigma, St. Louis, MO , USA), washed in water and mounted in 50% glycerol in PBS. The slides were examined on a Sigma Zeiss confocal microscope under 405 nm wavelength.

Cytoskeleton

Whole-Mount α-Tubulin Immunolabeling

Free-hand sections of shoot apices were fixed in 4% (w/v) paraformaldehyde in PEM buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO4, pH 6.8) for 1 h. After washing in PEM, cell walls were digested by a solution of 3% (w/v) macerozyme R10 and 3% (w/v) cellulase R10 (Duchefa) in PEM at room temperature for 1.5 h (modified according to Šamajová et al. 2014). The next steps were the incubation of the samples in absolute methanol (at -20°C) for 30 min and extraction with 5% (v/v) DMSO + 1% (v/v) Triton X-100 in PBS at room temperature for 1 h. Samples were subsequently transferred to blocking buffer for 30 min and after incubated overnight with rat anti-α-tubulin antibody diluted 1:40 in PBS. Following PBS washing, the cells were incubated overnight with FITC-anti-rat antibody (Invitrogen) diluted

1:40 in the same buffer. DNA was counterstained with DAPI (Sigma) in PMSO aliquoted and stored at -20ºC in the dark. Prepare working solucion by diluin 1:1000 with PBS for 10 min. The samples were mounted in an antifade VECTRASHIELD™.

RESULTS

Development and secretion mode of resin ducts

Resin ducts of *K. appariciana* originate from a single cell of the ground meristem (Fig. 1A). This initial cell has thin walls, dense cytoplasm and a prominent nucleus (Fig. 1A). Successive divisions of this cell form a rosette of still undifferentiated cells that remain in constant division, which later differentiate in the epithelium and sheath (Fig. 1B). The lumen of the duct is formed by schizogeny (cell separation) without signs of cell lysis in the early stages of duct development. The cytochemical analysis identified pectinase activity in the middle lamella between the central cells of the rosette, loosening themselves. Then, some points of greater concentration of the microtubules are observed in polarized regions of the cells (Fig. 1C-E) and they start to split away, forming an initial lumen. At the same time as the ducts develop and expand their lumen, the epithelial cells are constantly producing secretion which is temporarily storage within the cell. Mature ducts have a uniseriate secretory epithelium, constituted of thin-walled cells with dense cytoplasm and prominent nuclei (Fig. 1F). The ducts are axially elongated, have a uni- or biseriate sheath formed by cells with phenolic compounds and can vary in diameter and depth (Fig. 1G-H).

During the secretory phase, epithelial cells are elongated (Fig. 1H) and signs of cell death are observed in some of them evidenced by dark stained cells (Fig. 1E; H). DAPI assay also demonstrated that the nuclei of some cells showed a loss of shape, becoming more elongated and with many regions of heterochromatin (Fig. 1C-D). Finally, these cells rupture and release their content into the lumen.

Subcellular secretory machinery and holocrine secretion

Mature ducts have secreting cells and the lumen filled with a heterogeneous exudate (Fig. 2A). Epithelial cells have ribosome rich cytoplasm, abundant endoplasmic reticulum, many plastids, dictyosomes, conspicuous vacuoles and voluminous nucleus with decondensed chromatin and evident nucleolus (Fig. 2B-C).

The endoplasmic reticulum is especially extensive close to the plasma membrane and is associated with ribosomes (RER) in some points (Fig. 2B). Elaioplasts with few thylakoids containing starch grains and plastoglobules, small rounded mitochondria and vacuoles with secretion are prominent during secretory phase (Fig. 2C).

The secretion is mainly produced by elaioplasts and endoplasmic reticulum that show dilated cisterns in which secretion is accumulated (Fig. 2B). Many vesicles and small vacuoles with secretion are observed in the epithelial cells, mainly close to plasma membrane (Fig. 2B). These vesicles and vacuoles fuse each other forming larger structures with electron-dense or electron-opaque secretion (Fig. 2B; D-E).

Despite the parietal position of a large amount of secretory vesicles and vacuoles, there is no signs of their fusion to the plasma membrane to release the secretion. Rare plasmodesmata indicate little connection between adjacent epithelial cells and secretion is only released after lysis of the cell.

An increasing accumulation of electrodense material in the cytoplasm, condensation of chromatin (Fig. 1C) and rupture of the vacuole, plastids and mitochondria indicate the process of programmed cell death, associated with cellulase activity detected mainly in the periclinal cell wall facing the lumen (Fig. 1 I-J). The holocrine secretion mediated by programmed cell death and wall digestion of the epithelial cells is also related to the release of reactive oxygen species detected by DCFH-DA in some cells during the secretory activity (Fig. 1E).

DISCUSSION

Our study revealed that the resin ducts of *Kielmeyera appariciana* have their formation by a schizogenous process and the secretion release is holocrine. The formation of the lumen by cell separation is a complex and little explored process that involves digestion of the middle lamella between the initial cells of the duct (rosette) and participation of the cytoskeleton in the polarized growth of the cells that split away. Pectinase activity by loosening the rosette cells to form an intercellular space has already been detected during the formation of ducts and cavities of other species, such as *Citrus* and *Pinus* (Li et al. 2004; Liang et al. 2009). In other cases, pectinase and cellulase activities were related to secretion release into the duct lumen, as described in *Protium* (Palermo et al. 2017). The participation of the cytoskeleton has never been identified during the splitting the epithelial cells off but its reorganization to promote polarized growth is well-known in several cell types, especially during the growth of pollen tubes (Fu 2015). The cytoskeleton arrangement may also be involved with the secretory process, especially when it is granulocrine due to its coordination of the vesicle-trafficking within the cell during exocytosis (Zhang et al. 2019 and references therein). A higher amount of microtubules was observed near the outer periclinal wall of glandular trichomes in *Hyptis* (Tozin & Rodrigues 2016, 2019). This arrangement likely related to the release of secretion, due to the role of the microtubules and actin microfilaments in the transport of vesicles within the cell, was not observed in *K. appariciana*, since there is no exocytosis in the epithelial cells of this species.

Synthesis of secretion

The resin ducts of *K. appariciana* synthesize mainly terpenes, phenolic compounds, polysaccharides and proteins (Costa et al. 2020). The occurrence of these substances is directly related to our ultrastructural results, which include the predominance of organelles

involved in the production of lipids, such as plastids, smooth endoplasmic reticulum and mitochondria (Fahn, 1979). In addition, the presence of dictyosomes and rough endoplasmic reticulum are related to the production of polysaccharides (Young at al 2008). Similar subcellular characteristics have also been described for resin ducts of various species of Anacardiaceae (Joel & Fahn 1980 a; b; c; Nair et al. 1983; Venkaiah 1992; Carmello et al. 1995).

The epithelial cells of of the ducts of *Boswellia serrata* also secrete lipids in addition to polysaccharides (Nair & Subrahmanyan 1998) and the occurrence of glands that secrete exudates of mixed nature is not restricted to ducts of Calophyllaceae. Ducts of various species of Anacardiaceae and Burseraceae, in addition to glandular trichomes of *Inula viscosa* and *Fagonia* and laticifers of all plants secrete lipids together with polysaccharides and proteins (Werker & Fahn 1981; Fahn & Shimony 1988; Demarco 2015; Prado & Demarco 2018; Ramos et al. 2020).

The substances produced by ducts, which are resins, polysaccharides or a mixture of both, are highly viscous and have a high molecular weight. Therefore, they have some difficulty in freely passing through the plasma membrane and cell wall, which act as a mechanical barrier for the confinement of the synthesized substances inside the cells (Fahn 1979; Paiva 2016). There are several hypotheses that seek to explain how such substances can be released into the lumen but the most accepted hypothesis is the explanation that these substances of high molecular weight exert a turgor pressure within the protoplast, pushing the secretion in the periplasmic space to cross the cell wall, reaching outside the cell (Fahn 1979, 2000; Paiva et al 2008; Paiva 2016, Rodrigues & Machado 2012). However, the resin of the ducts of *K. appariciana* do not follow this pattern, since the secretion does not cross the plasma membrane nor the cell wall. The entire cell ruptures to release the secretion into the lumen (holocrine secretion).

Holocrine secretion

During the secretory activity, some epithelial cells of *K. appariciana* showed a cytoplasm darkening very similar to what happens in the resin ducts of *Mangifera indica* (Joel & Fahn 1980). For these authors, there is a synchrony in the secretory activity of the epithelium, which consists of storing secretion in the periplasmic space, releasing it into the lumen accompanied by the gradual darkening of the cytoplasm and, finally, the cell collapse. It may still happen that these cells do not undergo lysis and remain in the structure of the duct without secretory activity, i.e. in post-secretory phase, presenting vacuolated or distorted appearance of the cytoplasm. Cytoplasmic darkening does not happen uniformly in all secretory cells at the same time because the production and release cycles of secretion operate independently in each secretory cell and varies in duration according to the species and the developmental stage of the secretory structure (Schussler & Longstreth 1996; Paiva 2016).

The darkening of the cytoplasm in some cells in secretory activity of the ducts of *K. appariciana* coincides with the detection of reactive oxygen species, chromatin condensation, vacuole rupture, degradation of plastids and mitochondria, and cellulase activity especially concentrated in the periclinal wall inwards the lumen. All of these events demonstrate the occurrence of programmed cell death (Bosabalidis & Tsekos 1982; Willinghan 1999; Paiva & Machado 2007). Although there are many studies on ducts and secretory cavities that have programmed cell death as part of their formation (Palermo et al. 2017), this is the first study in which this highly coordinated process is related to the release of secretion. In the other glands with holocrine secretion, the exudate is released by cell disruption (Fahn 1979; Gaffal et al. 2007; Vesprini et al. 2008; Bosabalidis 2012), without observing the gradual and coordinated activity of cell autophagy that culminates in the collapse of the cell.

The secretion mode of oils and resins most commonly described in the literature is eccrine (Paiva et al., 2008; Batt, 1987; Milani et al., 2012; Rodrigues and Machado, 2012; Sá-Haiad et al. 2015; Palermo et al. 2017, Fahn, 1988; Fahn, 1979, Fahn, 2002; Gonçalves-Souza et al., 2018; Sá-Haiad et al, 2015). However, *Kielmeyera appariciana* appears against most of the studies carried out until the present moment when it was verified the holocrine release of lipophilic substances produced by epithelial cells.

Conversely, in the case of mucilage-secreting cells, the secretion is produced by dictyosomes (Lüttge and Schnepf 1976; Young at al 2008) is transferred to outside the protoplast via merocrine process in trichomes of *Ipomea cairica* (Paiva & Martins 2011), ducts of *Mangifera indica* (Joel & Fahn 1980c) and *Lannea coromandelica* (Venkaiah 1992). However, the end of the secretory phase in some mucilage-secreting cells is marked by cell death after the cell transfers the mucilage to a large periplasmic space which fills the cell lumen, as observed in the mucilage idioblasts of *Opuntia polyacantha* - Cactaceae (Mauseth 1980) *Araucaria angustifolia* (Mastrobert & Mariah 2008a) and exotesta of *Euphorbia milii* (Demarco & Carmello-Guerreiro 2011).

Our study opens new horizons for understanding the involvement of hydrolytic enzymes and the cytoskeleton in the process of schizogenous formation of secretory ducts and the holocrine secretion release. This process, although uncommon as it promotes the death of cells, derives from programmed cell death releasing the resin of *Kielmeyera*. PCD related to the release of secretion with the involvement of reactive oxygen species and cell wall digestion is described for the first time in a plant gland and may be an innovation of Calophyllaceae.

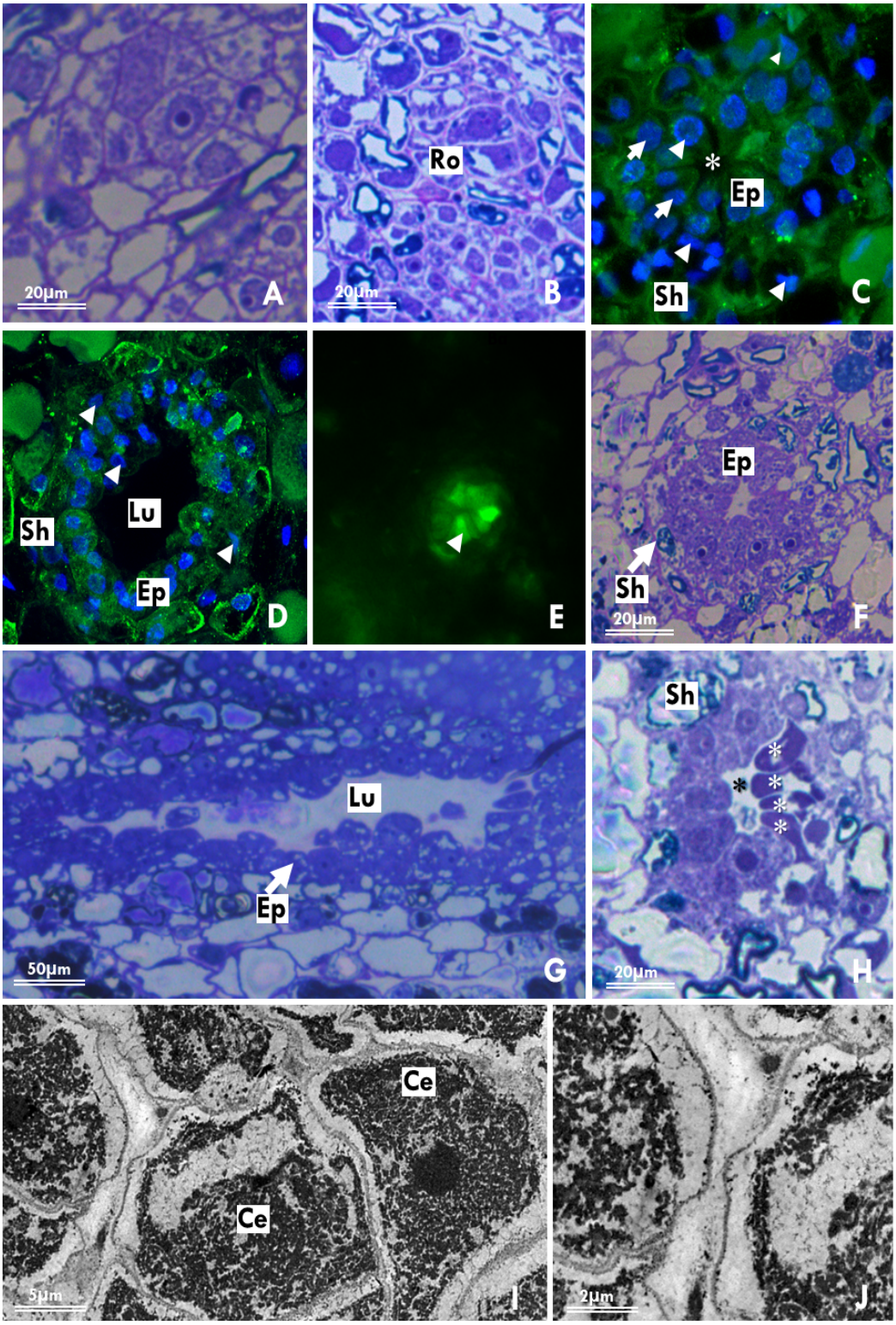

Fig. 1: Structure of the *Kielmeyera appariciana* ducts. A: Initial duct cell pointed with a short white arrow. B: Rosette. C: Ducto in the beginning of training. Cores marked in Dapi test. Apoptotic nuclei (White arrowhead) and intact nuclei (Long white arrows). D: Mature duct with marked nuclei after Dapi test. White arrowhead indicates apopitotic nuclei. E: DCFH-DA fluorescent dye with duct epithelial cell labeling. F: Ducto with lume formed and in secretory phase. G: Ducto ducts in longitudinal section. H: Duct with epithelial cells in the programmed cell death process (white

asterisk) and active cells (black asterisk). I: Cellulase. J: Details of the marking. (Ba: Sheath; Ce: Celullase; Ep: Epithelium; Lu: Lume; Ro: Rosette).

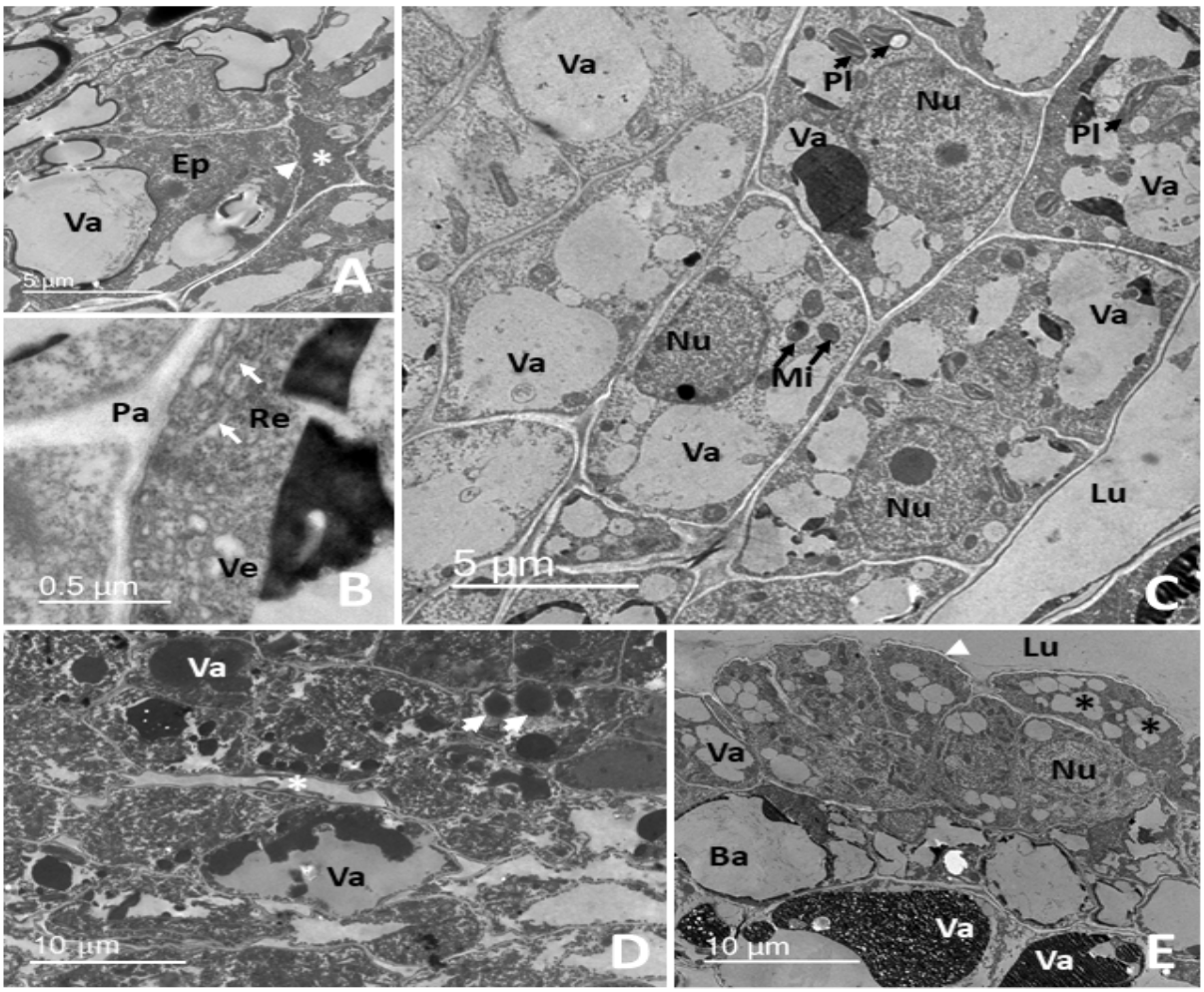

Fig. 2: Ultrastructure of the *Kielmeyera appariciana* ducts. A: Heterogeneous secretion inside the lime (white asterisk). White arrowhead points to the sinuous wall of the secretory epithelium. Asterisk. B: Endoplasmic reticulum with conspicuous cisterns (long white arrows) and production of small vesicles. C: Most abundant organelles in the epithelium and duct sheath. Long black arrows point to mitochondria, short black arrows point to plastids. D: Vacuoles with heterogeneous secretion. Detail for the electro-opaque drops inside the epithelium cell. Short white arrows pointing like elero-opaque. E: Fusion of vesicles inside the duct epithelial cells, secretory sheath with large vacuoles and containing granular material. Arrowhead points to sinuous wall of epithelium. Asterisk marks the fusion of small vesicles. (Ba: Sheath; Ep: Epithelium; Lu: Lume; Mi: Mitochondria Naked: Nucleus; Pa: Cell wall; Pl: Plastid;Re: Endoplasmic reticulum; Va: Vacuole; Ve: Vesicle).

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GENERAL CONCLUSIONS

This study demonstrates for the first time the occurrence of ducts with different secretions in the same organ of the same plant. The distinct origin of ducts from ground meristem in primary shoots and vascular cambium in secondary tissues might be related to the metabolic alteration which likely led to suppression of the biosynthetic pathway of terpenoids and phenolics in the secondary ducts. Further studies are needed to verify the occurrence of ducts with different origins in other groups of plants and the possible influence of their origin on the secretory activity. In this work, we also identified for the first time the participation of the cytoskeleton in the schizogeny of ducts, as well as programmed cell death as part of the process of holocrine secretion. Although programmed cell death is unprecedented for secretion release, the holocrine mechanism occurs in several types of gland, which should be investigated in search of the occurrence of programmed cell death mechanisms.