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Research article

True nectar or stigmatic secretion? Structural evidence elucidates an old controversy regarding nectaries in *Anthurium*

Élder Antônio Sousa Paiva^{1*}, Igor Ballego-Campos¹ and Marc Gibernau²

¹Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, MG, Brazil.

²CNRS-University of Corsica Pascal Paoli, UMR 6134 SPE, Equipe Chimie et Biomasse, Route des Sanguinaires - Vignola, 20000 Ajaccio, France

*Corresponding author. Email: epaiva@icb.ufmg.br

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Nectar and stigmatic secretion in *Anthurium*

ABSTRACT

PREMISE: Floral rewards are essential in the understanding of floral function and evolution of the relationships between flowers and pollinators. Due to the scarcity of structural studies, the presence of sugars in stigmatic exudates, as well as the presence of floral nectaries in *Anthurium*, is quite controversial. To solve this, we investigated the floral anatomy of *A. andraeanum* to elucidate whether (i) tepals are secretory organs, (ii) tepals possess a structurally recognizable nectary, and (iii) tepalar secretion differs from stigmatic secretion.

METHODS: Floral structure was assessed through light and electron microscopy on samples of immature, pistillate, and staminate flowers. The dynamic of the starch reserve was investigated, and the sugar content in the floral exudates was assessed using thin-layer chromatography.

RESULTS: Sugar analysis did not detect sucrose, glucose, or fructose in the stigmatic secretions, but confirmed their presence in the tepalar ones. Stigmatic secretion was produced by secretory stigmatic papillae, while tepalar exudates were produced by non-vascularized nectaries located in the apex of tepals. These nectaries were characterized by cells with cytoplasm rich in organelles, as well as a high content of calcium oxalate crystals and the presence of modified stomata.

CONCLUSIONS: Our results showed for the first time a nectary presence on tepals and true nectar secretion for *A. andraeanum*. Stigmatic secretion appears to be a distinct substance, and its often-reported sugar content seems to be a result of sample contamination. Nectar and stigmatic secretion have been often mistaken in other *Anthurium* species, and deserve a revision for this genus.

KEY WORDS: Araceae; floral ultrastructure; floral nectary; floral rewards; nectar secretion; stigmatic papillae; sugary secretions

INTRODUCTION

Floral rewards are essential in the understanding of floral function and evolution of plant-pollinator interactions (Baker and Baker, 1975; Cruden et al. 1983; Abrahamczyk et al., 2017). By exuding nectar, oils, scents or resins, floral glands are key features in plant reproductive success and in the development of the diverse relationships between flower and pollinators (Tölke et al., 2020).

Araceae, one of the most diverse monocot families, is mainly insect-pollinated (Gibernau 2011, 2016; Díaz-Jiménez et al., 2019), but surprisingly they are traditionally considered to be nectarless (Schwerdtfeger et al., 2002). Interestingly, sugars have been reported from secretions produced by the stigma in *Anthurium* (Bleiweiss et al., 2019), resulting in a significant controversy regarding floral exudates in the genus, especially concerning the occurrence of true nectar in its flowers. Quite straightforward terms, such as “nectaries”, “nectar”, and “pollination drops” have been indifferently used for *Anthurium* floral secretions leading to confusion not only of words, but also of the floral physiological processes. In fact, despite the presence of sugary secretion in the flowers of this diverse neotropical genus, nectary could not accurately point out. Indeed, we even surely answer whether or not such a nectary exists. Here, we have studied in detail the floral anatomy of *Anthurium*, and the chemical nature of its secretions to clarify these issues. This is a crucial point that may help to understand the floral biology and evolution of plant-insect interaction in aroids. On the other hand, there are gaps regarding the prospection of floral secretory structures in *Anthurium*, which precludes analyses concerning the homology of floral glands.

Most of the pollination interactions in Araceae are mutualisms (Chartier et al., 2014). Three types of rewarding mutualisms have evolved in this plant group; the

inflorescences “offering” a food reward, such as stigmatic exudates, nectar and/or pollen (Diaz and Kite, 2006; Gibernau, 2011); a sexual reward such as a liquid floral perfume for male euglossine bees (Hentrich et al., 2007, 2010; Etl et al., 2017); a mating site with food rewards (Maia et al., 2013) or a mating and oviposition site (Franz, 2007).

Anthurium is the largest aroid genus comprising 950 described and more than 2,000 estimated Neotropical species (Boyce and Croat, 2018). This taxonomically complex megagenus is one of the most morphologically and ecologically diverse aroid genera (Carlsen and Croat, 2019). Historically considered to be pollinated primarily by euglossine bees (Croat, 1980), *Anthurium* species also exhibit highly diverse pollination interactions, which include different kinds of bees (Apini, Augochlorini, Euglossini, Halictini, Meliponini, Tapinotaspidini), beetles (Curculionidae), and flies (Cecidomyiidae, Drosophilidae); but also lepidopterans (Lepidoptera), thrips (Thysanoptera) and even hummingbirds (see reviews Hartley and Gibernau, 2019; and Díaz-Jiménez et al., 2019). However, *A. andraeanum* has been reported to be visited by fragrance collecting male euglossine bees of *Eulaema seabrai* in wild Brazilian populations (Rocha-Filho et al., 2012).

All Araceae have a protogynous flowering sequence (Díaz-Jiménez et al., 2019), but its duration in *Anthurium* is quite variable, ranging from one week to over 30 days (Croat, 1980; Hentrich et al., 2010). Flowering starts with the female phase, with the production of drops of stigmatic secretion in some species, while in others, stigmas just have a moist appearance indicating their receptivity (Croat, 1980; Etl et al., 2017). Subsequently, the inflorescence enters a male phase, with the stamens emerging from several flowers in a progressive sequence (Croat, 1980; Hentrich et al., 2010).

111 It is well established that during the pistillate phase, the flowers exude a
112 secretion through the stigma that is the so-called pollination drop. Pollination drops are
113 better known as fluid secretions produced by the ovules of gymnosperms, which are
114 involved in reproduction (Coulter et al., 2012). However, stigmatic exudates are
115 reported in flowers of several angiosperm species, sometimes considered similar to
116 pollination drops of gymnosperms, in which they are related to male gamete transport
117 towards the megagametophyte (see Nepi et al., 2009 and references therein). From now
118 on, we will adopt the term stigmatic secretion, even though pollination drop is usually
119 used for stigmatic exudates in Araceae. In *Anthurium* species, the stigmatic secretion
120 can form conspicuous drops on the stigma surface of each flower and may contain
121 soluble sugars, being considered analogous to nectar (Bleiweiss et al., 2019) or even
122 considered nectar by some authors (Croat, 1980; Kraemer and Schmitt, 1999; Franz,
123 2007). However, not one of these studies interprets the stigma as a nectary.

124 In Araceae, the presence of sugars in the stigmatic secretion is reported for
125 genera other than *Anthurium*, as in *Monstera* (Ramirez and Gomez, 1978) and *Arum*
126 (Diaz and Kite, 2006), although in the latter the authors highlight the small proportion
127 of sugars, present at a lower concentration than observed in the nectar. The stigmatic
128 secretion is consistently sweet, and in the case of *A. seibertii* Croat & R.A.Baker, it was
129 reported to contain 8% sugar comprised of a combination of sucrose, glucose, and
130 fructose (in Croat, 1980). In *Arum maculatum* L., a terrestrial species widespread across
131 most of Europe and the Caucasus, the concentration of sucrose equivalent ranged
132 between 9–12.5% in the stigmatic secretions tested. This sugar concentration was only
133 slightly higher than that of the phloem in the same species (8% sucrose equivalent)
134 (Lack and Diaz, 1991). In *Arum hygrophilum* Boiss. of Israel, the stigmatic secretions
135 contained above 5% sugar (Koach, 1985).

The presence of true floral nectaries in *Anthurium* is quite controversial (see Hartley et al., 2017 and references therein). Aroids and the genus *Anthurium*, in particular, are supposed to be nectarless (Schwerdtfeger et al., 2002). But Daumann (1930) properly described tepalar sugary secretions in *A. digitatum*: “*The liquid droplets, which appear towards the end of the female phase, and especially during the male phase of a flower, on the free portion of each tepal, also contain monosaccharides (fructose and glucose) and disaccharide (sucrose). The sugar content... is higher than in the stigma secretion.*”. More recently, tepalar secretions or droplets called “nectar” are mentioned without any analysis for *A. concolor* K.Krause (Croat, 1991) and reported by Franz (2007), who stated: “*In the majority of species of Anthurium, the inflorescences are protogynous, producing stigmatic nectar for some time before the pollen is released (the tepals can also produce nectar during the emergence of the stamens)*”. Despite this statement, conspicuous “nectar” droplets were previously observed on the tepals of *A. amethystinum*, *A. cotobrusii* and *A. hacumense* (Croat, 1980). Observations of hummingbirds visiting inflorescences of different species of *Anthurium* suggest that the spadices are secreting large quantities of sugary liquid, estimated at 178 μ l/24h in *A. sanguineum* (Kraemer and Schmitt, 1999), and attracting animals with high energetic demands (Bleiweiss et al., 2019; Hartley and Gibernau, 2019).

In general, the nectar of angiosperms and pollination drops produced by gymnosperm ovules present a similar qualitative composition, noticeably regarding sugars, amino acids, and proteins (See Nepi et al., 2009 for details). Pollination drops usually possess a low concentration of sugars, being unattractive to most pollinators (Baker and Baker, 1983). The main functions of such pollination drops are pollen capture and germination, whereas nectar is a reward for interacting animals (Nepi et al.,

2009). Consequently, the two kinds of secretion differ primarily in their volume, as pollination drops (less than 0.25 μ l) are generally smaller than nectar secretions (Nepi et al., 2009). Secondly, they present different ranges of sugar concentrations: pollination drop sugar concentrations range from 5–10 %, whereas nectar sugar concentration is generally much higher (Nepi et al., 2009). Thirdly, of the three most common sugars – glucose, fructose, and sucrose – sucrose is the most common form found in nectar, whereas it is fructose in pollination drops (Nepi et al., 2009).

Although it remains unknown whether tepalar secretions in *Anthurium* are more similar to nectar or stigmatic drops, Daumann (1930) indicated a higher sugar content in tepalar secretions than in stigmatic ones. Further efforts are needed to determine if some *Anthurium* species produce floral secretions with high sugary content more related to true nectar. The presence of nectar in the absence of a nectary is not usual, although it can occur in some vegetative organs (Lortzing et al., 2016). Could this be the case with *Anthurium*? Would the supposed nectar be produced by an unstructured nectary, or would the stigma be involved in the synthesis and release of sugars through stigmatic secretion? Considering the relevance of the flowering behavior to the pollination biology of *Anthurium* species (Croat, 1980), we intend to answer the questions above by studying the structure and secretions of *A. andraeanum* inflorescences, shedding light on the current confusion between nectar and stigmatic secretion in *Anthurium*. The specific aims of this study were: i) Are the tepals gland-bearing organs? ii) If, so is the tepalar secretion different from that of the stigma? iii) Is there a structurally recognizable nectary?

MATERIALS AND METHODS

Plant material –

Ten individuals of *Anthurium andraeanum* Linden ex Andre, section *Calomystrium*, were observed for a year in order to assess all stages of inflorescence development and secretory activity. Samples of spadices, and secretions from both stigmas and tepals were collected from March to June, on cultivated plants growing in Belo Horizonte, Minas Gerais state, Brazil.

To recognize the pistillate and staminate phases of each inflorescence, we considered (a) the presence of stigmatic secretion and (b) the exertion of anthers and pollen release, respectively. It is important to emphasize that the time interval between these phases was not evaluated for two reasons. Firstly, this information was not relevant to our study focused on the female and male stages of anthesis and secondly because these stages are highly variable, and the transition between them difficult to recognize, as pointed by Croat (1980).

Light microscopy and starch dynamics –

Samples of spadices were taken from immature inflorescences immediately after spathe unfolding (i.e., previously to the observation of any sexual floral stages), and during both pistillate and staminate phases. In total, two inflorescences per individual from five individuals were sampled. Transverse and paradermal fragments of the spadices were subjected to a vacuum in Karnovsky solution (pH 7.2 in 0.1M phosphate buffer, modified from Karnovsky, 1965) for 5 minutes, and then left in this fixative for 24 hours at room conditions. Fixed samples were dehydrated in an ethanol series (Johansen, 1940) and subjected to pre-infiltration and infiltration in synthetic resin (2-hydroxyethyl methacrylate; Leica® Biosystems, Nussloch, Baden-Württemberg, Germany). Sections (5µm-thick) were obtained in a rotary microtome (Hyrax M40, Carl Zeiss Mikroskopie, Jena, Thuringia, Germany), stained with Toluidine blue pH 4.7

(modified from O'Brien et al., 1964), counterstained with ruthenium red (0.02%, aqueous solution) and mounted in synthetic resin (Entellan®, Sigma-Aldrich, St. Louis, Missouri, USA). In order also to detect the presence and dynamics of starch storage, unstained sections were tested using Lugol reagent (Johansen, 1940). To detect phenolic compounds and acidic polysaccharides, unstained sections were exposed to ferric chloride (Johansen 1940) and ruthenium red (Jensen 1962) respectively. All images were taken using a light microscope (CX41RF, Olympus Scientific Solutions, Waltham, Massachusetts, USA) equipped with filters to provide circularly polarized illumination, coupled with a digital camera and an image capturing system (TV0.5XC-3, Olympus Scientific Solutions, Waltham, Massachusetts, USA). To improve the observation of crystals within the tissues, sections were also analyzed under polarized light.

Electron Microscopy –

We applied both scanning (SEM) and transmission (TEM) electron microscopy in order to investigate the micromorphological arrangement of the inflorescence and the ultrastructural composition of the secretory cells, respectively.

For SEM analyses, samples of immature spadices and during both the pistillate and staminate phases were collected and subjected to fixation in Karnovsky solution (pH 7.2 in 0.1M phosphate buffer, modified from Karnovsky, 1965) for 24 hours. Fixed samples were dehydrated in an increasing ethanol series, submitted to critical-point drying, and affixed onto stubs to expose the spadices surface. The prepared materials/samples were then coated with a gold-palladium alloy and observed under a scanning electron microscope (Quanta 200, FEI Company, Eindhoven, North Brabant, Netherlands).

For ultrastructural analyzes, portions of the tepals and stigma were sampled at the beginning and at the end of the pistillate phase. The first was characterized by the presence of the stigmatic secretion and the absence of tepalar secretion, and the second by the peak of the tepalar secretion and interruption of the stigmatic exudation. Samples were removed using paradermal cuts to obtain 2mm-deep fragments from the tepalar surface, which were then subjected to vacuum in Karnovsky solution (pH 7.2 in 0.1M phosphate buffer, modified from Karnovsky, 1965) for 5 minutes, and left in this fixative for 24 hours. Fixed samples were post-fixed in 1% osmium tetroxide (pH 7.2 in 0.1 M phosphate buffer) for 2h, dehydrated in an increasing acetone series, embedded in epoxy resin (Spurr, 1969) and sectioned with the aid of an ultramicrotome (UC6, Leica Microsystems, Wetzlar, Hesse, Germany). Ultrathin sections were contrasted with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and analyzed under a transmission electron microscope (Tecnai G2–Spirit, Philips/FEI Company, Eindhoven, North Brabant, Netherlands).

Sugar analysis –

Secretions appearing on both the stigma and tepal surfaces were tested for the presence of sugar using thin-layer chromatography (TLC) and glucose strips tests (Alamar Tecno Científica, São Paulo, Brazil). Due to the small size of the flowers (~ 4 mm), samples of the exudates were collected under a stereomicroscope (Stemi 2000-C, Carl Zeiss Mikroskopie, Jena, Thuringia, Germany), 20 droplets per plant (10µL) on a same fine filter paper strip from five individuals, totaling five paper strips for each type of secretion, which were stored in sterile plastic vial tubes and immediately frozen. Secretions samples were (preferably) collected at the very beginning of the secretion release by the stigma or tepals, avoiding mixing these secretions. Flowers in which

stigmatic and tepalar secretions could not be precisely distinguished (either because of the collapse of stigmatic fluid drops or by the flow of secretions down the spadix) were not sampled. For analyses, strips containing secretions were unfrozen and carefully washed with distilled water (100 μ L) to obtain a diluted solution.

Thin-layer chromatography was carried out in 10 x 10 cm TLC plates (Silica gel 60, aluminum plates, Merck), using a mixture of 1% formic acid in chloroform: methanol (1:1) as a mobile phase and 15% sulfuric acid as the revealing solution (Stahl, 1969, modified). Sucrose, glucose, and fructose solutions (at 0.5%) were used as standards.

RESULTS

Stages of secretory processes –

In immature inflorescences, immediately after spathe unfolding, the spadix was at its final stage of expansion and differentiation, displaying a greenish-yellow coloration but bearing still immature flowers (Fig. 1A). After the full exposure of the spadix, flowering started in an acropetal sequence, with the basal flowers maturing first (Fig. 1A-C).

The first sign of secretory activity in the inflorescence was the accumulation of the stigmatic secretion as small drops (~ 0.5 μ L) on the stigma surface, which occurred concomitantly with the beginning of the pistillate phase, during the early stages of flower anthesis (Figs. 1B, D). The time delay for flower maturation on two successive rows along the spadix was extremely variable, ranging from one to more than 20 days.

The tepals started secreting later, around the second day of anthesis. At the same time, stigmatic secretion was still released/produced and lasted for about two days, totaling approximately three days of secretory activity during the pistillate phase. Some

inflorescences showed to be very productive during this secretion stage, resulting in tepalar secretions running down the spadix and accumulating at the base of the spathe (Fig. 1E). Exudation was easily noticeable on the surface of the tepals even after the secretion phase (Fig. 1F). In the same progression, as the stigmatic exudates, tepalar secretion occurred in an acropetal direction, concurrently with the flowering process. Anthers elongated from below the tepals and were exposed only later, after there was no more evidence of stigmatic fluid or of nectar secretion. Even without secretory activity, the remaining nectar can be observed, especially in the absence of nectar consumers. The anthers released pollen as soon as exposed.

Sugar analysis –

TLC analysis and glucose strips tests confirmed the presence of sugars in tepalar secretions. They showed that the exudate had a mixture of sucrose, glucose, and fructose, thus corroborating that the tepalar secretions are, in fact, nectar. Both types of analysis, however, failed in detecting sucrose, glucose, or fructose in the stigmatic secretion of the studied species.

Structural organization –

Floral nectaries –

The flowers of *A. andraeanum* were compactly arranged within the spadices (Figs. 2, 3A). On the spadix surface, the four tepals of a single flower formed a diamond shape, with the apex of the pistil and the stigmatic surface in its center (Fig. 2A). The flattened distal portion of the tepals makes the flower surface also flat and gives the spadix a smooth appearance at the final stage of expansion (Figs. 1A, 2A). With the

beginning of the pistillate phase, the protrusion of the apical portion of the pistil gives a rough appearance characteristic of spadices in this species (Fig. 1B-C).

Just after the spathe unfolding, the tepalar stomata were closed and covered by a cuticle, and only during the final expansion of the floral parts was evidence of stomatal opening observed (Fig. 2B-C). However, fully opened pores were not seen before the beginning of the pistillate phase. They remained open even in the staminate phase when the tepalar secretions were no longer perceived (Fig. 2D). It was observed that the stomata were randomly distributed, and their density ranged from 25 to 40 mm^{-2} ; each tepal had a surface of about 0.5 mm^{-2} .

The tepal epidermis was uniseriate, with stomata restricted to the flattened apical portion. In these floral parts, the entire mesophyll was parenchymatous, and there was only one vascular bundle, which corresponded to the midvein (Fig. 3A-D). The vascular bundle was collateral and did not branch, presenting few cells of both xylem and phloem (Fig. 3B). In the apical portion of the tepals, marked by the absence of vascular elements, the parenchyma underlying the epidermis had predominantly globose cells, which showed dense cytoplasm; these cells were interposed with vacuolated cells with phenolic content (Fig. 3C-D). In the basal two-thirds of the tepal length, the parenchyma cells showed a less dense cytoplasm and were slightly elongated in the axial direction. The vascular bundle ended before reaching the apical third of the tepal (Fig. 3D), comprising a apical, non-vascularized portion that extended over a depth ranging from 200 to 400 μm and corresponded to the nectary. In this region, the epidermis presented dense cytoplasm and juxtaposed cells covered by a thin cuticle, which was inconspicuous when observed under a light microscope (Fig. 3D). Thus, there is only one nectary per tepal, whose surface corresponds to the exposed portion of the tepal.

The tepals showed a clear distinction between the secretory portion located in the upper third, characterized by cells of greater cytoplasmic density, and the underlying non-secretory portion, with vacuolated cells (Fig. 3D), and a lower concentration of starch. It is also noteworthy that there was a strong asymmetry in the distribution of calcium oxalate crystals (druses) within these two regions. The density of cells containing crystals was higher in the secretory portion (Fig. 3E) than in the non-secretory portion of the tepals (Fig. 3F).

Nectary starch dynamics –

Starch was found in the tepals during the three floral phases analyzed (Fig. 4). It was noted to be more abundant towards the apical part of the tepals so that starch grains were rarely observed in their basal portion. However, the amount of starch was always much higher during anthesis, in both the pistillate and staminate phases, than during early developmental stages of immature flowers (Fig. 4A-F). In the latter case, few and very small starch grains were scattered throughout the tepal tissue, both in the subglandular parenchyma and in the nectary portion, but rarely in the epidermal cells (Fig. 4A-B).

In the flowers at staminate and pistillate phases, the starch grains were generally large and abundant (Fig. 4C-F). However, differences could be seen regarding the pistillate and staminate phases, especially in the nectary and the immediate subjacent tissue (Fig. 4D, F). Flowers at the beginning of the pistillate phase showed higher content of starch in the nectary portion (epidermis and immediate subepidermal cell layers), with larger conspicuous grains abundantly distributed (Fig. 4C-D). At the staminate phase, however, flowers usually displayed a lower amount of starch in the same zone, with gradually smaller grains towards the epidermis, where they were

usually hard to observe (Fig. 4E-F). These specific changes between pistillate and staminate phases resulted in a noticeable starch configuration in the nectary, which could be distinctly recognized in the staminate phase with a clearer, almost starch-free zone at the tepal apex (Fig. 4E-F).

Stigma and secretory stigmatic papillae –

The stigma of *A. andraeanum* flowers was brush-like (Figs. 2C, 5A) and presented several stigmatic papillae, which could be 250µm long (Fig. 5A) at the beginning of the pistillate phase. The papillae presented a dense cytoplasm and a thin pecto-celulosic cell wall, through which the exudate was released outwards to the external environment, leaving the surface of the papillae with several strands of secretion (Fig. 5B). These strands were heavily stained in pink by ruthenium red, and in magenta by toluidine blue, which strongly indicates secretion of mucilaginous nature. At the staminate phase, the stigmatic papillae began to show signs of senescence and necrosis, and the secretory process ended, culminating in a drastic volume reduction in the stigmatic region (Fig. 5C).

Ultrastructure of secretory cells –

The epidermal cells at the tepal apex presented a dense and organelle-rich protoplast, with conspicuous nuclei (Fig. 6A). These cells had cell walls with a remarkable asymmetry, being thicker at the outer periclinal face, which was covered by a thin cuticle (Fig. 6A). The subjacent parenchyma presented cells with thin walls, a dense and organelle-rich protoplast composed of plastids with starch grains, mitochondria, and vacuoles. Also, plasmodesmata were sparsely distributed, being

restricted to the rare primary pit field (Fig. 6B-D). These cells alternated with cells characterized by a large vacuole filled by phenolics.

In immature inflorescences, just before the pistillate stage and nectar secretion, the organelle-rich parenchyma cells presented plastids with large starch grains. These plastids exhibited a dense stroma and a poorly developed endomembrane system (Fig. 6C).

During the pistillate phase and nectar secretion, the parenchyma cells remained as described before, but mitochondria abundance was remarkably high (Fig. 6D). Indeed, evidence for starch hydrolysis and structural changes in the plastids were observed. In these organelles, the intermembrane space enlarged, and a well-developed endomembrane system was established at the plastid periphery in some of them (Fig. 6E). This plastid swelling seemed to evolve fast and producing a clearer and more sparse stroma. At this stage, plastids fused, and the product of this fusion was outlined only by the outer plastid membrane (Fig. 6F), resembling a large vacuole on which some starch grains remained. In some cells, vacuoles and swollen plastids were similar and presented a structurally similar content (Fig. 6F-G).

Stigmatic papillae at the secretory phase (i.e., pistillate phase) also showed a dense cytoplasm, with numerous dictyosomes, mitochondria, and plastids containing starch grains. Oil droplets and small vacuoles were also seen scattered throughout the entire cytoplasmic matrix; the latter usually highly juxtaposed or showing signs of fusion (Fig. 7A-B). In some papillae, a remarkable presence of secretion was observed within the periplasmic space, along with noticeable compression of the protoplast (Fig. 7C-D). Exudation of mucilage was seen due to the formation of strands of secretion crossing the cell wall (Fig. 7C, E).

DISCUSSION

Structure of nectary and secretory stigmatic papillae –

Despite some reports about the presence of soluble sugars in secretions found on the spadix of different species of *Anthurium*, nectar secretion has not been adequately demonstrated. This lack of structural studies of secretory activity has resulted in considerable controversy about the presence of floral nectaries or even true nectar secretion in this genus (see Hartley et al., 2017). The occurrence of secretory activity in Araceae inflorescences has been documented for some species, but only in rare studies is there a structural analysis that identifies the origin of the secretory product (Skubatz and Kunkel, 1999; Hadacek and Weber, 2002; Gonçalves-Souza et al., 2017; Gonçalves-Souza et al., 2018). Our study demonstrated the existence of active tepalar nectaries in *Anthurium andraeanum*.

The high cytoplasmic density of cells involved in the secretory process constitutes remarkable structural evidence that indicates secretory activity in a given plant structure (Lüttge, 1971; Fahn, 2000). For some glands, the presence of stomata is another indication of the release pathway of secretory products, a fact common in floral nectaries in which the release of nectar frequently occurs through modified stomata (Fahn, 1979; Paiva, 2017 and references therein). Thus, the presence of these characteristics in the apical portion of the tepals is the first evidence that these organs are involved in a secretory process in *A. andraeanum*. The record of secretory activity at the apex of the tepals is not unprecedented for Araceae; on the contrary, there are indications that it constitutes the most probable source of secretions related to the attraction of pollinators in this plant group (Gonçalves-Souza et al., 2017). As a key trait of floral nectaries (Paiva, 2017), the tepalar surface of *A. andraeanum* shows strong evidence of nectar release through modified stomata. Also, the tepal vasculature in *A.*

andraeanum, presenting a single vascular bundle that ends near to the secretory tissue, seems to reinforce that nectar is not directly derived from phloem. Stored starch in secretory parenchyma and evidences of its hydrolysis during the secretory phase allow us to infer that nectar is at least partially derived from starch, as commonly observed in floral nectaries (Nepi et al., 1966; Durkee et al., 1981; Ren et al., 2007; Paiva, 2012).

The presence of calcium oxalate crystals, as observed in the secretory portion of *A. andraeanum* tepals, has been reported to be widely distributed in Araceae (Keating 2004). In tepals of *A. andraeanum*, Higaki et al. (1984) reported that druse and raphide crystals occurred in a higher frequency than in other tissues of the plant. Calcium crystals are related to the maintenance of calcium regulation in the cytosol (see Paiva, 2019), which in turn seems to modulate the activity of H⁺-ATPases (Kinoshita et al., 1995) and some membrane transport processes. For instance, sucrose transport from the symplast to the apoplast, if against a concentration gradient, occurs employing antiporters (Vassilyev, 2010) and depends on ATPase activity. Therefore, as pointed out by Pireda et al. (2017), calcium regulation appears to be crucial to nectar secretion, which may explain why the presence of calcium oxalate crystals next to or inside nectar-secreting tissues has been reported in several plant species (See Paiva et al., 2007 and references therein; Konarska, 2014; Pereira et al., 2018). Accordingly, the presence of abundant crystals in the distal portion of *A. andraeanum* tepals must be regarded as another piece of evidence of nectar secretion capacity.

As expected, the stigma in *A. andraeanum* proved to be closely related to the secretory activity, which resulted in secretion release by the stigmatic papillae. Papillae are types of trichomes, characterized by a narrow length-width ratio. However, even though being long, in Araceae, these stigmatic trichomes are traditionally called papillae, a term that will be employed here. Some structural features of these papillae,

mainly the high protoplast density and the presence of mucilage residues, appeared to be coincident with stigmatic secretion release and denotes their ability for synthesis of mucilages (see Ballego-Campos and Paiva, 2018).

Our investigations of the ultrastructural cell features proved to be important for the location of secretory tissues in *A. andraeanum*, especially in the distal portion of the tepals. Plant secretion implies the synthesis and processing of different substances and usually occurs in cells with high metabolic activity. Thus, cytoplasmic density and abundance of organelles are good indicators of secretory activity. Besides the overall cytoplasmic density observed in the tepalar nectaries, we also noticed a remarkable presence of mitochondria in the secretory parenchyma of this portion, which denotes the intense metabolic demands involved in the nectar secretion (Roy et al., 2017).

During the stage of nectar production, the structural changes observed in plastids seem to be similar to those reported by Gonçalves-Souza et al. (2017) in the secretory portion of *Philodendron adamantinum* spadix, another Araceae species. This apparent conversion of plastids into vacuoles, although understudied, may be a shared process in plant secretory dynamics. Plastid changes commonly occur at the end of the secretory phase and have been reported in nectaries of some plant species (Peng et al., 2004; Paiva and Machado, 2008; Guimarães et al., 2016). Starch grains remnants inside the vacuoles, as observed by some authors (Paiva and Machado, 2008; Guimarães et al., 2016), should be considered as strong evidence of plastid incorporation into vacuoles or plastid conversion into a vacuole.

Regarding the stigma, the organelle-rich cytoplasm of the stigmatic papillae showed ultrastructural aspects highly related to those reported for colleters and other mucilage-secreting structures (Horner and Lersten, 1968; Fahn, 1979, 2000; Oliveira et al., 2017; Ballego-Campos and Paiva, 2018). The presence of numerous, juxtaposed

small vacuoles, along with their apparent fusion and the accumulation of secretion in the periplasmic space, evidences is consistent with the secretory mechanism proposed by Paiva (2016), which results in cell wall transposition by the produced mucilage by a cyclic action of compression and expansion of the protoplast.

Stigmatic secretion, nectar, and pollination –

There are, in the literature, several cases in which stigmatic secretion and nectar (e.g., tepalar secretions) in Araceae species are not distinguished, being referred only as nectar (Ramirez and Gomez, 1978; Croat, 1980, Kraemer and Schmitt, 1999; Diaz and Kite, 2006; Franz, 2007). For example, Kraemer and Schmitt (1999) reported, in *Anthurium sanguineum*, nectar droplets with an average volume of 13.7µl and an average sucrose content of 4.6%, but reaching 12%, with no indication of the secretion origin. However, our results for *A. andraeanum* show that claims of sugars in the stigmatic secretion of aroids should be taken with caution, as there is evidence that these may result from contamination with the true nectar. As pointed out by Croat (1980) for some *Anthurium* species, we observed that the accumulation of released secretions could result in its dripping through the spadix. Even when drops are not so large, the inflorescence visitors can spread these droplets through other parts of the spadix. Therefore, it is complicated to sample a pure drop of stigmatic secretion, and we believe that some contamination with true nectar must occur, resulting in sugar in the stigmatic fluid, a fact that we did not observe in our samples from *A. andraeanum*.

Interestingly, in the literature data, the composition of the stigmatic secretion has been reported as being the same as that observed in the nectar released by the tepals, both in *A. andraeanum*, and *A. seibertii*, for instance (see Croat, 1980). This reinforces the possibility of some contamination during sample collection. For some species,

nectar droplets are reported to be found scattered on the tepals (Daumann, 1930; Croat, 1980; Franz, 2007), which suggests a true nectary action, in a similar way that we describe here. Another interesting point is that when stigmatic secretion is reported as being nectar, the sugar concentration is usually lower than the true nectar, as reported for some *Arum* species (Diaz and Kite, 2006) and *Anthurium sanguineum* (Kraemer and Schmitt, 1999). However, although it remains obscure and understudied, the secretory activity of tepals in *Anthurium* was described a long time ago by Daumann (1930), who clearly described nectar secretion in *A. digitatum* in a pattern similar to the observed here. Additionally, some other reports suggest the involvement of tepals in nectar release. Croat (1980), for instance, reported such occurrence at least in three *Anthurium* species, namely *A. amethystinum*, *A. cotobrusii*, and *A. hacumense*.

Another interesting piece of evidence suggesting that the stigmatic exudate is distinct from nectar in *Anthurium* was recently presented by Bleiweiss et al. (2019) when reporting the attraction of birds to *Anthurium* (“Guango” *Anthurium*) inflorescences. In this case, the authors observed that sugary secretions produced at the beginning of the staminate phase favors visitation, but reported the absence of visits in the pistillate phase, during which stigmatic secretions were released.

Although both the floral phases occurred with a remarkable time interval, as pointed out by Croat (1980), uncollected nectar can persist on the spadix after the pistillate phase, as we have seen in *A. andraeanum*. Such residues tend to become very concentrated due to evaporation, which contributes to their conservation and increases the possibility that the nectar can act as a floral reward also in the staminate phase. However, it is necessary to emphasize that there is, in the literature, evidence of nectar secretion in *Anthurium* even during the staminate phase (Dauman, 1930; Bleiweiss et al., 2019).

It is important to emphasize that the nectar secretion observed in cultivated *A. andraeanum* is occasional and unpredictable. The same individual showed conspicuous nectar release at a specific flowering episode and then remained for several flowering events with no nectar secretion.

CONCLUSIONS

Our main result is that the *Anthurium* flowers produce two distinctive types of secretions with different chemical compositions and ecological functions. Still, due to the small flower size, these secretions can mix, generating confusion. Our findings regarding stigmatic secretion composition call attention to how important it is to accurately study the chemical nature of this exudate in other species of *Anthurium*, avoiding any kind of contamination. Considering that sugars were not detected in the stigmatic secretion, the term "stigmatic nectar" must be avoided. In addition to the possible contamination by other sources of sugars, the simple presence of traces of some sugar does not necessarily characterize such secretion as nectar.

Moreover, the production of secretion in specific secretory structures of the stigma (i.e., the stigmatic papillae) reinforces their distinct nature and function. Our results also highlight that the distal portion of the tepals has great secretory potential in Araceae, and in *A. andraeanum* this region comprises a floral nectary. True nectar secretion is demonstrated for the first time in Araceae through structural and chemical analyses. Once the controversial issue involving sugar secretion in *Anthurium* has been elucidated, and the nectary location and structure described, broader studies including related genera should be encouraged in order to better understand the evolution of floral rewards in the intricate plant-pollinator relationships in Araceae.

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AUTHOR CONTRIBUTIONS

E.A.S.P. conceived and designed the research. E.A.S.P. and I.B-C. did the structural and ultrastructural analyses. I.B-C did the chemical analyses. All authors collaborated to analyze the data, write and revise the text.

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Figure captions

FIGURE 1. Inflorescences of *Anthurium andraeanum* showing different functional stages and nectar release. **A.** Immature spadix, just after the spathe unfolding. **B-D.** Beginning of pistillate phase, showing secreting stigmas (arrows in D). Note that in B the spadix apex (yellow part) remained in pre-anthesis. **E.** Advanced pistillate phase during nectar secretion, notice the accumulation of nectar remnants observable at the base of the spathe (arrows) due to nectar dripping off from the spadix. **F.** Staminate phase, nectaries were no more active, but nectar residues were still present (arrows).

FIGURE 2. Spadix surface of *Anthurium andraeanum* showing different floral functional stages. **A-B.** Immature spadix, just after spathe unfolding. Notice that in B stomata are still closed or showing first evidence of guard cell expansion and pore opening. **C.** Flowers at the beginning of the pistillate phase, showing stigma with remnants of stigmatic secretion; remarkable stomata with large pores in the insert. **D.** Flowers during the staminate phase, with exposed anthers; the insert shows a stoma with large pore.

FIGURE 3. Flowers at the pistillate phase showing the overall structure and nectary anatomy. **A.** Transverse section of a flower (dotted area) showing its overall structure. The circles indicate tepal vascular bundles. **B-C.** Transverse section of the tepals

showing the region below the nectary (B), and the nectary tissue (C). Note the presence of a vascular bundle in B and its absence in C. **D.** Longitudinal section of a tepal showing the distinction of the nectary region (above the dotted line) and the ordinary mesophyll. Note the vascular bundle ending at the limits of the nectary tissue. The insert shows a modified stoma from the nectary region. **E-F.** Comparative distribution of crystals in the tepals at the level of the nectary tissue (E) and in the subjacent mesophyll (F). In E, the circles in the lower right corner indicate CaOx crystals in one of the tepals. (an, anther; ph, phloem; st, stigma; te, tepal; vb, vascular bundle; xy, xylem).

FIGURE 4. Starch content in the tepals of *Anthurium andraeanum* flowers at distinct phases. Starch grains stained in black, after reaction with Lugol's solution. **A-B.** A general overview of the tepal apex in an immature flower (A) and detail of its respective nectary portion (B). **C-D.** A general overview of the tepal apex in a flower at pistillate phase (C) and detail of its respective nectary region (D). **E-F.** A general overview of the tepal apex in a flower at staminate phase (E) and detail of its respective nectary region (F).

FIGURE 5. Overall structure of the stigma. **A-B.** Stigma at the pistillate phase showing the apical portion covered with stigmatic papillae (detail in B). Note the presence of mucilage filling the stylar channel in A, and the release of secretion as fine strands by the stigmatic papillae in B (arrows). **C.** Stigma at the staminate phase. Note the shrinkage of the stigma and stylar channel.

FIGURE 6. Ultrastructure of nectar-secreting cells during the pre-secretory (A-C) and the secretory phases (D-G). **A.** Epidermal cell with dense protoplast and conspicuous

nucleus. Note the thin and well-adhered cuticle. **B.** Detail of parenchyma cells on whose walls plasmodesmata can be seen (arrows); plastids and mitochondria appear to be the most representative organelles. **C.** Plastids with large starch grains. **D.** Parenchyma cell showing plastids and mitochondria. **E.** Detail of plastid with a multivesicular body from the endomembrane system. Arrows indicate the outer plastidial membrane. **F.** Final stages of plastid swelling and fusion; notice that the dotted line delimits a new area outlined only by the outer plastid membrane. **G.** Portion of cytoplasm with an intact plastid and a vacuole showing content structurally similar to the swollen plastids showed in F. (cu, cuticle; cw, cell wall; mb, multivesicular body; mi, mitochondria; nu, nucleus; pl, plastid; ps, phenolic substances, st, starch; va, vacuole).

FIGURE 7. Ultrastructural aspects of the stigmatic papillae at the pistillate phase. **A-B.** The overall composition of the cytoplasm showing organelle-rich matrix, numerous small vacuoles, and oil droplets. **C-D.** Cells showing a gradual compression of the protoplast and accumulation of mucilage in the periplasmic space. In D, median portion of the stigma, in which the papillae are juxtaposed; observe in the center a cell with dark protoplast and mucilage in the periplasmic space. **E.** Section of stigmatic papillae showing abundant mucilage exudation through the cell wall in the form of fine strands (also seen at the insert in C). (cw, cell wall; di, dictyosome; mi, mitochondria; mu, mucilage; od, oil droplet; pe, periplasmic space; pl, plastid; st, starch; va, vacuole).