ORIGINAL ARTICLE

Anatomical, histochemical, and developmental approaches reveal the long-term functioning of the floral nectary in *Tocoyena formosa* (Rubiaceae)

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Abstract

Tocoyena formosa has a persistent floral nectary that continues producing nectar throughout flower and fruit development. This plant also presents an intriguing non-anthetic nectary derived from early-developing floral buds with premature abscised corolla. In this study, we characterize the structure, morphological changes, and functioning of *T. formosa* floral nectary at different developmental stages. We subdivided the nectary into four categories based on the floral and fruit development stage at which nectar production started: (i) non-anthetic nectary; (ii) anthetic nectary, which follows the regular floral development; (iii) pericarpial nectary, derived from pollinated flowers following fruit development; and (iv) post-anthetic nectary that results from non-pollinated flowers after anthesis. The nectary has a uniseriate epidermis with stomata, nectariferous parenchyma, and vascular bundles, with a predominating phloem at the periphery. The non-anthetic nectary presents immature tissues that release the exudate. The nectary progressively becomes more rigid as the flower and fruit develop. The main nectary changes during flower and fruit development comprised the thickening of the cuticle and epidermal cell walls, formation of cuticular epithelium, and an increase in the abundance of calcium oxalate crystals and phenolic cells near the vascular bundles. Projections of the outer periclinal walls toward the cuticle in the post-anthetic nectary suggest nectar reabsorption. The anatomical changes of the nectary allow it to function for an extended period throughout floral and fruit development. Hence, *T. formosa* nectary is a bivalent secretory structure that plays a crucial role in the reproductive and defensive interactions of this plant species.

Keywords Nectar · Nectary lifespan · Non-anthetic nectary · Post-floral secretion · Ontogeny · Structure

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Introduction

In Rubiaceae, the gynoecium typically exhibits syncarpy and an inferior ovary, with the nectary often presented as a nectariferous disk positioned on top of the ovary, encircling the base of the style (Galetto 1998; Bernardello 2007; Judkevich et al. 2022). The cushion-like appearance of the floral nectary is a consistent trait within the family (Brown 1938). The position, morphology, and structure of the floral nectary in Rubiaceae species have been employed as crucial taxonomic characteristics (Brown 1938; Galetto 1998; Bernardello 2007; Florentin et al. 2016) and examined in many ecological investigations (Koptur 1992; Santos and Del-Claro 2001; Del-Claro et al. 2013; Falcão et al. 2014; Sanz-Veiga et al. 2017, 2021; Stefani et al. 2019). Structurally, the floral nectary in Rubiaceae comprises a uniseriate epidermis containing stomata and a multilayered parenchyma that may or may not have vascularization (Florentin

et al. 2016). Vascularized parenchyma may have both xylem and phloem bundles, or solely phloem bundles, which might penetrate deeply into the nectary base or the lower half of the nectary (Galetto 1998). Notably, investigations into nectary have been limited to only a few Rubiaceae species (Galetto 1998; Florentin et al. 2016; Judkevich et al. 2022).

Tocoyena formosa (Cham. & Schltdl.) K.Schum. (Rubiaceae) exhibits flowers with long hypocrateriform tubes, highlighting their close relation to long-tongued hawkmoths for sexual reproduction (Gottsberger and Silberbauer-Gottsberger 2006; Sanz-Veiga et al. 2021), and ranks among the 50 most common and widely distributed woody species in the Brazilian Cerrado (Ratter et al. 2003), a savanna vegetation. The substantial volume of nectar produced by floral nectary in the *T. formosa* attracts a diversity of long-tongued hawkmoth species, which exclusively function as pollinators for this specialized sphingophilous species (Silberbauer-Gottsberger 1972; Sanz-Veiga et al. 2021; Amorim et al. 2022). In *T. formosa*, the floral nectary is persistent, and following pollination and the abscission of the corolla tube, the nectary continues to produce nectar throughout fruit development (Sanz-Veiga et al. 2017). This post-floral secretion entices several ant species that consume the nectar but do not contribute to fruit protection against pre-dispersal seed predators (Sanz-Veiga et al. 2017, 2021). Intriguingly, even the nectary of non-pollinated flowers sustains nectar production for approximately 2–3 months after the detachment of the corolla tube (Sanz-Veiga et al. 2017).

A previous study reported the occurrence of floral bud abortion in *T. formosa*, where the base of the calyx continued to produce nectar (Santos and Del-Claro 2001). Our observations across various *T. formosa* populations in the Brazilian Cerrado have similarly indicated the corolla tube abscission in early-developing floral buds, followed by subsequent nectar secretion from the remaining non-anthetic nectary. These non-anthetic nectaries are attended by ants, which consume the small amounts of nectar produced before the emergence of the post-floral nectaries in the inflorescence (Fig. 1). Despite the recognized ecological significance of the floral nectary in *T. formosa*, which is persistent
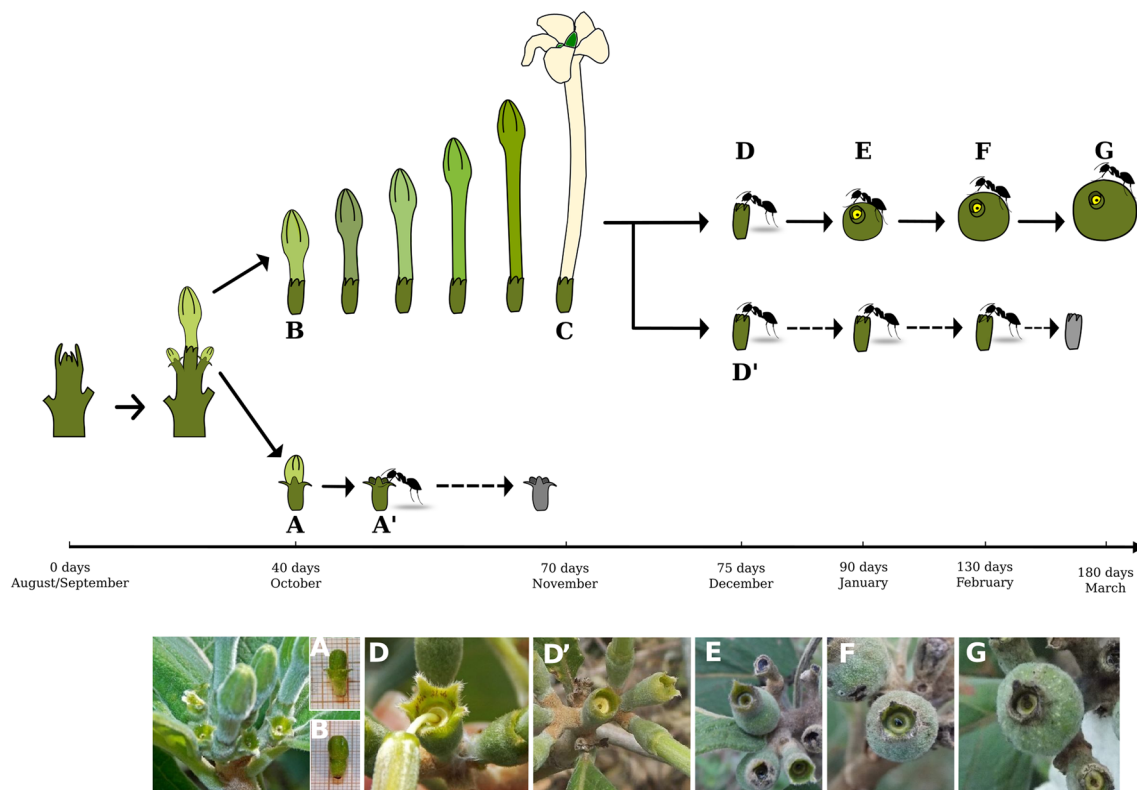
Fig. 1 Diagram of the nectary lifespan and reproductive phenology of *Tocoyena formosa* (Rubiaceae). A Floral bud with loosely arranged sepals. A' Floral bud with detached sepals after the corolla falls. The nectary was exposed to the environment (non-anthetic nectary). B Floral bud with adpressed sepals and normal development (floral nectary before anthesis). C During the anthesis, the nectary remains covered by nectar (anthetic nectary during anthesis). D Fertilized flowers after the corolla falls. D'. The nectary in non-fertilized flowers (post-anthetic nectary) produces nectar following the fruit maturation. E–G Fruit development. Note similar size of nectary in different fruit stages. E Pericarpial nectary in initial fruit (0.5–1.0 cm of diameter). F Pericarpial nectary in intermediated fruit (1.5–2.0 cm of diameter). G Pericarpial nectary in mature fruit (2.5–3.0 cm of diameter). Grey images = nectaries in senescence. Nectaries with ants =nectaries exposed to the environment

and functional as the fruit develops (Santos and Del-Claro 2001; Queiroga and Moura 2017; Sanz-Veiga et al. 2017), the anatomical changes of this organ during floral and fruit development have not been explored.

Considering that the floral nectary in *T. formosa* is persistent and produces nectar along the floral and fruit development, in this study, we subdivided it into four categories: (i) non-anthetic nectary, which results from the early-developing floral buds with abscised corolla (Fig. 1A, A'); (ii) anthetic nectary, which follows the regular floral development and is active during anthesis producing the nectar consumed by the long-tongued hawkmoth pollinators (Fig. 1B, C); (iii) pericarpial nectary, which occurs in developing fruits after successful pollination (Fig. 1D–G); and (iv) post-anthetic nectary that results from non-pollinated flowers after anthesis (Fig. 1D'). From a developmental perspective, we studied the anatomy and histochemistry of the floral nectary of *T. formosa*, aiming to characterize the structure, morphological changes, and functioning of this nectary at different developmental stages. This approach allows us to understand the link between the structural and histochemical features and the secretion of the long-term nectaries in *T. formosa*.

Material and methods

Study site

The study was conducted in a population of *T. formosa* in a private reserve of cerrado sensu stricto (22° 48′ 50″ S and 48° 44′ 40″ W) located in Pratânia municipality, São Paulo State, Brazil. The reserve is located at an elevation of approximately 720 m a.s.l. and encompasses a total area of 224 ha. The climate is warm temperate (Cwa, according to Koeppen 1948) and markedly seasonal, characterized by rainfall primarily in the spring and summer and drought in the fall and winter. The mean temperature during the warmest month is 22.8 °C, while the annual precipitation averages around 1450 mm (Cunha and Martins 2009).

Species characterization, field observation, and sampling

Tocoyena formosa, a deciduous shrub measuring 0.5–3.0 m in the study area, enters its reproductive season in September/October, coinciding with new leaf growth, and finishes in April/May as the fruits mature (Sanz-Veiga et al. 2017; 2021). The long and slender corolla tube spans 6 to 15 cm long, and flowers are arranged in dichasial cyme inflorescences at the terminal position (Oliveira et al. 2004). The species presents protandrous flowers that remain viable for up to 4 days (Oliveira et al. 2004). During the first night

(male phase), pollen is released onto the closed stigmatic lobes, a mechanism commonly observed in Gardenieae species, the tribe in which *T. formosa* is included, known as secondary pollen presentation. From the second night (female phase), the stigmatic lobes become wide open and receptive (Oliveira et al. 2004). Following the abscission of the corolla tube, the nectary remains active, continuously producing nectar (Fig. 1; Sanz-Veiga et al. 2021).

From October 2015 to February 2016, 10 individuals were selected for comprehensive observations on floral morphology, anthesis, fruit development, and morphology during the reproductive season. The focus was on understanding the diverse roles of the floral nectary, encompassing their overall morphology, nectar secretion, flower and nectary longevity, and fruit development. Notably, during our field observations, we found two morphotypes of early-developing floral buds based on calyx appearance (Fig. 1). Consequently, both morphotypes were collected for subsequent anatomical analyses.

For the anatomical analysis of the floral nectary, samples of 10 individuals were taken between October 2015 and February 2016, as well as between October 2016 and February 2017. These samples covered eight distinct stages of flower and fruit development (Table 1).

Structural and histochemical analyses

Samples were fixed in formaldehyde 4% and glutaraldehyde 1% in sodium phosphate-buffered 0.2 M, pH 7.2 (McDowell and Trump 1976), or FAA_{50} (Johansen 1940) for 48 h, dehydrated in an ethanol series and embedded in 2-hydroxyethyl-methacrylate (Leica Microsystems, Heidelberg, Germany). Serial transverse and longitudinal sections (5 μm thick) were cut using a rotary microtome (Leica RM2255), and they were stained in 0.05% toluidine blue, pH 4.7 (O'Brien et al. 1964). The slides were mounted in synthetic resin (Entellan, Merck KGaA, Darmstadt, Germany).

We investigated the main classes of compounds present in the protoplast and the cell walls of the nectariferous tissues (epidermis and parenchyma). For this purpose, the fresh material sections and the fixed and embedded in resin samples were used for histochemical analysis: a 0.02% aqueous solution of ruthenium red for pectin detection, Lugol reagent to detect starch grains, Sudan IV to detect total lipids, 10% aqueous solution of ferric chloride for phenolic compounds (Johansen 1940), NADI reagent for essential oils and oleoresin (David and Carde 1964), and bromophenol blue to detect the total proteins (Mazia et al. 1953).

Furthermore, to identify the content of the cell layers associated with vascular bundles surrounding the nectariferous parenchyma, we employed Dragendorff's reagent for alkaloids (Baerheim-Svendsen and Verpoorte 1983) and vanillin hydrochloride for tannins (Mace and Howell

Table 1 Morphology of the floral nectary in flower and fruit development of *Tocoyena formosa* (Rubiaceae). The number of signals indicates the amount of the nectar (+) little, (++) moderate, and (+++) abundant or (−) absent

Stages of development of the flowers and fruit			Nectar presence	Aspect and color of the nectary	Named
Floral buds	Calyx detached of the corolla (abortive buds)	Before the corolla falls (0.5–1.5 cm of length)	−	Greenish	Non-anthetic nectary before the corolla abscission
		After the corolla falls (1 cm of length) Nectary exposed	++	Swollen and yellow	Non-anthetic nectary in floral bud after the corolla abscission
	Calyx adpressed of the corolla (0.5–1.5 cm of length)		−	−	Pre-anthetic nectary
Flower in anthesis			+++	Swollen and light yellow	Anthetic nectary
Non-fertilized flowers	After the corolla falls; ovary does not develop in fruit (its diameter is similar to ovary in flower) Nectary exposed		+++	Swollen and yellow in center and green in border	Post-anthetic nectary
Fruits	0.5–1.0 cm of diameter Nectary exposed		++	Swollen and greenish yellow	Pericarpial nectary in initial fruit
	1.5–2.0 cm of diameter Nectary exposed		++	Dried with necroses in border and greenish yellow	Pericarpial nectary in intermediate fruit
	2.5–3.0 cm of diameter Nectary exposed		+	Dried with necroses in border and greenish yellow	Pericarpial nectary in mature fruit

1974). The comprehensive examination and documentation of all samples were undertaken using a light microscope (Olympus BX41) and a digital camera (Olympus C7070). We used a polarized light filter to observe the crystals within the nectary tissues.

Results

### Nectary morphological variation during floral development

Tocoyena formosa inflorescences presented two morphotypes of early-developing floral buds, discernible by the variation in calyx morphology (Fig. 1). The first morphotype exhibited loosely arranged sepals and a premature corolla tube abscission, giving rise to the non-anthetic nectary (Fig. 1A). The second morphotype featured sepals closely adpressed to the corolla tube, which follows the regular floral development (Fig. 1B). The floral buds that originate the non-anthetic nectary secreted minute quantities of nectar (less than 1 µL) that attract ants (Fig. 1A') before the emergence of the first post-anthetic nectaries following flower anthesis.

Typically, the oldest terminal floral buds proceeded with regular development, giving origin to the flowers (Fig. 1B). While the younger early-developing floral buds

exhibited premature corolla abscission (Fig. 1A'), accompanied by subsequent nectar production. We removed the early-developing corolla to assess the nectar production in the anthetic floral buds, yet no nectar was observed (Table 1).

After the regular development of these floral buds and subsequent flower anthesis, the corolla tube, whether from pollinated or non-pollinated flowers, abscised, unveiling the post-anthetic nectary to the environment (Fig. 1B–D'). Then, the nectary became swollen, yellowish, and covered with nectar (Table 1). Anthesis spanned 4 days, within which the nectary enlarged, becoming pale yellow, and produced abundant nectar with volume reaching up to 80 µL (Fig. 1C, D and Table 1). In this stage, the nectary was denominated as anthetic nectary. After pollination, the corolla abscised, and the fruits started development (Fig. 1E–G). Throughout the fruit development period (approximately 105 days), the post-floral pericarpial nectary persisted in producing nectar. During the fruit development, this nectary changed its appearance (from swollen to dry with necrosis in the borders) and coloration (from pale yellow to greenish yellow). At the final stages of fruit development, the nectar production became noticeably diminished (hindering volume measurement) until production ceased (Fig. 1F, G and Table 1).

In non-pollinated flowers, the nectary also continued producing nectar after the corolla had abscised, extending for approximately 90 days and accompanying the initial stages
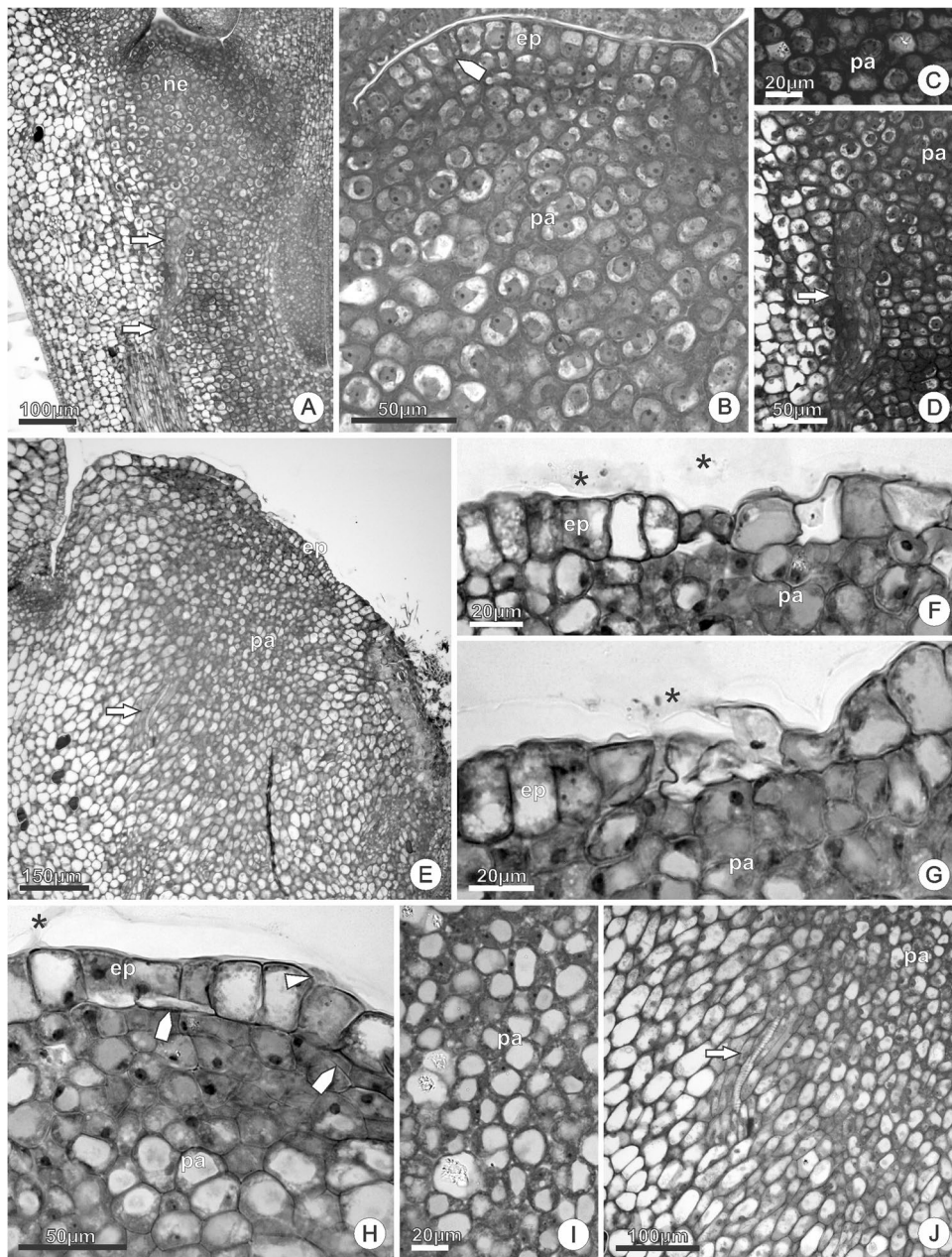
Fig. 2 Structure of the nectary (non-anthetic) in the floral bud with detached sepals to the corolla of *Tocoyena formosa*. **A–H**, **J** Longitudinal sections. **I** Transverse section. N–**D**. Non-anthetic nectary in floral buds with corolla. **A** Nectary (ne) localized in the top of the ovary. Note vascular bundles (arrows) near to the nectariferous parenchyma. **B** Nectary with a uniseriate epidermis (ep) and parenchyma (pa) with compactly arranged cells. Note epidermis recovered by a thin continuous cuticle and asymmetric anticlinal divisions (marker) in the epidermal cells. **C** Parenchyma cells with dense cytoplasm and central volume nucleus. Note some cells contain small calcium oxalate crystals. **D** Periphery of the nectariferous parenchyma with vascular bundles (arrow) containing predominantly phloem. **E–J** Non-anthetic nectary in the buds after the corolla falls. **E** Epidermal (ep) and parenchyma cells (pa) of the nectary had large vacuoles and prominent nucleus. Note vascular bundles (arrow). **F–G** Nectariferous epidermis and secretion (asterisk). Note thin cuticle, stomata (**F**) and cells with tortuous contour, disrupted walls and failures in the cuticle (**G**). **H** Secretion in the subcuticle space (arrowhead) and periclinal divisions occurred in subjacent epidermal cells. Note secretion (asterisk). **I** Parenchyma cells with large vacuoles and prominent nucleus. Note calcium oxalate crystals. **J** Periphery of the nectariferous parenchyma (pa) containing vascular bundle (arrow). ep, nectariferous epidermis; ne, nectary; pa, nectariferous parenchyma

of fruit development of pollinated flowers. This stage was designated the post-anthetic nectary (Fig. 1D' and Table 1).

Nectary anatomy in different flower and fruit stages

Throughout all the analyzed flower and fruit stages, the floral nectary consisted of a uniseriate epidermis with stomata and multiple layers of specialized nectar-secreting parenchyma. This parenchyma was vascularized mostly by phloem originating from vascular bundles located peripherally around the nectary.

Non-anthetic nectary before and after corolla abscission

In the floral bud with detached sepals to the corolla, the nectary exhibited an epidermis recovered by a thin continuous cuticle (Fig. 2A, B). The epidermis comprised rectangular cells with thin walls, dense cytoplasm, and the voluminous central nucleus (Fig. 2A, B). Asymmetrical anticlinal divisions were evident in the epidermal cells (Fig. 2B), likely precursor stages for stomata formation. The nectariferous parenchyma consisted of isodiametric cells arranged compactly, characterized by dense cytoplasm and voluminous central nucleus (Fig. 2B). Sparse parenchyma cells had tiny calcium oxalate crystals (Fig. 2C). Vascular bundles, predominantly containing phloem, were situated along the periphery of the nectariferous parenchyma (Fig. 2A, D).

After the corolla abscission, the nectary was exposed to the external environment and became covered with exudate (Figs. 1A' and 2E). The epidermal cells were covered by a thin cuticle (Fig. 2E–H). Stomata (Fig. 2F) and epidermal cells with irregular contours, disrupted walls, and cuticle irregularities (Fig. 2G) were observed. The accumulation of secretion was evident in the subcuticular space (Fig. 2H), on the surface near stomata (Fig. 2F), and in regions with disrupted epidermal cells (Fig. 2G). Periclinal divisions occurred underneath epidermal cells (Fig. 2H). Epidermal and parenchyma cells were voluminous and had large vacuoles and prominent nuclei (Fig. 2H, I). Parenchyma cells exhibited larger and more abundant calcium oxalate crystals (Fig. 2I). Vascular bundles, mainly composed of phloem, occurred in the periphery of the nectariferous parenchyma (Fig. 2E, J).

Pre-anthetic and anthetic nectary

In floral buds with adpressed sepals (Fig. 1B), the nectary structure (Fig. 3A) resembled the non-anthetic nectary. A continuous thin cuticle covered the epidermis, where the stomata were observed (Fig. 3B). Both epidermal and parenchyma cells had thin walls, dense cytoplasm, and central voluminous nuclei (Fig. 3B). Cell divisions in different planes were observed in nectariferous parenchyma (Fig. 3B).

Clusters of cells with dense cytoplasm, voluminous nuclei, and dense contents greenish stained with toluidine blue, indicating the presence of phenolic compounds, were located near vascular bundles at the nectariferous parenchyma periphery (Fig. 3C).

Throughout the 4-day anthesis, the nectary produced copious nectar (Fig. 1C). A continuous thick cuticle covered the epidermis surfaces, extending as rib-like projections into the anticlinal walls (Fig. 3E, F). Epidermal cells had thick walls, and their external periclinal walls bore outgrowths toward the cuticle (Fig. 3G). Extension of the cuticle into substomatal chambers and subepidermal cell walls formed a cuticular epithelium (Fig. 3E). Accumulations of secretion were observed in substomatal chambers and on the surface of the epidermal cells (Fig. 3F). Subepidermal cells were loosely arranged (Fig. 3E–G). Parenchyma cells exhibit thin walls and cell divisions in various planes (Fig. 3H). Numerous idioblasts with druse occurred in the nectariferous parenchyma, primarily associated with vascular bundles surrounding the nectary (Fig. 3I).

Post-anthetic nectary

After the corolla abscission in non-pollinated flowers, the exposed floral nectary continued producing nectar (Fig. 1D') and exhibited similar features to anthetic nectary (Fig. 4A). Certain features, however, were accentuated at this stage (Fig. 4A), such as the presence of the two layers of the cuticular epithelium (Fig. 4B), thick epidermal cell walls with outgrowths of the outer periclinal walls toward the cuticle (Fig. 4C, D), accumulations of secretions in substomatal chambers (Fig. 4C), and an increase in the occurrence of idioblasts with druse (Fig. 4C, D). Layers of voluminous cells filled with dense content, greenish stained with toluidine blue (indicating phenolic compounds), accompanied the vascular bundles surrounding the nectariferous parenchyma (Fig. 4A, E), which appear to derive from the cell clusters previously described in the nectary of the floral buds.

Pericarpial nectary in initial, intermediate, and mature fruits

We selected three stages of fruit development for anatomical analyses based on the fruit diameter, the aspect of the nectary, and nectar abundance: initial, intermediate, and mature stages (Table 1). The nectary remained intact, producing nectar along the fruit development (Fig. 1D–G). The nectary was structured (Fig. 4F) similarly to the post-anthetic stage in all the examined stages. In the nectary of initial fruits, epidermal cells with thick walls displayed prominent extensions of the outer periclinal walls toward the cuticle (Fig. 4G). The parenchyma cell walls thickened progressively, accompanied by an enhancement in intercellular spaces (Fig. 4G–I).
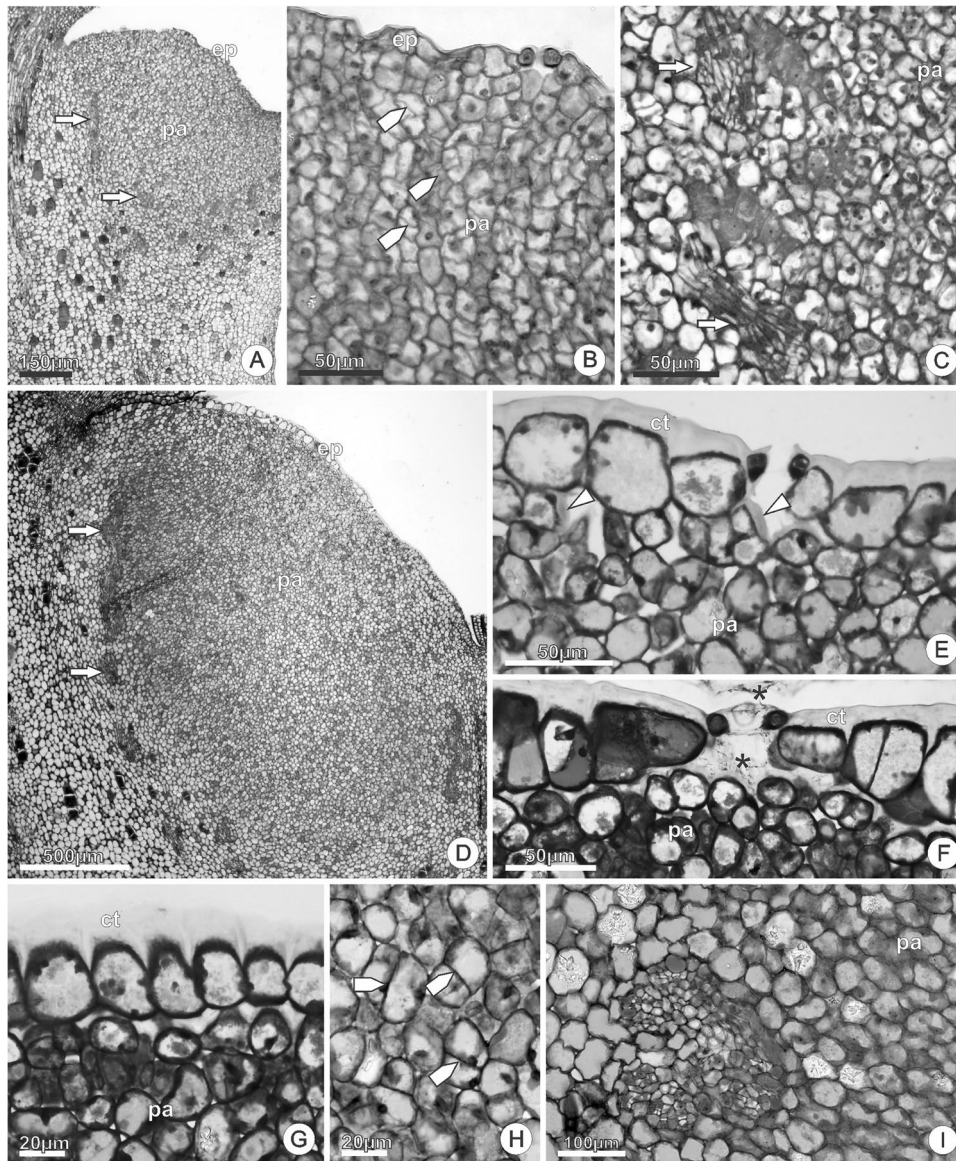
Fig. 3 Structure of the pre-anthetic and anthetic nectaries of *Tocoyena formosa*. **A–H** Longitudinal sections. **I** Transverse section. **A–C** Pre-anthetic nectary in floral buds with adpressed sepals. **A** Nectary with a uniseriate epidermis (ep) and parenchyma (pa). Vascular bundles (arrows) occurred in the periphery of the nectariferous parenchyma. **B** Epidermis (ep) covered by a thin cuticle and containing stomata. Parenchyma (pa) with compactly arranged cells and cell divisions in distinct planes (markers). Note small calcium oxalate crystals in some parenchyma cells. **C** Periphery of the nectariferous parenchyma (pa) showing clusters of cells with dense cytoplasm, voluminous nucleus, and greenish content stained with toluidine blue near to the vascular bundles (arrows). **D–I** Anthetic nectary in flowers in anthesis. **D** General aspect of the nectary. **E** Thick cuticle with riblike projections in the anticlinal walls and extension of the cuticle to the substomatal chambers and the subepidermal cell walls (arrowheads). Note subepidermal cells loosely arranged and intercellular spaces. **F** Accumulations of secretion (asterisk) in substomatal chambers and on the surface of the epidermal cells. **G** Thick walls and outer periclinal walls with outgrowths toward to the cuticle (ct) of the epidermal cells. **H** Cell divisions (markers) in the nectariferous parenchyma. **J** Vascular bundles on the nectariferous parenchyma periphery associated with numerous drusiferous idioblasts. ep, nectariferous epidermis; pa, nectariferous parenchyma; ct, cuticle

Within the parenchyma cell protoplast, Sudan IV reagent confirmed translucent inclusions as lipid drops (Fig. 4I). Cells enriched with dense content, strongly greenish stained by toluidine blue, exhibited numerous vacuoles accompanying the vascular bundles (Fig. 4J).

Histochemical characterization

Throughout different flower and fruit developmental stages, the cuticle that covered the floral nectary displayed a positive response to Sudan IV, indicating the presence of lipid
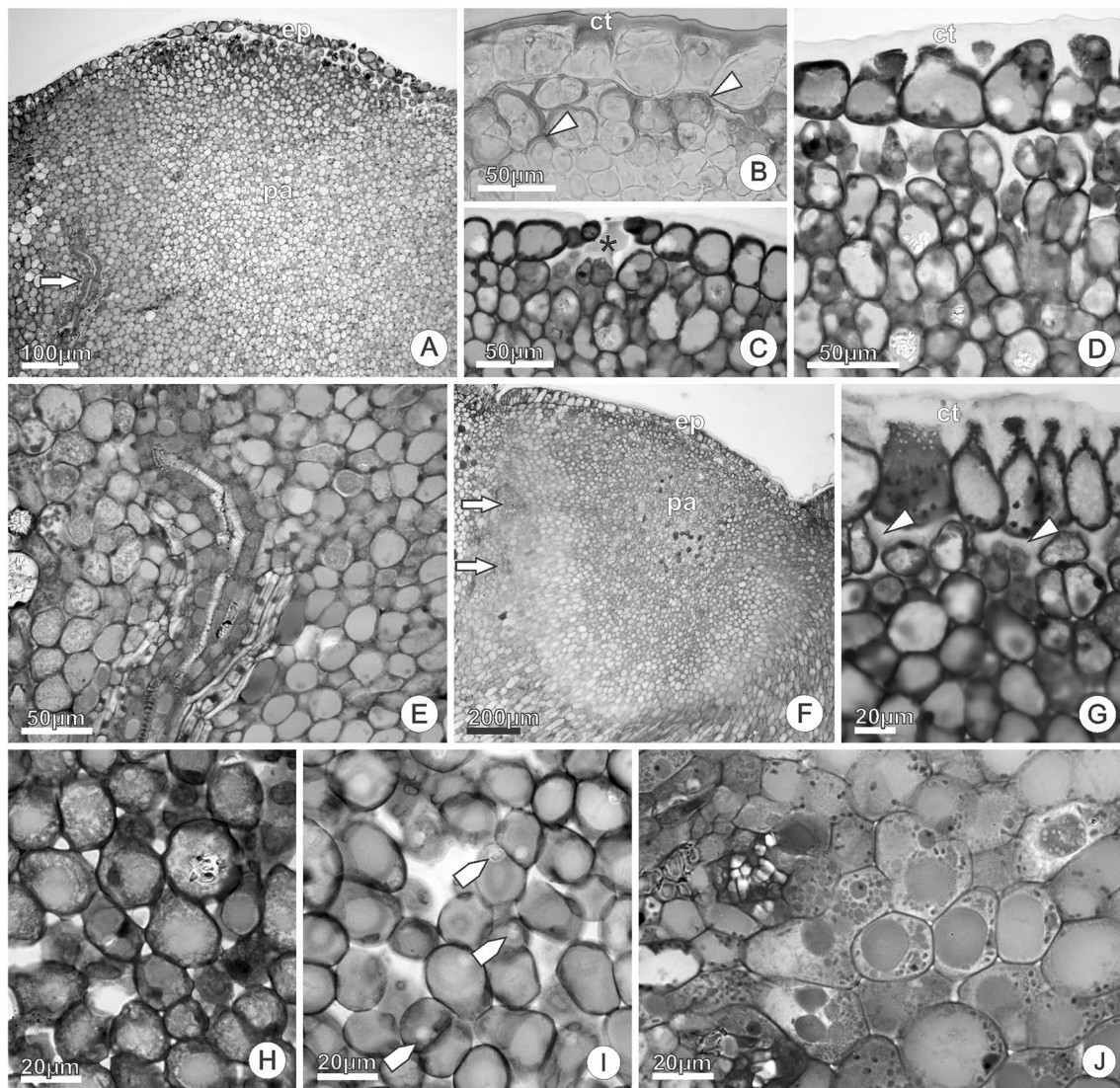
Fig. 4 Structure of the nectary non-pollinated flowers (post-anthetic) and in fruits (pericarpial) in different developmental stages of *Tocoyena formosa*. **A–I** Longitudinal sections. **J** Transverse section. **A–E** Post-anthetic nectary in non-fertilized flowers after the corolla falls. **A** Nectariferous epidermis (ep) and parenchyma (pa). Note vascular bundle (arrow). **B** NADI-stained section showing cuticular epithelium (arrowheads). **C** Accumulations of secretion in substomatal chambers (asterisk) and drusiferous idioblasts in the parenchyma. **D** Epidermal cells with thick walls and outgrowths of the outer periclinal walls toward the cuticle (ct). Note drusiferous idioblasts in the parenchyma. **E** Vascular bundles associated with cells filled by greenish content localized in the periphery of the nectariferous parenchyma. **F–J** Peri-carpial nectary in the initial, intermediated and mature fruit stages. **F** Nectariferous epidermis (ep) and parenchyma (pa). Note vascular bundle (arrow). **G** Nectary in the initial fruits containing epidermal cells with thick walls and developed outgrowths of the outer periclinal walls toward the cuticle. Note cuticular epithelium (arrowheads). **H** Parenchyma cells with thick walls loosely arranged and calcium oxalate crystal. **I** Wide intercellular spaces and translucent inclusions (markers) in the protoplast of the parenchyma cells. **J** Numerous vacuoles filled by greenish content (staining with toluidine blue) in the cells surrounding nectariferous parenchyma associated with vascular bundles. ep, nectariferous epidermis; pa, nectariferous parenchyma

substances (Fig. 5A–D), which facilitated the assessment of the thickness and integrity of the cuticle. The cuticular epithelium in the nectary of the flowers in anthesis (Fig. 5B), non-pollinated flowers after corolla abscission, and fruit development (Figs. 4B and 5D, I) was evidenced by the positive reactions of both Sudan IV and NADI reagent, indicating the presence of lipids and terpenes, respectively.

The nectariferous epidermis and parenchyma cells exhibited pectocellulosic primary cell walls across different floral and fruit stages, evidenced by the positive reaction to ruthenium red test and toluidine blue staining.

Sudan IV staining revealed the presence of total lipids in the protoplast of both epidermal and parenchyma cells within the nectary of flowers and fruits (Fig. 5A–D; Table 2).
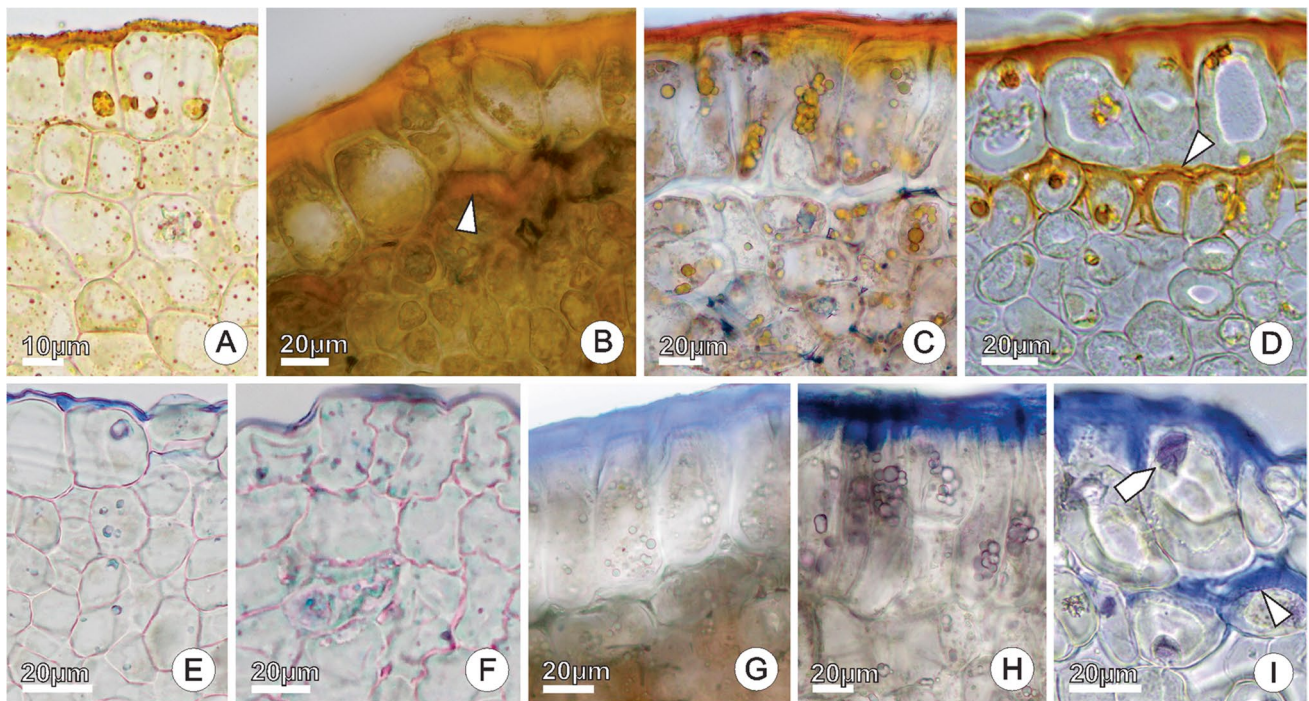
Fig. 5 Lipid substances in the floral nectary of *Tocoyena formosa* in flower and fruit different developmental stages. Longitudinal sections. **A–D** Detection of lipids with Sudan IV. **A** Lipid droplets in protoplast of the epidermal and subepidermal cells in the non-anthetic nectary before corolla abscission. Note thin cuticle. **B** Thick cuticle with rib-like projections in the anticlinal walls and extension of the cuticle to the substomatal chambers (arrowhead) in the floral nectary during anthesis. Note drops in the protoplast of the epidermal and parenchyma cells weakly labeled for lipids. **C** Lipid drops in epidermal and subepidermal cells in post-anthetic nectary. Note thick cuticle. **D** Larger lipid drops in the protoplast of the epidermal and parenchyma cells in the pericarpial nectary in mature fruit. Note thick cuticle with riblike projections in the anticlinal walls and cuticular epithelium (arrowhead). **E–I** Detection of terpenoids with NADI's reagent. **E** Drops of a mixture of essential oils and oleoresin in the protoplast of the epidermal and parenchyma cells in non-anthetic nectary after the corolla falls. **F** Anthetic nectary in floral buds; essential droplets were observed mainly in the protoplast of the epidermal cells. **F** Weak labeling for terpenoids in drops of the epidermal and subepidermal cells in the floral nectary during anthesis. **H** Numerous drops of a mixture of essential oils and oleoresin in the protoplast of the epidermal and subepidermal cells in post-anthetic nectary. **I** Large drops of a mixture of essential oils and oleoresin in the protoplast and in the subcuticular space (marker) in the pericarpial nectary in mature fruit. Note cuticular epithelium (arrowhead)

Tiny and sparse lipid droplets were detected in the non-anthetic nectary in floral buds before corolla abscission. In contrast, after the corolla abscission, these droplets enlarged notably (Fig. 5A), particularly within the epidermal cells. In the floral nectary before anthesis, sparse lipid droplets were present in the protoplast of both epidermal and parenchyma cells. In the floral nectary in anthesis, drops in the epidermal and subepidermal cells were weakly labeled (Fig. 5B). In the post-anthetic nectary, the lipid droplets were larger and more abundant (Fig. 5C). Lipid droplets were larger, but less numerous in the nectary during the fruit development (Fig. 5D). This positive reaction was consistent in terms of size and distribution of the translucent droplets in toluidine blue stained (Fig. 4G, I).

Using NADI reagent, essential oils (blue staining) were apparent (Fig. 5F), as well as a mixture of essential oils and oleoresin (purple staining, see Fig. 5E, H and I), within the protoplast of both epidermal and subepidermal cells across various flower and fruit stages. However, no such substances were detected in the non-anthetic nectary before corolla abscission, nor in the floral nectary before anthesis (Fig. 5G; Table 2). After corolla abscission, sparse droplets of a mixture of essential oils and oleoresin were detected in the non-anthetic nectary (Fig. 5E). Droplets of essential oils were observed in the floral nectary before anthesis (Fig. 5F). Within the post-anthetic nectary, drops of the essential oil and oleoresin mixture were more abundant (Fig. 5H). During fruit development, essential oils and oleoresin were larger and appeared less frequently in the protoplast of the nectary cells. Deposits of these substances were evident in the periplasmic spaces of the epidermal cells (Fig. 5I).

Lugol's reagent revealed numerous starch grains (granules of dark brown or purple color) within both the epidermal and parenchyma cells of the non-anthetic nectary before corolla abscission (Fig. 6A). After corolla abscission, starch grains were less abundant in epidermal and subepidermal

Table 2 Histochemical characterization of the nectariferous epidermis (Ep) and parenchyma (Pa) in flower and fruit development of *Tocoyena formosa*. Key to cell layers: *Ct* cuticle, *CE* cuticular epithelium, *SS* subcuticular space, *Ep* epidermal cells, *CW* cell wall, *Pt* protoplast, *Pa* parenchyma cells; [1]essential oils; [2]mixture of essential oils and oleoresin; – absence of reaction

Reagent	Substance	Non-anthetic nectary before the corolla abscission	Non-anthetic nectary after the corolla abscission	Pre-anthetic nectary	Anthetic nectary	Post-anthetic nectary	Pericarpial nectary in initial fruit	Pericarpial nectary in intermediate fruit	Pericarpial nectary in mature fruit
Ruthenium red	Pectin	CW (Ep, Pa)	CW (Ep, Pa)	CW (Ep, Pa)	CW (Ep, Pa)	CW (Ep, Pa)	CW (Ep, Pa)	CW (Ep, Pa)	CW (Ep, Pa)
Lugol	Starch	Pt (Ep, Pa)	Pt (Ep)	Pt (Ep)	–	Pt (Ep)	Pt (Ep, Pa)	Pt (Ep)	–
Sudan IV	Total lipids	Ct	Ct Pt (Ep, Pa)	Ct	Ct; CE Pt (Ep, Pa)	Ct; CE Pt (Ep, Pa)	Ct; CE Pt (Ep, Pa)	Ct; CE Pt (Ep, Pa)	Ct; CE Pt (Ep, Pa)
NADI reagent	[1]Essential oils [2]Mixture of essential oils and oleoresin	–	[2]Pt (Ep, Pa)	[1]Pt (Ep, Pa)	–	SS [2]Pt (Ep, Pa)	[2]Pt (Ep, Pa)	SS [2]Pt (Ep, Pa)	SS [2]Pt (Ep, Pa)
Ferric chloride	Phenolic compounds	–	–	–	–	–	–	–	Pt (Ep, Pa)
Bromophenol blue	Total protein	–	–	–	–	–	–	–	–

Fig. 6 Detection of the starch grains (black points) with Lugol's reagent in the floral nectary *Tocoyena formosa* in flower and fruit different developmental stages. **A**, **B** Non-anthetic nectary before (**A**) and after (**B**) the corolla falls. **C** Anthetic nectary in the floral bud. **D** Pericarpial nectary in intermediary fruit
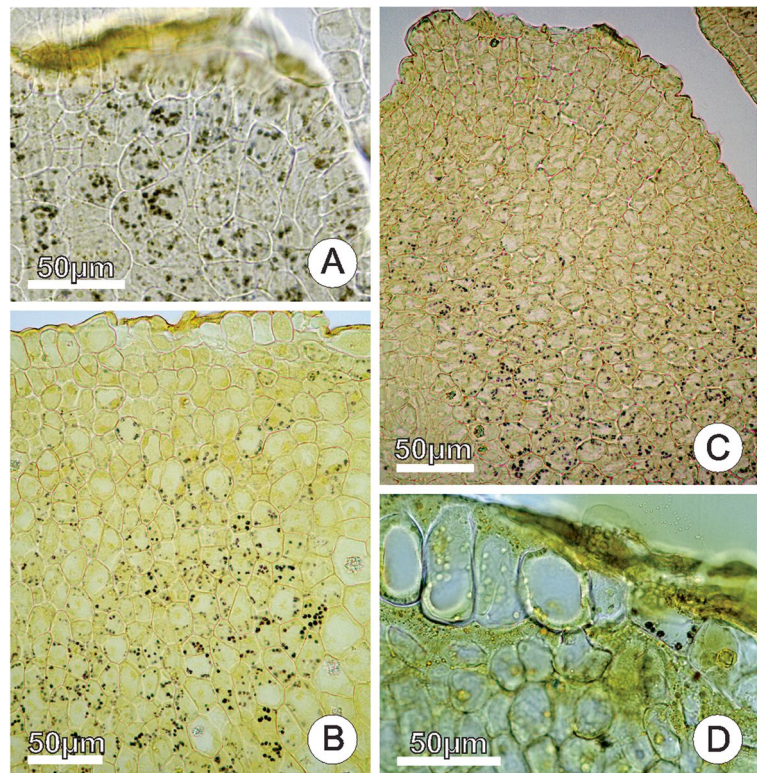
cells (Fig. 6B; Table 2). In the pre-anthetic nectary, starch grains occurred on the periphery of the nectariferous parenchyma, gradually diminishing toward the epidermis (Fig. 6C). In the anthetic post-anthetic and the pericarpial nectaries, starch grains were either scarce or absent within the nectariferous tissues (Fig. 6D; Table 2).

Phenolic compounds (green or dark brown color) were sporadically detected within the protoplast of the epidermal and parenchyma cells in the nectary of the mature fruit (Table 2). Interestingly, the greenish content densely stained by toluidine blue (an indication of phenols) within the cell layers adjacent to vascular bundles in the post-anthetic and pericarpial nectaries did not react to ferric chloride (indicative of phenolic compounds), Dragendorff's reagent (indicative of alkaloids), or vanillin hydrochloride (indicative of tannins).

Discussion

This study presents the first comprehensive comparative analysis of the morphology and functioning of the floral nectary throughout the reproductive development and the anatomical characterization of the non-anthetic nectary in a Rubiaceae species. The floral nectary of *T. formosa* was observed in two distinct morphotypes of floral buds, each having different functions. The abscission of the corolla in the younger early-developing floral buds of the dichasial cymose inflorescence leads to the initiation of nectar secretion. In contrast, the terminal floral buds follow their regular developmental course, originating new flowers. Consequently, the developing floral buds of the latter morphotype lack nectar secretion. However, nectar secretion begins immediately after corolla abscission in the younger early-developing floral buds. The presence of the floral nectary is a widely observed feature in numerous species within the Rubiaceae (*e.g.*, Amorim and Oliveira 2006; Del-Claro et al. 2013; Falcão et al. 2014; Chomicki et al. 2016). Our observations, which include the identification of the non-anthetic nectary in other Rubiaceae species like the hummingbird-pollinated *Palicourea rigida*, suggest that this classification can be extended to other species within the diverse Rubiaceae.

Tocoyena formosa is a deciduous shrub (Silberbauer-Gottsberger 1972) that exhibits a synchronization between the activity of its non-anthetic nectary and the sprouting of new leaves during early spring. This is a critical period for plants because the soft tissues are more susceptible to herbivore attacks (Korth et al. 2006). In this sense, the synchronization between nectar secretion of the non-anthetic nectary and leaf expansion further highlights the potential protective function of this nectary. Nectar production in post-anthetic nectary linked to defense mechanisms against herbivores has been previously observed in other Rubiaceae species (Del-Claro et al. 2013; Chomicki et al. 2016). However, the intriguing role of the floral nectaries observed in *T. formosa* adds further complexity to understanding plant defensive strategies against herbivores. The non-anthetic nectary attracts ants and may act

as a preemptive defense mechanism during the period of high plant susceptibility to herbivore attacks. This nectary precedes the beginning of nectar production by nectaries derived from both non-pollinated (post-anthetic nectary) and pollinated flowers (pericarpial nectary).

Morphologically, the non-anthetic nectary exhibits characteristics of immature tissues, including a thin cuticle and soft cell walls. The epidermal and parenchyma cells show dense cytoplasm, voluminous nuclei, and continuous cell divisions. All these features are typical of the pre-secretory stage (Fahn 1979; Machado et al. 2022). Therefore, we suppose that the exudate covering the nectary after corolla abscission might not constitute nectar but rather a pre-nectar released due to the exposure of the nectary of early-developing floral bud to a drier environment following premature corolla abscission. While flowers retain more moisture than the stem, water movement via the xylem is thermodynamically challenging (De la Barrera and Nobel 2004). Consequently, the water supply and photosynthates to flowers primarily occur through the phloem solution (De la Barrera and Nobel 2004). In this context, secretions observed in the non-anthetic nectary might have originated as leakage of phloem solution, resulting from structural weakness of developing tissues when subjected to elevated pressures within the phloem (De la Barrera and Nobel 2004). Apart from sugars and other nectar constituents, the water in the nectar can also serve as a valuable resource for ants associated with the plant (Galetto and Bernardello 1992).

Exudate release onto the surface of the non-anthetic nectary after corolla abscission seems to occur in two ways: disrupted epidermal cells and stomatal openings. The relatively young tissues of the nectary within floral buds are less resistant to mechanical damage, with their rigidity increasing progressively with age. The morphological changes in the floral nectary during *T. formosa* reproductive development, like cuticle thickening, lipid deposition in substomatal chambers, and cuticular epithelium formation, can act as mechanical protection and an effective strategy against continuous water loss. Additionally, rib-like projections in the epidermal cells of the floral nectary during anthesis provide a support function. In flowers, periclinal and anticlinal divisions enhance nectary volume, while epidermal cell anticlinal divisions ensure their coverage on the surface. Cell divisions, growth, and formation of new specialized cell walls (Calvin 1970), such as that occurring in tissues of the nectary during flower development, have been reported in different species (Nepi 2007), and it reveals the regenerative potential of secretory cells (Guimarães et al. 2018).

Our findings, particularly those concerning the characteristics of epidermal cell walls, suggest the participation of common epidermal cells in nectar release in addition to the stomata. Modified stomata are recognized as a

significant apoplasmic pathway for nectar release (Galetto 1995; Thomas and Dave 1992; Bernardello 2007; Guimarães et al. 2018; Judkevich et al. 2022). During initial fruit development, morphological changes are more pronounced in the post-anthetic and the pericarpial nectaries. In this last stage, the outer periclinal wall substantially thickens and projects toward the outer periclinal walls of the cuticle, potentially promoting solute transfer over short distances. Similar wall projections have been observed in the perigonal nectaries of *Fritillaria meleagris* (Liliaceae), where they played a role in nectar exportation and resorption (Stpiczyńska et al. 2012). In bracteal and circumbracteal nectaries of *Gossypium hirsutum* (Malvaceae), trichome head cell walls exhibit ingrowths toward the plasma membrane during the secretory stage, expanding the area of nectar secretion until fruit maturation (Chatt et al. 2021). The presence of epidermal cell projections in *T. formosa* might suggest nectar resorption during post-anthetic and pericarpial stages. However, detailed studies integrating nectar secretion dynamics and ultrastructural analyses are necessary to fully understand the role of these cell wall projections in nectar transport.

Progressive depletion of starch grains accumulated in the anthetic nectary of regular floral buds in *T. formosa* implies that these starch grains likely served as the primary sugar source for the pre-nectar, a phenomenon commonly observed in the floral nectaries of various species (Nepi et al. 1996; Durkee et al. 1981; Ren et al. 2007; Paiva 2012; Guimarães et al. 2018; Chatt et al. 2021; Paiva et al. 2021). Notably, the floral nectary remains consistently covered by nectar over an extended period (about 105 days), spanning flower anthesis and fruit development (Sanz-Veiga et al. 2017). The prevalence of phloem bundles in the periphery of the nectary observed in *T. formosa* strongly suggests that the phloem solution serves as a crucial source of nectar sugars during different phases of nectar production (Bernardello 2007; Nepi 2007; Vassilyev 2010).

The visual increase in both the quantity and size of calcium oxalate crystals within the parenchyma of the nectary, particularly in association with vascular bundles at the periphery of the nectary during the flowering and fruiting stages of *T. formosa*, may be linked to various factors. These include the regulation of cytosolic calcium levels, the maintenance of cell homeostasis, solute translocation in the phloem (Paiva and Machado 2005; Paiva 2019; Mesquita-Neto et al. 2020), and even the transport of sucrose from the symplast to the apoplast (as discussed in Paiva et al. 2021). This observed increase in calcium crystals may further indicate the participation of phloem solution in the origin of nectar.

The presence of clusters of cells exhibiting phenolic content near vascular bundles at the periphery of the nectariferous parenchyma, as observed in the nectary of regular floral buds, results in the formation of a contiguous layer of densely filled cells surrounding the post-anthetic and pericarpial nectaries of *T. formosa*. While our histochemical tests for directly detecting phenolic compounds were inconclusive, we posit that these cells contain such compounds based on the green staining with toluidine blue (Ramalingam and Ravindranath 1970). We hypothesize that these continuous cell layers may function as barriers, preventing the efflux of pre-nectar into adjacent nectary tissues. Vacuoles filled with phenolic would increase cell turgor and reduce intercellular spaces. Although the primary cell walls in these regions are permeable, the potential efflux of pre-nectar or nectar via the apoplast could be obstructed, aligning with Vassilyev's (2010) hypothesis regarding the mechanism of floral nectar transport. Although comparable features, such as a tannin-rich tissue layer beneath the secretory tissue in the nectaries of *Hiptage sericea* (Malpighiaceae) and tannin-containing idioblasts in the parenchyma of the elaiophores of *Krameria triandra* (Krameriaceae), have been reported (Subramanian et al. 1990; Vogel 1974), the specific functions of these specialized cell layers remain relatively unexplored.

The histochemical analysis, revealing the presence of neutral lipids and terpenes (essential oils and oil-resin) in epidermal and parenchyma cells during the functioning of the floral nectary in *T. formosa*, suggests other substances in nectar composition beyond sugars, as reported to different species (Fahn 1979, 2000; Baker and Baker 1973; Vesprini et al. 1999; Stpiczyńska et al. 2012; Tölke et al. 2015; Chatt et al. 2021). This multifaceted composition may play crucial roles in plant-animal interactions, encompassing both attraction and defense (Pacini et al. 2003; Koptur 2005; Heil 2011). A notable feature observed in the post-anthetic nectary of *T. formosa* was the predominance of lipophilic secretions in the epidermal and subepidermal cells. This indicates that the nectary has the potential to generate floral scents in the form of volatile compounds. Nectary acting as a scent gland, for instance, has been documented in the flower of the Bignoniaceae, *Jacaranda oxyphylla* (Guimarães et al. 2018). Furthermore, lipophilic substances within the periplasmic space of the epidermal cells in pericarpial nectaries in *T. formosa* may be associated with cuticle restoration (Paiva 2017).

A noteworthy aspect of *T. formosa* is the presence of early-developing floral buds that give origin to the non-anthetic nectaries, easily distinguished by the loose arrangement of sepals before premature corolla abscission. *Tocoyena formosa* blooming season begins at the end of the dry season, and the inflorescences are multiflowered, bearing large flowers. Furthermore, the relatively high water required for producing large flowers might contribute to the plant selection of floral buds. The impact of such water-related costs on flower morphology, including reductions in size and quantity, has been documented by

De la Barrera and Nobel (2004). This alteration in morphology is especially significant for species inhabiting water-limited environments (Galen et al. 1999), where many species flower during the dry season, reducing transpirational water loss in arid regions (Larcher 1980). To investigate these hypotheses, future research should concentrate on the vascularization patterns of terminal and lateral younger early-developing floral buds within the inflorescence and their connections to flower water relations, focusing on the timing of different maturation stages of floral structures.

The floral nectary of *T. formosa* was divided into four categories based on the stage of reproductive development at which nectar production was seen: non-anthetic nectary, anthetic nectary, post-anthetic nectary, and pericarpial nectary. This subdivision facilitated the correlation between the structural and histochemical changes of the nectary with the phases of reproductive phenology. The non-anthetic and post-anthetic nectaries share a common ontogenetic origin and potentially serve a similar defensive function within the plant. Nonetheless, they exhibit variations in tissue maturation and histochemical composition. The prolonged ability of the floral nectary in *T. formosa* to secrete nectar continuously for more than 105 days throughout various reproductive stages is facilitated by anatomical changes and vascular involvement in pre-nectar production, complemented by possible nectar reabsorption during post-anthetic stages. The floral nectary of *T. formosa* plays a crucial role in the reproductive and defensive interactions of this plant species with mutualist partners. Hence, it is a bivalent secretory structure and is an intriguing model for in-depth exploration spanning ultrastructural, chemical, and ecological aspects.

Declarations

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