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Floral resins of *Philodendron adamantinum* (Araceae): secretion, release and synchrony with pollinators

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ABSTRACT

Philodendron is the only genus of Araceae in which resin release occurs in the inflorescence. The resinous secretion adheres to the smooth body surface of the pollinating scarab beetles and allows attachment of pollen grains, making its transport possible. In order to understand the process of resin synthesis and release to the external environment, we used structural, ultrastructural and histochemical analyses at different stages of development of the inflorescences of *Philodendron adamantinum*. Two types of secretory canals were observed in the spathe: small caliber canals near the abaxial face, and larger caliber canals in the adaxial region. Only the latter canals release secretion into the external environment. The secretory epithelium in these canals is formed by a layer of cuneiform cells, and exhibits secretory activity throughout the development of the spathe. Resin exudation is a peculiar characteristic of these canals and appears to result from pressure exerted by the secretory epithelium and by structural modifications in the wall of cells adjacent to the epidermis, which allow the formation of a separation zone whereby the resin is released. The observed synchrony between anther dehiscence and resin exudation of *P. adamantinum* enhances the role of this secretion in the pollination process.

Keywords: Araceae, cantharophily, insect-plant interaction, resin exudation, resiniferous canals

Introduction

Plant resins are chemically complex substances synthesized, stored and sometimes released by plants. The function of resins, in most cases, is to protect the plant body against biotic agents, such as herbivores and pathogens (Dell & McComb 1978), due to its toxic and deterrent character (Langenheim 1990). Moreover, resins have high viscosity and the capacity for rapid polymerization, which assists in the cicatrization of injuries, prevents the entry of pathogens and increases the indigestibleness of plant tissues (Mithöfer & Boland 2012).

Resin production usually occurs in vegetative organs. The secretion is called superficial when produced by glandular trichomes, or internal when produced in structures such as ducts and cavities (Dell & McComb 1978).

In some cases, resins are produced in flowers or inflorescences and hold a function in the reproductive biology of the plant. This is the case in floral resin glands that produce these substances as floral rewards for female bees of the tribes Euglossini, Meliponini (Apidae), and Anthidiini (Megachilidae) which use them as raw material for nest construction (Simpson & Neff 1981; 1983; Michener 2007). These resin glands occur in the pseudanthium of *Dalechampia* (Euphorbiaceae) (Armbruster & Webster 1979; 1981; Armbruster 1984; 1996) and in staminate and pistillate flowers of *Clusia* (Clusiaceae) (Armbruster & Webster 1979; Bittrich & Amaral 1996; 1997; Lopes &

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Machado 1998; Carmo & Franceschinelli 2002; Sá-Haiad *et al.* 2015) and other species of this family (Hammel 1999; Bittrich *et al*. 2003).

Within Araceae, which is recognized as a family with numerous resin producers (French 1987; Mayo *et al.* 1997), only representatives of *Philodendron* possess resin secretion in the inflorescences (Mayo *et al*. 1997). This also actively released resin in *Philodendron* is not a floral reward nor is it involved in the attraction of pollinators, which are cyclocephaline beetles of the family Scarabaeidae (Gottsberger & Amaral 1984; Gottsberger 1986; 1990; Gibernau *et al*. 1999; 2000; Gibernau & Barabé 2002; Gottsberger & Silberbauer-Gottsberger 2006; Maia *et al*. 2010; Gottsberger *et al*. 2013). However, it makes part of the fine-tuned pollination mechanism. The resin secretion covers the smooth body of the hairless scarab beetles and acts as an adhesive for pollen grains (Gibernau *et al.* 1999; 2000; Gibernau & Barabé 2002; Pereira *et al*. 2014).

According to Mayo (1991), the release of resin in the inflorescences varies as to the location, structure and pattern of canal activity among species. Many species of the three subgenera of *Philodendron* release resin on the adaxial side of the spathe and, more rarely, on the stamens (Mayo 1991; Grayum 1996; Croat 1997). In *Philodendron adamantinum*, the pollinating scarab beetles *Erioscelis emarginata*, attracted by specific volatile compounds produced in the osmophores at the apex of sterile and fertile staminate flowers (Pereira *et al.* 2014; Gonçalves-Souza *et al.* 2017), enter the pollination chamber in the evening of the first day of flowering when pistillate flowers are viable. They remain in the chamber during the night and day two of inflorescence flowering. From the late morning on, the release of resin droplets on the adaxial side of the spathe has been observed to occur in the period immediately preceding dehiscence of the anthers. The chamber closes due to the gradually constricting spathe and the beetles are expulsed from dusk on, covered with resin and loaded with pollen-dust (Pereira *et al.* 2014).

The exudation of resin produced in ducts and cavities without the occurrence of injuries is an unusual phenomenon, since resins synthesized in these structures usually remain in the lumen. In the only other report, Sá-Haiad *et al.* (2015) demonstrated the release of resin by canals and cavities by means of ruptures in the apex of stamens and staminodes in flowers of *Clusia*.

Aiming to understand the production and, principally, the release of resin by the spathe of *Philodendron adamantinum,* an endemic species in which the resin plays an important role in pollination, the objectives of the present study were the anatomical characterization of the resin canals present in the adaxial face of the spathe, ultrastructural analysis of the secretory cells of these canals, and the investigation of the chemical groups of the substances that makeup the secretion.

Materials and methods

Collection

Samples of spathes of *Philodendron adamantinum* Mart. ex Schott were collected in Parque Estadual do Rio Preto (Rio Preto State Park), located in the municipality of São Gonçalo do Rio Preto, Minas Gerais, Brazil. Inflorescences at different stages of development, according to the need of each analysis, were located in different populations distributed throughout the park.

The collections were carried out from November to January, during the flowering period of *P. adamantinum*, from 2012 to 2016.

Voucher material was deposited in the herbarium BHCB of the Instituto de Ciências Biológicas of Universidade Federal de Minas Gerais under the number 161786.

Structural analysis and the nature of the secretion

Spathe fragments from three inflorescences were collected at various stages of development: inflorescences measuring 10 %, 30 %, 50 % and 70 % of final size, which reaches approximately 15cm in length, and fully developed inflorescences 15 hours prior to, and during, the time of resin release. Samples were taken from the basal, median and apical regions of the spathe and processed for light microscopy and histochemical tests.

All samples were subjected to vacuum in Karnovsky solution pH 7.2 in 0.1 M phosphate buffer (Karnovsky 1965) and fixed for 24 hours. The material was then dehydrated in an ethanol series and subjected to pre-infiltration and infiltration in synthetic resin (2-hydroxyethyl methacrylate) (Leica®) according to Paiva *et al.* (2011). The samples were sectioned (6µm thick) with a rotary microtome (Hyrax M40, Zeiss, Microm GmbH, Walldorf, Germany), stained with Toluidine blue solution, pH 7.4 (O'Brien *et al.* 1964), counterstained with ruthenium red solution, arranged on slides and mounted in Entellan® for photodocumentation and study by light microscopy.

Histochemical tests were employed on material fixed in Karnovsky solution, both in sections obtained by free hand and by microtome after inclusion in resin. The following histochemical tests were applied: Lugol for the identification of starch (Johansen 1940); NADI for the detection of resin or essential oils (David & Carde 1964); 10 % aqueous ferric chloride solution for phenolic compounds (Johansen 1940); 0.02 % aqueous solution of ruthenium red for the detection of pectic compounds (Jensen 1962); and Sudan red B for lipids in general (Brundrett *et al*. 1991).

Analyses by scanning electron microscopy were performed on spathe samples 15 hours prior to and during resin release. Samples were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer, dehydrated in an increasing

ethanol series, dried by the critical point method using liquid CO_2, sputter-coated with gold (Robards 1978), and observed using a Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands), at 12-20 kV.

For transmission electron microscopy, transverse fragments of the adaxial face of young spathes - about 10 % to 20 % of final size, 15 hours prior to, and during, the time of resin release were subjected to vacuum in Karnovsky solution pH 7.2 in 0.1 M phosphate buffer (Karnovsky 1965), fixed for 24 hours, post-fixed in 1 % osmium tetroxide (0.1 M phosphate buffer, pH 7.2) for two hours, washed in phosphate buffer (0.1 M, pH 7.2), dehydrated in an ethanol series and infiltrated with Araldite® resin (Roland 1978). The 50nm ultrafine sections obtained were contrasted with uranyl acetate and lead citrate and examined using a Tecnai G2-12-Spirit transmission electron microscope (Philips/FEI Company, Eindhoven, Netherlands) at 80 kV.

Results

Structural organization and nature of the secretion

The spathe of *Philodendron adamantinum* possesses a white, glabrous, smooth and shiny-looking adaxial face (Fig. 1A). The lower part of the spathe forms a large chamber that can house several beetles of *Erioscelis emarginata* (Cyclocephalini, Dynastinae) (Fig. 1B), the pollinating agent. During the second day of spathe opening, at the end of the morning, yellow-orange viscous resin drops are released on the adaxial face (Fig. 1B-C). The acropetal closure of the spathe expels beetles sheltered in the pollination chamber, forcing them to climb the spadix, at which time their body comes into contact with the resin, which will serve as an adhesive for the pollen grains produced by the fertile staminate flowers arranged in the distal portion of the spadix.

On the adaxial surface of the spathe the epidermis has juxtaposed cells with a smooth cuticle and numerous stomata. At the time of resin release, rupture and separation of the epidermal cells and underlying tissue were observed, giving rise to openings through which resin release occurs (Fig. 1D). These openings occur at specific points on the surface of the spathe and have a diameter slightly greater that 100μm, and rarely perceptible to the naked eye. On the other hand, the extravasated resin, whose droplets exceed 1mm in diameter, is easily observed in the field.

The mesophyll of the spathe is formed of three distinct regions. Two regions are portions underlying the epidermis of each face, comprising globular parenchyma cells with

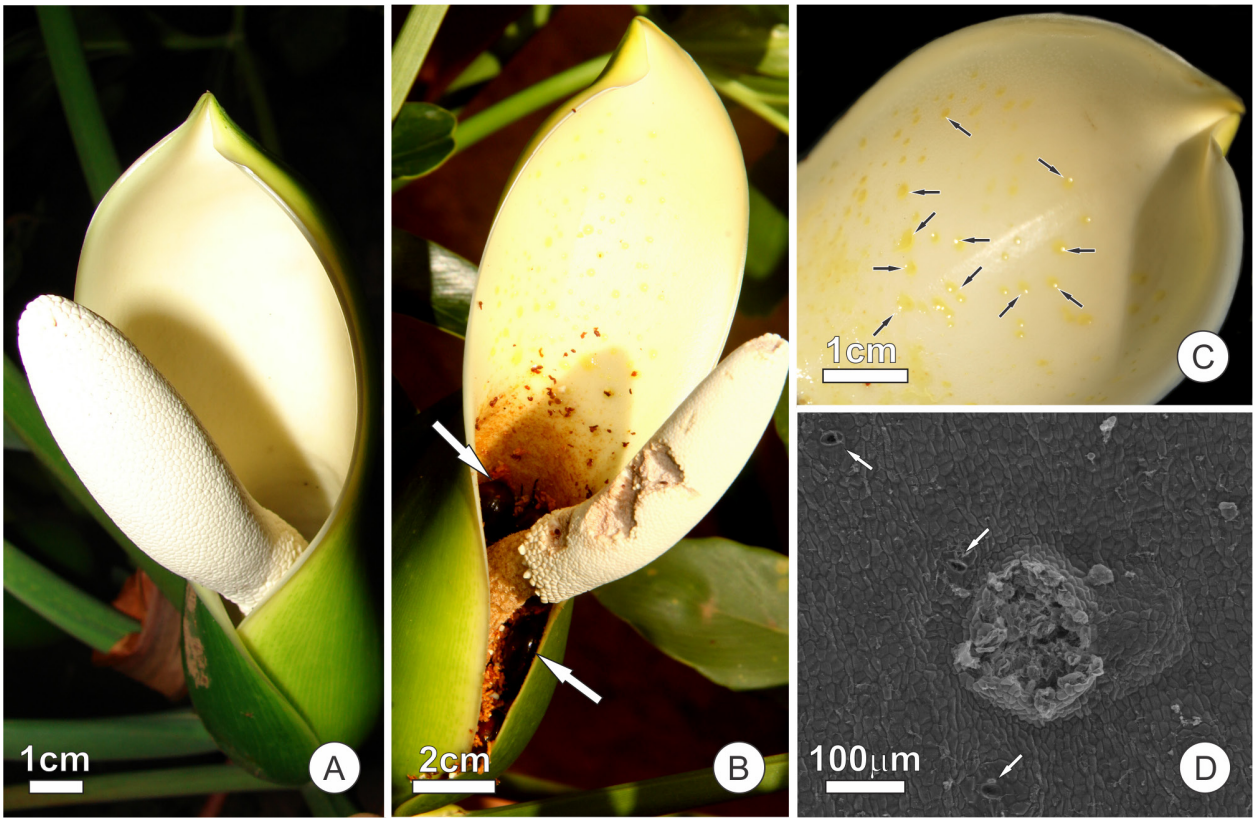

Figure 1. Inflorescences of *Philodendron adamantinum*. **A.** Prior to the release of resin. Note the homogeneous texture of the adaxial face of the spathe. **B.** Overview at the time of resin release. Note the beetles located in the chamber formed by the spathe (arrows). **C.** Apex of the spathe showing resin droplets (arrows). **D.** Adaxial face of the spathe highlighting the opening through which the resin is exuded. Note the absence of tissue organization and its great dimension, when compared to the stomata (arrows).

little intracellular space. Separating these two regions is the third region, a median portion consisting of several layers of larger, spaced cells forming aerenchyma. Cells with phenolic content and resin secretory canals can be observed distributed among the three regions (Fig. 2A-B).

Although distributed throughout the mesophyll, the resin secretory canals form two distinct types: a) small-caliber and b) large caliber secretory canals. The small caliber canals occur near the abaxial face in deeper layers of the mesophyll (Fig. 2A) and do not release secreted resin. The orientation of these canals is always parallel to the surface and they do not deflect their apex towards the epidermis. Moreover, they have only two cell layers surrounding the secretory epithelium (Fig. 2A).

The large-caliber secretory canals occur on the adaxial face and in the aerenchyma (Fig. 2B) and are capable of releasing resin on the adaxial face of the spathe. They form a complex anastomosed system (Fig. 2C), comprising a network of secretory ducts. All canals tend to be parallel to the epidermis, but their apex is always deflected towards the adaxial surface of the spathe (Fig. 2D). A set of parenchyma cells arranged between each end of the canal and the epidermis exhibits, on adjacent cell walls, an accumulation of pectic substances (Fig. 2D).

Three to four layers of parenchyma cells with large vacuoles involve the secretory epithelium of the large caliber canals. These cells are flattened in the younger phases, when resin accumulation occurs in the lumen of the canal (Fig. 2E). Upon release of the resin into the external medium, the subepithelial cells and those of the secretory epithelium increase in volume considerably, consequently reducing the volume of the canal lumen (Fig. 2F). On the morning of the second day of spathe opening, the cells from the secretory epithelium separate at the ends of the resin canals, which allows the resin to extravasate. The secretion accumulates in pockets formed in the intercellular space below the epidermis (Fig. 2G), whose rupture releases resin mainly in the region of the constriction of the spathe close to the staminode zone. When the opening of the duct coincides with the presence of a stomata, the secretion is released by it (Fig. 2H).

Starch grains are present in the mesophyll cells of the spathe, except in the layers underlying the epidermis and the cells of the resin secretory canals (Fig. 3A). In the early stages of spathe development, starch occurs in low concentrations, both in the vicinity of the secretory canals and in the rest of the mesophyll. During the development of the inflorescence, starch is accumulated and, 15 hours before the release of resin, a larger quantity of this reserve is observed (Fig. 3B). At the time of resin release by the spathe, a significant part of the starch has been consumed (Fig. 3C).

Material of lipid nature, revealed by the Sudan test, is restricted to the cuticle of the epidermal cells of the abaxial face (Fig. 3D), and secretion found in the lumen of the large caliber resin canals (Fig. 3E). The secretion of the smaller caliber canals, as well as the content of the secretory cells, did not test positive for lipids. The NADI test revealed the terpenic nature of the secretion exclusively in the larger caliber canals.

Ultrastructural organization

The secretory epithelium, composed of a layer of cuneiform cells, has secretory activity that extends from the initial stages of spathe formation until close to resin release. In all analyzed stages, the production of granular appearing secretion and its accumulation in the periplasmic space adjacent to the inner periclinal walls of the cells, close to the canal's lumen, were observed (Fig. 4A). No plasmodesmata were observed connecting the secretory cells to each other or to the other cells of the canal.

The set of organelles of the secretory cells is composed mainly of plastids, mitochondria, endoplasmic reticulum and dictyosomes (Fig. 4). Plastids are numerous, occupy a large part of the cytoplasm, and have a poorly developed internal membrane systems and translucent stroma (Fig. 4A-B); they are often associated with segments of the endoplasmic reticulum (Fig. 4B). The nucleus is voluminous (Fig. 4C) with an evident nucleolus.

The secretory cells do not have developed vacuoles during the secretory period (Fig. 4A), except at the end of this period, when large vacuoles can be seen filled with granular material in large cells (Fig. 4E). This content resembles the product accumulated in the periplasmic space (Fig. 4A, C-D). Drops of material of lipid nature are observed in the lumen of the canal, as well as inside the secretory cells (Fig. 4C-E). The material accumulated in the periplasmic space is forced against the wall and through it, as a result of protoplast expansion (Fig. 4C-E).

Near the moment of resin release to the external environment, the parenchyma cells that surround the secretory epithelium are vacuolated, with organelle composition similar to the secretory cells, but they are not very numerous. In these cells the vacuome is developed, generally formed by a large central vacuole filled with granular material (Fig. 5A). In the phase immediately preceding the release of the resin to the external environment, dictyosomes exhibit the release of vesicles and small vacuoles fuse with the plasma membrane releasing its contents into the periplasmic space (Fig. 5A, D). At the end of the canal, the de-structuring of the wall of these cells can be observed, along with the swelling of the middle region of the lamella (Fig. 5B-D).

Discussion

Inflorescences of *P. adamantinum* present two types of secretory canals with different structure, size, content, distribution and function. Type I, the non-resin releasing one, is very abundant in Araceae and occurs in several parts

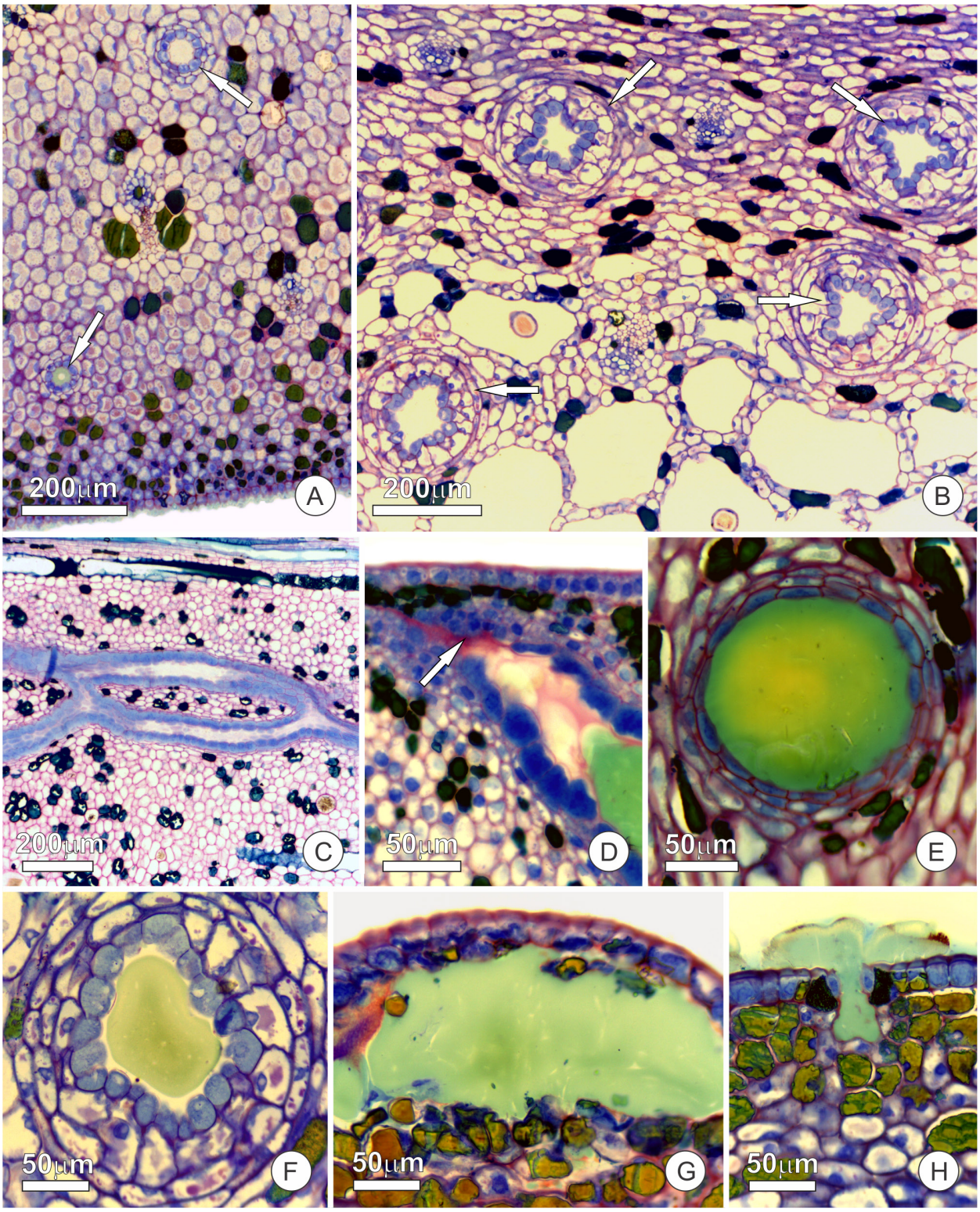

Figure 2. Sections of spathes of *Philodendron adamantinum*. **A-C, E, G-I.** At the time of resin release. **A.** Transverse section in the abaxial region. Arrows highlight smaller caliber canals. **B.** Transverse section close to the adaxial face of the epidermis. Note the larger caliber canals (arrows). **C-D.** Longitudinal section of the adaxial region. **C.** Resin secretory canals in anastomosis. **D.** Spathe at 20 % development exhibiting apex of the secretory canal in the direction of the adaxial side of the spathe. Arrow indicates region with accumulation of pectic material. **E.** Secretory canal in transverse section 15 hours before exudation, showing resin-filled lumen and flattening secretory and subepithelial cells. **F.** Secretory canal in transverse section showing voluminous secretory and subepithelial cells and a reduced lumen. **G.** Accumulation of resin in the subepidermal intercellular space, displacing the epidermis. **H.** Resin being released to the external medium through open stomata.

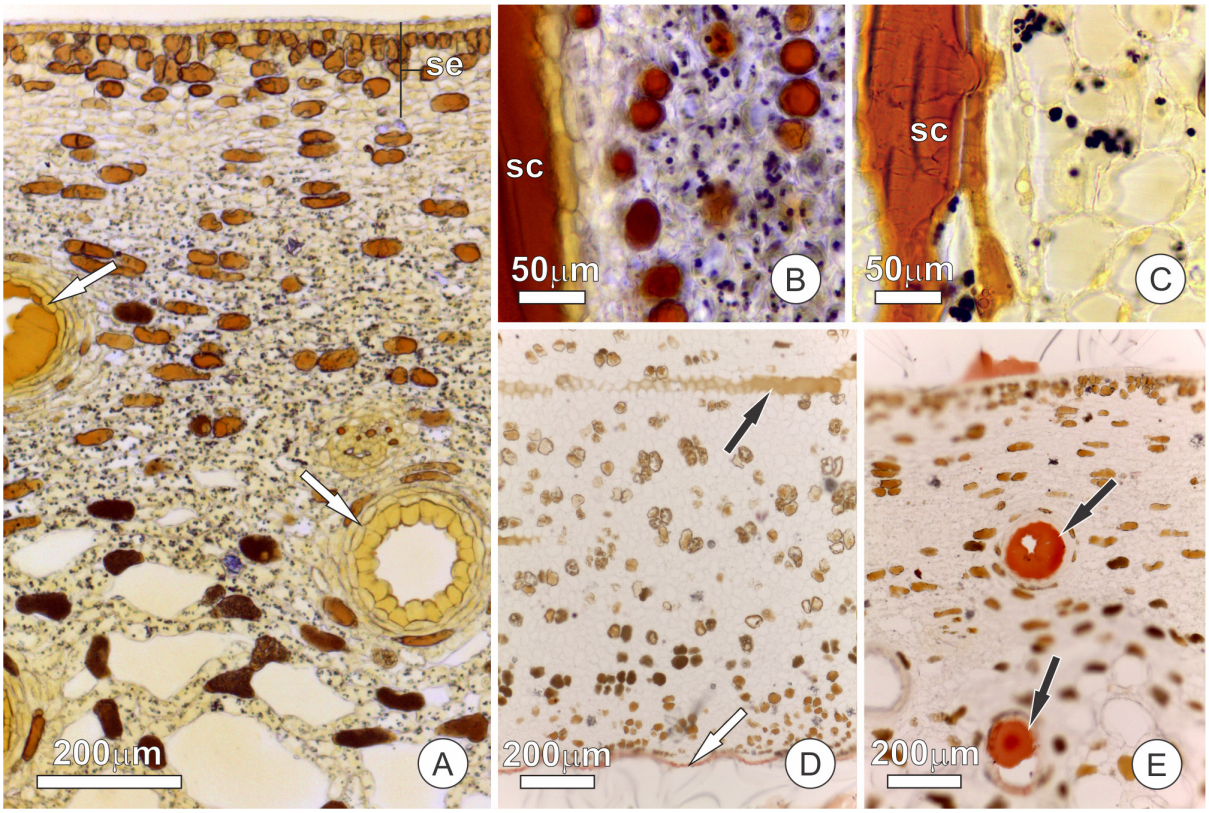

Figure 3. Histochemical tests performed on sections of spathes of *Philodendron adamantinum*. **A-C.** Lugol test. **A.** Spathe 15 hours before the release of resin. Note the distribution of starch grains throughout the region of the mesophyll near the adaxial side of the epidermis. Also note the absence of grains in the subepidermal layers and the cells of the secretory canals (arrows). **B.** Region near the resin secretory duct, 15 hours prior to release. **C.** Region near the secretory duct at the time of release, showing the consumption of starch grains. **D-E.** Sudan red test. **D.** Region of mesophyll near the abaxial face of the epidermis. Note a positive result for only the cuticle (white arrows); the black arrow points to a smaller caliber secretory canal. **E.** Region of mesophyll near the adaxial face of the epidermis, highlighting the lipidic nature of the secretion of the larger caliber ducts (black arrows); there was no reaction in the epidermis (white arrows). **sc –** secretory duct; **se –** subepidermic layers.

of the plant with the general function of cicatrization, acting against herbivores and type II, the resin releasing type, which is specific for the adaxial part of the spathe, having the function to guarantee pollen adherence to the smooth cuticule of the scarabs and thus is directly related to the pollination mechanism.

According to French (1987), the genera *Cercestis*, *Culcasia*, *Furtadoa*, *Homalomena* and *Philodendron* possess resiniferous canals in their roots. In these genera, with the exception of *Furtadoa*, there are also records of secretory cavities in the stem, while in *Culcasia* and *Philodendron* conspicuous canals are observed in the leaves; possibly these canals are of the type I, the non-resin releasing type.

Although there have been some works reporting the presence of resin secretory canals in species of the family Araceae (French 1987; Mayo *et al.* 1997), Langenheim (2003) treats this information with caution. Several substances are reported in the literature as resin and, according to Langenheim (2003), only the detailed study of the chemical composition of these substances allows them to be recognized as such. Although an accurate analysis of the constitution of the secretion was not part of the objective of the present study, some characteristics allow us to label the secretion of the larger caliber canals of the spathe of *P. adamantinum* as resin.

The anatomical structure of these canals is consistent with the morphology of other resin secretory canals and cavities: epithelium containing a layer of secretory cells and some layers of underlying parenchymal cells (Gilliland *et al.* 1988; Ciccarelli *et al.* 2001; Machado & Carmello-Guerreiro 2001). This composition was also observed among the resin canals of the inflorescence axis of *P. adamantinum* (personal observations) and in the roots of other species of the genus (French 1987).

The organelle composition of the epithelial cells of the canals of *P. adamantinum*, composed predominantly of plastids, mitochondria and smooth endoplasmic reticulum, is compatible with the synthesis of terpene resins, as observed in the secretory structures of these resins in other plant species (Gilliland *et al.* 1988; Langenheim 2003; Rodrigues *et al.* 2011; Sadala-Castilho *et al.* 2016).

The small diameter secretory canals (type I) present in the spathe of *P. adamantinum* have similar behavior to that observed for the root canals of other species of the

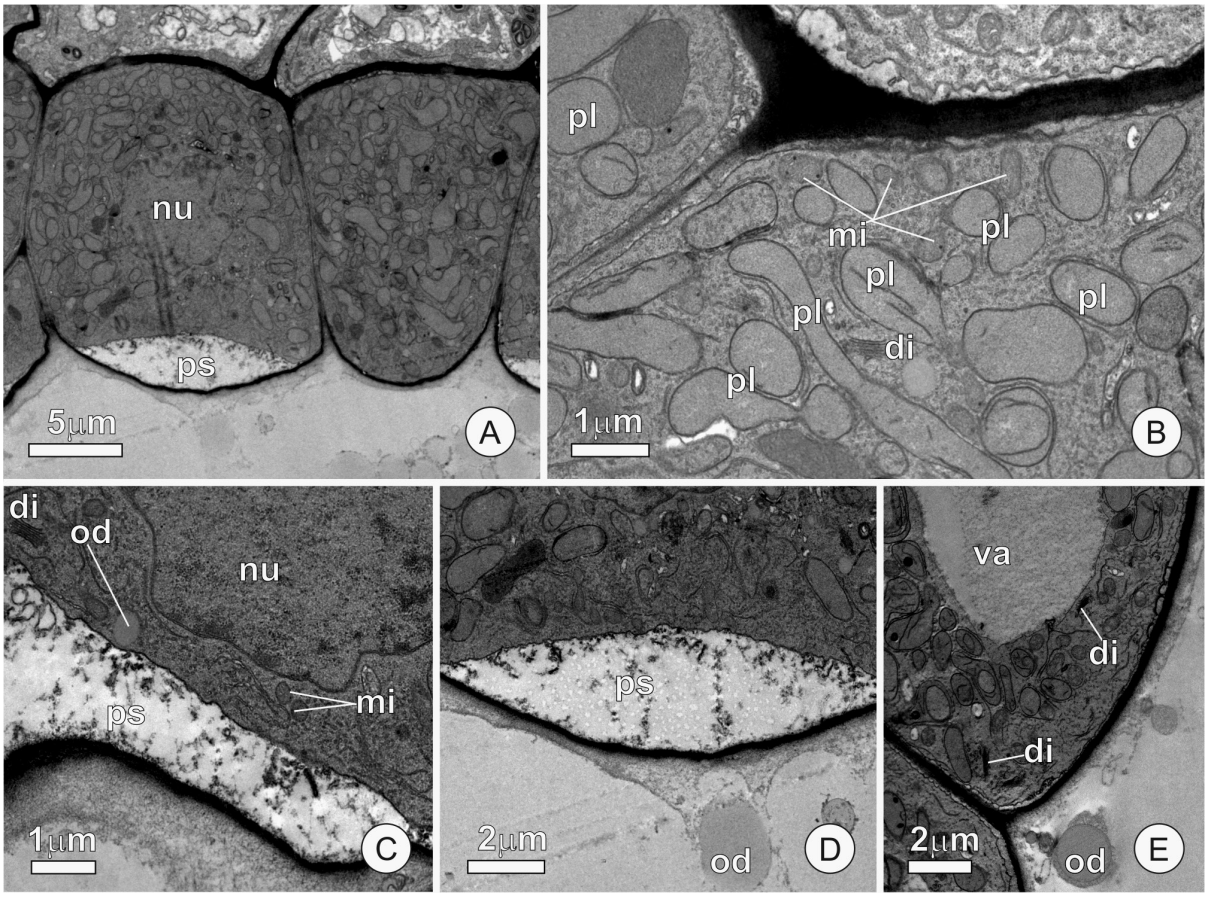

Figure 4. Sections of resin secretory canals located in the mesophyll of spathes of *Philodendron adamantinum*. **A-D.** Spathe at approximately 10 % of the final size. **A.** Part of a secretory canal in transverse section showing the epithelial cells. Note the periplasmic space filled with secretion. **B.** Secretory cell highlighting the high organelle population. **C.** Detail of the periplasmic space of a secretory cell. **D.** Secretory cell and part of the lumen of the canal. Note the droplet of lipidic material. **E.** Secretory cell 15 hours before resin release, showing the vacuole. **di** – dictyosome; **mi** – mitochondria; **nu** – nucleus; **od** – oil droplet; **pl** – plastid; **ps** – periplasmic space; **va** – vacuole.

genus *Philodendron* (French 1987) and in the axis of the inflorescence of *P. adamantinum* (P Gonçalves-Souza *et al.* unpubl. res.): secretion accumulates in the lumen of the ducts and release occurs only in the case of injury. This is the pattern observed for internal resin secretory structures – canals and cavities, which usually do not actively release secretion into the external environment (Dell & McComb 1978).

In *P. adamantinum*, the release of resin to the adaxial surface of the spathe is extremely synchronized, and initiates with the beginning of the functional male phase of the inflorescence on day two of the flowering cycle, some hours before the release of the chains of pollen grains from the fertile staminate flowers, and thus requires a control mechanism. The enlargement of the subepithelial cells of the secretory canals disposed on the adaxial side of the spathe reduces the size of the lumen of the canal and causes rupture of subepidermal layers, forming pockets that force the epidermis to rupture, similar to the mechanism of rupture of the subcuticular pockets described by Paiva (2017). Lorio & Hodges (1968) observed that the oil-resin exudation pressure in *Pinus taeda* reaches 10atm. Even under such pressure there is a need for injury to exudate, demonstrating that the pressure generated by the resin accumulation is not sufficient to break the tissues and leak the contents of the lumen into the external environment. Therefore, this distinct canal type described for *P. adamantinum* presents different physiological behaviour, and only the type II canals which are restricted to the adaxial face of spathe, react to the pressure by cell rupture and consequently allow resin extravasation.

But some questions remain to be answered regarding this precise system of resin releasing. Being cell turgor involved in the resin release, and the mechanical movements in plants mainly related to turgor pressure, we can hypothesize that the closure of the pollination chamber caused by the narrowing spathe might also be linked to the extravasation of the resins on the adaxial face of the spathe. Most of the plant organ movements originate from turgor changes of specialized cells (Scorza *et al.* 2014 and references therein), and in *P. adamantinum*, this turgor appears to act simultaneously on both pollination chamber and

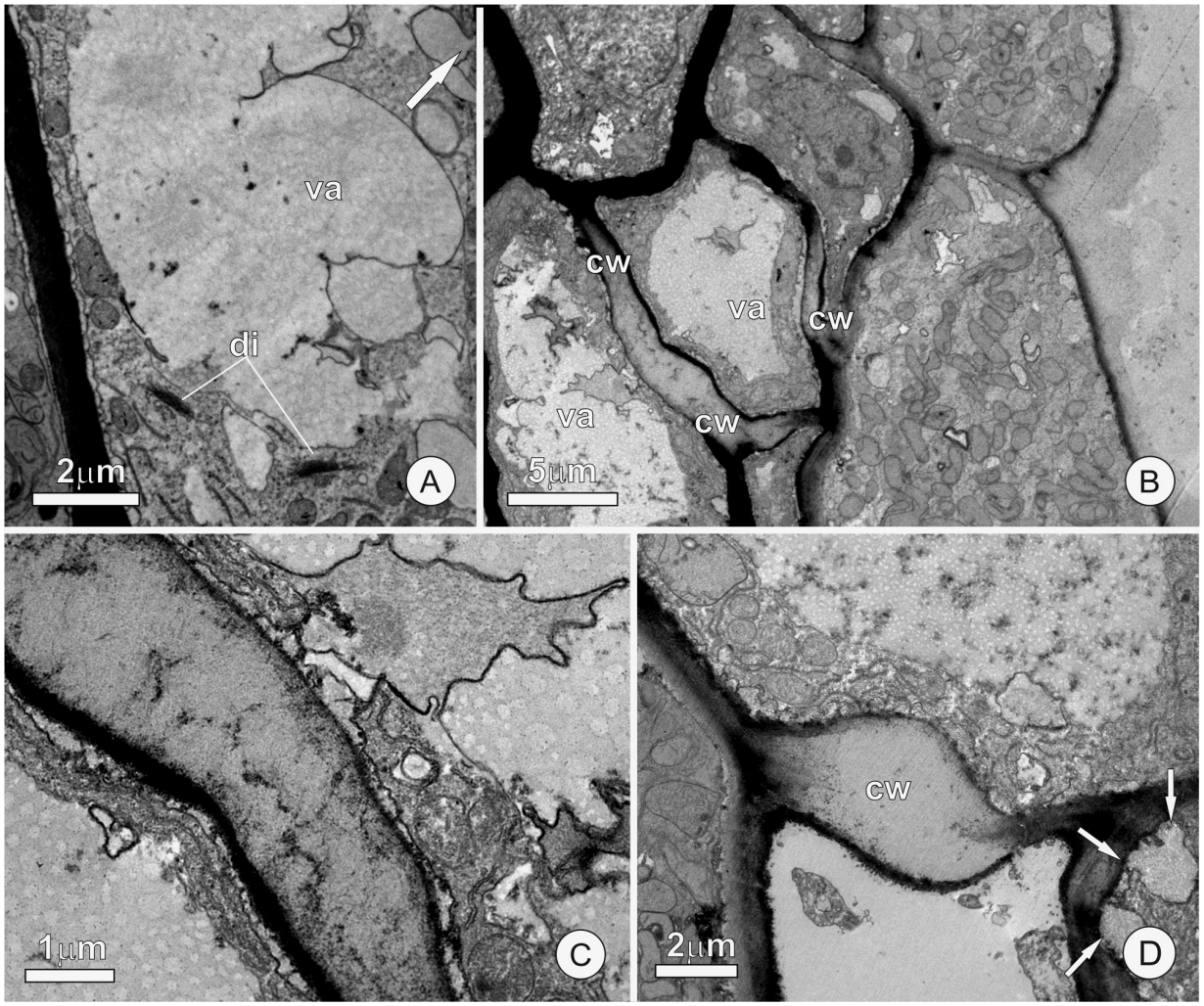

Figure 5. Sections of resin secretory canals located in the mesophyll of spathes of *Philodendron adamantinum*. **A.** Secretory cell 15 hours before the release of resin to the external medium. The arrow highlights the vesicle being incorporated into the plasma membrane. **B-D.** Apical region of canal in a spathe two hours before resin release. **B.** Secretory canal showing epithelium and underlying cells containing large vacuoles. Note the thickening of the wall. **C.** Tissue adjacent to secretory epithelium showing cell wall disorganization. **D.** Detail of swollen wall. Arrows indicate periplasmic space. **cw** - cell wall; **di -** dictyosome; **va -** vacuole.

resin release, contributing to the observed synchrony between closure of the pollination chamber and the resin release.

The presence of starch grains in the spathe does not seem to be related to resin synthesis, since the secretory phase of the canals starts early in spathe development, while the accumulation of this reserve takes place in more advanced stages. However, it is possible that the hydrolysis of starch acts on the cells of the canal by generating an osmotic gradient that contributes to the increased volume of these cells at the time of exudation.

The pectic nature of the cell wall near the apex suggests a pre-established route for canal opening. The separation of cells is probably due to the action of pectinases, which are essential for the breakage of the middle lamella and the consequent weakening of this layer (Roberts *et al*. 2000). This enzymatic action is common, for example, in areas of fruit dehiscence (Petersen *et al*. 1996).

Thus, there is strong evidence that resin release to the external environment occurs as a result of a) increased pressure on the resin accumulated in the lumen of the secretory canal and b) weakening of the middle lamella of the parenchyma cells near the apex of the canal.

Resin release through stomata, however, seems to be insignificant, given the limitation imposed by the tiny stomatal pore, when compared to the wide space formed by the rupture of the epidermis.

In *P. adamantinum*, the resin released in the adaxial surface of the spathe covers the entire body surface of the large *Erioscelis emarginata* beetles. The pollen grains released in long chains glue to the resin-sticky cuticle of the beetles when they search their way out the closing pollination chamber. Due to the smooth surface of the beetle's body surface and absence of hairs to which pollen grains would adhere, the released resin appears to be necessary to make the beetles efficient pollen carriers and the pollination

mechanism work. In this context, it would be interesting to experimentally quantify Philodendron-pollen adherence to beetles with dry to those with resin covered body surface. Furthermore, it could also be tested whether the released resin diminishes pollen viability or not, a vital feature of beetle transported pollen to conspecific stigmas.

Other substances seem to be enrolled in adhesion of pollen, as reported by Schnetzler *et al*. (2017) for polysaccharides in trichomes of *Dorstenia cayapia* (Moraceae). In oil-producing Cucurbitaceae, Possobom & Machado (2017) reported the adhesive properties of oil from male flowers, which adhere the pollen grain to the body of floral visitors.

It is important to pay attention to the cellular mechanisms that control the process of resin release, especially with regard to precision and timing. These mechanisms ensure that the resin is available at the moment when the pollinators leave the spathe chamber and pass through the newly released pollen, allowing it to act as a fixation element for pollen on the body of pollinators. Such specificity in this insect-plant relationship evidences the close relationship between such pairs throughout evolution.

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