

NATALIE DO VALLE CAPELLI

ULTRASTRUCTURE AND CHEMICAL ANALYSIS OF OSMOPHORES IN
APOCYNACEAE

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ULTRAESTRUTURA E ANÁLISE QUÍMICA DE OSMÓFOROS EM
APOCYNACEAE

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A Deus, por ter sustentado o meu ser durante toda a trajetória.

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Abstract

Apocynaceae presents the flowers with the highest degree of synorganization among the eudicots and highly elaborated pollination mechanisms associated with the high diversity of glands. The osmophore stands out as responsible to produce a floral scent which attracts pollinators and, despite its fundamental relevance for pollination, its structure and mechanism of production and release of the perfume is essentially unknown in Apocynaceae. This present work aims to characterize morphologically and ultrastructurally the osmophores of Apocynaceae, besides chemically identifying the compounds that constitute the floral scent. Species from two subfamilies were selected to describe the diversity of osmophores and types of scent in the family. The osmophores were firstly located histochemically and, later, this region was processed for transmission electron microscopy. Micromorphological analysis was performed by scanning electron microscopy, and the identification of volatile oils made by GC-MS. Osmophores are located on the adaxial surface of the free portion of the petals. They varied in the shape of epidermal cells, striation of the cuticle and presence of trichomes. This gland is mostly formed by secretory epidermis and parenchyma, except in *Plumeria*, where the osmophores are exclusively epidermal. The secretory cells presented thick walls in the Asclepiadoideae and secretion produced by plastids and SER in all species. Several vesicles perform the intercellular transport of secretion, as well as its release to the environment. However, *Plumeria* and *Ditassa* transfer the secretion produced to the vacuole before releasing it, and *Tabernaemontana* has a mixed release process. The composition of the scent varied among species, with great production of hydrocarbons, alcohols, ketones or monoterpenes depending on the species. Apocynaceae presents a high morphological and metabolic diversity in the osmophores which cannot be correlated with their morphology, subcellular organization, period of secretion release or pollination syndrome. This is the first comprehensive structural study of osmophores in this family that points out to very distinct evolutionary processes that may be related to multiple emergences in the phylogeny and species-specific associations with pollinators.

Keywords: Apocynaceae, evolution, flower, odour bouquet, osmophore, secretory process, ultrastructure.

Resumo

Apocynaceae apresenta as flores com o maior grau de sinorganização das eudicotiledôneas e mecanismos de polinização altamente elaborados associados à mais alta diversidade de glândulas. O osmóforo destaca-se como responsável pela produção do perfume floral para atração dos polinizadores e, a despeito de sua importância fundamental para a polinização, a sua estrutura e mecanismo de produção e liberação do perfume é praticamente desconhecido nas Apocynaceae. O presente trabalho tem o propósito de caracterizar morfológica e ultraestruturalmente os osmóforos de Apocynaceae, além de identificar quimicamente os compostos que constituem o perfume floral. Espécies de duas subfamílias foram selecionadas, visando descrever a diversidade de osmóforos e tipos de perfume na família. Primeiramente, os osmóforos foram localizados histoquimicamente e, posteriormente, essa região foi processada para microscopia eletrônica de transmissão. Análise micromorfológica foi realizada através de microscopia eletrônica de transmissão. Análise micromorfológica foi realizada através de microscopia eletrônica de varredura e a identificação dos óleos voláteis, através de GC/MS. Os osmóforos estão localizados na superfície adaxial da porção livre das pétalas. Eles variaram quanto ao formato das células epidérmicas, ornamentação da cutícula e presença de tricomas. Esta glândula é, em sua maioria, formada por epiderme e parênquima secretores, exceto em *Plumeria*, onde os osmóforos são exclusivamente epidérmicos. As células secretoras apresentaram paredes espessas nas Asclepiadoideae e secreção produzida pelos plastídeos e REL em todas as espécies. Diversas vesículas realizam o transporte intercelular da secreção, assim como a sua liberação para o meio externo. Contudo, *Plumeria* e *Ditassa* transferem a secreção produzida para o vacúolo antes de liberá-la e *Tabernaemontana* possui processo de liberação misto. A composição do perfume variou entre as espécies, havendo grande produção de hidrocarbonetos, alcoóis, cetonas ou monoterpenos dependendo da espécie. Apocynaceae apresenta uma alta diversidade morfológica e metabólica em seus osmóforos que não pode ser correlacionada à morfologia, organização subcelular, período de liberação da secreção ou síndrome de polinização. Esse é o primeiro estudo estrutural abrangente sobre osmóforos na família e aponta para processos evolutivos muito distintos que podem estar relacionados a múltiplos surgimentos na filogenia e associações espécie-específicas com os polinizadores.

Palavras-chave: Apocynaceae, evolução, flor, odor, osmóforo, processo secretor, ultraestrutura.

Background

Pollination may be such a specialized event that, in some cases, implies not only in the need for a specific pollinator but also for a series of secretory structures which allows the flower to be pollinated (Meve and Liede 1994; Borba and Semir 1998; Demarco 2014, 2017a; Monteiro and Demarco 2017).

In plants that have several secretory structures, mutualistic relationships seem to be more common (Fahn 1979). When the floral glands are analyzed, many of them produce substances that act as attractive to pollinators or produce reward substances for them (Nishida 2014), aid in the dispersal of seeds and act as a protection against herbivores and pathogens (Mithöfer and Boland 2012; Vitarelli *et al.* 2015). The participation of these substances in interactions between plants and animals is undeniable, acting as mediators of a range of ecological responses, and may act as attractive or repellent (Dusenbery 1922; Harborne 1994).

Among the main floral traits the color and the odor are usually the main responsible for pollinator attraction but the smell of the insects is apparently much more accurate than the vision (Kevan and Baker 1983). The presence of scent is related to the production of volatile chemical substances by secretory structures located inside or on the surface of vegetative and/or reproductive organs (Svendsen and Scheffer 1985).

In pollination, the volatile floral substances can play a role as a signal for visitors or even act as the sole available reward of the flower. In addition, some flowers can mimic pheromones of female bees and wasps, attracting the respective males that establish a pseudocopula with flowers, performing a mistake pollination (Silva 1992).

The release of scent by flowers can be highly complex, exhibiting dynamic emission patterns and chemical composition of the volatile compounds. The petals are the main producers of odors and the gland responsible for their production is called osmophore (Vogel

1990). This gland has a restricted occurrence and are often located at the distal portion of the perianth and usually have secretory epidermis (Vogel 1990).

Plants with floral scent may present different pollination syndromes within the same group or may be related to a major type of syndrome. Studies that address these relationships are very informative providing excellent opportunities to determine if pollinator changes are correlated with parallel variations in floral bouquet chemistry (Dobson 2006), since similarities between visual attractant plus the chemical composition of the floral scent, independently of the phylogenetic relationship between the species compared, may suggest that they have the same group of pollinators (Knudsen *et al.* 1993). These similarities have been found, for example, in plants pollinated by butterflies (Knudsen and Tollsten 1995; Raguso and Pichersky 1995; Jürgens *et al.* 2002, 2003), bats (Knudsen and Tollsten 1995; Bestmann *et al.* 1997), beetles (Thien *et al.* 1975; Yasukawa *et al.* 1992; Jürgens *et al.* 2000) and female euglossine bees (Williams and Whitten 1983; Sazima *et al.* 1993).

Increase in floral temperature up to 22 °C higher than the environmental temperature may also be related to osmophore activity, forming an intrafloral microclimate that is attractive to insects both as a shelter and as a mating site (Meeuse 1975). In addition, flowers that produce scent as a resource for the pollinator have their oils collected and transferred to a dilatation of the tibia of posterior leg in Euglossini tribe (Apidae), whose internal structure is glandular. There are speculations that the collected oils are used since as a territorial marker, also as defensive kairomones, or even transferred to the respective females at the moment of the copula (Williams 1983, Williams & Whitten 1983; Roubik 1989).

The diversity of volatile compounds emitted by flowers can be grouped as derivatives of fatty acids, terpenoids and benzenoids, as well as compounds containing nitrogen and sulfur. Some compounds are present in almost all floral scents, others are found only at certain species (Dobson 2006). Despite the greater or lesser resemblance between the bouquet

of each flower, there must be a synchronization between the moment of insect activity and the emission of the fragrance, as well as other parameters of the floral development. Phenomena such as the anthesis occurring at a certain period of the day, changes in the presentation of nectar and pollen, thermogenesis, and changes in position of moving organs are essential to ensure pollination in several groups (Bünning 1967; Hess 1983; Proctor *et al.* 1996).

Floral scents are particularly important in flowers opening at night, where olfactory signals attract pollinators over long distances in deceit pollination systems where odors mimic sex hormones, the scent of flowers that supply nectar, or even the smell of decaying organic matter in sapromiophilic systems (Proctor *et al.* 1996).

All these factors demonstrate the great diversity of osmophore activity that can be reflected in a great morphological diversity, since it is possible to observe flowers emitting distinct scents at different times of the day related to various syndromes of pollination within a single group of plants. Therefore, this dissertation presents a study of the Apocynaceae with aromatic flowers, aiming to analyze, for the first time, the diversity of scent glands in this family. This present work is structured according to the format in which it will be submitted to the Annals of Botany.

ORIGINAL ARTICLE

Scent glands and the nature of the floral perfume in Apocynaceae*Natalie do Valle Capelli^{1*}, Diego Demarco²**^{1, 2} Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo,**São Paulo, 05508-090, Brazil**For correspondence. E-mail na.capelli@usp.br*

Introduction

Apocynaceae is one of the largest families of angiosperms with 366 genera (Endress *et al.* 2014) and about 5000 species (Endress 2004; Endress *et al.* 2007), which have flowers with an extreme degree of sinorganization that highlight them as the most morphologically complex flowers among eudicots (Kunze 1991; Endress 2016). This morphological complexity is directly related to highly elaborated pollination mechanisms and to the occurrence of the largest diversity of glands in the same flower (Demarco 2017a). Thirteen types of floral glands were described for this group: colleter, glandular trichome, secretory idioblast, laticifer, style head, nectary (primary and secondary), osmophore, tapetum, staminal wing gland, stylar canal, extragynoecial compitum and obturator (Woodson Jr. and Moore 1938; Fallen 1986; Vogel 1990; Galetto 1997; Torres and Galetto 1998; Lin and Bernardello 1999; Gagliardi *et al.* 2016; Demarco 2017; Monteiro and Demarco 2017). Some of these glands have recognized taxonomic and phylogenetic importance (Woodson Jr. and Moore 1938; Simões *et al.* 2007) (Demarco 2017a), besides representing the floral defensive system of flower and allow to understand the mechanisms or evolutionary strategies related to the different mechanisms of pollination.

Although the perfume production is well known in flowers of Apocynaceae flowers (Stevens 1988; Vogel, 1990; Silva 1992; Marcondes-Ferreira & Kinoshita 1996; Rohrbeck *et al.* 2006; Setzer 2013; Shuttleworth 2016; Burger *et al.* 2017; Heiduk *et al.* 2017) and the first description of the structure of a scent gland or osmophore has been made in *Ceropegia elegans* Wall. (Vogel 1963), only almost fifty years later, another osmophore study was published for the family using two species: *Orbea variegata* Haw. and *Boucerosia indica* (Wight. & Arn) Plowes (Ceropegieae, Asclepiadoideae) (Płachno *et al.*, 2010). Other studies only reported the existence of osmophores, without describing them (Endress 1994; Torres and Galetto 1998).

Some Apocynaceae flowers produce a sweet odor, while others produce a fetid odor (Stevens 1988; Vogel 1990; Silva 1992; Marcondes-Ferreira & Kinoshita 1996; Płachno *et al.* 2010; Setzer 2013). Among the main chemical compounds identified in species of Asclepiadeae (Asclepiadoideae) are the sesquiterpenoids, derived from fatty acids, monoterpenoids, benzenoids and nitrogen compounds (Jürgens *et al.* 2006, 2009; Vitarelli and Santos 2009; Burger *et al.* 2017; Heiduk *et al.* 2017). The different types of scent are usually associated with a characteristic type of coloration, such as the flowers of the sapromiophilic Ceropegieae that may have a dark brown, red or yellow color. In addition, volatile compounds produce a stool-like smell (Meve and Liede 1994).

Flowers with very intense scent are frequently visited by insects and all of their pollen is removed in a short period of time but for this, it is necessary that there is an adequate relation between the size of the pollinator and the pollen, which allows the pollination to occur successfully, since removing the pollen requires some strength (Meve and Liede 1994). Apocynaceae pollinators are not usually uniform in size. In the tribe Asclepiadeae, flowers are predominantly visited by Hymenoptera (some bees and wasps) and butterflies (Jürgens *et al.* 2009). In Ceropegieae, in contrast, pollination is characteristically carried out by flies (Vogel 1963) and the female ones usually fly searching for a substrate to lay their eggs (Meve and Liede 1994).

The present work aimed to characterize the Apocynaceae osmophores using species with flowers having varied floral morphologies and offering distinct rewards to the pollinators. We analyzed the morphology, ultrastructure and secretory activity of this gland and the chemical composition of the floral scent, helping to understand the diversity of osmophore types in the family and the pollinator attractive strategies.

Material and methods

The species were selected based on the subfamily that they belong to, aiming to obtain a greater morphological and functional diversity. The type of aroma was also used for this selection. The species studied are:

Aspidosperma australe Müll. Arg (Rauvolfioideae) occurring at Campinas (SP), which produces a sweet aroma;

Plumeria rubra L. (Rauvolfioideae) from São Paulo (SP), which produces a sweet aroma;

Tabernaemontana catharinensis A.DC. (Rauvolfioideae) located in Campinas (SP), which produces a sweet aroma;

Ditassa gracilis Hand.-Mazz. (Asclepiadoideae) from Santana do Riacho (MG), which produces a sweet aroma;

*Hoya carnos*a (L. f.) R. Br. (Asclepiadoideae) occurring at São Paulo (SP), which produces a sweet aroma;

Stapelia hirsuta L. (Asclepiadoideae) from São Paulo (SP), which produces a fetid aroma.

All flowers analyzed in the present study were collected at 10 o'clock.

Location of osmophores

To identify the position and distribution of the osmophores, flowers in anthesis were submerged in neutral red for 30 minutes and then washed in distilled water for observation of the stained regions, indicating the possible location of the osmophore (Vogel 1990).

Micromorphology of osmophores

For the micromorphological study, anthetic flowers were fixed in FAA (formalin, acetic acid and ethyl alcohol) for 24 h (Johansen 1940). Osmophores were isolated, dehydrated in an ethyl series, dried by the critical point method, mounted on aluminum stubs and covered with gold, with subsequent observation in a Sigma VP Carl Zeiss scanning electron microscope.

Anatomy of osmophores

For the anatomical analysis, flowers in three developmental stages were used: floral bud (about half of the final length), pre-anthetic and anthetic flowers. The material was fixed in Karnovsky's solution, dehydrated in an ethyl series, embedded in methacrylate (Gerrits 1991), and sectioned transversely and longitudinally with 12 µm thick in a Microm HM340E rotary microtome (Microm International, Walldorf, Germany). The sections were stained with toluidine blue (O'Brien *et al.* 1964) and the photomicrographs were obtained in a Leica DMLB light microscope (Leica Microsystems Inc., Heidelberg, Germany).

Ultrastructure and secretory activity

For the ultrastructural study, flowers in the three developmental stages previously fixed in Karnovsky's solution were postfixed in 1% osmium tetroxide, dehydrated in a graded ketone series and included in Spurr resin. The sectioning was performed in a Leica Ultracut UCT (Leica Microsystems Inc., Heidelberg, Germany), and the ultrathin sections were stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963) with subsequent observation in a Zeiss EM900 transmission electron microscope.

Composition of the secretion

As the neutral red test is not specific for volatile oils, the dye-retaining region was isolated, sectioned on a freezing microtome and subjected to tests to verify the presence or absence of major chemical classes in the composition of the secretion (Table 1).

Table 1. Histochemical tests used to detect compounds present in the secretion of osmophores.

<i>HISTOCHEMICAL TEST</i>	<i>COMPOUND</i>	<i>REFERENCE</i>
Sudan Black B	Lipids	Pearse, 1985
Sudan IV	Lipids	Pearse, 1985
Neutral red	Lipids	Kirk, 1970
Nile blue	Acidic and neutral lipids	Cain, 1947
Nadi reagent	Essential oils and resins	David and Carde, 1964
Copper acetate and rubeanic acid	Fatty acids	Ganter and Jollés, 1969, 1970
Ruthenium red	Acidic mucilage	Johansen, 1940
Alcian blue	Acidic mucilage	Demarco, 2017b
PAS reaction	Carbohydrates	McManus, 1948
Aniline blue black	Proteins	Fisher, 1968
Wagner's reagent	Alkaloids	Furr and Mahlberg, 1981
Dragendorff's reagent	Alkaloids	Svendsen and Verpoorte, 1983
Ferric chloride	Phenolic compounds	Johansen, 1940
Lugol's reagent	Starch	Johansen, 1940

Control of hydrophilic and lipophilic substances was performed according to Demarco (2017). All photomicrographs were carried out using an Olympus BX51 light microscope (Melville, USA).

Analysis of the chemical composition of volatilizable samples

The chemical constituents present in the volatile oil samples were analyzed using a gas chromatograph (Shimadzu Model QP 2020) equipped with the headspace module (HSS, Model AOC-5000 Plus from Shimadzu Co.) and the RTX- 1MS (fused silica in dimethylpolysiloxane with a length of 30m, internal diameter of 0.25mm, film thickness of 0.25µm and helium entrainment gas, with flow of 2 mL.min⁻¹), automatic injector (split mode) 1: 3) and an electronic integrator, coupled to the mass spectrometer operating by electronic impact (70 eV).

The chromatographic conditions of the analysis were programmed in: the sample was submitted to the HSS oven, whose temperature was 60 °C (vial temperature), maintained for 30 minutes, followed by the injection of 2.5mL of the air sample trapped in the vial. The analysis time was 35 minutes per sample and the oven temperature of the column was 40-250 °C, with an initial temperature of 40 °C held for 1min. (0-1min \therefore 40 °C) and, subsequently, heating rate of 5 °C.min⁻¹. up to 150 °C (1-23min \therefore 40 °C \rightarrow 150 °C), after which the temperature was maintained at 150 °C for a further 2 min. (23-25 min \therefore 150 °C), followed by a new heating rate of 20 °C.min⁻¹ to 250 °C (25-30 min \therefore 150 °C \rightarrow 250 °C) which was maintained for 5min. (30-35min \therefore 250 °C) until finalization of the analysis. The temperatures of the injection syringe and the injector were 80 °C and 200 °C, respectively.

In the mass spectrometer the temperatures of the ion source and interface were 250 °C and the range of masses for acquisition of the spectra were from m/z 33.0 to m/z 350.0. The component concentrations were calculated based on the peak areas in the chromatograms. The identification of the substances was confirmed by the Kovats retention index (IK) of the constituents obtained by co-injecting the sample with a homologous series of n-alkanes (C₉H₂₀ - C₂₀H₄₂, Sigma-Aldrich, 99%) (Adams, 2001) and posterior comparison of mass spectra with the CG-EM system database (Mclafferty and Stauffer, 1989).

Results

In all the studied species, the scent begins to be released in the pre-anthesis, with an enhanced release in the anthesis phase. The cells that compose the osmophore have a basipetal differentiation and are always found in different secretory phases along the gland.

Location and structural organization

All the analyzed flowers are pentamerous, gamosepalous, gamopetalous and also have epipetalous stamens and two carpels. The flowers of *Aspidosperma*, *Plumeria* and

Tabernaemontana (Rauvolfioideae) have a long floral tube and short corolla lobes, unlike *Ditassa*, *Hoya* and *Stapelia* (Asclepiadoideae) whose floral tube is very reduced and the corolla lacinia are very long. Although the extent of fusion between petals is variable, the osmophores were always located on the adaxial surface along the entire free portion of the petals, reaching almost to their margins. There is a great morphological diversity in the osmophores of the species analyzed, being composed of epidermis or epidermis and parenchyma, and varied shapes of epidermal cells, where some of them may not be secretory.

1. Rauvolfioideae

1.1 *Aspidosperma australe*

Aspidosperma flowers have a greenish-colored corolla (Fig. 1A), and exude a sweet odor. The osmophore, in this species, is constituted by the adaxial epidermis of the corolla lobes, which is specifically located in the free portion of the petals. The secretory surface extends almost to the involute margins of each lobe (Fig. 1B). The epidermal osmophore cells are all similar in shape, composed of rounded apex papillae (Fig. 1C, D) which have a very striated cuticle (Fig. 1D). Three to four layers of subepidermal parenchyma are secretory and also take part in the osmophore (Fig. 1E). The cells of the secretory parenchyma are isodiametric and resemble the non-secreting parenchyma cells in size. In the osmophore surface there are also trichomes (Fig. 1B, C), however they are not secretory.

Ultrastructure

Cell wall

Epidermal secretory cells have projections in their outer periclinal wall, which is thicker than the wall of parenchyma cells (Fig. 2A). Several plasmodesmata (Fig. 2B) were observed in the walls of the secretory cells, connecting their cytoplasms. The epidermis is

covered by a striated cuticle that accompanies the cell wall projections, and this cuticle has pectin projections traversing it almost completely (Fig. 2C).

Cytoplasm

The cytoplasm of both the epidermal cells and the secretory parenchyma are enriched in ribosomes and has many elaioplasts in the epidermis (Fig. 2D) or chloroplasts in the parenchyma (Fig. 2E), besides smooth endoplasmic reticulum (SER) (Fig. 2F). Dictiosomes and mitochondria were more rarely visualized. Several secretory vesicles are observed in the cytoplasm (Fig. 2G-H), carrying the secretion through the cytoplasm. The secretion of the epidermis and secretory parenchyma is produced in the elaioplasts and SER.

Between the cells of the secretory parenchyma and between the parenchyma and the epidermis, the secretion is transferred from one cell to another either by plasmodesmata (Fig. 2B) or through vesicles that merge to the plasma membrane (exocytosis) (Fig. 2G-H), being encompassed by the adjacent cell in reverse mechanism (endocytosis). There is no accumulation of secretion inside the central vacuole (Fig. 2A).

Secretion release

In epidermal cells, vesicles with secretion fuse to the plasma membrane in the distal portion of the cell, transferring the secretion to a small periplasmic space (Fig. 2H). This secretion will then be forced through the wall and the cuticle by the pressure exerted by the active movement of the protoplast due to the turgor pressure. The volatilization of the secretory components assists in their release to the environment. This release occurs without rupture of the cell wall or the cuticle.

Composition of the secretion

The histochemical analysis identified only lipids in the secretion of the osmophore, including terpenes (Table 2-3, Fig. 4).

Table 2. Results of the histochemical tests used to detect the compounds present in the secretion of osmophores in the species studied.

<i>Histochemical test</i>	<i>Compound class</i>	<i>Aa</i>	<i>Pr</i>	<i>Tc</i>	<i>Hc</i>	<i>Sh</i>
Neutral red	Lipids	+ (3D)	+	+	+ (3A)	+
Sudan Black B	Lipids	+	+	+ (3B)	+	+
Sudan IV	Lipids	+	+	+	+	+ (3C)
Nile blue	Acidic lipids	+ (3E-F)	+	+	+	+
NADI reagent	Essential oils	+	+ (3G)	+	+	+
Copper acetate and rubeanic acid	Fatty acids	-	-	-	-	-
Ruthenium red	Acidic mucilage	-	-	-	-	-
Alcian blue	Acidic mucilage	-	-	-	-	-
PAS reaction	Carbohydrates	-	-	-	-	-
Aniline blue black	Proteins	-	-	-	-	+ (3H)
Wagner's reagent	Alkaloids	-	-	-	-	-
Dragendorff's reagent	Alkaloids	-	-	-	-	-
Ferric chloride	Phenolic compounds	-	-	-	-	-
Lugol's reagent	Starch	-	-	-	-	-

+ = positive; - = negative; () = figure; Aa = *Aspidosperma australe*; Pr = *Plumeria rubra*; Tc = *Tabernaemontana catharinensis*; Hc = *Hoya carnosa*; Sh = *Stapelia hirsuta*.

Flower scent

Fig 4. Chromatogram amplification of *Aspidosperma* sample - time interval of 2.0 - 30.0 min.

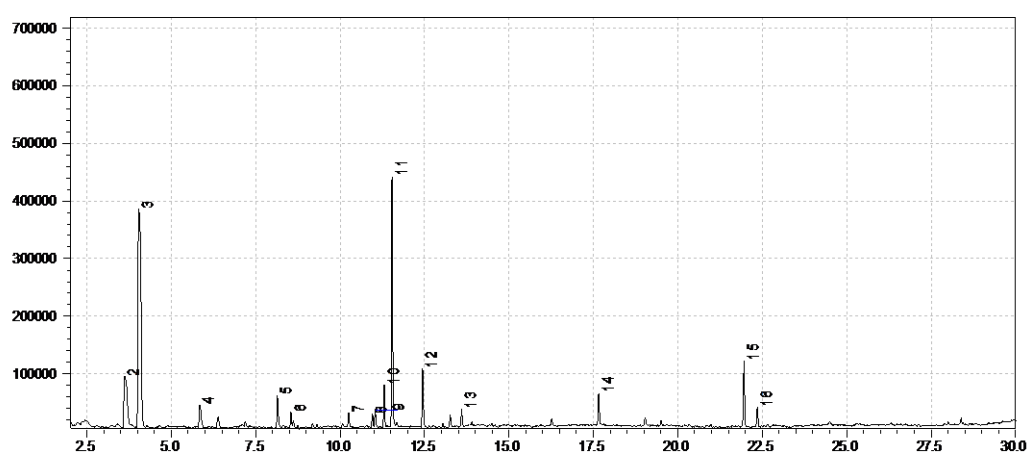


Table 3. Chemical composition and relative percentage of the volatile fraction obtained by headspace from *Aspidosperma* sample.

	KRI	Number in the chromatogram	Relative %
<i>Hydrocarbons</i>			
4-Methyl-pent-2-(Z)-ene	726	1	7,26
2,4-Dimethyl-heptane	853	3	39,81
<i>Aldehydes, Ketones and short chain Furan Derivatives</i>			
Hexanal	807	2	9,71
Heptanal	899	4	2,70
6-Methyl-hept-5-en-2-ona	985	5	2,68
2-Penyl-furan	1038	6	1,00
Nonanal	1102	11	17,20
<i>Monoterpenoids</i>			
(E)- β -Ocimene	1050	7	0,89
exo-Isocitral	1073	8	0,79
Camphor	1143	12	4,20
Menthol	1173	13	1,22
<i>Sesquiterpenoids</i>			
β -Farnesene	1098	10	2,73
Bicyclogermacrene	1494	16	1,41
<i>Aromatic Derivatives</i>			
Methyl benzoate	1091	9	1,12
<i>Nitrogen-containing compounds</i>			
Methyl anthranilate	1337	14	2,20
<i>Unknowns</i>			
m/z 204, 161, 85, 71, 57*, 43	1415	15	5,08
<i>Hydrocarbons</i>			47,07
<i>Aldehydes, Ketones and short chain Furan Derivatives</i>			33,29
<i>Monoterpenoids</i>			7,10
<i>Sesquiterpenoids</i>			4,14
<i>Aromatic Derivatives</i>			1,12
<i>Nitrogen-containing compounds</i>			2,20
<i>Unknowns</i>			5,08

* The mass value marked with asterisk refers to the base peak of the mass spectrum that has a relative abundance of 100%; KRI: Kovats retention Index.

1.2 *Plumeria rubra*

Plumeria flowers have pink-colored corolla, being yellow in the center-basal portion (Fig. 5A), exuding a mild, sweet odor. The osmophore of this species is constituted by a

papillose epidermis with cells of acute apex (Fig. 5B). The secretory surface covers almost the entire adaxial face of the corolla lobes and presents a smooth cuticle (Fig. 5C). The subepidermal parenchyma does not present secretory activity (Fig. 5D).

Ultrastructure

Cell wall

The outer periclinal wall of the papilla has no projections and is covered by a thin, smooth cuticle (Fig. 6A). The epidermis have plasmodesmata in the anticlinal walls (Fig. 6B).

Cytoplasm

The cytoplasm of the epidermal secretory cells is dense (Fig. 6A) due to a large number of ribosomes and polyribosomes, and many elaioplasts (Fig 6A, C), mitochondria (Fig. 6D) and SER are observed. Secretory vesicles formed by plastids and SER are observed in the cytosol (Fig. 6E-F), in addition to large starch grains within the elaioplasts (Fig. 6A, C).

The secretion is produced mainly by SER and elaioplasts, packaged by vesicles produced by the same organelles (Fig. 6 B-C, E) that transfer the metabolites to the central vacuole, where it will be stored (Fig. 6A). Secretion droplets free in the cytosol can reach the plasmodesmata and cross to the adjacent cell. Stromules were observed connecting the elaioplasts (Fig. 6F), and connections between mitochondria were also frequent.

Secretion release

After being temporarily stored in the vacuole, vesicles formed by protrusions of the vacuole transport the secretion to the plasma membrane (Fig. 6A), where they merge transferring their contents into a narrow periplasmic space. This secretion crosses the wall and the cuticle through the pressure exerted by the active movement of the protoplast and by the volatilization of its components.

Composition of the secretion

Only lipids are present in the osmophore secretion, including terpenes (see Table 2, 4; Fig. 7).

Flower scent

Fig 7. Chromatogram amplification of *Plumeria* sample- time interval of 2.0 - 30.0 min.

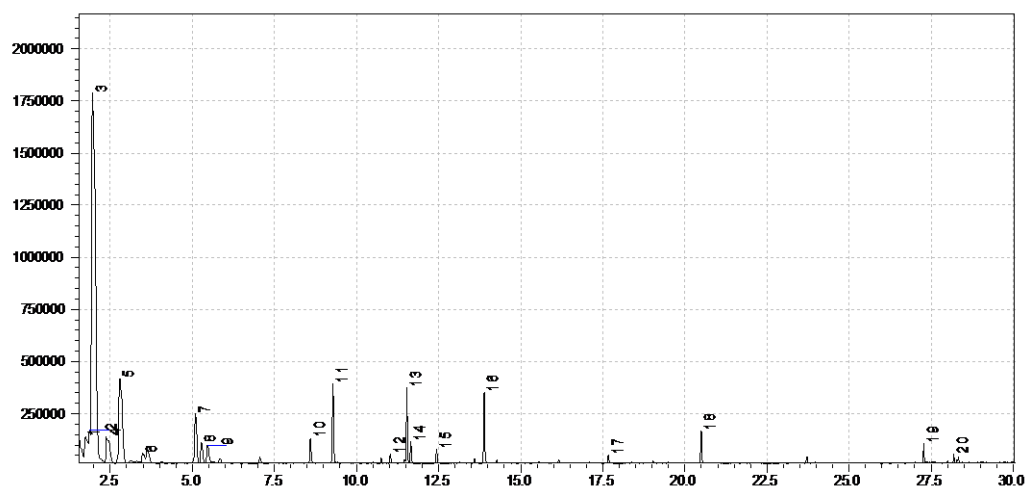


Table 4. Chemical composition and relative percentage of the volatile fraction obtained by headspace from *Plumeria* sample.

	KRI	Number in the chromatogram	Relative %
<i>Alcohols, aldehydes and short chain ketones</i>			
2-Methyl-propanol	597	1	2,23
3-Methyl-butanal	643	2	1,74
2-Methyl-butanal	650	3	57,73
2-Methyl-butanol	705	5	10,51
Octanal	1001	10	1,22
Nonanal	1102	13	3,29
<i>Monoterpenoids</i>			
Camphor	1143	15	0,64
<i>Nitrogen-containing compounds</i>			
2-Methyl-butanonitrila	679	4	3,70
Butanal-O-metiloxima	798	6	0,62
(Z)-2-Methyl-butylalldoxima	815	7	4,08
(E)-2-Methyl-butylalldoxima	830	8	1,57
Benzilnitrila	1105	14	0,95
O-3-methylbutyl-hidroxilamine	873	9	1,48
<i>Aromatic Derivatives</i>			
Phenylacetaldehyde	1043	11	3,96
Methyl benzoate	1091	12	0,39

Methyl salicylate	1205	16	3,23
Isobutyl benzoate	1350	17	0,32
Isoamyl benzoate	1409	18	1,51
Benzyl benzoate	1762	19	0,58
2-Phenylethyl benzoate	2113	20	0,24
<i>Alcohols, aldehydes and short chain ketones</i>			76,72
<i>Monoterpenoids</i>			0,64
<i>Nitrogen-containing compounds</i>			12,40
<i>Aromatic Derivatives</i>			10,23

* The mass value marked with asterisk refers to the base peak of the mass spectrum that has a relative abundance of 100%; KRI: Kovats retention Index.

1.3 *Tabernaemontana catharinensis*

Tabernaemontana flowers have white corolla (Fig. 8A) and exude a mild and sweet odor. The osmophore is located in the adaxial side of the corolla lobes and the whole surface that extends almost until their margin is formed by secretory cells (Fig. 8B). These epidermal cells are papillose with rounded apex which are arranged in several pairs or even quartets of juxtaposed cells (Fig. 8C). The osmophore also consists of three to four layers of transversely elongated secretory parenchyma cells (Fig. 8D).

Ultrastructure

Cell wall

The walls of the secretory epidermis and parenchyma are thin, and the epidermis is covered by a smooth cuticle (Fig. 9A). There are no pectin projections traversing the cuticle and no plasmodesmata have been observed between the secretory cells.

Cytoplasm

In the cytoplasm there is a high density of ribosomes dispersed in the cytosol, and the predominant organelles are the elaioplasts, mitochondria and SER (Fig. 9B), which is often in periplastidial position. Dictiosomes (Fig. 9C) and rough endoplasmic reticulum (RER) were rarely visualized. Many secretory vesicles (Fig. 9D) are dispersed in the cytoplasm, in

addition to the central vacuole, indicating the high secretory activity of the cell. Associations between SER, mitochondria and elaioplasts (Fig. 9B) with large starch grains (Fig. 9A) were frequent. In secretory cells of the epidermis and also in the secretory parenchyma, secretion is mainly produced by SER (Fig 9D) and elaioplasts, being observed as secretory droplets in the cytosol or as plastoglobules (Fig. 9B), respectively.

Secretion is transported between the epidermal and parenchyma cells through vesicles (exocytosis and endocytosis), and the dictiosomes (Fig. 9C) are the main responsible for the production and targeting of these vesicles, although their production from SER (Fig. 9D) has also been observed. Likewise the vesicles are responsible for the intercellular transport of the secretion, part of the vesicles is directed to the central vacuole, merging with the tonoplast and transferring the secretion into its interior (Fig. 9A).

Secretion release

Two releasing routes of granulocrine secretion were observed in the epidermal cells. The predominant mode of secretion release was through vesicles directed straight from the dictiosomes or SER to the plasma membrane at the distal portion of the cell. However, transport of the secretion stored inside the vacuole (Fig. 9A) from vesicles originated from vacuolar protrusions (Fig. 9E) was also observed. In both cases, the vesicles fuse to the plasma membrane, transferring their content into small periplasmic spaces (Figs. 9E-F). The secretion is forced to pass through the wall by the turgor pressure that actively moves the protoplast, and the secretion is accumulated in a cupule (Fig. 9F) formed by a depression of the outer periclinal wall at the papilla tip. Subsequently, this secretion is released to the gland surface through the pressure exerted by the secretory process itself and volatilization of the secretory components.

Composition of the secretion

The secretion consists exclusively of volatile oils, including terpenes (see Table 2, 5; Fig. 10).

Flower scent

Fig 10. Chromatogram amplification of *Tabernaemontana* sample - time interval of 2.0 - 30.0 min.

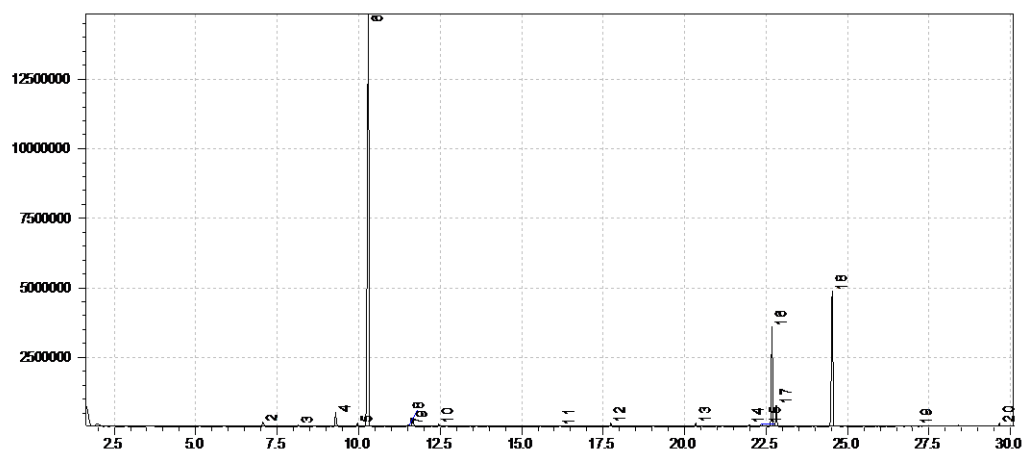


Table 5. Chemical composition and relative percentage of the volatile fraction obtained by headspace from *Tabernaemontana* sample.

	KRI	Number in the chromatogram	% Relative
<i>Alcohols, aldehydes and short-chain ketones</i>			
Butan-2-ol	610	1	8,34
6-Methyl-Hept-5-en-2-ona	985	3	0,24
Nonanal	1102	8	0,14
<i>Hydrocarbons</i>			
Tridecane	1299	12	0,31
Tetradecane	1400	13	0,34
Pentadecane	1500	17	2,24
Hexadecane	1600	19	0,09
<i>Monoterpenoids</i>			
(Z)- β -Ocimene	1040	5	0,34
(E)- β -Ocimene	1050	6	55,23
Rosefuran	1095	7	0,14
Camphor	1143	10	0,21
<i>Sesquiterpenoids</i>			
β -Copaene	1376	14	0,23

Bicyclogermacrene	1494	15	0,23
α -Farnesene	1498	16	10,43
<i>Aromatic Derivatives</i>			
Benzaldeide	961	2	0,72
Phenylacetaldehyde	1043	4	1,58
<i>Nitrogen-containing compounds</i>			
Phenylethanonitrile	1131	9	0,84
2-phenyl-nitroethane	1205	11	0,12
<i>Unknows</i>			
m/z 203, 136, 94, 81, 69*, 41	1545	18	18,08
m/z 230, 107, 93, 81, 69*, 41	2192	20	0,17
<i>Alcohols, aldehydes and short-chain ketones</i>			8,72
<i>Hydrocarbons</i>			2,98
<i>Monoterpenoids</i>			55,92
<i>Sesquiterpenoids</i>			10,89
<i>Aromatic Derivatives</i>			2,30
<i>Nitrogen-containing compounds</i>			0,96
<i>Unknows</i>			18,25

* The mass value marked with asterisk refers to the base peak of the mass spectrum that has a relative abundance of 100%; KRI: Kovats retention Index.

2. Asclepiadoideae

2.1 *Ditassa gracilis*

The flowers of *Ditassa* have white corolla (Fig. 11A) and exude a mild and sweet odor. The osmophore is located on the adaxial side of the corolla lobes (Fig. 11B) and is formed by papillose epidermal cells with a rounded apex (Fig. 11C), which have lamellate walls and are covered by a smooth cuticle (Fig. 11C). In addition, three to four layers of subepidermal parenchyma are also secretory, taking part in the osmophore (Fig. 11D). Few trichomes (Fig. 11B) are present in the region of the osmophores, however, they are not secretory.

Ultrastructure

Cell wall

The cell walls of the secretory cells are thicker than those of other osmophores (Fig. 12A), especially the outer periclinal wall of the papillae (Fig. 12B), which has a fenestrated

internal portion (Fig. 12C). Plasmodesmata are present in the anticlinal walls (Fig. 12D) and assist in the transference of secretion between cells. The epidermis is covered by a mostly smooth cuticle, which has projections of pectin crossing all the cuticle extension (Fig. 12B). The cells of the secretory parenchyma are transversely elongated and have similar subcellular organization to the epidermal cells.

Cytoplasm

The cytoplasm of the secretory cells is rich in ribosomes and has a large amount of elaioplasts and mitochondria (Fig. 12E-F). Elaioplasts have many small plastoglobules and large starch grains and are frequently associated with mitochondria (Fig. 12E-F) and SER. Close to the association of these three organelles, it is possible to observe vesicles formed by SER with secretion inside them. The secretory vesicles are routed toward the vacuole, where the secretion is stored (Fig. 12A).

Secretion release

Although some osmiophilic droplets are observed free in the cytosol (Fig. 12G) and can be released via eccrine secretion, the granulocrine mode of release predominates. After the storage of the secretion inside the vacuole, vacuolar vesicles derived from protrusions of the tonoplast (Fig. 12G) transport the secretion to the distal portion of the cell and merge with the plasma membrane, transferring the secretion to the apoplast, forming large periplasmic spaces (Fig. 12G-H), mainly near the fenestrated regions of the cell wall, where osmiophilic droplets can be observed in intramural spaces (Fig. 12C). Then, the secretion is pushed by the active movement of the protoplast, traversing the cell wall and cuticle, and volatilizing on the osmophore surface to the environment.

Composition of the secretion

Only volatile oils are present in the osmophore secretion, including the terpenes (see Table 2).

2.2 *Hoya carnososa*

The flowers of *Hoya* have white corolla (Fig. 13A) and exude a mild and sweet odor. The osmophore is located along the entire free portion of the petals (Fig. 13B) and is formed by the epidermis of the adaxial side (Fig. 13C) and three to four layers of transversely elongated parenchyma cells (Fig. 13D). The secretory epidermis extends almost to the margin of the corolla's lobes and is composed of cubic cells covered by a smooth cuticle (Fig. 13C). The whole lobe of the corolla presents a dense indumentum; however, these trichomes are not secretory and have no relation with the secretory process.

Ultrastructure

Cell wall

The wall of the secretory cells is lamellate and particularly thicker in the outer periclinal portion of the epidermal cells, which are covered by a smooth cuticle and show no pectin projections (Fig. 14A). All secretory cells communicate through a large amount of plasmodesmata (Fig. 14B).

Cytoplasm

The secretory cells have a large number of ribosomes and have the predominance of elaioplasts with plastoglobules and starch grains (Fig. 14C, E), dictiosomes (Fig. 14D) and SER. The secretory activity is characterized by an intense production of vesicles by the dictiosomes (Fig. 14D) and by elaioplasts filled with droplets of secretion (Fig. 14C, E). The

vesicles of the dictiosomes carry the secretion through the cytoplasm towards the distal portion of the epidermal cell. There is no accumulation inside the vacuole (Fig. 14A, F).

Secretion release

The transport of the secretion inside the cell and between different cells until its release on the surface of the osmophore occurs through vesicles. In the epidermal cells, the vesicles fuse with the plasma membrane both adjacent to the outer periclinal wall and adjacent to the distal portion of the anticlinal walls (Fig. 14F). The secretion transferred to the periplasmic space crosses the wall and the cuticle through the pressure exerted by the active movement of the protoplast and by volatilization of the oils of the secretion, reaching the environment.

Composition of the secretion

Only lipids are present in the osmophore secretion, including terpenes (see Table 2, 6; Fig. 15).

Flower scent

Fig 15. Chromatogram magnification of *Hoya* sample - time interval of 2.0 - 30.0 min.

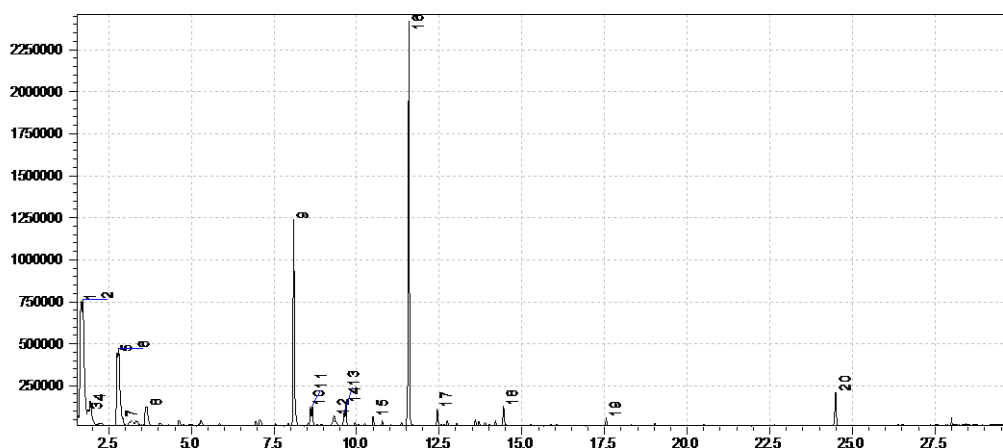


Table 6. Chemical composition and relative percentage of the volatile fraction obtained by headspace from *Hoya* sample.

	KRI	Number in the chromatogram	Relative %
<i>Primary alcohols, aldehydes and short chain ethers</i>			
Ethyl acetate	586	1-2	26,06
3-Methyl-butanal	643	3-4	3,64
3-Methyl-butanol	697	5-6	14,09
Hexanal	807	8	2,88
Octanal	1001	10	1,13
<i>Monoterpenoids</i>			
Sabinene	976	9	15,90
β -Mirceno	1003	11	1,36
α -Terpinene	1018	12	0,59
β -Felandreno	1031	13	0,84
Limonene	1033	14	1,40
γ -Terpinene	1062	15	0,53
Linalol	1098	16	25,85
Camphor	1143	17	1,03
2-Hydroxy-1,8-cineole	1247	18	1,29
<i>Aromatic Derivatives</i>			
Methyl 2-methoxybenzoate	1249	19	0,43
<i>Unknows</i>			
<i>m/z</i> 77*, 62, 59, 45	735	7	0,40
<i>m/z</i> 203, 81, 79, 69*, 41	2094	20	2,59
<i>Primary alcohols, aldehydes and short chain ethers</i>			47,80
<i>Monoterpenoids</i>			48,79
<i>Aromatic Derivatives</i>			0,43
<i>Unknows</i>			2,99

* The mass value marked with asterisk refers to the base peak of the mass spectrum that has a relative abundance of 100%; KRI: Kovats retention Index.

2.3 *Stapelia hirsuta*

The flowers of *Stapelia* have atro-vinaceous corolla with bands of darker coloration alternate with lighter ones and very long petals when compared to the other species studied, besides being succulent, (Fig. 16A). The odor is fetid, resembling decaying organic matter. As in other species, the osmophore extends along almost the entire adaxial surface of corolla lobes, except on their margins. The gland is composed of papillose epidermal cells with

mammillate apex (Fig. 16B-C) and two to three layers of longitudinally elongated secretory parenchyma cells (Fig. 16D). Among the papillae, short trichomes are observed alternating with long trichomes (+ 1cm) (Fig. 16B, D); however, these trichomes are not secretory. Both the secretory epidermal cells and the trichomes are covered by a finely striated cuticle. This is also the only species that has vascularized osmophores. The vascularization is formed by small bundles derived from the petal veins that contain both xylem and phloem (Fig. 16D).

Ultrastructure

Cell wall

Secretory cells have a thick, lamellate cell wall (Fig. 17A-B), whose epidermis is covered by a striated cuticle and have no pectin projections. All secretory cells have a large number of plasmodesmata (Fig. 17C), connecting the osmophore cells.

Cytoplasm

The cytoplasm of the secretory cells have a high density of ribosomes and polyribosomes, where elaioplasts and mitochondria are the predominant organelles (Fig. 17D-E). Mitochondria have a large number of cristae (Fig. 17C, E, H) and the elaioplasts have a profusion of plastoglobules (Fig. 17D). These two organelles are generally found associated in the cytoplasm (Fig. 17E) and secretory droplets are observed in their vicinity free in the cytosol (Fig. 17D). In addition to the elaioplasts, chloroplasts were observed in the secretory parenchyma (Fig. 17F). The secretory activity of epidermal and parenchyma cells involves a high production of vesicles formed by SER (Fig. 17G). The vesicles transport the secretion between parenchyma cells and epidermal cells via exocytosis/endocytosis (Fig. 17H). In the epidermis, the vesicles are directed through the cytoplasm towards the distal portion of the cell, without transferring its contents to the central vacuole.

Secretion release

The volatile oils are released into the environment through granulocrine process of vesicle production from the SER (Fig. 17G), which carry the secretion until merge to the plasma membrane in the distal portion of the cell. The secretion transferred to small periplasmic spaces is pressed through the wall and cuticle by the movement of the protoplast due to the entrance of water in the vacuole that regenerates the cell turgor. The volatilization of the secretory compounds also assists in the release of the scent, which occurs without rupture of the cell wall or cuticle.

Composition of the secretion

Only lipids are present in the secretion of the osmophore and, among them, the terpenes. Protein and/or amino acid clusters were also found in some regions within the epidermal cells (see Table 2, 7; Fig. 18).

Flower scent

Fig. 18. Chromatogram magnification of *Stapelia* sample- time interval from 2.0 to 30.0 min.

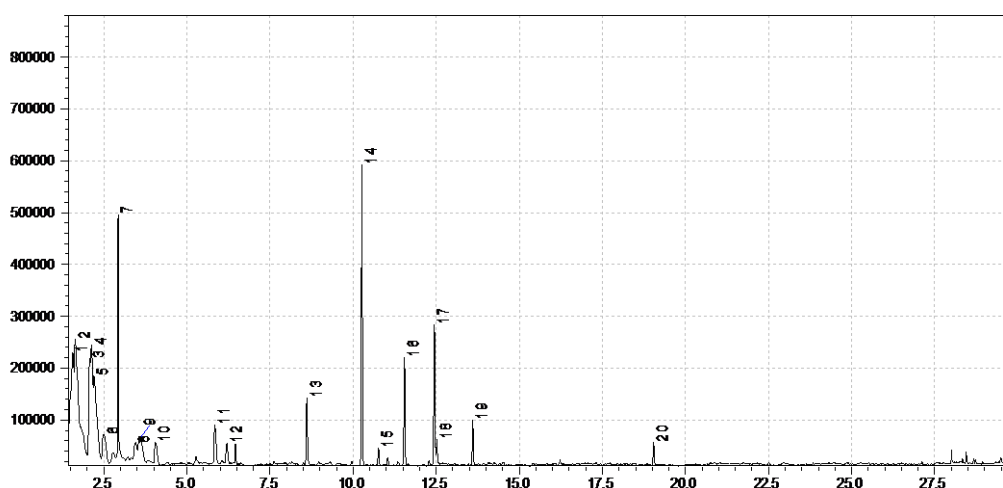


Table 7. Chemical composition and relative percentage of the volatile fraction obtained by headspace from *Stapelia* sample.

	KRI	Number in the chromatogram	Relative %
<i>Hydrocarbons</i>			
2,3-Dimethyl-Pentane	575	1	3,69
3-Methyl-Pentane	620	2	8,08
Cyclohexane	653	3-4	18,04
Heptane	700	6	3,88
2-Methyl-heptane	801	8	2,07
3,4-Dimethylhexane	805	9	3,73
2,4-Dimethylheptane	853	10	2,29
<i>Alcohols, aldehydes and short chain ketones</i>			
Hexan-3-one	667	5	3,88
Heptanal	899	11	3,04
Octanal	1001	13	3,58
Octanol	1070	15	0,85
Nonanal	1102	16	5,26
<i>Monoterpenoids</i>			
(Z)- β -Ocimene	1040	14	14,61
Camphor	1143	17	7,20
Cosmene	1170	18	1,17
Menthol	1173	19	2,16
Isobornyl acrylate	1395	20	1,13
<i>Aromatic Derivatives</i>			
Anisol	917	12	1,61
<i>Unknowns</i>			
m/z 77*, 62, 59, 45	735	7	6,24
<i>Hydrocarbons</i>			41,78
<i>Alcohols, aldehydes and short chain ketones</i>			16,61
<i>Monoterpenoids</i>			26,27
<i>Aromatic Derivatives</i>			1,61
<i>Unknowns</i>			6,24

* The mass value marked with asterisk refers to the base peak of the mass spectrum that has a relative abundance of 100%; KRI: Kovats retention Index.

Discussion

In this work, we have demonstrated the great morphological and functional diversity of osmophores in Apocynaceae. Each of the six species analyzed has osmophores with particular characteristics, presenting different morphology, secretory activity and chemical composition of their perfume, regardless of whether the scent is sweet or fetid. The only

characteristics common to all the osmophores analyzed are their location in the free portion of the petals and the production of the secretion by plastids and SER (Table 8).

Table 8. Comparison between osmophore characteristics in Apocynaceae.

		Rauvolfioideae			Asclepiadoideae			
		<i>Aa</i>	<i>Pr</i>	<i>Tc</i>	<i>Dg</i>	<i>Hc</i>	<i>Sh</i>	
Floral characteristics	Sympetaly	+	+	+	+	+	+	
	Long floral tube	+	+	+	-	-	-	
	Short floral tube	-	-	-	+	+	+	
	Sweet odor	+	+	+	+	+	-	
	Fetid odor	-	-	-	-	-	+	
Location and structural organization	Adaxial side of the corolla lobe	+	+	+	+	+	+	
	Rounded apex papillae	+	-	+	+	-	-	
	Acute apex papillae	-	+	-	-	-	-	
	Mammillate apex papillae	-	-	-	-	-	+	
	Cubic cells	-	-	+	-	-	-	
	Striated cuticle	+	-	-	-	-	+	
	Smooth cuticle	-	+	+	+	+	-	
	Epidermis + parenchyma	+	-	+	+	+	+	
	Only secretory epidermis	-	+	-	-	-	-	
	Non-glandular trichomes	+	-	-	+	+	+	
Ultrastructure	Cell wall	Projections of the OPW	+	-	-	-	-	-
		Thick outer periclinal wall	-	-	-	+	+	+
		Fenestrated wall	-	-	-	+	-	-
		Plasmodesmata	+	+	-	+	+	+
		Projections of pectin into cuticle	+	-	-	+	-	-
	Cytoplasm	Ribosome rich cytoplasm	+	+	+	+	+	+
		Elaioplasts producing secretion	+	+	+	+	+	+
		Starch within the elaioplasts	-	+	-	+	-	-
		Chloroplasts	+	-	-	-	-	+
		SER producing secretion	+	+	+	+	+	+
		Droplets free in the cytosol	-	+	+	+	-	+
		Dictiosomes producing vesicles	-	-	+	-	+	-
	Storage in the central vacuole	-	+	+	+	-	-	
	Release of secretion	Vacuolar vesicles	-	+	+	+	-	-
		Vesicles from elaioplast, SER and/or dictiosome	+	-	+	-	+	+
Cupule formation		-	-	+	-	-	-	
Intramural space		-	-	-	+	-	-	
Chemical composition of the secretion (%)		Hydrocarbons	47,07	76,72	2,98	NP	47,8	41,78
	Alcohols, Aldehydes and Esters	33,29	-	8,72	NP	-	16,61	
	Monoterpenoids	7,1	0,64	55,92	NP	48,79	26,27	
	Sesquiterpenoids	4,14	-	10,89	NP	-	-	
	Aromatic Derivatives	1,12	10,23	2,3	NP	0,43	1,61	
	Nitrogen-containing compounds	2,2	12,4	0,96	NP	-	-	

Structural location and organization

The osmophores are located on the adaxial surface of the petal lobes in all Apocynaceae species, as well as in the vast majority of the osmophores already described for other families (Pridgeon and Stern 1983; Stpiczyńska 2001; Ascensão *et al.* 2005; Baudino *et al.* 2007; Wiemer *et al.* 2008; Teichert *et al.* 2009; Pansarin *et al.* 2009; Melo *et al.* 2010; Płachno *et al.* 2010; Marinho *et al.* 2014; Gagliardi *et al.* 2016; Maiti and Mitra 2017). The location of this gland in another floral whorl, axis of the inflorescence, bracts or even leaves has also been reported and may be related to the absence of corolla in some flowers or, commonly, to a specific pollination mechanism (Ormond *et al.* 1981; Pridgeon and Stern 1983, 1985; Vogel 1990; Sazima *et al.* 1993; Bergstrom *et al.* 1995; Skubatz *et al.* 1996; Stpiczyńska 2001; Vogel and Hadacek 2004; Caissard *et al.* 2004; Trujillo and Sérsic 2006; García *et al.* 2007; Bolin *et al.* 2009; Melo *et al.* 2010; Freitas *et al.* 2017). In some groups, the presence of diffuse osmophores in more than one floral whorl can act as an odor guide for pollinators (Bergstrom *et al.* 1995).

Histologically, osmophores can be constituted by epidermis or by secretory epidermis and parenchyma (Vogel 1990). Both types were found in Apocynaceae, not having a relation with the amount of scent produced or the period of the day of their release to the environment. The predominant type of the family seems to be composed of epidermis and parenchyma, and was found in *Aspidosperma*, *Tabernaemontana*, *Ditassa*, *Hoya* and *Stapelia*, in addition to the previous descriptions made for *Boucerosia* and *Orbea* (Płachno *et al.* 2010). *Plumeria* was the only one to present an exclusively epidermal osmophore.

The osmophores varied in surface morphology and may be glabrous (*Plumeria* and *Tabernaemontana*) or possess trichomes (*Aspidosperma*, *Ditassa*, *Hoya* and *Stapelia*). Epidermal secretory cells may be (1) rounded apex papillae (*Aspidosperma*; *Tabernaemontana*; *Ditassa*); (2) acute apical papillae (*Plumeria*; *Stapelia*); (3) cubic cells

(*Hoya*); (4) mammillate apex papillae (*Stapelia*). Furthermore, the cuticle varied from striated (*Aspidosperma*; *Stapelia*) to smooth (*Tabernaemontana*; *Plumeria*; *Ditassa*; *Hoya*). None of these surface characteristics can be correlated with the composition of the perfume or the secretory process, although the presence of an indumentum especially tomentose in *Hoya* may reduce or delay the release of the scent to the environment by retaining part of the volatiles close to the flower. Different epidermal cell shapes and cuticle ornamentation were observed in *Boucerosia* and *Orbea* by Płachno *et al.* (2010), fitting into our types described as acute apex papillae and mammillate apex papillae.

The morphological variation of the osmophores and the impossibility of relating the structure to the secretory process or to the floral bouquet seems to be a constant in other groups. Although the release of the scent seems to vary rhythmically (Matile and Altenburger 1988), all species analyzed released it throughout the day.

Ultrastructure and secretory process

Secretory cells usually have thin walls (Fahn 1979) but the osmophores of Asclepiadoideae (*Ditassa*, *Hoya* and *Stapelia*) have thick, often lamellate walls. The outer periclinal wall is the thicker portion, *i.e.*, the main region through which the secretion diffuses to reach the environment without any cell rupture. The thickening of these walls is unrelated to the secretory process and seems to be a feature of the subfamily Asclepiadoideae.

The production of the oils begins in the intermediary phase of the floral development in flowers with long corolla and in the pre-anthesis of the smaller flowers. In all species analyzed, intense secretory activity of elaioplasts and SER was observed, as reported by Płachno *et al.* (2010) for other Apocynaceae, and also in studies with Araceae (Skubatz *et al.* 1993, 1996; Skubatz and Kunkel 1999), Orchidaceae (Pridgeon and Stern 1983; Stern *et al.* 1987; Melo *et al.* 2010; Kowalkowska *et al.* 2012, 2015) and Passifloraceae (García *et al.*

2007). The secretion composed exclusively of lipids, detected in all the osmophores studied, is directly related to these organelles, since the plastids and endoplasmic reticulum are responsible for the production of all types of lipids in the cell (Buchanan *et al.* 2015). Additionally, the observation of periplastidial reticulum reinforces the importance of the interaction between these two organelles in the production of volatile oils and the association of these organelles with mitochondria in certain regions of the cytoplasm, demonstrates the energy requirement spent in this secretory process (Gama *et al.* 2015).

Compartmentalized production becomes more evident when we consider that monoterpene production occurs mainly in elaioplasts, while sesquiterpenes occurs mainly in SER (Buchanan *et al.* 2015). In addition, the three most abundant organelles of the Apocynaceae osmophores are the same, as observed in the cells involved in the synthesis of volatiles in osmophores of *Stanhopea anfracta* (Orchidaceae; Curry 1987). Based on the location of the hydroxymethyl-glutaryl-CoA synthase activity, the enzyme indicative of the mevalonate pathway, Curry (1987) demonstrated that it was localized primarily in the intermembrane space of mitochondria and in the SER. Secondly, this enzyme was also found in the plastids, characterizing a pronounced compartmentalization during the synthesis of terpenoids.

The energy reserve for the whole secretory process was found in the form of starch grains in the plastids of the secretory epidermis itself or the secretory parenchyma. The only exception was *Plumeria*, where the epidermal osmophore has its starch reserve in the non-secretory parenchyma. The presence of starch in subepidermal tissue has been related to thermogenesis in several types of osmophores (Vogel 1990) and new studies are needed to determine if this process occurs in Apocynaceae.

Secretory cells in all analyzed osmophores were also highlighted by the presence of a highly developed vacuome. A profusion of vesicles can be observed from the beginning to the

end of the secretory phase, even after the formation of a large central vacuole. Although secretory droplets have been observed free in the cytosol in some species, the production and transport of secretion through vesicles produced by SER and/or dictiosomes is predominant. Vesicles formed by the elaioplasts were also identified, transferring the osmiophilic secretion, observed as plastobobules, to the cytosol. The predominance of membrane-bound secretion explains the constant observation of intercellular transport occurring through exocytosis and endocytosis, in addition to granulocrine (or exocytic) release to the surface of the gland.

The occurrence of vesicles being incorporated into the plasma membrane, related to granulocrine secretion, has previously been described in Orchidaceae osmophores (Stpiczyńska 2001; Kowalkowska *et al.* 2012, 2015) and is the most common mechanism of lipid secretions release in secretory cells. The release of secretion occurs mediated by vesicles in two distinct processes in Apocynaceae. *Aspidosperma*, *Hoya* and *Stapelia* produce a secretion that is immediately released into the environment, while *Plumeria* and *Ditassa* produce a secretion that is transported to the central vacuole, where it is stored temporarily. Subsequently, vesicles formed from vacuolar protrusions direct the secretion until its release in the distal portion of the cell. *Tabernaemontana* presented a mixed process, where part of the secretory vesicles is released soon after their production, while others transfer their contents to the vacuole and then, the vacuole releases it to the outside. This storage in the vacuole is not related to a protection against the volatilization of the compounds or storage for release in a restricted period of the day, since the perfume was detected throughout the day. However, the transference to the intravacuolar environment may be related to some chemical modification of the secretory compounds, generating the volatile compound that is released by the flower.

Regardless the origin of the vesicles, their fusion with the plasma membrane in the distal portion of the epidermal cells transfers the secretion into a periplasmic space. In all

species, the periplasmic space has always been reduced, showing that the secretion crosses the wall and the cuticle shortly after its release into this space, despite its predominantly hydrophobic composition as opposed to that of the cell wall, which is hydrophilic. The passage of these compounds through the wall seems to be forced by the active movement of the protoplast that pushes the secretion through it, as proposed by Paiva (2016). However, the cyclic movement of the protoplast to release the secretion proposed by this author was not observed. The rapid protoplast movement makes impossible the formation of large periplasmic spaces and is probably related to changes in the turgor pressure of the cell, as proposed by other researchers (Lüttge and Schnepf 1976).

After crossing the wall, the secretion must pass through the cuticle before it is released to the environment. The thickness of the cuticle varied among the analyzed species, as well as their ornamentation, but no distension or rupture was observed in any of them. *Aspidosperma* and *Ditassa* presented pectin projections traversing most of the cuticle, which may assist in the conduction of, at least, part of the secretion (Gama *et al.* 2016). The secretion may cross the cuticle due to the lipophilic nature of the cutin via diffusion, aided by the secretory flow, being pushed by the protoplast and/or by microchannels within the cuticle (Paiva 2016). This seems to be the standard process of releasing of the osmophore secretion to the gland surface (Pridgeon and Stern 1983; Stern *et al.* 1987; Kowalkowska *et al.* 2015).

Chemical composition of volatile oil

Among the floral volatiles, several types of hydrocarbons, benzenoids, ethers, esters, aldehydes, ketones and amino terpenoids have already been registered (Williams and Whitten 1983; Knudsen *et al.* 1993). In the present work, the perfume released by Apocynaceae flowers also has diverse origin and presents a high diversity, not being possible to correlate with the secretory process or period of fragrance releasing. Among the Rauvolfioideae, the

scent released by *Aspidosperma* showed about 80% of hydrocarbons plus aldehydes, ketones and furan derivatives and *Plumeria* has almost 77% of primary alcohols, aldehydes and ketones, while *Tabernaemontana* has almost 67% of mono- and sesquiterpenes composing the perfume. Among the Asclepiadoideae, *Hoya* presented similar values between monoterpenes and primary alcohols, aldehydes and esters, which together added almost 96% of the floral bouquet, while *Stapelia* produced around 58% hydrocarbon plus primary alcohols, aldehydes and ketones and about 26% monoterpenes, as major compounds.

Attraction of pollinators

The presence of volatile nitrogen compounds may be related to the attraction of flies (Dobson, 2006), often producing a scent that attracts *Calliphoridae* and *Sarcophagidae* flies, which identify the flowers as oviposition sites (Kuglel, 1956). This identification was observed for several species in several other families (Araceae, Aristolochiaceae, Burmanniaceae, Malvaceae, Orchidaceae, Rafflesiaceae, and Taccaceae) that are under sapromiophylic syndrome (Faegri and van der Pijl 1979). However, the three genera of Rauvolfioideae analyzed (*Aspidosperma*, *Plumeria* and *Tabernaemontana*) are pollinated by moths or bees during the night or early morning, while *Stapelia*, which is pollinated by blowflies, has not presented nitrogen compounds among its volatile compounds.

The immense diversity found in the Apocynaceae osmophores for all analyzed aspects prevents the correlation of their characteristics with any structural characteristics of the secretory process or with the pollination biology of the species analyzed up to the present moment. New studies are essential to try to determine which evolutionary factors have influenced the appearance of such distinct glands in subfamilies with relatively constant floral morphology and similar pollination syndromes.

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Figures

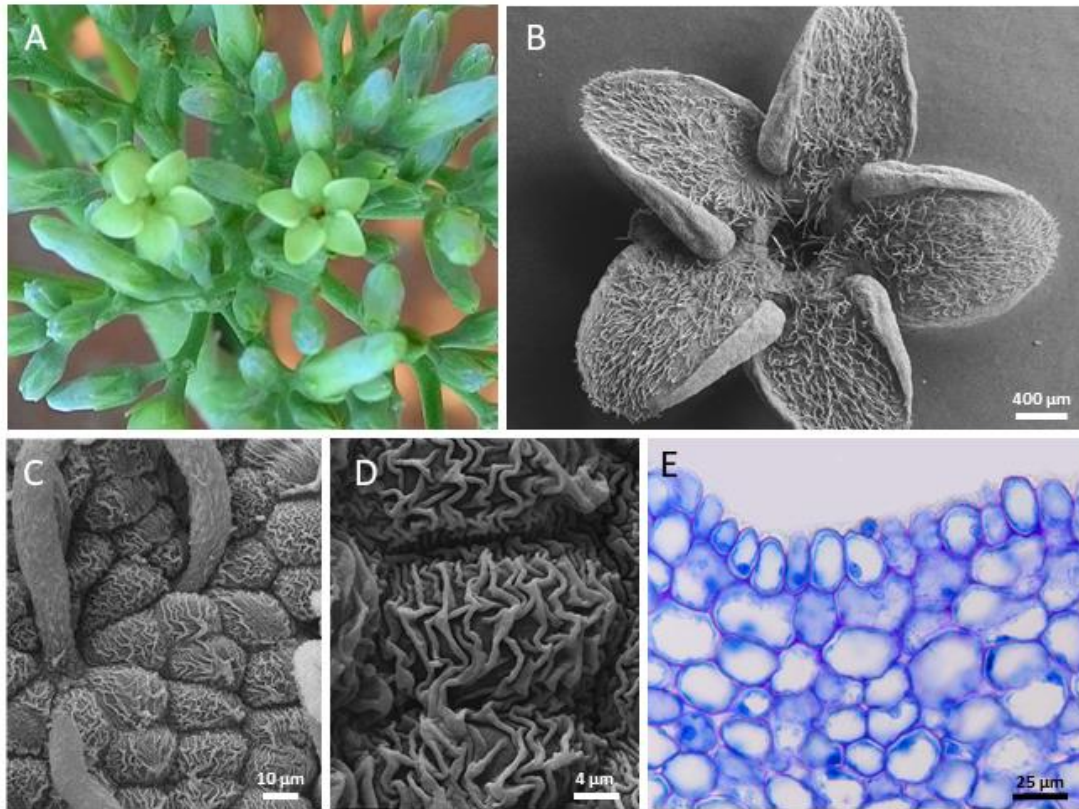


Fig 1. A-E. *Aspidosperma australe* **A.** Anthetic flowers **B-D.** Morphology of osmophores; Scanning electron microscopy **B.** Detail of the corolla lobes. Secretory surface extends along the whole flat surface almost to the revolute margins of each lobe **C-D** Osmophore papillae with rounded apex **D.** Cuticle striations in the osmophore cells **E.** Osmophore anatomy. Epidermis and three to four layers of secretory parenchyma layers in the adaxial side of the petal.

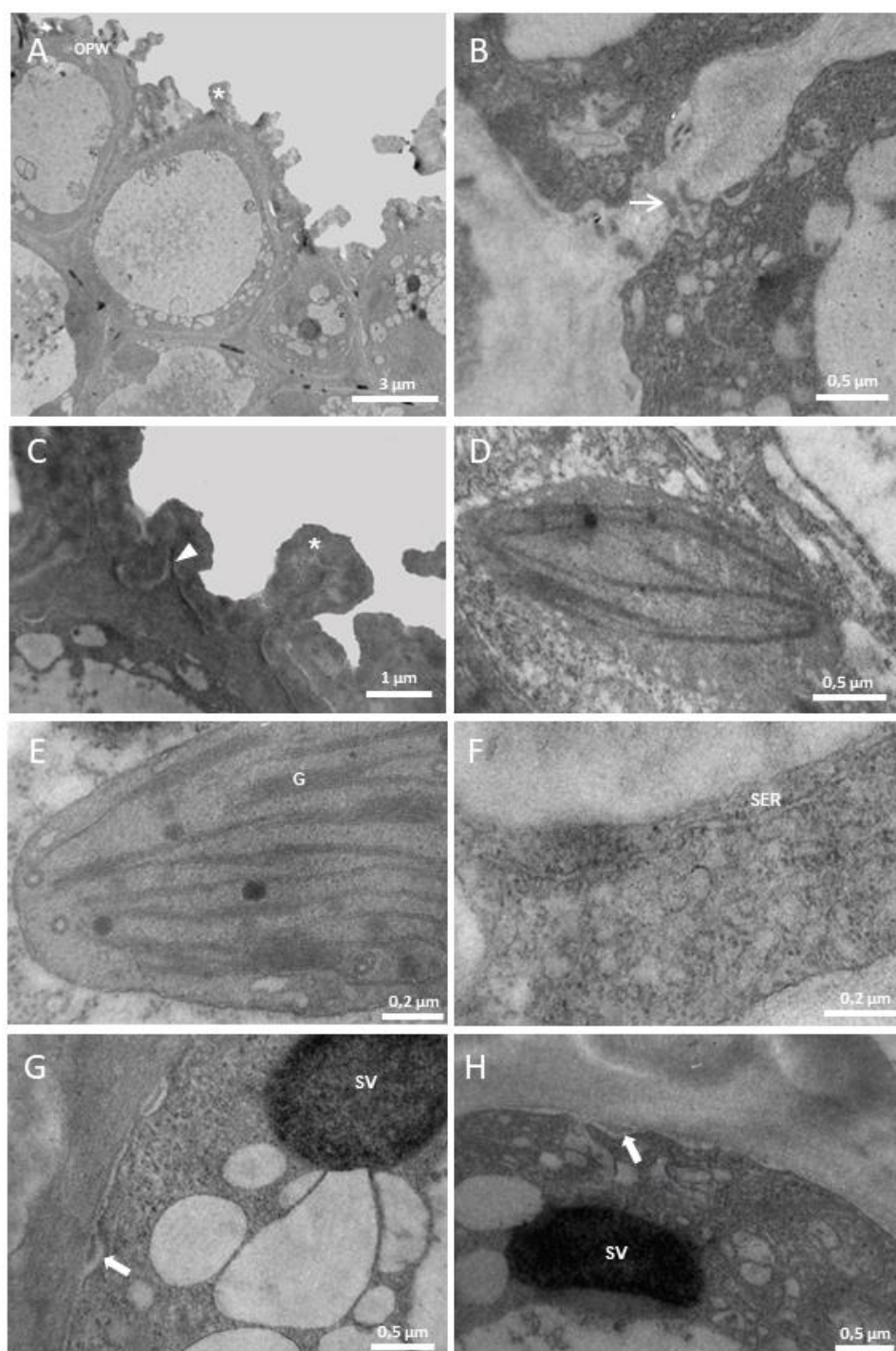


Fig 2. A-H. Ultrastructure of the osmophores in *Aspidosperma australe* **A.** Epidermal secretory cells with projections of their outer periclinal wall and cuticle striations **B.** Plasmodesmata observed in the secretory cells **C.** Cuticle striations accompany the wall projections. Note thin pectin projections traversing the cuticle almost completely **D.** Elaioplast **E.** Chloroplast **F.** Smooth endoplasmic reticulum in the periphery of the cell **G-H.** Secretory vesicles and their fusion with plasma membrane. asterisk, cuticle striation; thin arrow, plasmodesma; wide arrow, vesicle fused to plasma membrane forming the periplasmic space; arrow head, pectin projection; G, granum; OPW, outer periclinal wall; SER, smooth endoplasmic reticulum; SV, secretory vesicle.

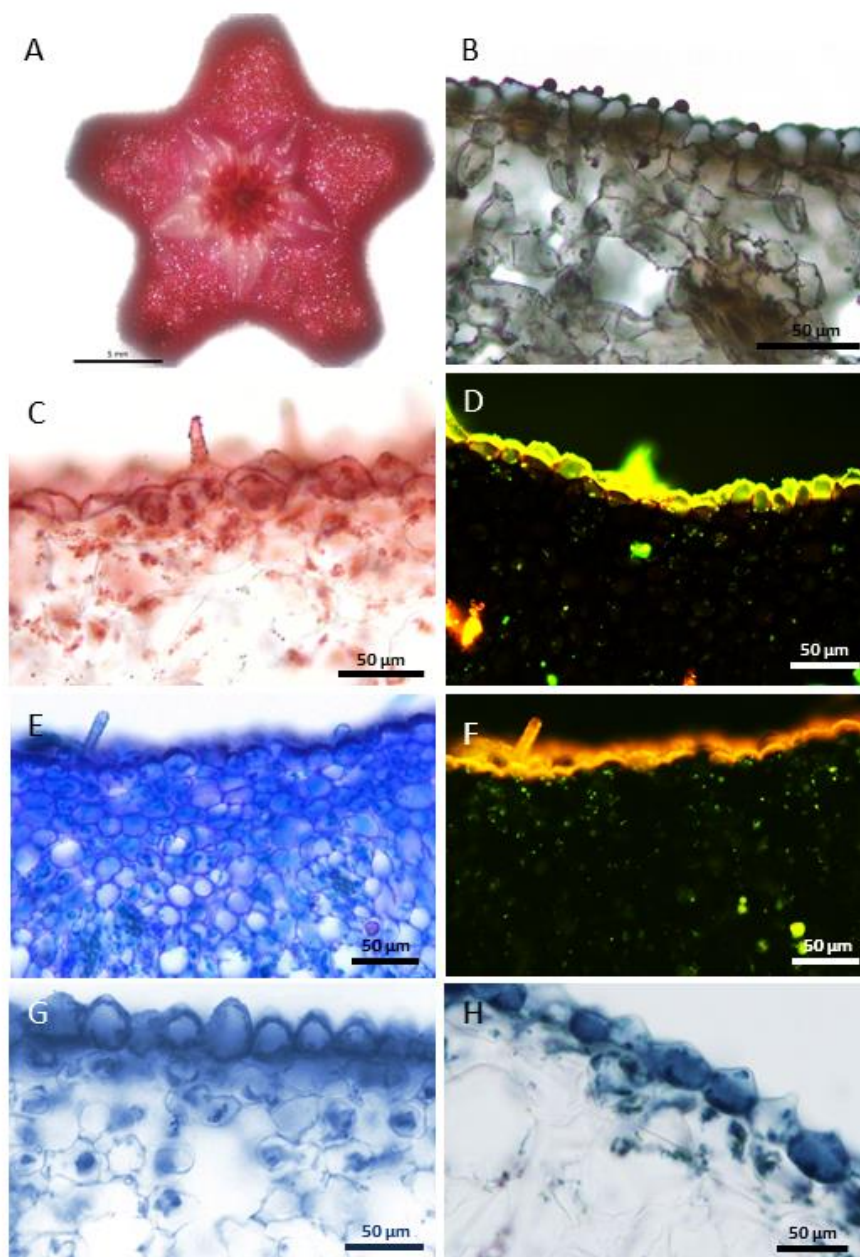


Fig 3. A-H. Results of histochemical tests applied to osmophores of Apocynaceae A-F. Lipid detection A. Neutral red. *Hoya carnososa* B. Sudan Black B. *Tabernaemontana catharinenses* C. Sudan IV. *Stapelia hirsuta* D-F. *Aspidosperma australe* D. Neutral red under blue light E. Nile blue F. Nile blue under blue light G. Detection of essential oils with NADI reagent. *Plumeria rubra* H. Proteins stained with aniline blue black. *Stapelia hirsuta*.

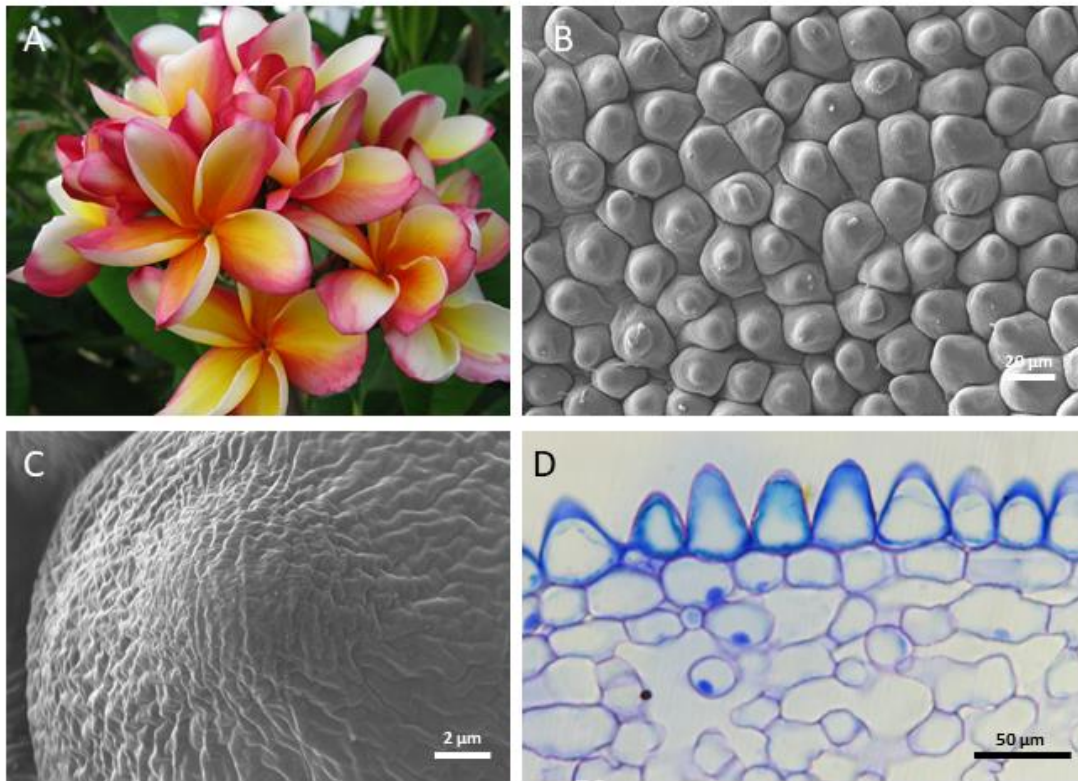


Fig 5. A-D. *Plumeria rubra* **A.** Anthetic flowers **B-C.** Osmophore morphology. Scanning electron microscopy. **B.** Papillose secretory epidermis with cells of acute apex **C.** Smooth cuticle covering the papilla **D.** Osmophore anatomy. Osmophore composed exclusively of epidermis.

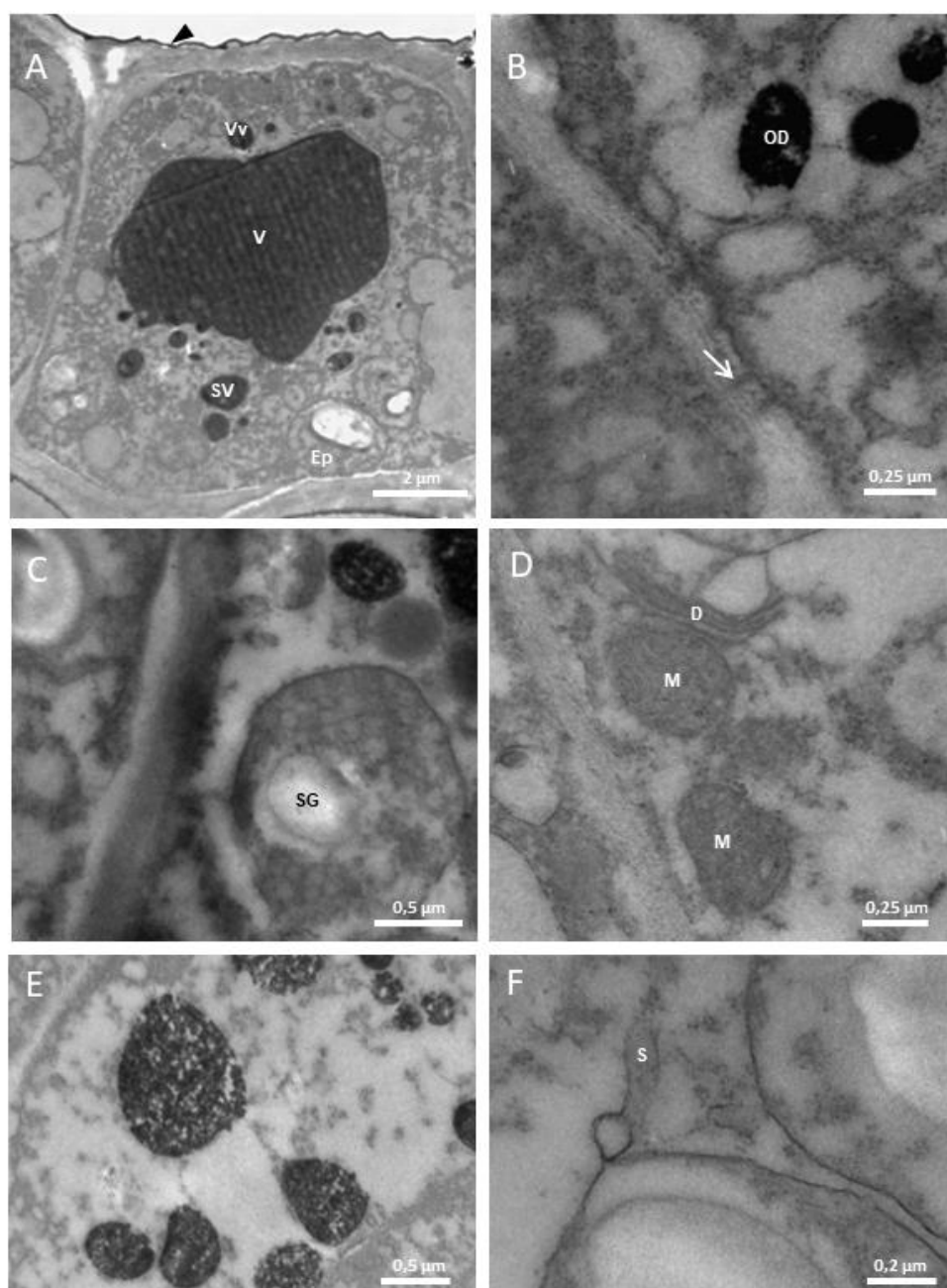


Fig 6. A-F. Ultrastructure of the osmophores in *Plumeria rubra* **A.** Secretory epidermis covered by a thin, smooth cuticle. Note the central vacuole filled with secretion, the secretory vesicles fusing with the vacuole and the releasing of vacuolar vesicles in the distal portion of the cell **B.** Plasmodesmata in the anticlinal walls and osmiophilic droplets inside a vesicle **C.** Elaioplast with starch grain **D.** Mitochondria and a dictyosome **E.** Secretory vesicles formed by plastids and SER **F.** Stromule between two elaioplasts. Arrow, plasmodesma; Arrowhead, cuticle; D, dictyosome; Ep, elaioplast; M, mitochondrion; OD, osmiophilic droplet; S, stromule; SG, starch grain; SV, secretory vesicle; V, vacuole; Vv, vacuolar vesicle.

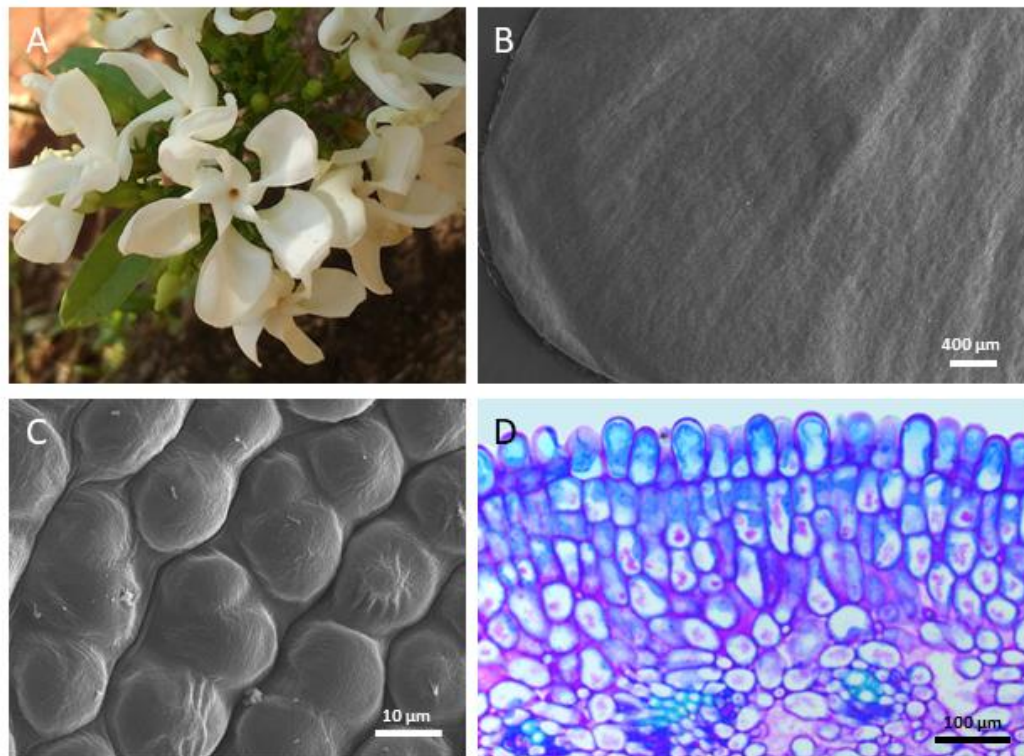


Fig 8. A-D. *Tabernaemontana catharinenses* **A.** Anthetic flowers **B.** Osmophore located in the adaxial face of the corolla lobe **C.** Secretory papillae with rounded apex, arranged in several pairs or even quartets of juxtaposed cells **D.** Osmophore anatomy. The secretory tissues consist of epidermis and three to four layers of transversely elongated parenchyma cells.

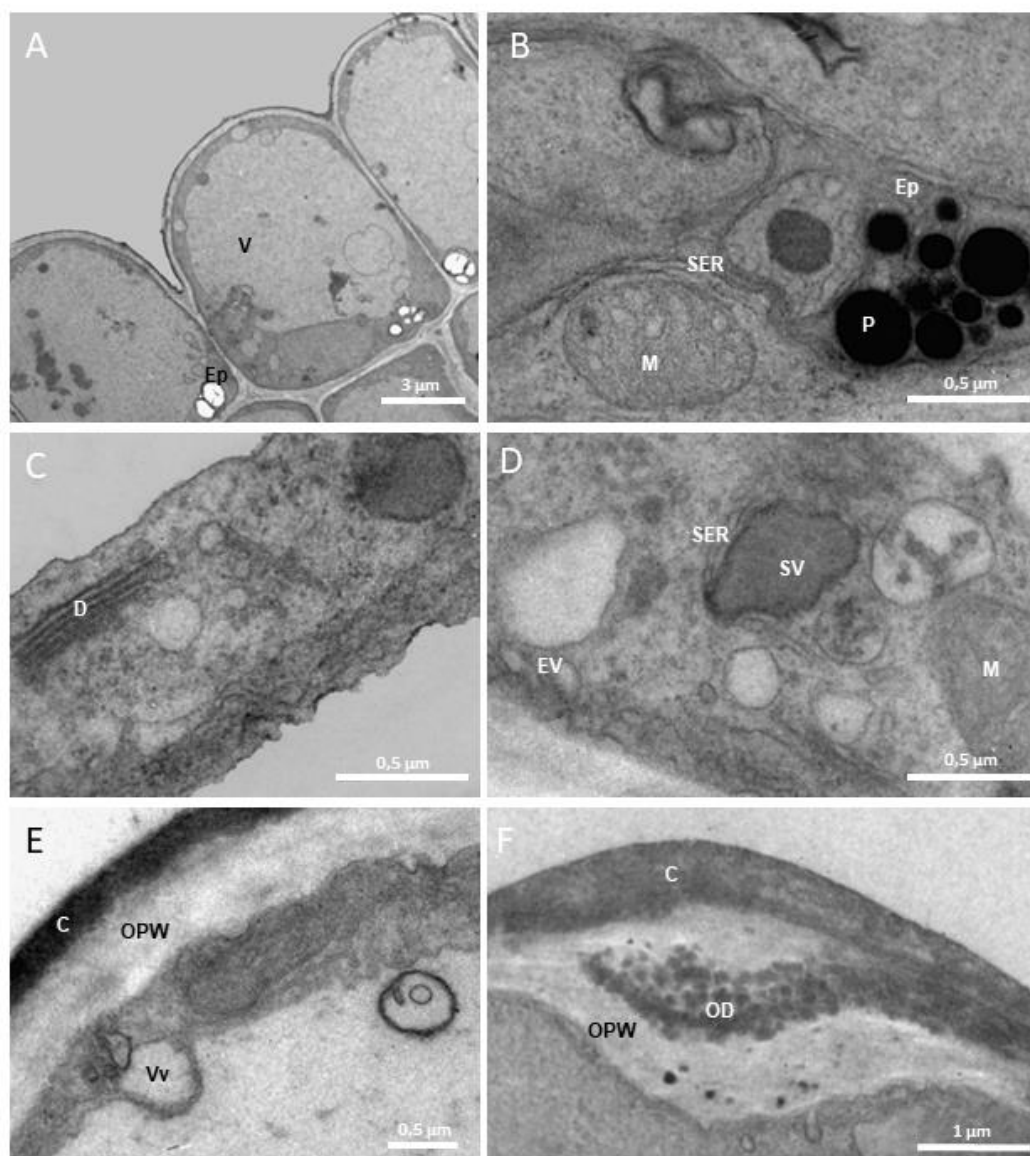


Fig 9. A-F. Ultrastructure of the osmophores in *Tabernaemontana catharinenses*. **A.** Secretory epidermis covered by a smooth cuticle. Note the presence of secretion inside the central vacuole and elaioplasts with large starch grains **B.** Elaioplasts with many plastoglobules, mitochondrion and periplastidial SER **C.** Dictyosome producing vesicles **D.** Secretory vesicles produced by SER and endocytic vesicles formed by plasma membrane associated to a mitochondrion **E.** Vacuolar vesicle originated by a protrusion of the vacuole near the outer periclinal wall of the epidermis **F.** Osmiophilic droplets in a cupule formed by a depression of the cell wall tip during the secretion release. C, cuticle; D, dictyosome; Ep, elaioplast; Ev, endocytic vesicle; M, mitochondrion; OD, osmiophilic droplet; OPW, outer periclinal wall; SER, smooth endoplasmic reticulum; SV, secretory vesicle; V, vacuole; Vv, vacuolar vesicle.

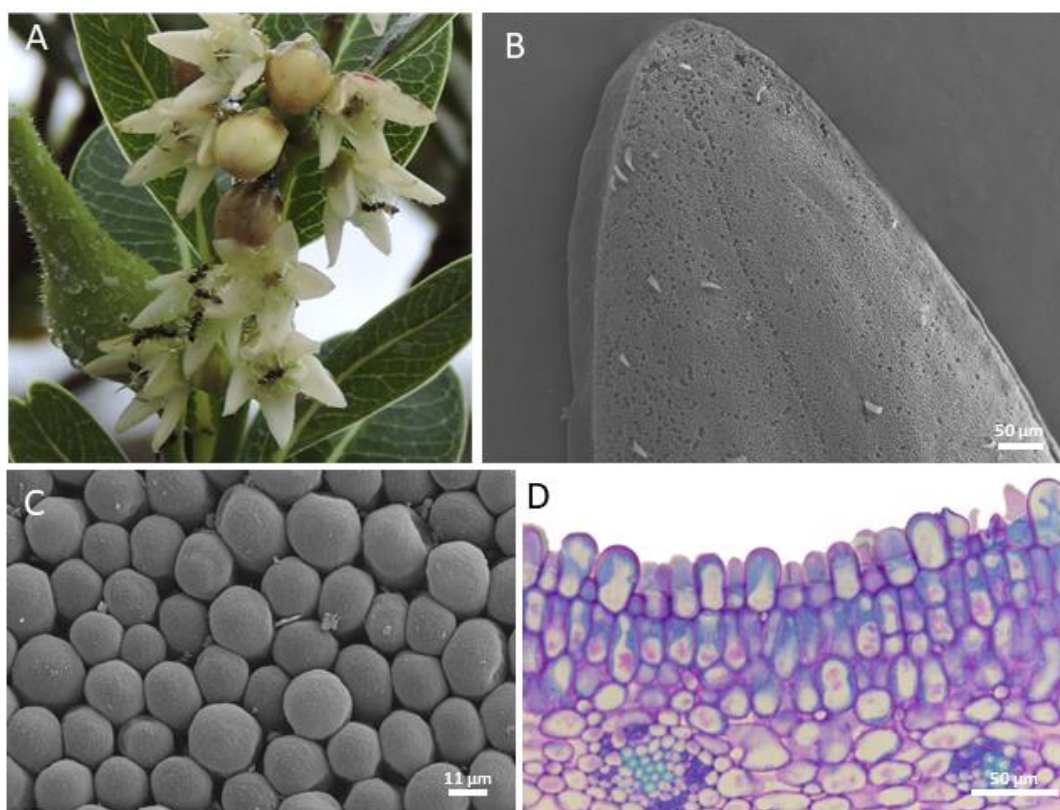


Fig 11. A-D. *Ditassa gracilis* **A.** Anthetic flowers **A-B.** Scanning electron microscopy **B.** Morphology of the osmophore located in the adaxial side of the corolla lobe. Note few non-glandular trichomes present in the region of the osmophore **C.** Papillose secretory epidermal cells with rounded apex and smooth cuticle **D.** Osmophore anatomy. Secretory tissues composed of epidermis and three to four layers of transversely elongated parenchyma cells.

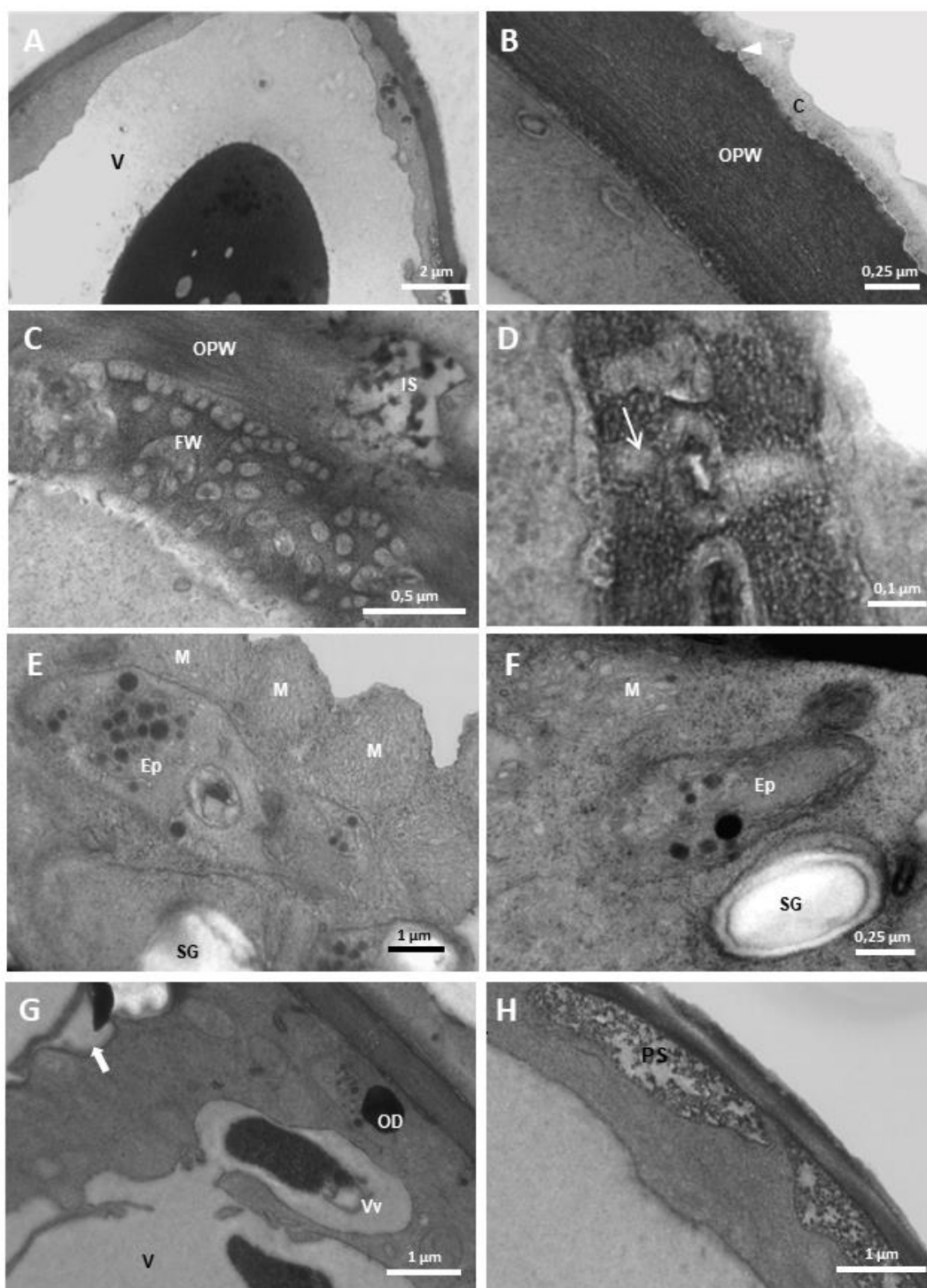


Fig 12. A-D. Ultrastructure of the osmophores in *Ditassa gracilis* **A-C.** Secretory epidermal cell. **A.** Note secretion stored inside the central vacuole. **B.** Outer periclinal wall covered by a thin cuticle **C.** Fenestrated internal portion of the outer periclinal wall and presence of osmiophilic droplets in an intramural space **D.** Plasmodesmata **E,F.** Elaioplasts with plastoglobules and large starch grains associated with many mitochondria **G.** Vesicles derived from vacuolar protrusions which transfer the secretion from vacuole to the periplasmic space. Note an osmiophilic droplet free in the cytosol **H.** Secretion in the periplasmic space. Arrowhead, pectin projection; thin arrow, plasmodesma; wide arrow, vesicle fused to plasma membrane forming the periplasmic space; C, cuticle; Ep, elaioplast; FW, fenestrated wall; IS, intramural space; M, mitochondrion; OPW, outer periclinal wall; PS, periplasmic space; SG, starch grain; V, vacuole.

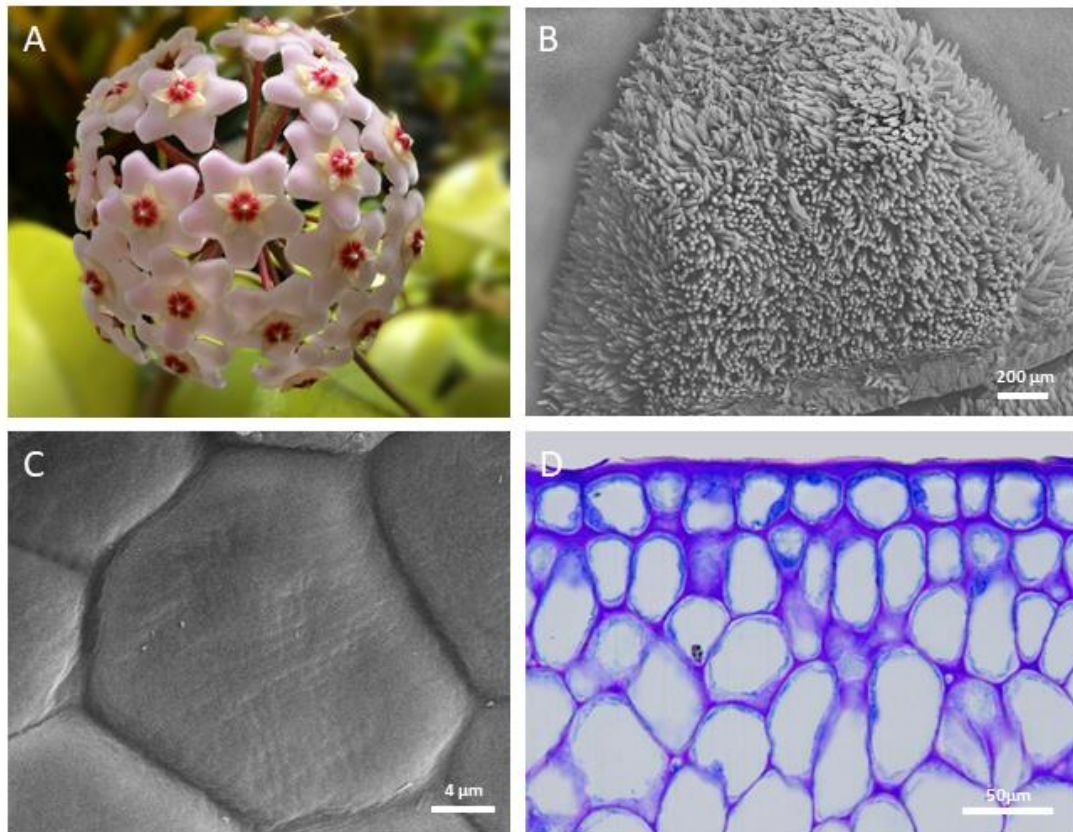


Fig 13. A-D. *Hoya carnosae* A. Anthetic flower B, C. Morphology of the osmophore. Scanning electron microscopy B. Adaxial surface of the corolla lobe, showing the high amount of trichomes on the petal. C. Secretory epidermal cells covered by a smooth cuticle D. Osmophore anatomy. Secretory tissues composed of epidermis and three to four layers of parenchyma.

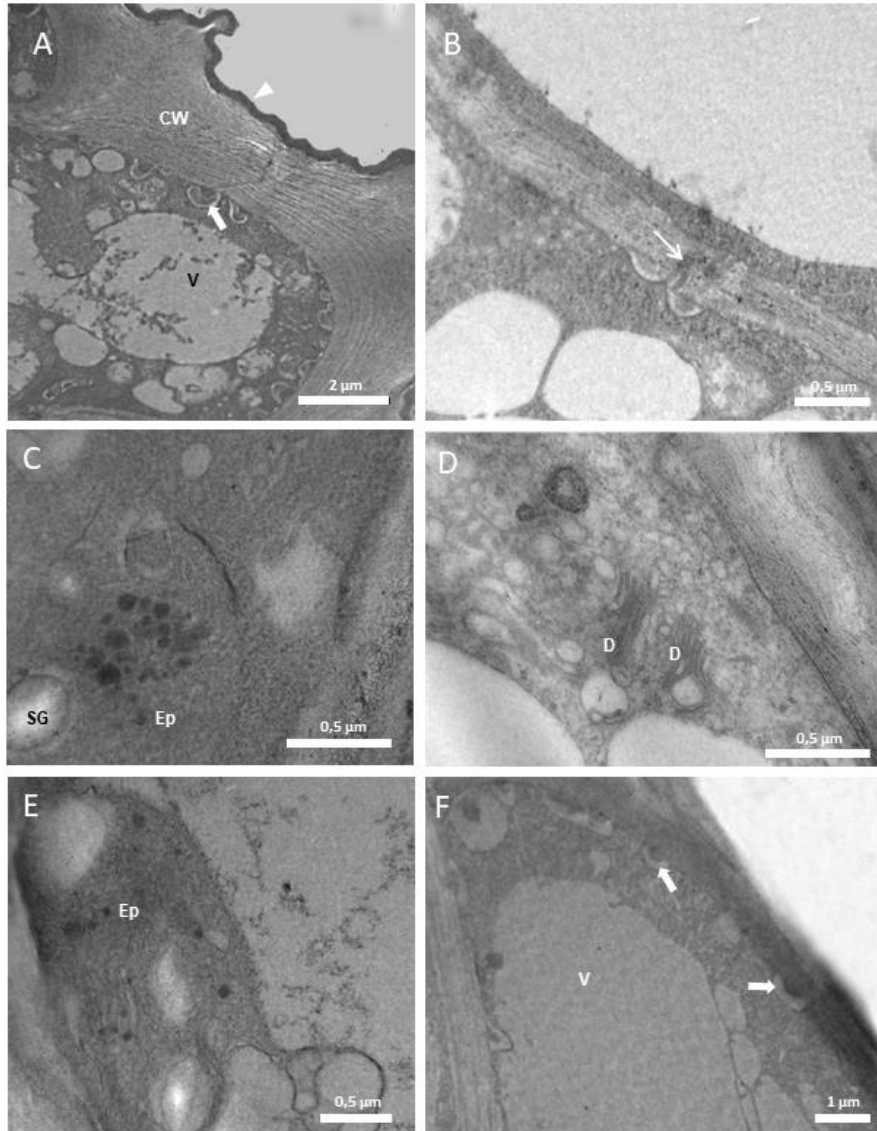


Fig 14. A-F. Ultrastructure of the osmophores in *Hoya carnosae* **A.** Lamellate, thick cell walls in the secretory epidermal cells. A thin cuticle covers the epidermis, which is striated due to projections of the outer periclinal wall **B.** Plasmodesmata communicating adjacent secretory cells **C, E.** Elaioplasts with small plastoglobules and starch grains **D.** Abundance of dictyosomes producing a high amount of vesicles **F.** Secretory vesicles fused to plasma membrane, transferring the secretion to the periplasmic space. Arrowhead, cuticle; thin arrow, plasmodesmata; wide arrow, vesicle fused to the plasma membrane forming the periplasmic space; CW, cell wall; D, dictyosome; Ep, elaioplast; SG, starch grain; V, vacuole.

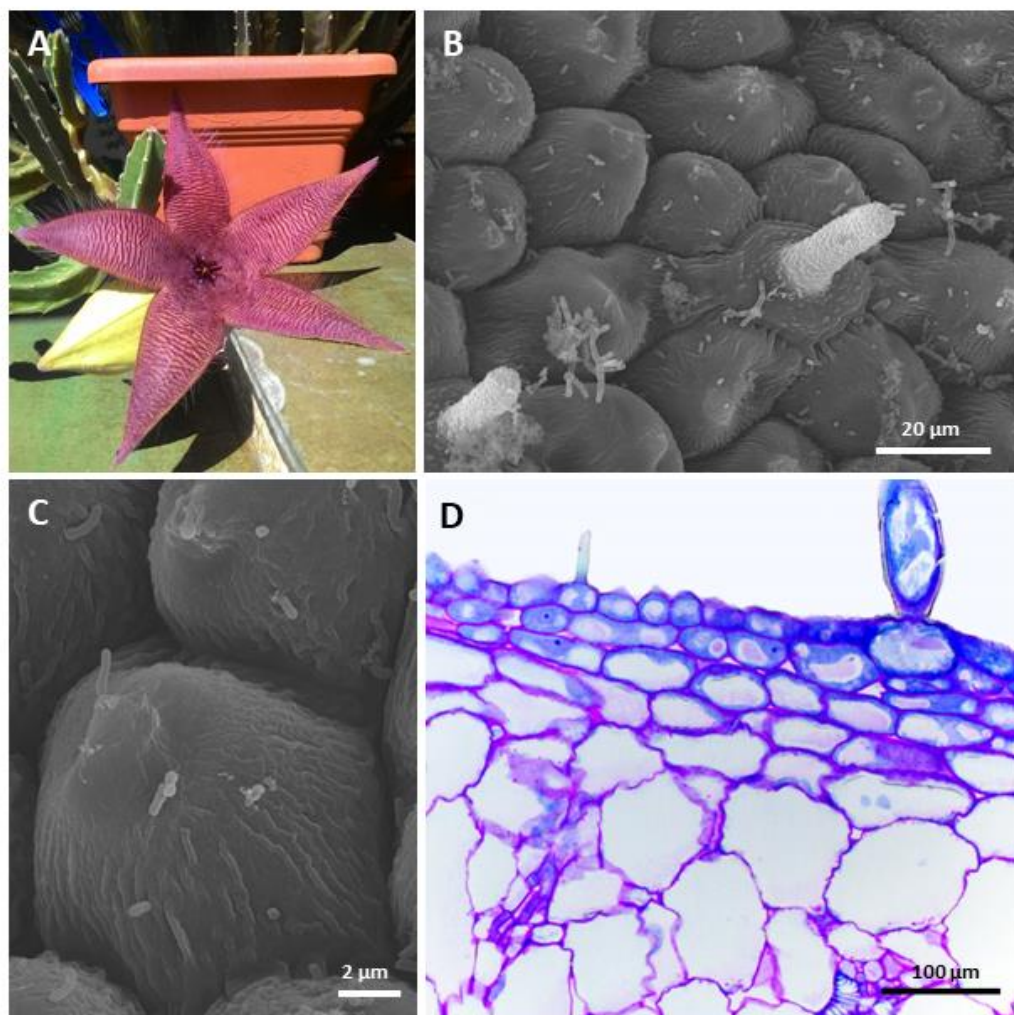


Fig 16 A-D. *Stapelia hirsuta* A. Anthetic flower B,C. Secretory epidermal papillae with mammillate apex C. Cuticle striations on the papillae D. Osmophore anatomy. Secretory tissues composed of epidermis and two to three layers of horizontal elongated parenchyma cells. B,D. Presence of non-glandular trichomes among secretory cells of the osmophore epidermis.

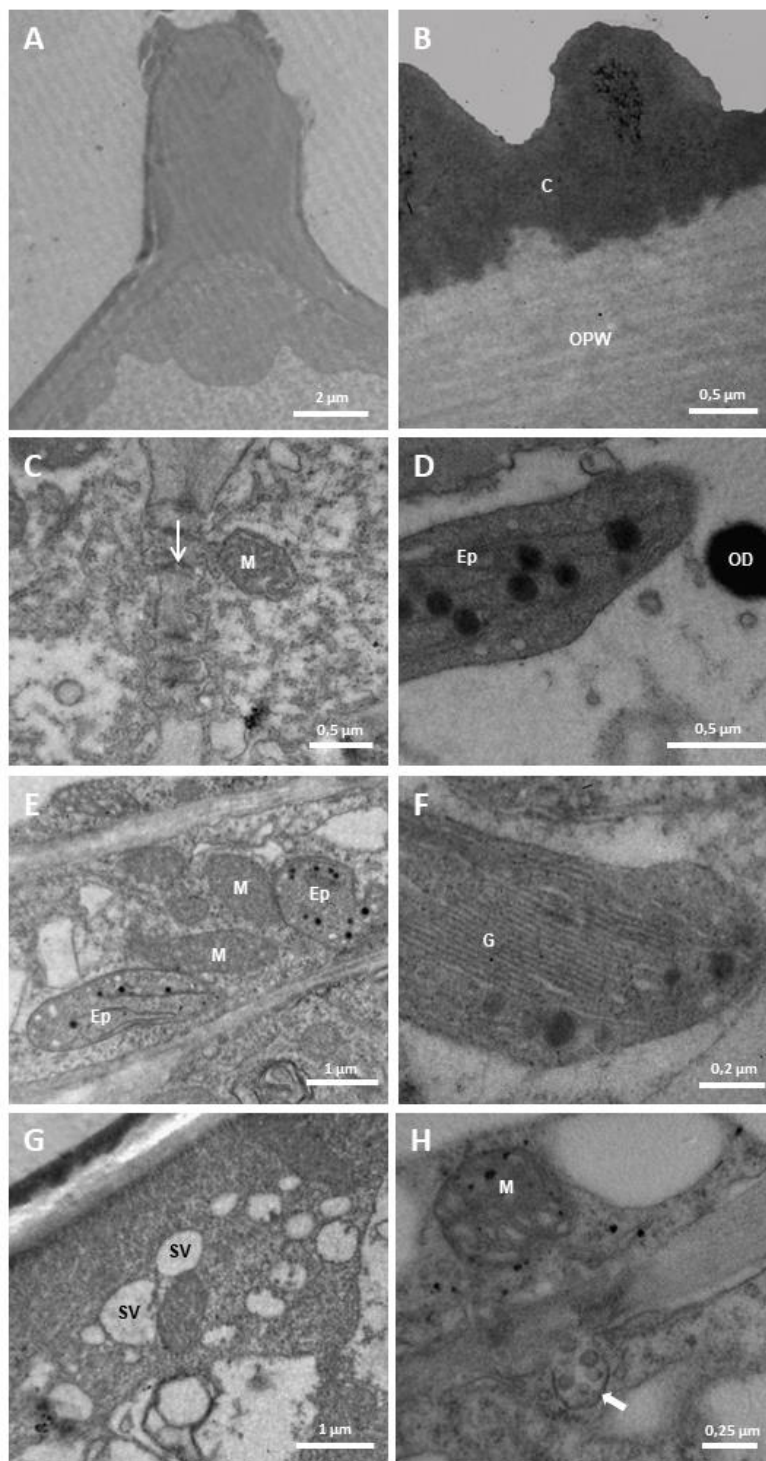


Fig 17. A-H. Ultrastructure of the osmophores in *Stapelia hirsuta* **A.** Mammillate epidermal cell covered by a thin cuticle **B.** Cuticle striations on the outer periclinal wall **C.** Plasmodesmata connecting two secretory cells **D.** Elaioplast with large plastoglobules. Note a osmiophilic droplet free in the cytosol **E.** Elaioplasts associated to mitochondria **F.** Chloroplast in the secretory parenchyma **G.** Secretory vesicles formed by SER **H.** Transference of secretion between osmophore cells via exocytosis. Thin arrow, plasmodesma; Wide arrow, exocytic vesicle fused to the plasma membrane; C, cuticle; Ep, elaioplast; G, granum; M, mitochondrion; OD, osmiophilic droplet; OPW, outer periclinal wall; SV, secretory vesicle.