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

Development, secretory activity and chemical composition of the resin in the ducts of *Kielmeyera appariciana* Saddi (Calophyllaceae)

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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.

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.

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