



Structure and ultrastructure of nuptial and extranuptial nectaries explain secretion changes throughout flower lifetime and allow for multiple ecological interactions

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1 **Structure and ultrastructure of nuptial and extranuptial nectaries explain secretion**
2 **changes throughout flower lifetime and allow for multiple ecological interactions**

3
4 **Running Title:** Structure and ultrastructure of nuptial and extranuptial nectaries

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1 **Abstract**

2 Nectaries are specialized nectar-producing structures. Nectar traits affect animal behaviour
3 and ecological and evolutionary processes, such as pollination and biotic defence. We
4 previously found that there are differences in the characteristics of nuptial and extranuptial
5 nectar and in the types of animals that visit each nectary in *Amphilophium mansoanum*
6 (Bignoniaceae) flowers. We now hypothesize that nectar traits reflect the anatomical,
7 histochemical, and subcellular characteristics of each nectary type. Using routine light
8 microscopy, scanning electron microscopy, and transmission electron microscopy methods,
9 we studied nuptial and extranuptial nectaries (NN and ENN, respectively) in young flower
10 buds, pre-anthesis buds, and first- and second-day flowers. NN was a prominent annular disk,
11 whereas ENN was a concave, patelliform trichome. Only ENN contained alkaloids, while
12 both nectaries contained starch grains, lipid droplets, proteins, terpenes and phenolic
13 compounds. Both nectaries showed subcellular organization consistent with hydrophilic and
14 lipophilic secretion, the latter being predominant in second-day flowers. In NN, the
15 subnectary parenchyma had phloem and amyloplasts until pre-anthesis. Starch grains
16 decreased and tracheary elements were seen in newly opened flowers. ENN are not
17 vascularised, with vascular bundles from the calyx approaching the base of the nectary.
18 Starch grains were scarce and very small in the ENN secretory head cells. Fibrillary proteins
19 were found only in NN and periplastidial reticulum was observed only in ENN. In NN, nectar
20 secretion begins shortly before anthesis, being released through the raised stomata and the
21 reticulate cuticle. In ENN, secretion extends from the young flower bud stage to senescent
22 flowers, accumulating in small subcuticular spaces and being continuously released through
23 the intact cuticle. Temporal differences in nuptial and extranuptial nectar production, as well
24 as in the structural characteristics and nectar release mechanisms between nuptial and
25 extranuptial nectaries, may explain the differences in nectar characteristics of *A. mansoanum*.

26
27 **Key words:** ant, bees, extranuptial nectary, mutualism, nuptial nectary, ultrastructure.

28 **Introduction**

29 Nectar, a sugar-rich secretion, is a highly energetic and ready-to-consume primary
30 resource for several animals (Nepi *et al.* 2018; Nicolson 2007, 2022). When these animals
31 forage among different plant species, they come across nectar with different volume,

1 chemical profiles (including sugars, amino acids, proteins, lipids, organic acids and
2 secondary compounds), and concentrations (Calixto *et al.* 2021; Barberis *et al.* 2023; Zhou
3 *et al.* 2024). Due to the effects of these traits on animal's foraging behaviour and movement,
4 nectar is an important mediator of mutualisms between plants and animals, including
5 pollination and indirect defence against herbivores (Nicolson 2007, 2022; Heil 2015; McPeck
6 *et al.* 2021; Mogensen *et al.* 2024). When plants and animals exert selective pressures on
7 each other, these interactions can either increase or decrease reproductive and survival
8 success, thereby affecting ecological and evolutionary processes (Nepi 2017; Nepi *et al.*
9 2018; Suissa *et al.* 2024).

10 Nectaries, *i.e.*, structures specialized in nectar production, show a wide range of sizes,
11 morphology, anatomy, and locations (Erbar 2014 and references therein), which may
12 influence nectar traits (Petanidou *et al.* 2000; Nepi 2007). Based on their topography, they
13 are further categorized as floral or extrafloral nectaries (Caspary 1848). From a functional
14 perspective, nectaries can be classified as nuptial, when associated with pollination, or
15 extranuptial, when associated with defence (Delpino 1873).

16 Considering that nectaries have evolved independently across angiosperms (Weber
17 and Keeler 2013; Phukela *et al.* 2020), including different lineages of Bignoniaceae
18 (Bignoniaceae) (Nogueira *et al.* 2013), and the cost of nectar production for plants
19 (Southwick 1984; Pyke 1991), it is expected that mechanisms of nectar synthesis and release
20 would be variable among plant organs (Nepi 2007; Chatt *et al.* 2021) and flower
21 developmental stages (Nepi 2007; Guimarães *et al.* 2016). Besides that, traits such as
22 vascularisation, may severely affect nectar traits (Frey-Wyssling 1955; Galetto 1995).
23 Therefore, studying nectary micromorphology, anatomy, and ultrastructure is essential to
24 understand the relationships between plants, visiting animals, and the environment (Kostroyco
25 and Chwil 2022; Almeida *et al.* 2023).

26 Members of the Bignoniaceae family, including *Amphilophium mansoanum* (DC.)
27 L.G. Lohmann [sin. *Distictella mansoana* (Vahl) Urb.], commonly have two nectary types in
28 the same species, nuptial and extranuptial, which are usually morphologically different
29 (Galetto 1995; Balduino *et al.* 2023) and have distinct evolutionary origins (Lohmann and
30 Taylor 2014). Nuptial nectaries are typically annular disks at the ovary base (Thomas and

1 Dave 1992; Galetto 1995, 2009; Bernardello 2007; Rivera 2000a; Guimarães *et al.* 2016,
2 2018), composed of a unistratified epidermis, nectary and subnectary parenchyma supplied
3 with phloem (*sensu* Nepi 2007). Their secretion begins before anthesis, so that nectar is
4 already available when a flower opens (Guimarães *et al.* 2016, 2018). Nectar-secreting cells
5 contain abundant mitochondria, rough endoplasmic reticulum, Golgi bodies with numerous
6 vesicles, and amyloplasts, which degrade as anthesis progresses (Guimarães *et al.* 2016,
7 2018; Machado *et al.* 2017). Secretion may shift from hydrophilic to lipophilic during
8 anthesis, which is distinguished by an increase in plastoglobuli, and is released through
9 modified stomata, cuticular pores, or microchannels (Machado *et al.* 2016; Guimarães *et al.*
10 2016, 2018). In contrast, extranuptial nectaries are glandular trichomes that vary in shape,
11 size, number, and location (Seibert 1948; Rivera 2000b; Nogueira *et al.* 2013; Fróes *et al.*
12 2015; Miranda *et al.* 2024). These trichomes are composed of a stalk and a multicellular
13 secretory head, with cells showing abundant mitochondria, endoplasmic reticulum,
14 leucoplasts, few dictyosomes, and wall ingrowths with plasmodesmata between the stalk and
15 the secretory head cells (Gama *et al.* 2016). Different types of nectar-secreting trichomes
16 (putative nectaries) may occur adjacently on the same perianth whorl (Elias and Gelband
17 1976; Galetto 1995; Nogueira *et al.* 2013; Fróes *et al.* 2015).

18 In a previous study with *A. mansoanum*, two types of nectaries were found within a
19 given flower, nuptial (annular disk) and extranuptial (patelliform glands near the calyx
20 margins), which nectar differs in composition, volume, concentration, and secretion
21 dynamics (Balduino *et al.* 2023). Furthermore, since nuptial nectaries are visited by medium
22 and large bees, while extranuptial nectaries are visited mainly by ants. Nectar from both
23 nectary types differed regarding chemical composition, being nuptial nectar sucrose-
24 dominated and extranuptial nectar hexose-rich, the first had a more variable amino acid
25 profile than the latter and had a theophylline-like alkaloid, which was exclusive from nuptial
26 nectar (Balduino *et al.* 2023).

27 While this previous study focused on the ecological and chemical aspects of nectar,
28 in this research we aim, to expand upon those findings by investigating the anatomy,
29 ultrastructure and histochemistry of the nuptial and extranuptial nectaries that co-reside in *A.*
30 *mansoanum* flowers from a developmental perspective. Studying the structure and
31 functioning of the different nectaries within a given flower expands our understanding on the

1 relationships between nectaries and their secretions, which mediate plant-animal interactions.
2 We hypothesize that the specificities of nectar from nuptial and extranuptial nectaries of *A.*
3 *mansoanum* (shown in Balduino *et al.* 2023) reflect underlying subcellular specializations.
4 Beyond that, nectaries that coexist within the same flower are subjected to the same
5 environmental and physiological conditions. Therefore, by comparing the structural
6 mechanisms that govern the nectar secretion process in distinct nectaries and their respective
7 functioning, we can gain deeper insights into how nectary features relate to their ecological
8 functions.

10 **Material and Methods**

11 *Plant material and study site*

12 *Amphilophium mansoanum* is a liana native to Brazil but not endemic, exhibiting a
13 broad geographic distribution across the country, particularly on the edges of tropical forests
14 and savannas (Lohmann and Taylor 2014). The zygomorphic, pentamerous flowers are
15 arranged in racemose panicles, although simple racemes may occasionally occur (Pool, 2009)
16 (Fig. 1A). The dome-shaped calyx is coriaceous with a cluster of glands near its margins
17 (Fig. 1B). The corolla is infundibuliform, bent at ca. 90° above the base, coriaceous, with
18 five lobes, imbricate, white and internally yellow (Fig. 1A). The androecium has four stamens
19 and included anthers. The ovary is sessile, and the nectary disk is annular (Fig. 1C). Drops
20 of viscous secretion (Fig. 1D) occur on the calyx surface from young floral buds to open
21 flowers.

22 We sampled flowers (n= 3 flowers per stage, 4 plants) at four different stages of development:
23 young buds (10-20 mm), pre-anthesis buds (40-50 mm), freshly opened flowers (first-day
24 flowers) and flowers around 24 hours from opening (second-day flowers). The samples of
25 the nuptial nectary (NN) and extranuptial nectary (ENN) were collected in the morning hours
26 from natural populations of *A. mansoanum* at the edge of cerrado vegetation in the Midwest
27 of São Paulo State, Brazil (22°53'56"S and 48°29'68"W; 966 m), from November 2021 to
28 February 2023. A voucher specimen was deposited at the BOTU herbarium (Águas de Santa
29 Bárbara, São Paulo, Brazil) under number 41575. The collection was made by T.C. Monteiro
30 and H.K. Balduino.

31

1 *Structural, histochemical and ultrastructural studies*

2 For general histological characterization, samples were fixed in 2.5% glutaraldehyde
3 in 0.025 M phosphate buffer (Feder and O'Brien 1968) in field conditions and maintained
4 overnight at 4°C in the laboratory. Specimens were dehydrated through graded series of
5 ethanol solutions (60-100%), and embedded in glycol methacrylate historesin (Leica
6 Microsystems, Nussloch/Heidelberg, Germany). Thin sections (4-6 µm) were obtained using
7 a semiautomatic microtome (Leica RM2245), placed on microscopic glass slides, stained
8 with 1% toluidine blue in 1% aqueous sodium tetraborate solution (O'Brien *et al.* 1964) and
9 mounted in synthetic resin (Entellan, Merck KGaA, Darmstadt, Germany). The results of the
10 light microscopic examinations were documented with a photomicroscope (Leica DMR)
11 equipped with a digital camera (Leica DFC 500).

12 For histochemical studies, fresh NN and ENN samples were hand-cut using a razor
13 blades and subjected to the following treatments: Alcian blue for polysaccharides and acid
14 polysaccharides (Figueiredo *et al.* 2007); Lugol's reagent for alkaloids and starch grains
15 (Johansen 1940); ferric chloride for phenolic compounds (Johansen 1940); mercuric
16 bromophenol blue for proteins (Mazia *et al.* 1953); NADI reagent (α -naphthol + dimethyl-
17 p-phenylenediamine) for essential oils and oleo-resins (David and Carde 1964); ruthenium
18 red for pectin (Jensen 1962); and Sudan IV for total lipids (Johansen 1940). When required,
19 standard control sections were prepared at the same time. All observations and
20 documentation were performed using a light microscope (Leica DMR) with a digital camera
21 (Leica DFC 500).

22 For surface examination, portions of the flowers were cut with razor blades in the
23 median longitudinal and transversal planes. The samples were fixed in 2.5% glutaraldehyde
24 (0.1 mol. L⁻¹ phosphate buffer, pH 7.2), dehydrated in an aqueous ethanolic series, critical-
25 point dried (Bal-Tec CPD 030), mounted on a metallic support with adhesive, and sputtered
26 coated with gold (10 nm) (Bal-Tec SCD 050) following Bozzola and Russel (1992). The
27 material was then examined with a scanning electron microscope (SEM Quanta 200, Fei
28 Company, FEI, Gräfelfing, Germany) at 20 kV.

29 For cellular ultrastructure examination, the fragments were fixed in 2.5%
30 glutaraldehyde (0.1 M sodium phosphate buffer pH 7.3, overnight at 4° C) and post-fixed
31 with 1% osmium tetroxide (OsO₄) in the same buffer for 2 h at room temperature (Hayat

1 1989). After washing in distilled water, the material was dehydrated in aqueous acetone and
2 embedded in Araldite resin (Araldite 502, Electron Microscopy Sciences, Hatfield, USA).
3 Semi-thin sections (0.5 μm) were obtained using an ultramicrotome (Leica Reichert) and
4 stained with 0.05% toluidine blue (O'Brien *et al.* 1964). Ultrathin sections (60 nm) were
5 stained with 2% uranyl acetate solution for 15 min and lead citrate for 15 min (Reynolds
6 1963) and observed with a transmission electron microscope (TEM Tecnai Spirit, FEI
7 Company, Germany) at 60 kV.

8 We also employed the zinc iodide–osmium tetroxide (ZIO) method for
9 endomembrane impregnation (Reinecke and Walther 1978). After primary fixation, the
10 samples were incubated in a solution containing Zn, I, TRIS-aminomethane and 1.0%
11 osmium tetroxide (Reinecke and Walther 1978), and processed conventionally for TEM.

13 Results

14 *Nuptial nectary structure, ultrastructure and secretion*

15 The nuptial nectary (NN) is an annular disk located below the ovary (Fig. 1C, 2A).
16 The expanded base of the stamen filaments creates a constriction that surrounds the nectary
17 and forms a nectar chamber (Fig. 1C), where the secreted nectar builds up. Nectar secretion
18 begins in pre-anthesis floral buds. At this developmental stage, nectary surface had common
19 epidermal cells with different sizes and shapes, with anticlinal walls that were undulating to
20 different degrees and external periclinal walls sticking out (Fig. 2B-C). Numerous stomata
21 also with different shapes and sizes were distributed across the surface of the nectary (Fig.
22 2B-C). The curvature of the external periclinal wall varied from flat to slightly convex to
23 rounded (Fig. 2C). These included ordinary stomata with a long narrow opening and an outer
24 stomatal rim, as well as stomata that were sunken or raised (Fig. 2B-C). Giant and raised
25 stomata with a large opening and stomatal border, elevated by their subsidiary cells, were
26 commonly observed (Fig. 2D). First-day flowers showed flocculent secretion residues
27 covering the nectary surface (Fig. 2E). At this point, the giant raised stomata had a bigger
28 round opening that was full of secretion residues (Fig. 2F).

29 Cross-sections of the NN of pre-anthesis buds showed three different regions under a
30 light microscope (Fig. 3A). They featured a single-layered epidermis, a nectary parenchyma
31 situated beneath the epidermis, and a large subnectary parenchyma that was densely

1 vascularised by vascular bundles originating from the flower receptacle (Fig. 3A). At this
2 stage, a clear separation of the nectary and subnectary parenchyma was evident (Fig. 3A):
3 the nectary parenchyma was made up of conspicuous cells with an ovoid to isodiametric
4 shape that were arranged in 3-5 nonvascularised parenchymal layers (Fig. 3B); the subnectary
5 parenchyma was made up of ovoid cells that were arranged in several layers with
6 proliferating vascular bundles, mostly phloem (Fig. 3C). On the first day of anthesis, flowers
7 had an increase in voluminous starch grains in the subnectary parenchyma cells (Fig. 3D),
8 while in the nectary parenchyma, the intercellular spaces developed and were filled with
9 secretions (Fig. 3E). Stomata with large open pores are evident at this stage (Fig. 3F). On the
10 second day of anthesis, the epidermal cells and parenchyma cells displayed dense
11 accumulations (Fig. 3G-H), while the starch grains were either absent or scarce (Fig. 3I). At
12 this stage, parenchyma cells contained homogenous content (Fig. 3I), probably phenolic
13 compounds, and tracheary elements in the subnectary parenchyma (Fig. 3J).

14 The histochemical characterization of the disk tissues of first-day flowers revealed
15 positive reaction for hydrophilic and lipophilic substances (Fig. 4; Table 1). Tests with
16 mercury blue bromophenol detected basic proteins stained in blue in the subnectary
17 parenchyma cells, mainly in the vascular bundles (Fig. 4A), and in nectary parenchyma cells
18 (Fig. 4 B). Alcian blue highlighted acid polysaccharides substances (Fig. 4C). Tests with
19 NADI reagent also showed a positive result, developing both a pink coloration (characteristic
20 of oil-resins) and a blue colouration (characteristic of essential oils) in the parenchyma cells
21 (Fig. 4D). Tests with Lugol revealed accumulation of big starch grains in all disk tissues, but
22 more numerous in the subnectary parenchyma (Fig. 4E). Tests with Sudan IV stained in red
23 lipid droplets (Fig. 4F) and accumulations of lipid material in the intercellular spaces (Fig.
24 4G). With ferric chloride, all the nectary cells were stained brown (Fig. 4H), indicating the
25 presence of phenolic substances. Tests with ruthenium red stained mainly cell walls (Fig. 4I).

26 Under TEM, NN epidermal cells of the pre-anthesis buds displayed thick external
27 periclinal walls covered by a thin cuticle (Fig. 5A). There were a lot of free ribosomes,
28 mitochondria, plastids (Fig. 5B-C) and rough endoplasmic reticulum with cisternae and
29 vesicles filled with dense content, which was marked by ZIO (Fig. 5C). Amyloplast
30 containing starch grains and leucoplasts with dense matrix devoid of inner membranes and
31 containing a large osmiophilic inclusion occurred side by side in the epidermal cell (Fig. 5B).

1 The cytosol displayed oil drops (Fig. 5A, C). The vacuoles contained osmiophilic inclusions
2 and flocculent materials (Fig. 5C). Accumulations of similar materials also occurred within
3 the periplasmic space (Fig. 5A-C). In the nectary parenchyma (Fig. 5D-F), the cells exhibited
4 compact arrangement, thin walls, large nuclei, and vacuoles of varied sizes (Fig. 5D), along
5 with amyloplasts exhibiting different sizes and signs of degradation (Fig. 5E). These cells
6 regularly displayed leucoplasts containing small starch grains and osmiophilic globules
7 (terpenes/oils/phenols) (Fig. 5F). Inside the vacuoles, flocculent contents and membrane
8 debris were observed (Fig. 5D, F). In the subnectary parenchyma, the cells were larger and
9 contained numerous amyloplasts (Fig. 5G-I), as well as prominent nucleus (Fig. 5I). The
10 vacuoles varied in size and contained flocculent materials (Fig. 5H-I). The intercellular
11 spaces were filled with flocculant materials (Fig. 5I). Plasmodesmata were seen in the regions
12 of contact between parenchyma cells (Fig. 5H). At this stage, the phloem was the main
13 vascular tissue in the subnectary parenchyma (Fig. 5J), with sieve tube elements displaying
14 a wide calibre (Fig. 5K), and parenchyma cells showing amyloplasts with voluminous starch
15 grains (Fig. 5L).

16 The nuptial nectaries of first-day flowers contained epidermal cells characterized by
17 large vacuoles at the distal pole (Fig. 6A) and densely granulated cytoplasm (Fig. 6B).
18 Amyloplasts were localized at the base of the epidermal cells and were characterized by very
19 electron-dense stroma and broken-down starch grains (Fig. 6A). Lipid droplets occurred
20 dispersed in the cytoplasm and near the plasma membrane (Fig. 6B). The cuticle featured a
21 large cuticular layer with a network of electron-dense ramifications that does not extend to
22 the cuticle itself (Fig. 6C). This ultrastructural organization of the cuticle suggests a pattern
23 resembling microchannels, through which secretion reaches the surface. Secretion residues
24 were observed on the cuticle (Fig. 6C). At this stage, the nectary (Fig. 6D) and subnectary
25 parenchyma (Fig. 6E) cells showed increased vacuolization and a higher number of
26 amyloplasts, which consist of starch grains being broken-down. Small vacuoles were either
27 juxtaposed or fused with larger vacuoles (Fig. 6D-E), which contained fibrillary or
28 flocculated materials, membrane debris, and osmiophilic inclusions (Fig. 6E). Intercellular
29 spaces were wider and filled with flocculant material (Fig. 6E). In the phloem parenchyma
30 cells, signs of degradation were noted in the amyloplasts (Fig. 6F). Additionally, we observed
31 sieve tube elements with callose-plugged sieve plates (Fig. 6F-G). In the cytoplasm of

1 phloem parenchyma cells, bundles of fibrillar protein were abundant, especially near the
2 vacuoles (Fig. 6H). These cells had globular mitochondria, endoplasmic reticulum segments,
3 dictyosomes, and ZIO marker-improved vesicles view (Fig. 6I).

4 In the nuptial nectary of second-day flowers, cytoplasm was reduced to a thin parietal
5 layer, and a central vacuole had been formed in epidermal (Fig. 6J), nectary parenchyma
6 (Fig. 6 K), and subnectary parenchyma cells (Fig. 6L). Amyloplasts (Fig. 6J) exhibited
7 residual starch grains, dense stroma and large osmophilic inclusions (like terpenes/phenols).
8 Subnectary parenchyma cells showed a big nucleus, larger vacuoles, and amyloplasts that
9 were full of osmophilic substances (e.g. phenols and terpenes) and broken-down starch grains
10 (Fig. 6L–M). These cells also exhibited segments of endoplasmic reticulum and
11 mitochondria (Fig. 6M). Intercellular spaces were wider (Fig. 6L) and filled with flocculent
12 material (Fig. 6N). Osmiophilic bodies were observed attached to the tonoplast (Fig. 6N).

13 14 *Extranuptial nectary structure, ultrastructure and secretion*

15 Under SEM, the secretion-producing areas on the calyx exhibited patelliform-
16 concave glands surrounded by simple, uniseriate and pluricellular non-glandular trichomes
17 with pointy apical cells and warty ornamentations, as well as peltate glandular trichomes
18 featuring a flat head and raised stomata (Fig. 7A-C). The extended margins of the patelliform
19 glands protruded above the calyx surface (Fig. 7B, D). Transversal sections through the gland
20 revealed that the concave portion was aligned with the epidermis, while the foot was
21 immersed in the calyx mesophyll (Fig. 7D). The glands of young buds (Fig. 7E-F) did not
22 possess fully developed edges, but displayed three distinct areas: i) a concave, multicellular
23 secretory head composed of very long, narrow, thin-walled cells covered with a thick cuticle;
24 ii) a short stalk that extends for the length of the secretory head; and iii) a foot, or base,
25 comprised of numerous tangentially expanded cells. A single layer of flattened, rectangular
26 cells with thick anticlinal walls constitutes the stalk, connecting the secretory head to the
27 nectary foot (Fig. 7E-F). The foot of the gland is not vascularised and consisted of 3-5 layers
28 of cells with varied sizes and shapes, which contents are strongly stained with toluidine blue
29 (Fig. 7E-F). Vascular bundles from the calyx mesophyll circumscribe the gland foot, but they
30 do not extend through it (Fig. 8A).

1 In young buds, these glands are already secreting material despite the edges not being
2 fully developed yet. The exudate was stored beneath the cuticle in the centre of their concave
3 head (Fig. 7G). As differentiation progressed, the edge of the concave gland widened, and all
4 gland cells, except for the stalk cells, became denser and more toluidine blue-stained (Fig.
5 7H). Progressively, the edges become more extended and the vacuoles in the head and foot
6 cells become more developed (Fig. 7I) on first-day flowers. Additionally, the head secretory
7 cells exhibit thicker walls. They possess a conspicuous nucleus with well-organized
8 nucleolus, less cytoplasm, and several small vacuoles (Fig. 7J). The thick-walled cells in the
9 gland stalk contained large a vacuole and reduced cytoplasm (Fig. 7J).

10 Histochemical analysis of mature ENN in first-day flowers revealed positive reaction
11 for hydrophilic and lipophilic substances (Fig. 8; Table 1). With ferric chloride, phenolic
12 substances were stained brown and were detected in all nectary cells (Fig. 8A), with a higher
13 concentration in the gland's apex (Fig. 8B). After Sudan IV tests, the thick cuticle and lateral
14 cell walls of the stalk were better delimited (Fig. 8C). These tests also revealed that head
15 secretory cells (Fig. 8D) and foot cells (Fig. 8E) contained abundant lipid droplets. Basic
16 proteins were abundant in the head cells (Fig. 8F). Oil-resins, typically stained in pink with
17 NADI reagent, were also found in the head cells (Fig. 8E). With Lugol's reagent, small
18 amounts of starch grains were detected in the head cells, whereas the contents of the foot
19 cells acquired a reddish-brown colour (Fig. 8F), indicating the presence of alkaloids.
20 Ruthenium red treatment evinced an abundance of pectin in the thick cell walls of the gland
21 foot and of the central region of the secretory head (Fig. 8I).

22 From young floral buds to second-day flowers, ENN were active in secretion. Under
23 TEM, the secretory head cells in young buds were featured with large nuclei with organized
24 nucleoli, dense cytoplasm, poorly developed or absent vacuoles (Fig. 9A), and amyloplasts
25 with abundant starch grains (Fig. 9B-C). ZIO reaction showed that the dictyosomes were
26 well-developed and active in vesicle production (Fig. 9C). In pre-anthesis buds (Fig. 9D-I),
27 the head cells had a lot of mitochondria and long rough endoplasmic reticulum profiles (Fig.
28 9D). Leucoplasts had residual starch grains and big osmiophilic inclusions (Fig. 9E).
29 Elongated plastids devoid of inner membranes and presenting homogeneous stroma and
30 periplastidial reticulum (Fig. 9F) were found in the head cells. Plasmodesmata were seen in
31 the anticlinal walls connecting the secretory head cells (Fig. 9E-F). Golgi bodies cisternae

1 and vesicles with electron-dense material (Fig. 9G), marked by ZIO, were found in the head
2 cells. Stalk cells had thicker lateral walls and very thin anticlinal walls (Fig. 9H). They also
3 had large nuclei with evident nucleolus, dense cytoplasm with a lot of rough endoplasmic
4 reticulum, and small vacuoles (Fig. 9H). Gland foot cells were tangentially expanded and
5 had small vacuoles with dark inclusions, as well as cytoplasmic oil bodies (Fig. 9I).

6 In first-day flowers, cuticle was thicker and contained accumulations of lipids and an
7 electron dense material (Fig.10A). The nuclei were central and spherical, with abundant
8 cytoplasm displaying large lipid droplets (Fig. 10B). At this stage, there were periplasmic
9 spaces and paramural bodies along the anticlinal walls (Fig. 10C), which exhibited areas with
10 lipid inclusions (Fig. 10D). These cells contained polymorphic plastids with finely granular
11 stroma and osmiophilic inclusions (Fig. 10E), likely terpene/phenols. In stalk cells, there
12 were also lipid bodies, mitochondria, and small vacuoles (Fig. 10F). In the gland foot,
13 intercellular spaces increased in size and were filled with flocculent material (Fig. 10G). In
14 foot cells, the vacuoles were small, contained electron-dense bodies (Fig. 10G) and numerous
15 mitochondria (Fig. 10H). In the secretory head of second-day flowers there were more dark
16 cells (Fig. 10I) when compared to the previous stage (Fig. 10A). In addition, large
17 accumulations of electron dense material were visible in the cuticle (Fig. 10I-J). The dark
18 cells contained abundant cytoplasm with intact organelles and small vacuoles (Fig. 10K),
19 globose mitochondria, well-developed Golgi bodies with numerous cisternae, sparse profiles
20 of endoplasmic reticulum, and oil bodies (Fig. 10L).

21 **Discussion**

22 The location and morphology of the nectary disk (nuptial nectary, NN) of *A.*
23 *mansoanum* follow the pattern observed in other Bignoniaceae species (Gentry 1974; Elias
24 and Gelband 1976; Galetto 1995; Rivera 2000; Guimarães *et al.* 2016; Frazão *et al.* 2020),
25 as do the location and morphology of patelliform-concave glands (extranuptial nectary,
26 ENN), as reported in different Bignoniaceae taxa (Subramanian and Inamdar 1985; Galetto
27 1995; Rivera 2000b; Díaz-Castelazo 2005; Nogueira *et al.* 2013; Gama *et al.* 2013; Fróes *et*
28 *al.* 2015). The morphology and anatomy of the annular disks of *A. mansoanum* flowers aligns
29 with the pattern suggested by Nepi (2007), being featured with uniseriate epidermis, nectary
30

1 parenchyma, and a wider densely vascularised subnectary parenchyma. The patelliform
2 glands in *A. mansoanum* flowers have extended margins that project above the calyx surface.

3 Both nectaries exhibit cellular apparatus typical of nectar secretion; however, NN and
4 ENN display distinct structural characteristics, which likely lead to varied nectar features
5 (volume, concentration, and chemical composition).

6 Patelliform glands of *A. mansoanum* have a complex structure consisting of three
7 specialized regions: nonvascularised foot, stalk, and secretory head. For some authors, such
8 glands represent a symplesiomorphy of the group and are derived from peltate trichomes,
9 showing similar anatomical structure, but different shapes, differential proliferation and cell
10 expansion (Rivera 2000b; González 2013; Nogueira *et al.* 2013). Moreover, their stalk layers
11 are considered an equivalent feature, differentiated by cell number and shape and by the
12 number of cell layers (Fróes *et al.* 2015; Machado and Rodrigues 2021).

13 Similarly to other Bignoniaceae species (Machado *et al.* 2016; Guimarães *et al.* 2016,
14 2018), we saw changes in NN and ENN ultrastructure along flower development. These
15 changes likely contribute to the differences in nuptial and extranuptial nectar traits of *A.*
16 *mansoanum* recorded by Balduino *et al.* (2023).

17 Nuptial and extranuptial nectaries showed a significant difference in their sugar
18 sources. In NN, starch grains, stored in the parenchyma cells during flower lifetime, are
19 depleted during the pre-anthesis stage and on the first day of anthesis, as reported by Pacini
20 *et al.* (2003). This information associated with NN vascularisation indicates that NN can get
21 their energy directly from the phloem. In tun, starch grains are absent or present in minimal
22 amounts in ENN. Thus, the nectar sugar may originate directly from photosynthesis within
23 the nectary or from the calyx tissues surrounding the gland foot, as described in
24 nonvascularised nectaries (Nepi 2007). It is noticeable that the foot cells of the patelliform
25 glands (immersed in the calyx mesophyll) have more abundant plasmodesmata when
26 compared to other gland regions. The calyx's vascular tissues end at the base of the gland,
27 and do not penetrate the foot of the gland. Pre-nectar likely exits the sieve tubes through an
28 apoplastic route via intercellular spaces, reaches the gland foot, and follows a symplast route
29 in an ascending Trajectory through plasmodesmata, reaching the stalk cells. The apoplasmic
30 barrier in the lateral cell walls of the stalk cells directs the pre-nectar towards the secretory
31 head (Fahn 1979; Machado and Rodrigues 2021). In *A. mansoanum*, there is a substantial

1 difference in nectar volume between the two types of nectaries (Balduino *et al.* 2023).
2 Although this is not a general rule, this pattern is consistent with what has been reported by
3 Díaz-Castelazo *et al.* (2005) for other ENN, as NN are directly vascularised, while ENN are
4 smaller and nonvascularised. However, the clustered ENN, considered an adaptative trait
5 (Elias and Newcombe 1979), may collectively produce enough nectar to ensure ant visitation
6 to the calyx surface.

7 It is noticeable that the development level of the phloem and xylem in NN varies
8 between pre-anthesis buds and open flowers, with a predominance of phloem in pre-anthesis
9 buds. During this stage, phloem was characterized by large-calibre sieve tubular elements,
10 indicating that non-reducing sugars (and other solutes) are being unloaded into the subnectary
11 parenchyma where they will be stored as starch grains (Taiz *et al.* 2013). In most
12 Bignoniaceae species, nuptial nectary is supplied by phloem elements, except for *Tabebuia*
13 *heptaphylla* (Vellozo) Toledo, in which they are vascularised by both xylem and phloem
14 (Galletto 1995). A correlation between nectar concentration and nuptial nectary
15 vascularisation was highlighted by Frey-Wysling (1955) but remains poorly investigated.
16 Our study demonstrated that the nuptial nectary of *A. mansoanum* is vascularised with
17 phloem and xylem. The appearance of tracheary elements in the subnectary parenchyma of
18 first-day flowers indicates a change in the translocation route and pre-nectar components,
19 which is common depending on the stage of organ development and on the vascular
20 connections between source and sink (Taiz *et al.* 2013). This change means that water,
21 supplied both by phloem and xylem, is being used to compose nectar, which may result in a
22 more diluted secretion with lower sugar concentration. This is consistent with the
23 characteristics of *A. mansoanum* nuptial nectar reported by Balduino *et al.* (2023), which was
24 found to be less concentrated and more abundant than that of other Bignoniaceae species
25 (Galletto 1995, 2009). Both bee pollinator species reported visiting *A. mansoanum* flowers
26 are from the Centridini tribe (Yanagizawa and Gottsberger 1982; Balduino *et al.* 2023).
27 Although they prefer nectar with higher sugar concentrations (Roubik *et al.* 1995), it's
28 possible that they can use physiological and behavioural adaptations to concentrate diluted
29 nectar (Nicolson 2009), which may allow them to explore a wider range of nectar
30 concentrations (Galletto 2009), as well as different volumes.

1 The flowers of *A. mansoanum* quickly release a lot of nuptial nectar (Balduino *et al.*
2 2023). This might happen because of the large amyloplasts in the parenchyma cells, which
3 differentiate before secretion and lose most of their starch grains. Thus, starch grains were
4 either used to make nectar sugars or to provided energy for the secretory process (Fahn and
5 Shimony 2001; Stpiczyńska *et al.* 2005; Heil 2011; Guimarães *et al.* 2018). Based on a disk
6 surface SEM study, it appears that the modified and raised stomata of *A. mansoanum*
7 primarily facilitate nectar release (Gaffal *et al.* 1998; Wist and Davis 2006; Tölke *et al.* 2015).
8 TEM examination of the cuticle that cover the disk epidermis revealed a reticulated structure,
9 which may enhance permeation and facilitate the release of fat-soluble substances (Paiva
10 2016, 2017). The observation of secretion residues on the cuticle adds to the evidence that
11 lipophilic components of secretion are also being released through microchannels. Ascensão
12 *et al.* (1997) suggested that microchannels constitute the exit route of volatile substances. On
13 the other hand, secretion accumulates in small subcuticular spaces in patelliform glands,
14 particularly within the central epidermal cells, and is released through the cuticle or small
15 pores. These findings suggest that extranuptial nectar release occurs continuously, while
16 nuptial nectary releases secretion all at once through raised stomata. These findings align
17 with the variation of nuptial and extranuptial nectar production in space and time. Nuptial
18 nectary has a short lifespan and is active in secretion from pre-anthesis buds to second-day
19 flowers, while extranuptial nectary is active from young buds to senescent flowers, and
20 apparently to young fruits.

21 In general, the ultrastructural organization of the epidermal secretory cells in both NN
22 and ENN is made up of a complex system of endomembranes, including the endoplasmic
23 reticulum, dictyosomes, and vesicles. Dictyosomes in the gland cells are associated with the
24 production of hydrophilic substances, while free ribosomes and rough endoplasmic reticulum
25 elements can be related to the synthesis of enzymes involved in starch grain hydrolysis and
26 degradation processes, which were observed during nectar secretion (Paiva and Machado
27 2008; Possobom *et al.* 2010; Tölke *et al.* 2015; Guimarães *et al.* 2016, 2018). However, the
28 nectaries of *A. mansoanum* differ in abundance and volume of amyloplasts, mitochondria,
29 oil droplets, and degree of RER development. Moreover, the presence of fibrillar proteins
30 was observed only in NN, while plastids with periplastidial reticulum were observed only in
31 ENN. Recent findings highlight how mitochondria, along with plastids and the cytosol, help

1 produce terpenes (Pazouki and Niinemets 2016). Thus, the increase in number of
2 mitochondria observed here can be indicative of their involvement in terpene synthesis in
3 NN. The increase in the presence of lipids is commonly reported throughout nectary
4 development (Tölke *et al.* 2015; Guimarães *et al.* 2016, 2018). The presence of oil droplets
5 in vacuoles, scattered throughout the cytosol and within the periplasmic spaces, which are
6 more abundant in ENN than in NN, is ultrastructural evidence of lipidic secretion (Canaveze
7 *et al.* 2021; de Deus Bento *et al.* 2024). Plastid morphology in the secretory cells is quite
8 similar to that of plastids involved in the synthesis of terpenes/phenol in lipophilic glands
9 (Kowalkowska *et al.* 2012, 2015; Machado *et al.* 2023), but is uncommon in nectariferous
10 tissues (Guimarães *et al.* 2018). Numerous studies have indicated that plastids are involved
11 in the synthesis of volatile compounds, primarily of phenolic and terpenoid compounds
12 (Pridgeon and Stern 1985; Stern *et al.* 1987; Pais and Figueiredo 1994; Kowalkowska *et al.*
13 2012; Stpiczyńska and Davies 2016; Wiśniewska *et al.* 2018; Guimarães *et al.* 2018). The
14 presence of polymorphic plastids containing lipophilic inclusions provides strong evidence
15 that these structures are involved in scent production (Antón *et al.* 2012; Kowalkowska *et al.*
16 2015; Guimarães *et al.* 2018). Additionally, periplastidial reticulum contributes to the
17 translocation and/or temporary concentration of lipophilic secretions (Sá-Haiad *et al.* 2015;
18 Canaveze *et al.* 2021). It's possible that the osmium tetroxide-darkened material in the
19 plastids, cytoplasm, RER, and vacuoles is made up of unsaturated lipids, terpenes, and
20 phenolic compounds (Kowalkowska *et al.* 2015; Naczka *et al.* 2018), since osmium tetroxide
21 is known to have better penetration properties and a strong affinity for double bonds
22 (Angermuller and Fahini 1982). This is consistent with our histochemical tests that revealed
23 lipids, terpenes, and phenolic compounds in the secretory cells. Despite the low specificity
24 of the histochemical tests, Sudan IV and NADI reagent have been widely used to,
25 respectively, locate total lipids and terpenes in secretory tissues (Figueiredo *et al.* 2007).
26 Lugol's reagent, containing potassium iodide, can mark alkaloids in plant cells, in addition
27 to staining starch grains (Figueiredo *et al.* 2007).

28 In NN, the presence of polysaccharides throughout the nectary, in cell walls and
29 cytoplasm, keeps the tissue hydrated and may facilitated pre-nectar transport through nectary
30 cells. As in NN, polysaccharides may also be related to nectar pathways in ENN. Their
31 presence in the secretory head may also contribute to the viscosity of nectar (Fahn and

1 Benouaiche 1979), which is a feature that affects ant foraging behaviour (Lois-Milevicich *et*
2 *al.* 2021) and may be related to the protection of nectar from desiccation and pathogens.
3 Additionally, although further investigation is needed, polysaccharides could modulate
4 volatile organic compound (VOC) liberation due to their distinct lipophilic nature. Thus, this
5 could be an ecologically relevant feature, as ants rely on chemical cues for their activities
6 (Knaden and Graham 2016; Nelson *et al.* 2019).

7 Both types of nectaries produce hydrophilic and lipophilic secretion at different
8 developmental stages as reported in other bee-pollinated Bignoniaceae species (Baker 1977;
9 Guimarães *et al.* 2018), which reveals the plastic potential of secretory cells. Some authors
10 associate the presence of lipids to environmental conditions, since they may help prevent
11 water loss and increase nectar's availability period (Cobert *et al.* 1979; Stpiczyńska *et al.*
12 2021; da Silva *et al.* 2025). Although this rationale applies to ENN, one can also interpret
13 this result in terms of how valuable lipophilic compounds are for plants, as they can be floral
14 scent compounds related to either attraction or repellence of animals, which may be
15 mutualists or antagonists (Slavković and Bendahmane 2023). For animals, they can
16 constitute part of dietary requirements that enhances their health (Barberis *et al.* 2023),
17 movement (Gilbert *et al.* 1965; Levin *et al.* 2017), development and reproduction (Stone *et*
18 *al.* 1985; Canavoso *et al.* 2001; Vaudo *et al.* 2015). Additionally, finding a source of nectar
19 that contains lipids can benefit bees, such as *A. mansoanum* pollinators, that collect oil or
20 mix oil with nectar to feed their larvae, since lipophilic compounds are more caloric than
21 carbohydrates. Therefore, the presence of lipids and terpenes/phenols in both types of floral
22 nectaries helps explain the diversity of animals with varying needs and ecological roles that
23 visit *A. mansoanum* (Balduino *et al.* 2023).

24 Proteins are generally associated with plant defence (Schmitt *et al.* 2021) and their
25 absence in ENN is a remarkable trait, since the proportion of protein amino acids in
26 extranuptial nectar is higher than in nuptial nectar (Balduino *et al.* 2023). High enzyme
27 activity could explain this result, especially because ENN is more subjected to
28 microorganism arrival (Nepi *et al.* 2012). The presence of alkaloids in *A. mansoanum* nectary
29 tissues is aligned with its nectar composition (Balduino *et al.* 2023) and may also protect
30 against pathogens and antagonist herbivores (González-Teuber *et al.* 2009). Besides,

1 alkaloids also have neuroactive effects on visitors' foraging behaviour (Barberis *et al.* 2023;
2 Mogonsen *et al.* 2024).

3 Nuptial nectaries suddenly release nectar right before anthesis starts, so when the
4 flowers open, pollinators can immediately obtain nectar. This implies that the nectar volume
5 available in nuptial nectaries is negatively linked to the number of bee visits received by a
6 given flower, showing the ephemerality of this trophic resource associated to pollination
7 mutualism. In contrast, extranuptial nectaries are active for a longer period of time. They
8 produce nectar daily from the early bud stage until the flower dies, which may take up to six
9 days (*unpublished data*). Even though nectar production stops in the afternoon (Balduino *et*
10 *al.* 2023), extranuptial nectaries resume nectar production again early in the morning of the
11 following day. This mechanism of long-lasting nectar production and secretion allows
12 extranuptial nectaries to be explored by visiting insects for several days in a single flower,
13 and even for several weeks, if one considers that there are floral buds in different stages
14 within a given inflorescence.

15 **Conclusion**

16 In *A. mansoanum*, nuptial and extranuptial nectaries coexist in the same flower and
17 are exposed to similar environmental and physiological conditions. Both nectaries exhibit
18 cellular apparatus typical of nectar secretion; however, they also display distinct structural
19 characteristics. These differences are especially clear when considering for how long each
20 type of nectary secretes and the different groups of animals that visit each nectary. Thus, the
21 changes in both nuptial and extranuptial nectary cellular machinery through flower lifetime
22 reflect the functioning of both nectaries, especially the duration of their secretory activity and
23 the interactions in which each nectary type is involved. Therefore, understanding how the
24 different nectaries within a flower function can enhance our comprehension of their
25 ecological roles in mediating interactions between plants and animals.

26 **Data availability statement**

27 The data underlying this article are available in the article.

28

29 **Conflict of Interest Statement**

1 None declared.

2

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19

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Figure legends

Figure 1. Inflorescence and flowers of *Amphilophium mansoanum* (Bignoniaceae). (A) Inflorescence with flowers in different developmental stages. Note ants on the calyx margin in the pre-anthesis bud. (B) Clustered prominent pores arranged in longitudinal bands at the distal region of the young bud. (C) Pre-anthesis bud sectioned longitudinally in the median plane showing a nectary at the base of the ovary and nectariferous chamber. (D) Extranuptial nectar droplets at the calyx margin of the young and pre-anthesis buds.

ALT TEXT: Photographs labelled A to D. A shows an inflorescence with flowers in different developmental stages with ants on the calyx margin in the pre-anthesis bud. B shows clustered prominent pores arranged in longitudinal bands at the distal region of the young bud. C shows a pre-anthesis bud sectioned longitudinally in the median plane showing a nectary at the base of the ovary and nectariferous chamber. D shows extranuptial nectar droplets at the calyx margin of the young and pre-anthesis buds.

Figure 2. Micrographs of the nuptial nectary of *Amphilophium mansoanum* (Bignoniaceae) (A) Appearance of the nectary disk in pre-anthesis buds seen under a stereomicroscope. (B-F) SEM micrographs. (B) Stomata irregularly distributed on the nectary disk surface. (C) Detail showing ordinary stomata together with prominent large stomata with wide pores. Note the guard cells with cuticular ledges. (D) Giant raised stoma. (E) First day flower; nectary disk covered with flocculant secretion residues. (F) Raised stoma with wide circular pore filled with secretion residues.

ALT TEXT: Micrographs of the nuptial nectary of *Amphilophium mansoanum* labelled A to F. A shows the nectary disk in pre-anthesis buds seen under a stereomicroscope. B-F are SEM micrographs. B shows stomata irregularly distributed on the nectary disk surface. C shows ordinary stomata together with prominent large stomata with wide pores, which guard cells have cuticular ledges. D shows giant raised stoma. E shows the nectary disk of a first day flower covered with flocculant secretion residues. F shows raised stoma with wide circular pore filled with secretion residues.

Figure 3. Light micrographs of cross-sections of the nuptial nectary of *Amphilophium mansoanum* (Bignoniaceae). (A-F) Pre-anthesis buds. (A) General view of the nectary with epidermis, nectary parenchyma underneath the epidermis, and subnectary parenchyma. (B) Detail showing scarce starch grains in the parenchyma. (C) Vascular bundles in the subnectary parenchyma. Note predominance of phloem. (D) Abundance of starch grains in the subnectary parenchyma. (E) Intercellular spaces filled with secretions in the nectary parenchyma. (F) Stoma with a wide opening. (G-J) First-day flowers. (G)

1 Nectary parenchyma showing rounded cells with large vacuole and dense accumulations. (H) Subnectary
 2 parenchyma with vacuolated cells. (I) Parenchyma cells with phenolic compounds and sparse
 3 voluminous starch grains. (J) Tracheary elements in the subnectary parenchyma. Key to figures: ep,
 4 epidermis; ie, intercellular space; np, nectary parenchyma; sp, subnectary parenchyma; vb, vascular
 5 bundles; xy, xylem.

6 **ALT TEXT:** Light micrographs of cross-sections of the nuptial nectary of *Amphilophium mansoanum*
 7 labelled A to J. A-F show light micrographs of pre-anthesis buds. A shows a general view of the nectary
 8 with epidermis, nectary parenchyma underneath the epidermis, and subnectary parenchyma. B shows
 9 scarce starch grains in the parenchyma. C shows vascular bundles in the subnectary parenchyma with
 10 predominance of phloem. D shows abundance of starch grains in the subnectary parenchyma. E shows
 11 intercellular spaces filled with secretions in the nectary parenchyma. F shows a stoma with a wide
 12 opening. G-J show light micrographs of first-day flowers. G shows nectary parenchyma presenting
 13 rounded cells with large vacuole and dense accumulations. H shows subnectary parenchyma with
 14 vacuolated cells. I shows parenchyma cells with phenolic compounds and sparse voluminous starch
 15 grains. J shows tracheary elements in the subnectary parenchyma.

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 17 **Figure 4.** Histochemical characterization of the nuptial nectary of *Amphilophium mansoanum*
 18 (Bignoniaceae); cross-sections. (A-B) Positive reaction to test with mercury blue bromophenol reagent,
 19 evidencing the presence of basic proteins in the vascular bundles (vb), and in the parenchyma cells
 20 (arrowhead). (C) Positive reaction to test with Alcian Blue, evidencing the presence of acid
 21 polysaccharides. (D) Positive reaction to the test with NADI reagent (arrowhead), evidencing the
 22 presence of essential oils (blue staining) and oils-resin (pink staining). (E) Positive reaction to test with
 23 Lugol reagent (arrowhead), evidencing the presence of starch grains. (F-G) Positive reaction to test with
 24 Sudan IV (arrowhead), evidencing the presence of lipids. (H) Positive reaction to test with ferric chloride
 25 reagent (arrowhead), evidencing the presence of phenolic compounds.

26 **ALT TEXT:** Histochemical characterization of cross sections of the nuptial nectary of *Amphilophium*
 27 *mansoanum* labelled A to H. A-B show positive reaction to test with mercury blue bromophenol reagent,
 28 evidencing the presence of basic proteins in the vascular bundles (vb), and in the parenchyma cells
 29 (arrowhead). C shows positive reaction to test with Alcian Blue, evidencing the presence of acid
 30 polysaccharides. D shows positive reaction to the test with NADI reagent (arrowhead), evidencing the
 31 presence of essential oils (blue staining) and oils-resin (pink staining). E shows positive reaction to test
 32 with Lugol reagent (arrowhead), evidencing the presence of starch grains. F-G show positive reaction to

1 test with Sudan IV (arrowhead), evidencing the presence of lipids. H shows positive reaction to test with
 2 ferric chloride reagent (arrowhead), evidencing the presence of phenolic compounds.

3
 4 **Figure 5.** Transmission electron micrographs of the nuptial nectary of *Amphilophium mansoanum* in pre-
 5 anthesis buds. (A-C) Epidermal cells. (A) Part of an epidermal cell with thick outer periclinal wall and
 6 thin cuticle, abundant cytoplasm, endoplasmic reticulum, starch grains and oil drops. Note the
 7 osmiophilic body in the periplasmic space (arrow). (B) Endoplasmic reticulum profiles at the peripheral
 8 cytoplasm, dictyosomes and vesicles marked by ZIO method, rounded leucoplast with a large
 9 osmiophilic inclusion, and ellipsoid amyloplast. Note electron dense granules in the periplasmic space
 10 and attached to the tonoplast. (C) Abundant vesicles from dictyosomes filled with dense content marked
 11 by ZIO method, and dense material inside the vacuole. Note the lipid drop at the peripheral cytoplasm.
 12 (D-F) Nectary parenchyma. (D) Parenchyma cells with very thin walls (arrowhead), nucleus and large
 13 vacuoles filled with flocculant materials. (E) Starch grains with different sizes and degradation signals.
 14 (F) Plastids with starch grains and osmiophilic globules. Note osmiophilic bodies attached to the
 15 tonoplast. (G-I) Subnectary parenchyma. (G) Phloem and vacuolated parenchyma cells with big
 16 amyloplasts. (H) Plasmodesmata in the transversal walls connecting parenchyma cells (arrow heads). (I)
 17 Parenchyma cells with conspicuous nucleus, abundant cytoplasm, big amyloplasts and vacuoles with
 18 flocculent materials. Note the intercellular spaces with fibrillar materials. (J-L) Phloem in the subnectary
 19 parenchyma. (J) Several phloem cells in the subnectary parenchyma. (K) Sieve tube elements of large
 20 caliber. (L) Phloem cells with voluminous amyloplasts with intact starch grains. Key to figures: am,
 21 amyloplast; cc, companion cell; ct, cuticle; cw, cell wall; is, intercellular space; le, leucoplast; nu,
 22 nucleus; ol, oil drop; pe, periplasmic space; pp, phloem parenchyma; pl, plastid; sg, starch grain; sp,
 23 subnectary parenchyma; ste, sieve tube element; va, vacuole.

24 **ALT TEXT:** Transmission electron micrographs of the nuptial nectary of *Amphilophium mansoanum* in
 25 pre-anthesis buds labelled A to L. A-C TEM micrographs show epidermal cells. A shows part of an
 26 epidermal cell with thick outer periclinal wall and thin cuticle, abundant cytoplasm, endoplasmic
 27 reticulum, starch grains, oil drops and osmiophilic body in the periplasmic space (arrow). B shows
 28 endoplasmic reticulum profiles at the peripheral cytoplasm, dictyosomes and vesicles marked by ZIO
 29 method, rounded leucoplast with a large osmiophilic inclusion, ellipsoid amyloplast and dense granules
 30 in the periplasmic space and attached to the tonoplast. C shows abundant vesicles from dictyosomes
 31 filled with dense content marked by ZIO method, dense material inside the vacuole, and a lipid drop at
 32 the peripheral cytoplasm. D-F TEM micrographs show nectary parenchyma. D shows parenchyma cells
 33 with very thin walls (arrowhead), nucleus and large vacuoles filled with flocculant materials. E shows

1 starch grains with different sizes and degradation signals. F shows plastids with starch grains and
 2 osmiophilic globules, and osmiophilic bodies attached to the tonoplast. G-I TEM micrographs show
 3 subnectary parenchyma. G shows phloem and vacuolated parenchyma cells with big amyloplasts. H
 4 shows plasmodesmata in the transversal walls connecting parenchyma cells (arrow heads). I shows
 5 parenchyma cells with conspicuous nucleus, abundant cytoplasm, big amyloplasts and vacuoles with
 6 flocculent materials, and intercellular spaces with fibrillar materials. J-L TEM micrographs show phloem
 7 in the subnectary parenchyma. J shows several phloem cells in the subnectary parenchyma. K shows
 8 sieve tube elements of large caliber. L shows phloem cells with voluminous amyloplasts with intact
 9 starch grains

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11 **Figure 6.** Transmission electron micrographs of the nuptial nectary of *Amphilophium mansoanum*
 12 (Bignoniaceae). (A-I) First-day flowers; (J-N) Second-day flowers. (A-C) Epidermal cells. (A) Large
 13 vacuole in the apical pole and clustered dense plastids with small starch grains in the basal pole. (B)
 14 Granulated cytoplasm with scattered oil drops, and vacuoles with flocculant material. Note the
 15 periplasmic space along the outer cell wall. (C) Secretion residues (arrow) on the cuticle. Note
 16 microchannels projecting from the cell wall to the cuticle. (D) Nectary parenchyma cell with collapsed
 17 amyloplasts and broken starch grains. (E) Subnectary parenchyma with vacuoles full of fibrillar or
 18 flocculant materials, membrane debris, and osmiophilic inclusions. Note larger intercellular spaces filled
 19 with fibrillar material. (F-I) Phloem features in the subnectary parenchyma. (F) Vacuolated parenchyma
 20 cell containing broken starch grains. (G) Sieve plate plugged with amorphous deposits, typically callose.
 21 (H) Parenchyma cell with spindle-shaped protein bodies scattered in the cytoplasm. (I) Parenchyma cell
 22 showing dictyosomes and vesicles marked by ZIO reaction. (J) Epidermal cells with reduced cytoplasm
 23 and big vacuoles with fibrillar inclusion and membrane debris. Note denser clustered plastids with tiny
 24 starch grains. (K) Nectary parenchyma showing vacuolated cells with reduced cytoplasm, and
 25 intercellular spaces. (L) Subnectary parenchyma with wider intercellular spaces; cells showing nucleus,
 26 reduced cytoplasm and big amyloplasts. Note dark inclusion in the vacuole. (M) Detail showing part of
 27 the nucleus, endoplasmic reticulum element, mitochondria, amyloplast containing one starch grain and
 28 osmiophilic bodies, and vacuole. (N) Detail of the subnectary parenchyma showing intercellular space
 29 filled with fibrillar material. Note dark inclusion in the vacuole (arrow). Key to figures: ct, cuticle; cw,
 30 cell wall; di, dictyosome; er, endoplasmic reticulum; ie, intercellular space; mi, mitochondria; nu,
 31 nucleus; ol, oil drop; pb, protein bodies; pp, phloem parenchyma; pl, plastid; ste, sieve tube element; va,
 32 vacuole.

1 **ALT TEXT:** Transmission electron micrographs of the nuptial nectary of *Amphilophium mansoanum*
 2 labelled A to N. A-I TEM micrographs show first-day flowers; J-N TEM micrographs show second-day
 3 flowers. A-C show epidermal cells. A shows large vacuole in the apical pole and clustered dense plastids
 4 with small starch grains in the basal pole. B shows granulated cytoplasm with scattered oil drops,
 5 vacuoles with flocculant material, and periplasmic space along the outer cell wall. C shows secretion
 6 residues (arrow) on the cuticle, and microchannels projecting from the cell wall to the cuticle. D shows
 7 nectary parenchyma cell with collapsed amyloplasts and broken starch grains. E shows subnectary
 8 parenchyma with vacuoles full of fibrillar or flocculant materials, membrane debris, osmiophilic
 9 inclusions, and larger intercellular spaces filled with fibrillar material. F-I TEM micrographs show
 10 phloem features in the subnectary parenchyma. F shows vacuolated parenchyma cell containing broken
 11 starch grains. G shows sieve plate plugged with amorphous deposits, typically callose. H shows
 12 parenchyma cell with spindle-shaped protein bodies scattered in the cytoplasm. I shows parenchyma cell
 13 showing dictyosomes and vesicles marked by ZIO reaction. J shows epidermal cells with reduced
 14 cytoplasm and big vacuoles with fibrillar inclusion and membrane debris, and denser clustered plastids
 15 with tiny starch grains. K shows nectary parenchyma showing vacuolated cells with reduced cytoplasm
 16 and intercellular spaces. L shows subnectary parenchyma with wider intercellular spaces; cells showing
 17 nucleus, reduced cytoplasm, big amyloplasts, and dark inclusion in the vacuole. M details part of the
 18 nucleus, endoplasmic reticulum element, mitochondria, amyloplast containing one starch grain and
 19 osmiophilic bodies, and vacuole. N details the subnectary parenchyma showing intercellular space filled
 20 with fibrillar material, and dark inclusion in the vacuole (arrow).

21
 22 **Figure 7.** Extranuptial nectary of *Amphilophium mansoanum* (Bignoniaceae). (A-D) Scanning electron
 23 micrographs of the calyx surface. (A) Clustered concave glands at the surface of young flower buds
 24 surrounded by trichomes. (B) Gland with extended margin that projects above the surface of the calyx
 25 forming a wide central cavity. (C) Non-glandular trichomes, peltate glandular trichome, and raised
 26 stomata (arrow). (D) Gland sectioned longitudinally showing the concave portion located above the
 27 calyx surface and the portion immersed in the calyx mesophyll. (E-J) Light micrographs of the transversal
 28 section, stained with toluidine blue, showing glands at different developmental stages. (E-G) 6 μm thick;
 29 (H-J) 60 μm thick. (E-F) Young bud. (E) Gland formed by secretory head with undeveloped margin,
 30 uniseriate stalk, and parenchyma foot. (F) Detail showing head cells covered with a thick cuticle, and
 31 stalk cells with thick lateral walls. (G) Small subcuticular space in the central region of the secretory
 32 head with secretion accumulation (arrow). (H) Pre-anthesis bud; head secretory cells and foot cells with
 33 strongly stained dense cytoplasm. (I) First-day flowers; gland with expanded margin perpendicular to the

1 surface of the calyx. Note that all gland cells have less dense content and more developed vacuoles. (J)
 2 Detail showing thicker cuticle, head cells with thick walls, prominent nucleus and vacuoles with different
 3 sizes. Note the thick-walled stalk cells and dense granulations in the foot cells. Key to figures: pg,
 4 patelliform-gland; pt, peltate glandular trichome; nt, non-glandular trichome; sh, secretory head; st, stalk;
 5 ft, foot.

6 **ALT TEXT:** Extranuptial nectary of *Amphilophium mansoanum* labelled A to J. A-D shows scanning
 7 electron micrographs of the calyx surface. A shows clustered concave glands at the surface of young
 8 flower buds surrounded by trichomes. B shows gland with extended margin that projects above the
 9 surface of the calyx forming a wide central cavity. C shows non-glandular trichomes, peltate glandular
 10 trichome, and raised stomata (arrow). D shows gland sectioned longitudinally showing the concave
 11 portion located above the calyx surface and the portion immersed in the calyx mesophyll. E-J show light
 12 micrographs of the transversal section, stained with toluidine blue, showing glands at different
 13 developmental stages. E-F shows a young bud. E shows gland formed by secretory head with
 14 undeveloped margin, uniseriate stalk, and parenchyma foot. F details head cells covered with a thick
 15 cuticle, and stalk cells with thick lateral walls. G shows small subcuticular space in the central region of
 16 the secretory head with secretion accumulation (arrow). H shows pre-anthesis bud; head secretory cells
 17 and foot cells with strongly stained dense cytoplasm. I shows first-day flowers; gland with expanded
 18 margin perpendicular to the surface of the calyx. All gland cells have less dense content and more
 19 developed vacuoles. J details thicker cuticle, head cells with thick walls, prominent nucleus, vacuoles with
 20 different sizes, thick-walled stalk cells and dense granulations in the foot cells.

21
 22 **Figure 8.** Histochemical characterization of the extranuptial nectary of *Amphilophium mansoanum*
 23 (Bignoniaceae); cross-sections. (A-B) Positive reaction to starch grains and to alkaloids with Lugol. (A)
 24 Note the vascular bundles in the calyx mesophyll (arrowhead) ending at the foot of the gland. (B) Positive
 25 reaction more evident in the foot cells underlying the gland stalk and in the upper region of the secretory
 26 head. (C-E) Positive reaction to test with Sudan IV. (C) Presence of lipids in the lateral walls of the stalk
 27 cells and cuticle. (D) Lipid droplets (arrowhead) in the head cells. (E) Lipid droplets (arrowhead) in the
 28 foot cells. (F) Positive reaction to test with mercury blue bromophenol reagent, evidencing the presence
 29 of basic proteins in the head cells. (G) Positive reaction to test with NADI reagent (arrowhead),
 30 evidencing the presence of oils-resin (pink staining) in the head cells. (H) Positive reaction to test with
 31 ferric chloride reagent, evidencing the presence of alkaloids (stained reddish brown) in the foot cells. (I)
 32 Positive reaction to ruthenium red test, evidencing the presence of pectin in the cell walls of the central

1 region of the secretory head and in the foot of the gland. Key to figures: ft, foot; sh, secretory head; st,
2 stalk.

3 **ALT TEXT:** Histochemical characterization of the extranuptial nectary of *Amphilophium mansoanum*
4 labelled A to I. A-B show positive reaction to starch grains and to alkaloids with Lugol. A shows the
5 vascular bundles in the calyx mesophyll (arrowhead) ending at the foot of the gland. B shows positive
6 reaction more evident in the foot cells underlying the gland stalk and in the upper region of the secretory
7 head. C-E show positive reaction to test with Sudan IV. C shows presence of lipids in the lateral walls
8 of the stalk cells and cuticle. D shows lipid droplets (arrowhead) in the head cells. E shows lipid droplets
9 (arrowhead) in the foot cells. F shows positive reaction to test with mercury blue bromophenol reagent,
10 evidencing the presence of basic proteins in the head cells. G shows positive reaction to test with NADI
11 reagent (arrowhead), evidencing the presence of oils-resin (pink staining) in the head cells. H shows
12 positive reaction to test with ferric chloride reagent, evidencing the presence of alkaloids (stained reddish
13 brown) in the foot cells. I shows positive reaction to ruthenium red test, evidencing the presence of pectin
14 in the cell walls of the central region of the secretory head and in the foot of the gland.

15
16 **Figure 9.** Transmission electron micrographs of the extranuptial nectary of *Amphilophium mansoanum*
17 (Bignoniaceae). (A-C) Glands in young buds; (D-I) Glands in pre-anthesis buds. (A) Head cells with
18 spherical and organized nucleolus, dense cytoplasm, and poorly developed vacuoles. (B) Amyloplasts
19 with lots of starch grains, mitochondria, and small vacuole. (C) Amyloplasts, mitochondria, and
20 dictyosomes with adjacent vesicles containing electron-dense material, marked by ZIO. (D)
21 Mitochondria and extensive endoplasmic reticulum profiles. (E) Plastid with residual starch grains and a
22 big osmiophilic inclusion. Note the endoplasmic reticulum around the plastid, and the plasmodesmata in
23 the anticlinal walls of the head cells. (F) Elongated leucoplast devoid of inner membranes with
24 homogeneous stroma surrounded by endoplasmic reticulum. (G) Dictyosomes and vesicles, marked by
25 ZIO reaction, and endoplasmic reticulum profiles close to plasmodesmata (arrowhead). Note the dense
26 materials close to the plasma membrane and in the periplasmic space (arrows). (H) Stalk cell with thin
27 anticlinal walls and thickened lateral walls showing central nucleus and endoplasmic reticulum. (I) Gland
28 foot formed by tangentially expanded cells with dense inclusions in the vacuoles. Note cytoplasmic oil
29 body. Key to figures: cw, cell wall; di, dictyosome; er, endoplasmic reticulum; is, intercellular space; le,
30 leucoplast; mi, mitochondria; nu, nucleus; ol, oil body; pl, plastid; sg, starch grain; sh, secretory head; st,
31 stalk; va, vacuole.

32 **ALT TEXT:** Transmission electron micrographs of the nuptial nectary of *Amphilophium mansoanum*
33 labelled A to I. A-C shows glands in young buds; D-I shows glands in pre-anthesis buds. A shows head

1 cells with spherical and organized nucleolus, dense cytoplasm, and poorly developed vacuoles. B shows
 2 amyloplasts with lots of starch grains, mitochondria, and small vacuole. C shows amyloplasts,
 3 mitochondria, and dictyosomes with adjacent vesicles containing electron-dense material, marked by
 4 ZIO. D shows mitochondria and extensive endoplasmic reticulum profiles. E shows plastid with residual
 5 starch grains and a big osmiophilic inclusion, endoplasmic reticulum around the plastid, and the
 6 plasmodesmata in the anticlinal walls of the head cells. F shows elongated leucoplast devoid of inner
 7 membranes with homogeneous stroma surrounded by endoplasmic reticulum. G shows dictyosomes and
 8 vesicles, marked by ZIO reaction, endoplasmic reticulum profiles close to plasmodesmata (arrowhead),
 9 and dense materials close to the plasma membrane and in the periplasmic space (arrows). H shows stalk
 10 cell with thin anticlinal walls and thickened lateral walls showing central nucleus and endoplasmic
 11 reticulum. I shows gland foot formed by tangentially expanded cells with dense inclusions in the
 12 vacuoles. Note cytoplasmic oil body.

13

14 **Figure 10.** Transmission electron micrographs of the extranuptial nectary of *Amphilophium mansoanum*
 15 (Bignoniaceae). (A-H) First-day flowers; (I-L) Second-day flowers. (A) General view showing thick
 16 cuticle with accumulations of electron dense material. Note the dark and clear cells with thicker walls.
 17 (B) Head cells with voluminous nucleus, abundant cytoplasm, and large lipid drops. (C) Periplasmic
 18 spaces along the anticlinal walls containing paramural bodies. (D) Lipid inclusions in the anticlinal walls
 19 of the head cells. (E) Polymorphic plastids with finely granular stroma and osmiophilic inclusions (likely
 20 terpene/phenols). (F) Stalk cell with large lipid bodies, mitochondria, and small vacuoles. (G) Gland foot
 21 with wider intercellular spaces filled with fibrillar material, and cells with prominent nucleus, abundant
 22 cytoplasm and small vacuoles with electron dense bodies. (H) Detail of a foot cell with numerous
 23 mitochondria, electron-dense plastids and small vacuoles. (I) Apical region of the head with abundant
 24 dark cells. (J) Larger accumulations of electron-dense material in the cuticle. (K) Dark cells with
 25 abundant cytoplasm, rich in intact organelles and small vacuoles. (L) Dark cell showing well-developed
 26 Golgi bodies with numerous cisternae, sparse profiles of endoplasmic reticulum, oil bodies, and vacuoles
 27 with membrane debris. Key to figures: ct, cuticle; cw, cell wall; di, dictyosome; dc, dark cells; er,
 28 endoplasmic reticulum; ie, intercellular space; mi, mitochondria; nu, nucleus; ol, oil drop; pl, plastid; ps,
 29 periplasmic space; sg, starch grain; st, stalk; va, vacuole.

30 **ALT TEXT:** Transmission electron micrographs of the extranuptial nectary of *Amphilophium*
 31 *mansoanum* labelled A to L. A-H show first-day flowers; I-L show second-day flowers. A shows aeneral
 32 view showing thick cuticle with accumulations of electron dense material, and dark and clear cells with
 33 thicker walls. B shows head cells with voluminous nucleus, abundant cytoplasm, and large lipid drops.

1 C shows periplasmic spaces along the anticlinal walls containing paramural bodies. D shows lipid
2 inclusions in the anticlinal walls of the head cells. E shows polymorphic plastids with finely granular
3 stroma and osmiophilic inclusions (likely terpene/phenols). F shows stalk cell with large lipid bodies,
4 mitochondria, and small vacuoles. G shows gland foot with wider intercellular spaces filled with fibrillar
5 material, and cells with prominent nucleus, abundant cytoplasm and small vacuoles with electron dense
6 bodies. H details a foot cell with numerous mitochondria, electron-dense plastids and small vacuoles. I
7 show apical region of the head with abundant dark cells. J shows larger accumulations of electron-dense
8 material in the cuticle. K shows dark cells with abundant cytoplasm, rich in intact organelles and small
9 vacuoles. L shows dark cell showing well-developed Golgi bodies with numerous cisternae, sparse
10 profiles of endoplasmic reticulum, oil bodies, and vacuoles with membrane debris.
11

1 **Table 1.** Sites of histochemical reaction in nuptial and extranuptial nectaries of *Amphilophium*
 2 *mansoanum* flowers. These results are shown in Figures 4 and 8. NA, not applicable; inconclusive results;
 3 NN, nuptial nectary; ENN, extranuptial nectary; ep, epidermis; np, nectary parenchyma; snp, subnectary
 4 parenchyma; ft, foot cells; st, stalk cells; sh, secretory head; cw, cell wall; is, intercellular space; ic,
 5 intracellular; ex, extracuticular; cu, cuticle; scu, subcuticular space; vb, vascular bundles.

Stage	Nectary type	Reagents and target compounds							
		Ferric chloride (Phenolic substances)	Lugol (Starch grains)	Lugol (Alkaloids)	NADI (Terpenes)	Ruthenium red (Pectin)	Sudan (Lipids)	Aleian Blue (Acid polysaccharides)	Mercuric bromophenol blue (Proteins)
Young bud	NN	snp (ic)	snp (ic)	snp (ic)	NA	ep (na) np (cw) snp (cw)	snp (ic)	NA	NA
	ENN	NA	ft (ic) st (ic) sh (ic)	NA	NA	sh (ic)	st (cw) sh (cu)	NA	NA
Pre-anthesis bud	NN	ep (ic) np (ic) snp (ic)	ep snp (ic)	ep (ic)	ep (ic; ex) np (ic) snp (ic)	ep (cu; ic; cw;) np (is; cw; ic) snp (cw; vb)	ep (cu; ic) np (ic) snp (ic)	NA	NA
	ENN	sh (ic) ft (ic)	ft (ic)	ft (ic)	NA	ft (cw; ic), st (ic) sh (cw; ic)	ft (ic) st (cw; ic) sh (ic; cu; scu)	NA	NA
First-day flower	NN	ep (ic) np (ic) snp (ic)	ep (ic) np (ic) snp (ic)	np (ic) snp (ic)	ep (ex) np (ic) snp (ic; is)	ep (cw) np (cw) snp (cw)	ep (na) np (na) snp (is; ic)	ep (cw; ic) np (cw; ic) snp (cw; ic)	ep (ic) np (ic) snp (ic; vb)
	ENN	ft (ic)	ft (ic) sh (ic)	ft (ic) st (ic) sh (ic)	sh (ic)	ft (cw; is; ic) st (ic) sh (cw; ic)	st (cw) sh (cu)	sh (cw; scu) ft (is; cw)	sh (ic)
NN	np (ic) snp (ic)	ep (ic) np (ic)	snp (ic)	ep (ic) np (ic)	ep (cw) np (cw; ic)	ep (cu; ic)	NA	NA	

			snp (ic)		snp (ic)		snp (cw; vb; ic)		np (ic) snp (ic)
Second- day flower	ENN	ft (ic) sh (ic)	sh (ic) ft (ic)	ft (ic)	NA		sh (ic) ft (ic; cw)		ft (ic) st (cw) sh (cu; ic)
								NA	NA

1

ACCEPTED MANUSCRIPT

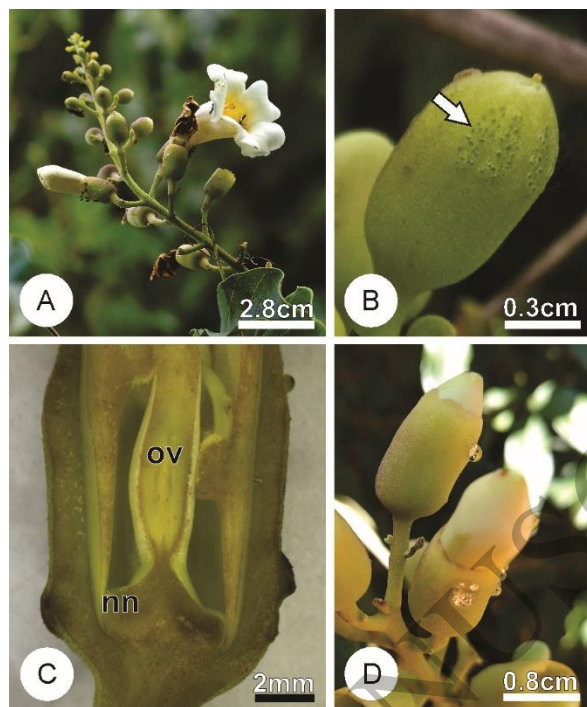


Figure 1
77x93 mm (x DPI)

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ACCEPTED MANUSCRIPT

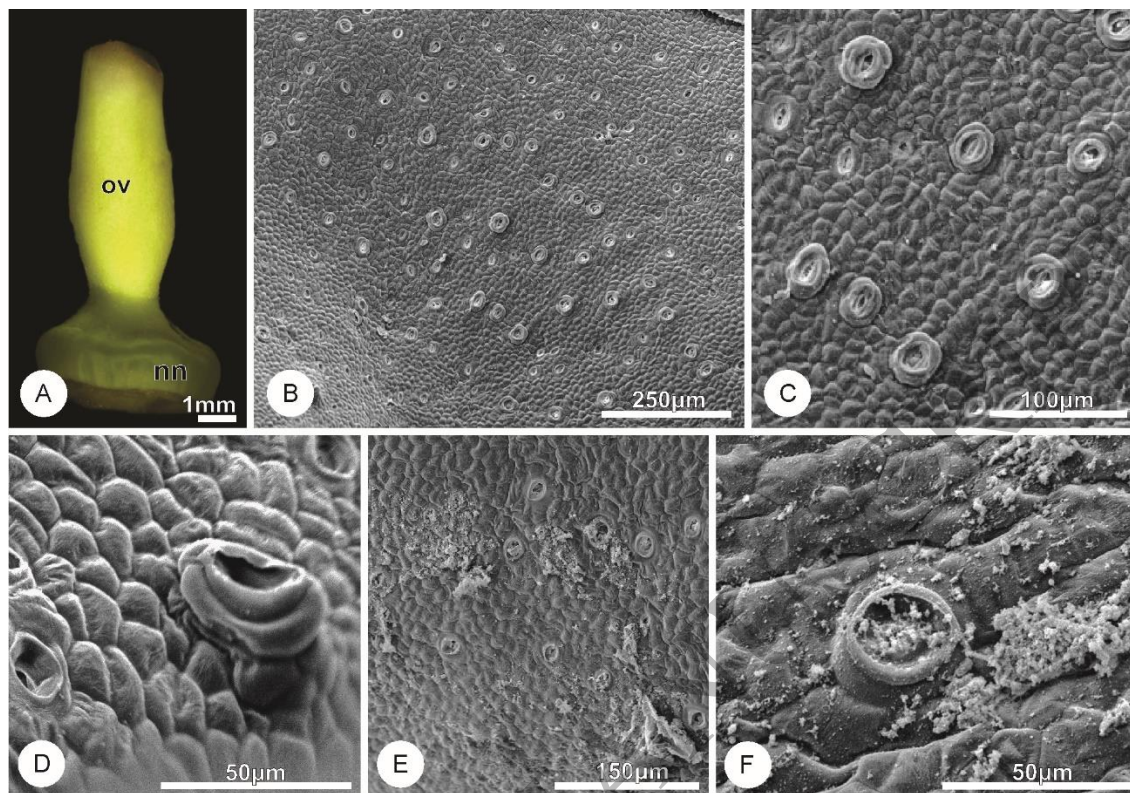


Figure 2
150x104 mm (x DPI)

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ACCEPTED MANUSCRIPT

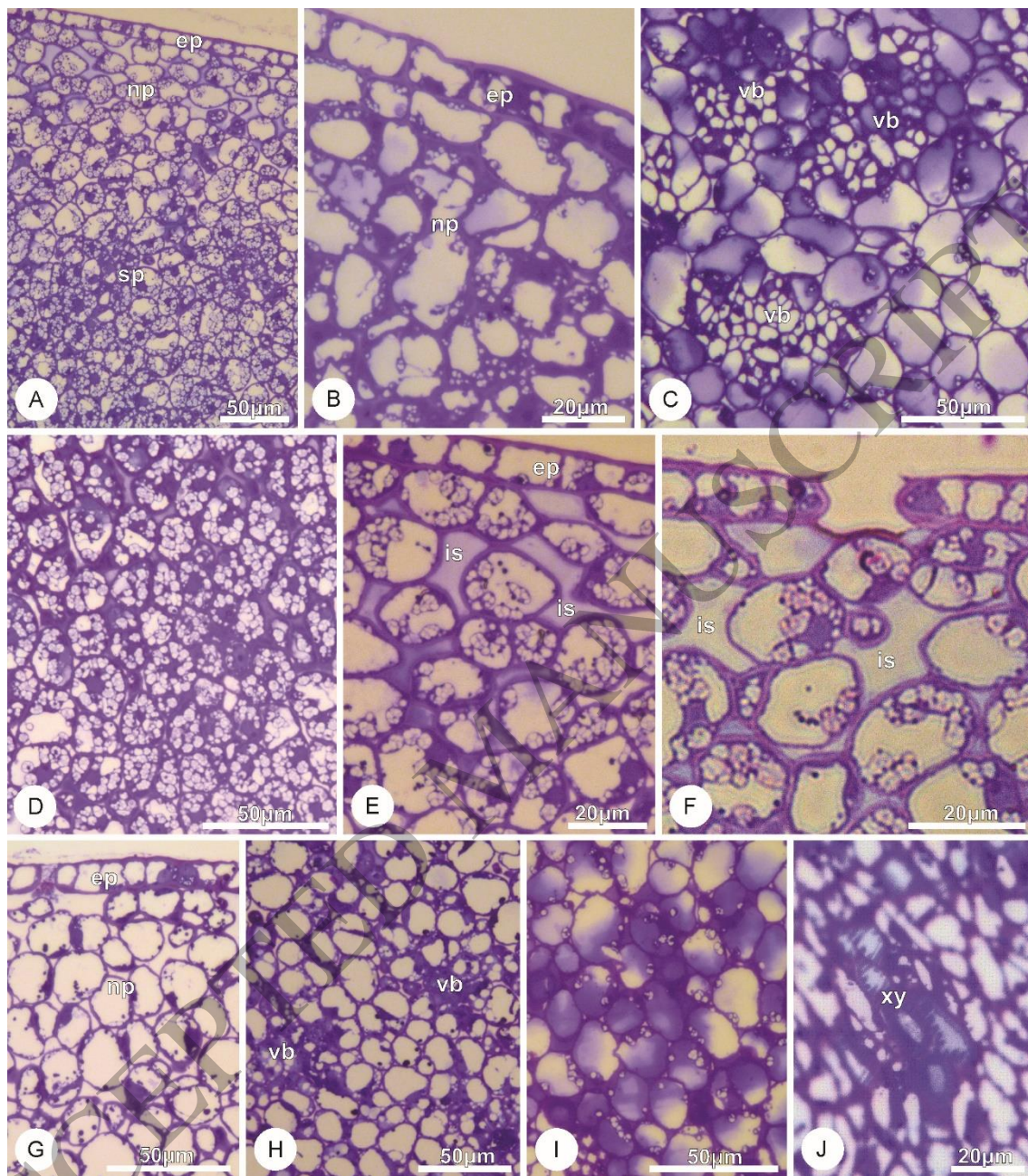


Figure 3
150x171 mm (x DPI)

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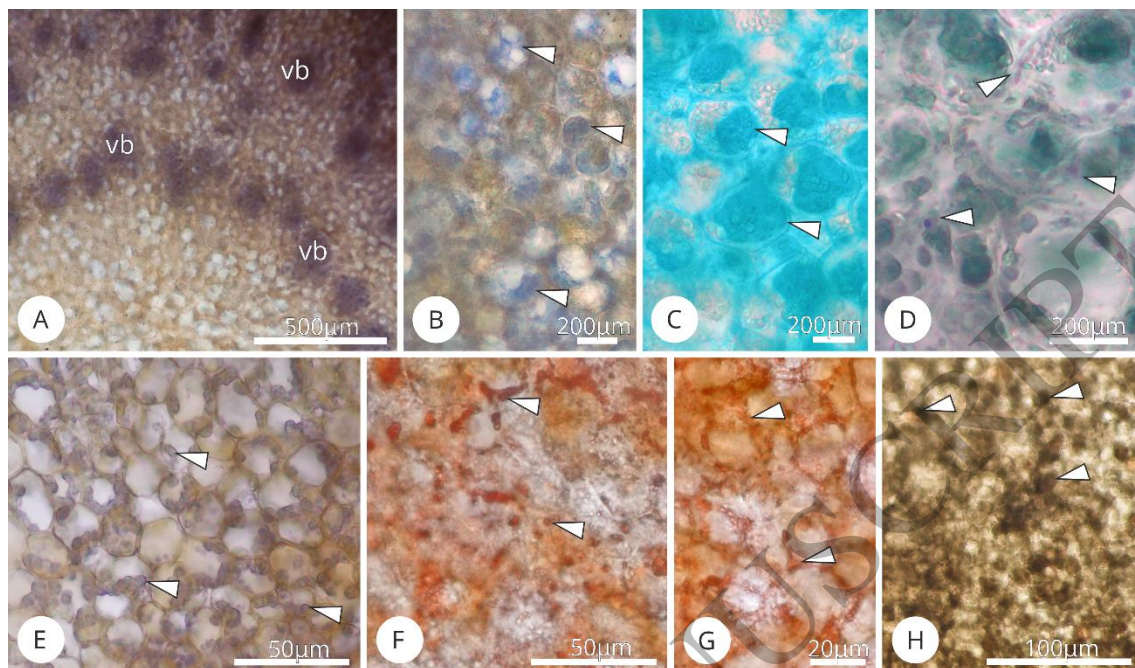


Figure 4
150x88 mm (x DPI)

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ACCEPTED MANUSCRIPT

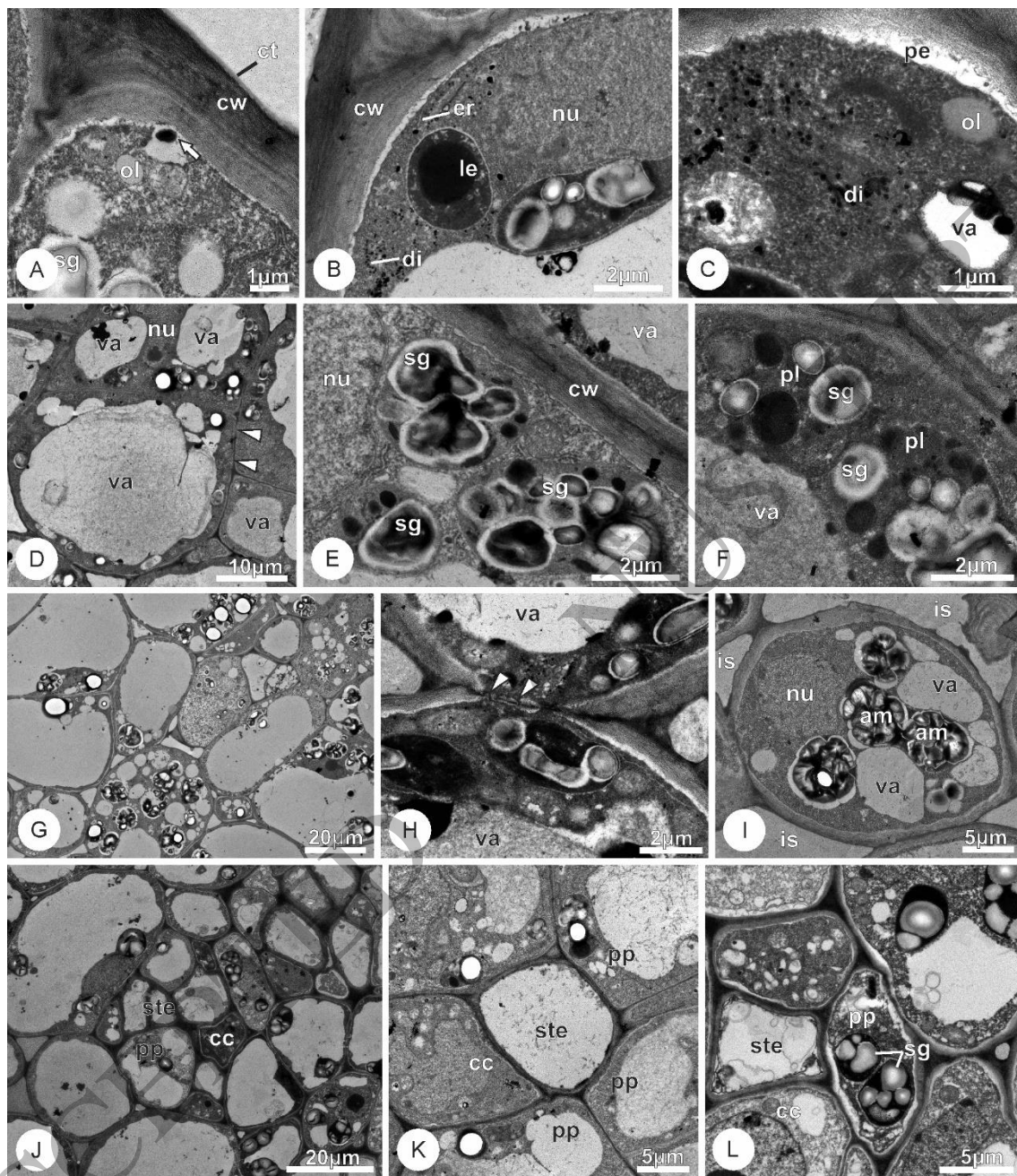


Figure 5
150x173 mm (x DPI)

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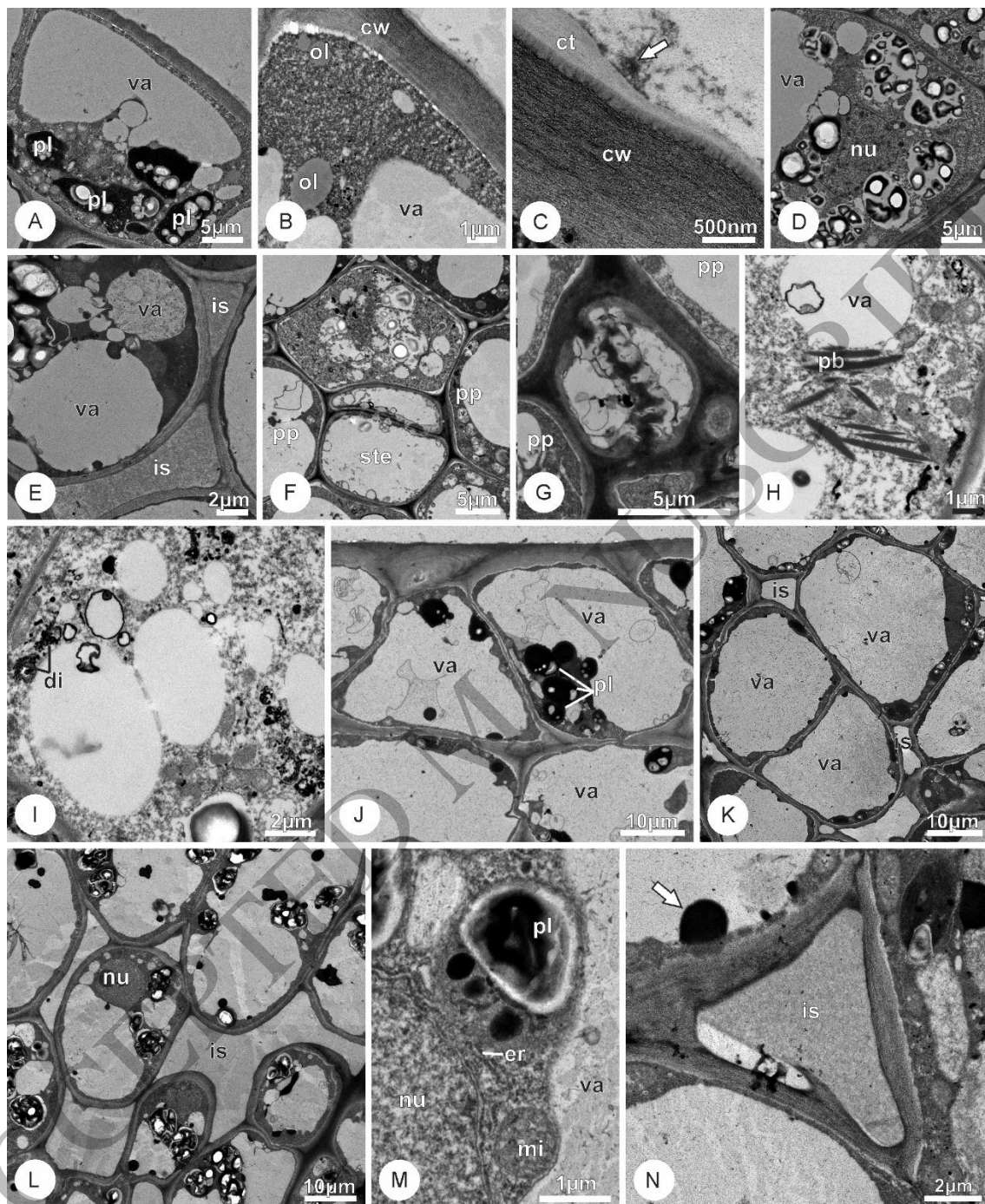


Figure 6
150x183 mm (x DPI)

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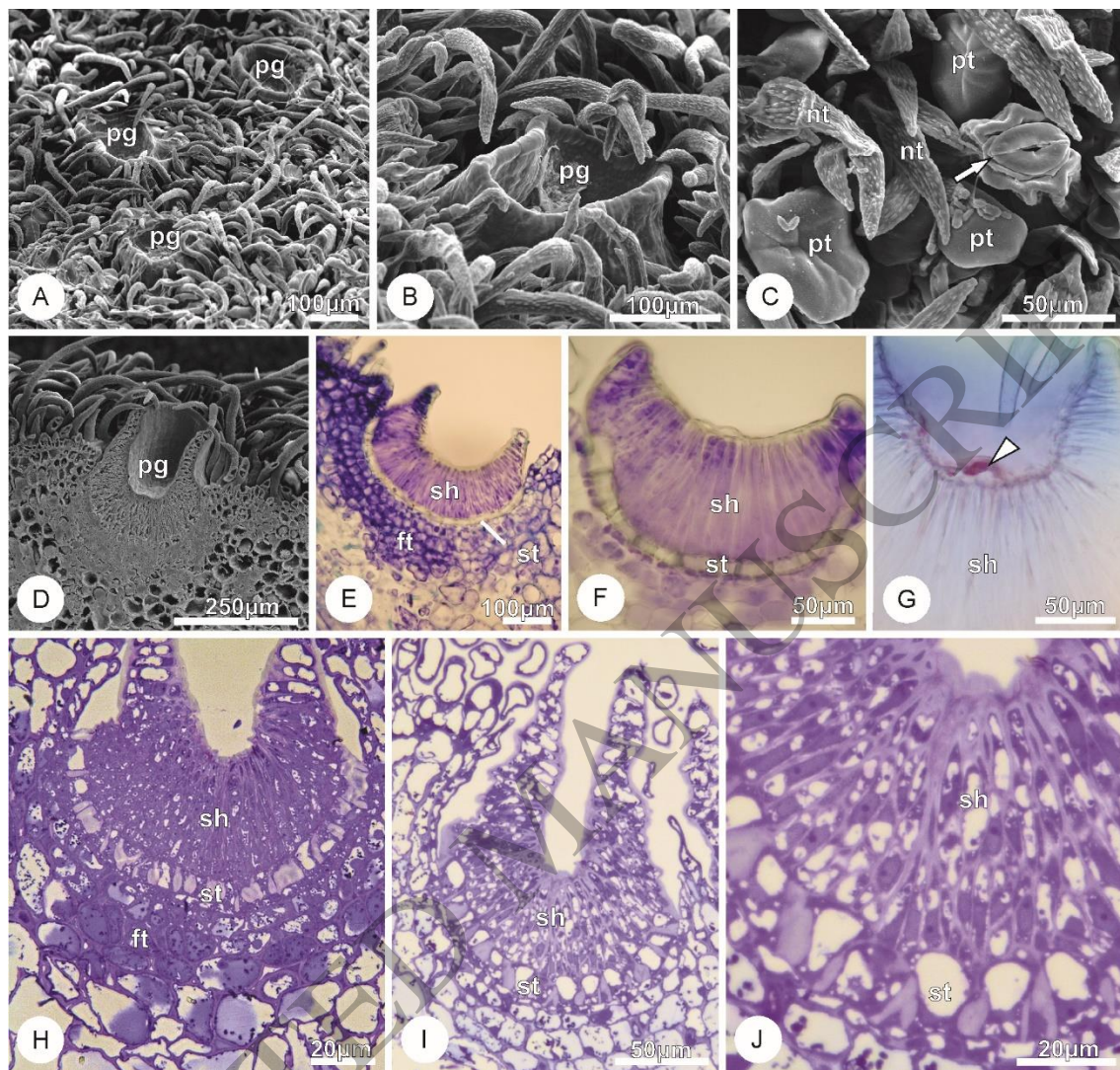


Figure 7
150x143 mm (x DPI)

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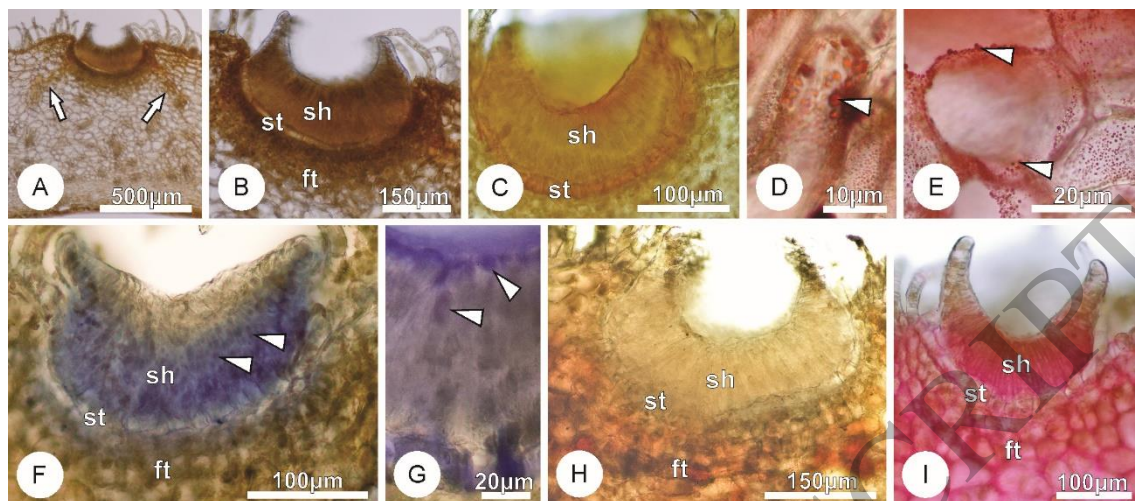


Figure 8
150x66 mm (x DPI)

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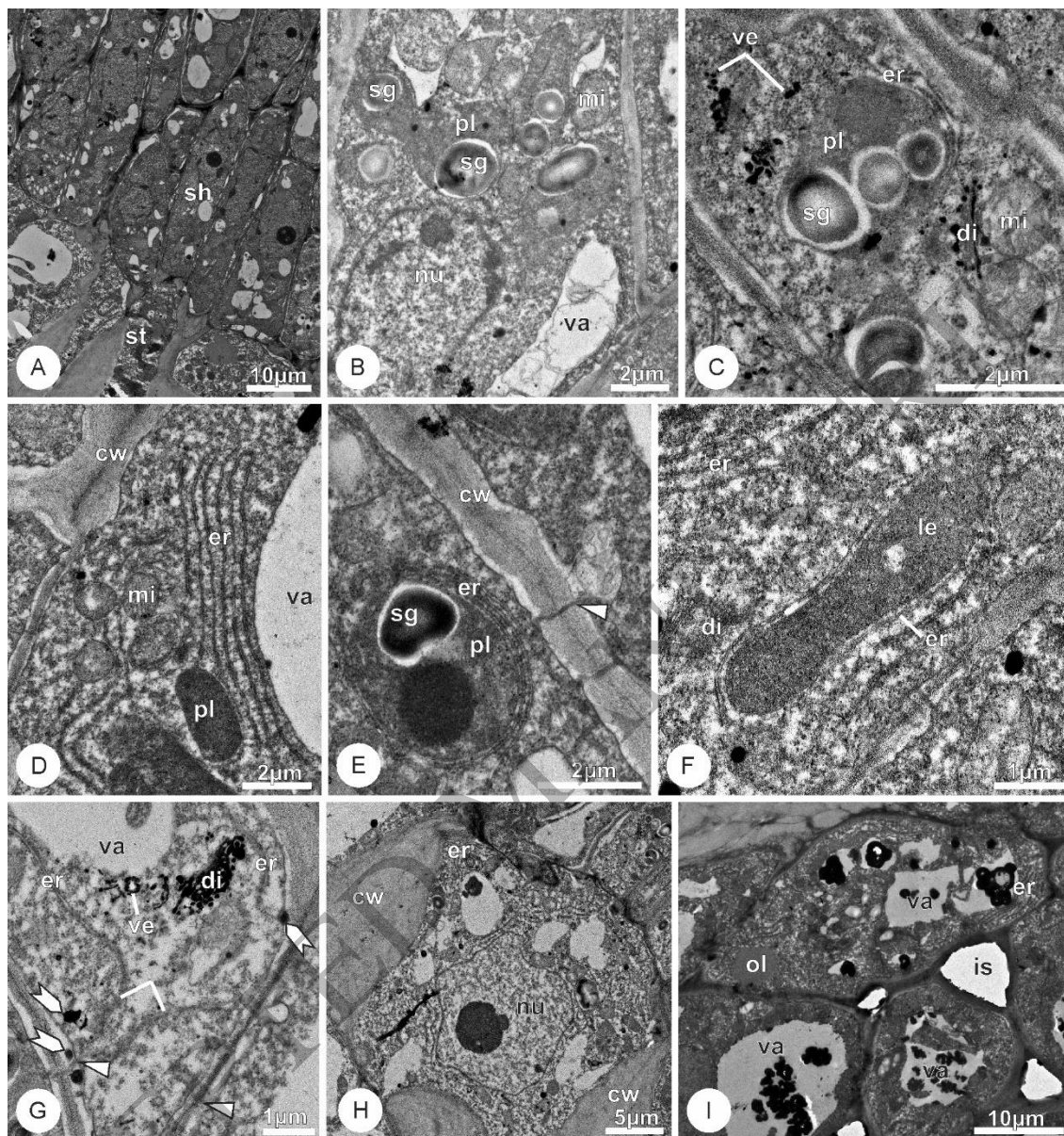


Figure 9
150x159 mm (x DPI)

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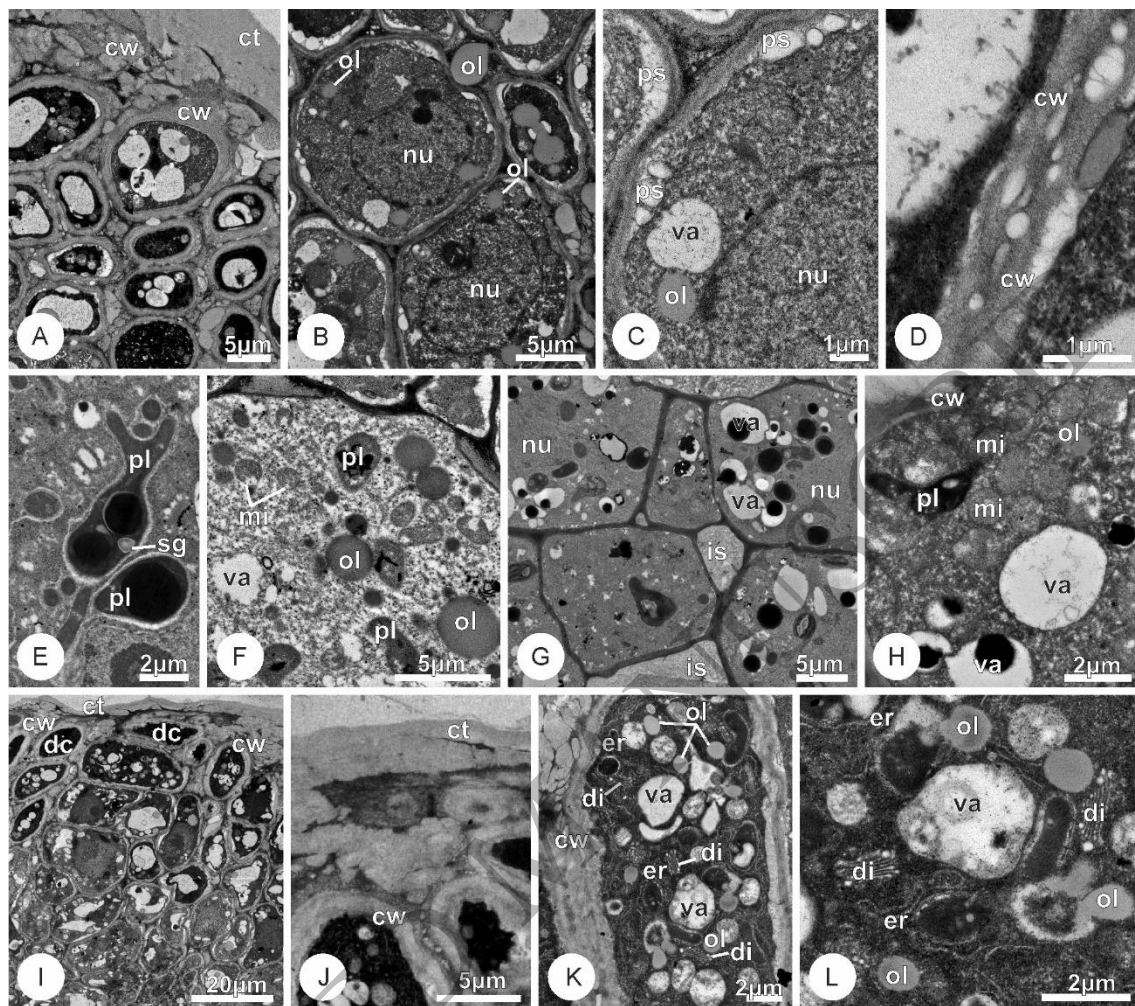


Figure 10
150x132 mm (x DPI)

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ACCEPTED