STRUCTURE AND HISTOCHEMISTRY OF THE FLORAL NECTARY OF Bauhinia monandra (FABACEAE)

Estructura e histoquímica del nectario floral de Bauhinia monandra (Fabaceae)

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ABSTRACT
The structure and histochemistry of the floral nectary of Bauhinia monandra Kurz (Fabaceae) were investigated. Besides possessing medicinal properties, this tree is also used in the recovery of degraded areas and urban arborization. Nectary samples were obtained from newly bloomed flowers. The nectary was located on the tubular hypanthium. This tube was partially coated by a nectary epidermis, whose cells had secretory features such as a relatively large nucleus, a dense cytoplasm, and small vacuoles. Subjacent to the nectary epidermis, there was a nectary parenchyma with eight to fifteen layers of cells which also have secretory features. Both the nectary epidermis and nectary parenchyma possessed starch grains. Subjacent to the nectary parenchyma passed collateral to amphicribral concentric vascular bundles constituted by more phloem than xylem. Although these vascular bundles did not emit terminations directed to the nectary parenchyma, the arrangement of the latter about the former suggests the supply of nectar precursors by the vascularisation. In the basal region of the hypanthium tube occurred modified stomata which were probably the main route of nectar release; and tector trichomes, possibly involved in the nectar retention.

Keywords: anatomy, flower, Leguminosae, medicinal plant, secretory structure.
INTRODUCTION

Fabaceae Lindl. comprises about 19,500 species distributed in approximately 770 genera, being considered the third largest family of Angiosperms. Bauhinia L. is a pantropical genus, one of the largest of Fabaceae, comprising about 150-160 species, in addition to several undescribed ones (Lewis et al., 2005; LPWG, 2017). Traditionally, it was placed in the paraphyletic Caesalpinioideae subfamily (Lewis et al., 2005). Recently, after the taxonomic reorganization of Fabaceae, Bauhinia was placed in the newly circumscribed subfamily Cercidoideae (LPWG, 2017). Bauhinia is represented by wood species, sometimes multi-stemmed, either trees, shrubs, and sub-shrubs, as well as a few semi-succulent species. The branches may have pairs of welldescribed thorns and extrafloral nectaries. The flowers have a long and tubular hypanthium, and the fruit is a dehiscent woody or coriaceous legume (Moreira et al., 2013; Castellanos and Forero, 2019).

Bauhinia monandra Kurz is a perennial tree native from Madagascar, Africa (Sinou et al., 2009). Like other species of the genus, it is used as a medicinal plant (Salatino et al., 1999). Different parts of the plant are used in folk medicine as anti-diabetic, anti-nephritic, diuretic, digestive, anti-hemorrhoidal, and anti-inflammatory, among other applications (Quijano et al., 2018).

Due to its rapid growth and lush flowers, B. monandra is commonly used in forest reestablishment as well as urban afforestation (Burnie et al., 2004; Santos et al., 2014). The inflorescences of this species are short racemes composed of 1-5 zygomorphic flowers displaying a cream or pale-pinkish claw and light pink petals tinged with dark pink spots (macula), specially on the banner (Torres-Colín et al., 2013). The hypanthium is tubular and accumulates a sweetened solution.

According to Fahn (1979), nectaries are nectar-producing structures, a sugary solution consisting basically of sucrose, fructose, and glucose. They occur in several species, in different plant organs, and can present variable structures (Bentley and Elias, 1983; Nepi, 2007). When located in the vegetative organs, they generally provide recompenses for animals, mainly ants, which confer them protection against herbivorous predation and pathogens attacks (Heil, 2011; Beaumont et al., 2016). If present in the flower, the produced nectar often serves as a reward for pollinators (Heil, 2011; Etcheverry et al., 2012).

Ecological studies on Bauhinia have shown the diversity of pollinating animals for this genus, including bats, bees, hummingbirds, moths, and butterflies (Hokche and Ramirez, 1990; Lau et al., 2009). Nectar is the main resource, exposed to specialized or non-specialized pollen vectors, such as in Bauhinia championii (Benth.) Benth., B. corymbosa Roxb., and B. glauca Benth. (Lau et al., 2009). However, little is known about the anatomical structure of the nectary in Bauhinia.

Considering the importance of B. monandra, to know a key structure in its sexual reproduction and thus offer elements for taxonomy studies, reproductive biology, and ecology of this species, the present work aimed at describing the anatomy of the floral nectary of B. monandra and detecting, by using histochemical tests, the different chemicals present in the nectary tissues.

MATERIALS AND METHODS

Fully opened flowers of Bauhinia monandra, which were pre-anthetic the day before, were collected in January 2014 from cultivated trees growing in the Universidade Estadual do Sudoeste da Bahia (UESB), Campus of Vitória da Conquista, Bahia State, Brazil (14°53’28” S; 40°48’14” W), at 823 meters above sea level. The voucher specimens, obtained by the second author of the present work, were deposited in the Herbarium of the Universidade Estadual de Santa Cruz (UESC), Bahia State, Brazil, under the no 21964, 21965, and 21966. The nectar samples of three flowers of three individuals were tested with Keto-Diabur-Test® (Roche Diagnostics; Hoffmann-La Roche, Risch-Rotkreuz, Switzerland). The hypanthium samples containing the nectary were manually removed from at least nine flowers of each of the three individuals studied here. Each hypanthium was then subdivided into three parts (basal, middle, and apical) by using a razor blade, and was immediately fixed in a solution containing glutaraldehyde and paraformaldehyde at pH 7.2 (Karnovsky, 1965) and stored in 70 % ethanol.

LIGHT MICROSCOPY

Three samples of each of the three different parts of the hypanthium from each individual, which were stored in 70 % ethanol, were progressively dehydrated to 95 % ethanol and then embedded in glycol-methacrylate resin (Historesin® Leica; Leica Microsystems, Heidelberg, Germany). Serial sections optimized for histochemistry studies (Leitão, 2018) 3 µm thick were obtained with a Leitz 1212 rotary microtome (Ernst Leitz Optical Works, Wetzlar, Germany). The sections were placed on slides and submitted to different contrasting and histochemical techniques: for structural characterization and metachromasy observation was used 0.025 % Toluidine blue O was in McIlvane buffer at pH 4.0 (Vidal, 1977); for total carbohydrates detection, the Periodic Acid Schiff reaction (Maia, 1979); to demonstrate the presence of mucilage, 5 % tannic acid and 3 % ferric chloride (Pizzolato and Lillie, 1973) or 0.05 % Alcian blue at pH 2.5 (Whiteman 1973); to identify the presence of total proteins 0.1 % Ponceau Xyline 2R at pH 2.5 (Vidal, 1970); for phenols, 10 % potassium dichromate (Gabe, 1968) and to locate lipids, Sudan black B or Sudan IV solution saturated in 70 % ethanol (Pearse, 1980).
The temporary slides were made in water (Johansen, 1940). For the analysis and photographic documentation, a Leica DM750 light microscope equipped with Leica ICC50HD digital image capture system (Leica Microsystems, Wetzlar, Germany) and the polarized light feature were employed. The anatomical descriptions were made according to terminologies proposed by Nepi (2007).

**SCANNING ELECTRON MICROSCOPY**

Three samples of each of the three different parts of the hypanthium from each individual, which were stored in 70 % ethanol, were dehydrated in an ethanol series (85 %, 95 %, and 100 % ethanol), for 15 min in each solution. After being placed three times in 100 % ethanol, the samples were submitted to ethanol/acetone (3:1, 1:1, 1:3) and 100 % acetone three times, and submitted to CO2 critical point drying, by using a Bal-Tec CPD030 (Balzers, Schaan, Liechtenstein). The cathodic deposition of gold was carried out in a Bal-Tec SCD050 (Balzers, Schaan, Liechtenstein) sputter coater. For observation and photographic documentation, a Quanta 250 (FEI Company, Oregon, USA) scanning electron microscope equipped with a digital image capture system was used at 10 kV.

**RESULTS**

The secretion could fill the hypanthium tube. It was colorless and had a sweet taste, reacting positively to the Keto-Diabur-Test®, attesting that it was nectar, and therefore, it was exuded by a nectary.

The newly opened flowers of Bauhinia monandra had lighter petals, which became pinker as the flowers aged (Fig. 1a). The receptacle was characterized by the tubular and constricted hypanthium measuring about 2.5 cm in length and with a discreet opening (Fig. 1b-d). The nectar accumulated inside the hypanthium (Fig. 1e). The nectary tissues were present, dorsally involving the basal third of the tube (Fig. 2a, b) and acropetally bifurcating in “Y”, forming two branches that run on the sides of the tube up to its opening, where the insertions of the floral parts were (Fig. 2c-f).

The epidermis of the tube was single-layered and slightly papillose (Figs. 2g-j, 3a-c). In the secretory portions of the tube, the nectary epidermis consisted of smaller cells with dense cytoplasm, strongly stained by Toluidine blue O pH 4.0 (TBO) (Fig. 2g) and Ponceau Xylidine (Fig. 2h). The vacuole was relatively smaller than in the other epidermal cells of the tube (Fig. 2i). Some cells contained intracellularly accumulations of secretion, which stained with TBO (Fig. 2g), but these cells did not possess well-formed secretory cells in association with nearby vascular bundles (Fig. 2h). These corpuscles could also be found in the non-nectary epidermis of the tube (Fig. 2i). The nectary epidermis presented an especially thin cuticle in the basal portion of the tube, not always detectable with Sudan (Fig. 3a). However, in the nectary epidermis at the upper portions of the tube, the cuticle was thicker (Fig. 3b), although thinner compared to the non-nectary epidermis (Fig. 3c).

Modified stomata occurred only in the basal third of the length of the tube (Fig. 4a) and distributed in a non-oriented manner (Fig. 4b). At the basal two-thirds of the tube, as well as close to its opening, there were normal uniseriate multicellular trichomes, with a tapered extremity and a dilated middle portion, ornamented by a rugosity like micro verrucas on their surface (Figs. 3d, 4c-d). Similar trichomes occurred in abundance on the outer surface of the hypanthium (Fig. 4c).

The nectary parenchyma was characterized by juxtaposed cells, organized in eight to fifteen layers (Fig. 2b, d, f), being thicker in the basal region of the tube (Fig. 2b). The cells were smaller, with cytoplasmic content more intensely stained with TBO (Figs. 2g, 3e) and Ponceau Xylidine (Fig. 2h) when compared to the non-nectary parenchyma cells (Fig. 2d, f, i). In longitudinal cuts, the cells were anticlinal elongated, and the stratification of the tissue was evident. Also, some cells organized in a cords-like pattern were visible crossing several layers of the tissue (Fig. 3f).

In the nectary parenchyma, the cell walls were stained purple with TBO, that is, they were metachromatic. The vacuoles were small and numerous (Fig. 3e). In the cytoplasm of these cells (Figs. 2j, 3g), as well as in the nectary or non-nectary parenchyma cells adjacent to the vascular bundles, large amounts of starch grains occurred, especially in the phloem proximities (Fig. 3g, h). In the adjacent non-nectary parenchyma cells as in the nectary and non-nectary epidermis cells, starch grains also occurred, but they were smaller than those found in the nectary parenchyma cells (Figs. 2j, 3g).

The vascular bundles were of the collateral or amphicribral concentric type, with a “C” or circular shape, respectively (Fig. 2a-f). They had more phloem than xylem (Fig. 3h-j). In the apical half of the hypanthium, larger vascular bundles occurred in two large concentric rings. The outer ring had ten vascular bundles. The inner one was incomplete, in horseshoe format. It had nine vascular bundles located in opposition to the bundles of the outer ring. Between the most ventral vascular bundle of the outer ring and the nectary tube, there was a small ring of smaller vascular bundles that were directed towards the fertile whorls of the flower (Fig. 2a, c, e).

Only the vascular bundles of the inner ring passed close to the nectary parenchyma (Fig. 2a, c, e), without, however, emitting vascular terminations to itself (Fig. 3i). Nevertheless, an arrangement of nectary parenchyma cells in association with nearby vascular bundles was notorious (Figs. 2b, d, 3i). In the basal third of the hypanthium, the five most dorsal vascular bundles passed beneath the nectary parenchyma (Fig. 2a). In the upper two-thirds (middle and apical), one...
to three bundles on each side were the closest to the nectary (Fig. 2c, e).

Idioblasts containing calcium oxalate crystals of the druse type occurred around the vascular bundles, especially in the phloem proximities (Fig. 3i-k). The results of the potassium dichromate and tannic acid/ferric chloride tests were negative, so no phenolic compounds or mucilages were detected in the investigated tissues, respectively.

DISCUSSION

The floral nectary of *Bauhinia monandra* is in the receptacle, a common feature in the Fabaceae family (Fahn, 1952; Paiva and Machado, 2008). Structurally, this is a modification in the hypanthium, which has developed the property of secreting and accumulating nectar and is therefore classified as a hypanthial nectary (Bernardello, 2007). The hypanthium is tubular in shape and the nectary tissues occur in specific regions of the tube. In the other regions, there is an ordinary epidermis without stomata, subtended by a non-nectary (fundamental) parenchyma.

Both nectary epidermis and nectary parenchyma cells have a relatively small vacuole(s) and a large nucleus, and cytoplasm heavily stained with Ponceau Xylidine. Such characteristics indicate high metabolic activity in the tissues (Nepi, 2007) during the process of nectar secretion. In the
Figure 2. *Bauhinia monandra* hypanthium and nectary tissues. (a-f) Cross-section of the basal (a, b), middle (c, d), and apical (e, f) third parts of the hypanthium. In smaller magnification (a, c, e), hypanthium overview, evidencing the nectary tissues (ellipses) and vascular bundles (arrows). In greater magnification (b, d, f), regions with nectary tissues, indicated by ellipses in a, c, e. (g-j) Cross-sections of the hypanthium in high magnification, stained with Toluidine blue O (g), Ponceau Xylidine (h, i) and Periodic Acid Schiff reaction (j), in the nectary tissues (g, h, j) and non-nectary tissues (i). Intravacuolar corpuscles (arrows) (g-j) and some starch grains in a nectary parenchyma cell are in evidence (ellipse) (j). Legends: NE- nectary epidermis, NNE- non-nectary epidermis, NNP- non-nectary parenchyma, NP- nectary parenchyma, VB- vascular bundle.
Figure 3. Details of the Bauhinia monandra hypanthium and nectary tissues. (a-c) Cross-sections of the nectary (a, b) and the non-nectary (c) epidermis of the tube stained with Sudan black B in the basal (a) and middle (b, c) thirds of the hypanthium, for better cuticle viewing (arrows). The regions of apparent discontinuity of the cuticle on the nectary epidermis in the basal third of the hypanthium (a) are indicated (brackets). (d) Detail of a tector trichome in the basal third of the tube, stained with Sudan IV. (e) Detail of nectary parenchyma cells, showing small and numerous vacuoles (asterisks). (f) Longitudinal section of nectary tissues, showing some cells organized pattern of cords (brackets). (g) Cross-section of the hypanthium submitted to the Periodic Acid Schiff reaction. (h) Longitudinal section of a vascular bundle subjacent to the nectary parenchyma, showing starch-containing cells. Periodic Acid Schiff reaction. (i) Detail of a vascular bundle in cross-section, subjacent to the nectary parenchyma, showing a druse (arrow). The vessel element groups are indicated by ellipses. (j, k) Longitudinal section of a vascular bundle subjacent to the nectary parenchyma, under non-polarised (j) and polarised light (k), where k corresponds to the rectangle in j. Arrows indicate druses. Legends: NE- nectary epidermis, NNP- non-nectary parenchyma, NP- nectary parenchyma, Ph- phloem, SCC- starch-containing cells, VB- vascular bundle, Xy- xylem.
vacuole of some *B. monandra* nectary cells occurs a corpuscle that stains with TBO, which demonstrates the presence of anionic radicals, that is, acid nature (Ribeiro and Leitão, 2020), and reacts positively to Periodic Acid Schiff reaction, confirming the presence of insoluble polysaccharides. The positive, although weak, result of Ponceau Xylidine also indicates the presence of proteins. Similar corpuscles were described for orchid nectaries (Stpiczynska et al., 2003, 2005; Leitão et al., 2014), without, however, concluding their function.

The nectary parenchyma is the most voluminous secretory tissue of the *B. monandra* nectary and, consequently, is the most active in the process of nectar secretion. Its cells are slightly larger than the epidermal ones and have relatively large starch grains. These structures are common in the nectary parenchyma of several species. In comparative observations of *Hymenaea stignocarpa* Mart. ex Hayne nectaries, before and after secretion, it was found that starch grains are hydrolyzed during nectar production (Paiva and Machado, 2008). Starch hydrolysis leads to the increase of osmotic pressure within the cells resulting in water inflow along the sugar concentration gradient, from vascularization to the nectary parenchyma (Stpiczynska et al., 2012). Many authors also inferred that starch hydrolysis in the nectary cells contributes directly to the carbohydrate

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**Figure 4.** Scanning electron micrographs of the *Bauhinia monandra* floral nectary. (a) General view of the nectary epidermis at the basal third of the hypanthium, showing the stomata (arrows). (b) Detail of the stomata (arrows) from the rectangle in a. (c) Longitudinal section of the hypanthium, showing the tector trichomes (arrows) inside and outside the tubular hypanthium (asterisk). (d) Detail of the tector trichomes (arrows) inside the tube.
content of nectar (Rachmilevitz and Fahn, 1973; Nepi et al., 1996; Nepi, 2007; Paiva, 2012; Paiva and Martins, 2014).

The vascular bundles in contact with the nectary parenchyma are in the inner ring of the hypanthium vascularisation. Although these vascular bundles do not emit vascular terminations directed towards the nectary parenchyma, this last tissue has its innermost layers arranged to involve some of these vascular bundles. This structural pattern is also reported for the hypantal nectary of *Caesalpinia gilliesi* (Wall. ex Hook) Dietrich (Fabaceae) (Cocucci et al., 1992). This association between nectary parenchyma and vascular bundles suggests that the phloem is a significant supplier of photoassimilates for nectar production. This phloem function is widely reported (Coutinho and Meira, 1996; Nepi, 2007; Paiva, 2012; Paiva and Martins, 2014).

Concerning the upper two-thirds of the nectary, where the nectary epidermis is covered by a distinct cuticle, no subcuticular nectar accumulation nor cuticle detachment were observed (Subramanian and Inamdar, 1989; Stpiczyńska et al., 2011; Paiva, 2012; Gonzalez and Marazzi, 2018), nor cuticle ruptures (Paiva and Machado, 2006; Vespriini et al., 2012), as described for several nectaries in different families, whose epidermis has noticeable secretory activity. There are nectaries whose nectary epidermis has a permeable cuticle, which remains intact after nectar secretion (Coutinho et al., 2010). Thus, we may infer that all the nectary epidermis cells from the *B. monandra* nectary are very likely to contribute to the nectar secretion in the tube lumen since they have characteristics of secretory cells.

The trichomes found inside the tube of the *B. monandra* floral nectary are of the tector type, that is, they do not have secretory activity and consequently do not participate in the nectar secretion. Tector trichomes are reported for the nectaries of *Solanum stenomorphum* Jacq. (Solanaceae) which apparently are involved in protecting these secretory structures (Falcão et al., 2003). In *Prunus persica* L. Batsch (Rosaceae), it is speculated that they attenuate the nectar evaporation (Radice and Galati, 2003), which may perhaps make sense for the floral nectary of *B. monandra*. In this species, although the nectar is retained within a tube, its flowers are agitated by the wind, thus becoming susceptible to nectar loss. Thus, it can be speculated that these trichomes act as an aid to the nectar retention inside the tube.

The length of the floral tube of many groups of plants is correlated with the length of the buccal parts (proboscis) of several pollinating insects (Nilsson, 1988). For the *Bauhinia* genus, a huge diversity of pollinators is reported, such as bats, birds, bees, butterflies, and moths (Hokche and Ramirez, 1990; Lau et al., 2009). However, in the present study, no information was found about the *B. monandra* pollinator. Nevertheless, the constricted tube form of the *B. monandra* nectary is typical of flowers pollinated by moths or butterflies (Nilsson, 1988; Yamasaki and Sakai, 2013).

**CONCLUSIONS**

It is concluded that the secretory tissues of *B. monandra* floral nectary are a continuum of epidermal and parenchymal cells that partially involves the tubular hypanthium, whose nectar precursors are mainly from vascular bundles crossing this region. Modified stomata at the base of the tube would be the main route of nectar elimination, although nectary epidermal cells appear to participate in this process.

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