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Development and differentiation of the extrafloral nectaries from flower buds in *Vigna luteola* (Leguminosae, Phaseolinae)

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Abstract: To study the ontogeny of the extrafloral nectaries present in the inflorescences of *Vigna luteola* (Jacq.) Benth (Leguminosae, Phaseolinae), the location, morphology, anatomy of the earliest stages, histology of the definitive structures and ultrastructure of the secretory stage were analyzed. The extrafloral nectaries at different developmental stages were examined with light microscopy and scanning electron microscopy. The secretory stage was also examined with transmission electron microscopy. The racemose inflorescence of *V. luteola* has six nodes. At each node, a short globose secondary axis bears two flowers and one to three extrafloral nectaries. Each extrafloral nectary originates from the abscission of a flower bud and is formed by two differentiated zones: a ring of epidermal cells surrounding a group of longitudinally enlarged papillose central cells, both with underlying secretory parenchyma. The primary secretory tissue consists of the central cells, while the ring contributes to secretion to a lesser degree. Secretion is granulocrine, by means of exocytotic vesicles and plasmalemma invaginations. Four developmental stages succeed; the third one being the secretory. The extrafloral nectaries activity period starts when the flowers of the same secondary axis open and ceases before fruit development.

Key words: Extrafloral nectaries, Leguminosae, ontogeny, Phaseolinae, *Vigna luteola*.

INTRODUCTION

The extrafloral nectaries (EFNs) can be distinguished from the floral ones either for the position they occupy or their function. Caspary (1848) distinguished them for the position, naming "floral" those that are placed somewhere in a flower and "extrafloral" the ones located in vegetative structures. Delpino (1875) classified them according to their function, calling "nuptial" the ones involved directly in pollination and "extranuptial" the ones associated with other functions. As there are intermediate situations, such as the ones on bracts, inflorescence rachis, or "outside" the flowers in the abaxial face of sepals, Schmid (1988) proposed the terms "reproductive" and "extra reproductive". Even though, the "floral" and "extrafloral" denomination continues to be the traditional use (Bernardello 2007). Characterization of EFNs considering their morphology, and, in some instances, their origin was performed by Zimmermann (1931), commented and accepted by Elías (1983) and updated by Díaz Castelazo et al. (2005).

The EFNs associated with inflorescences or flowers not directly implied with the process of pollination are common (Elías 1983). They can be positioned along the inflorescence axis (Bentley 1977a), in the abaxial face of the bracts (Rickson & Rickson 1998), in the pedicels (Keller 1977), near the base of the flowers (Elías 1983)

or in the abaxial face of the sepals (Anderson & Simon 1985).

EFNs in association with flowers or inflorescences have been found in several Leguminosae (Elías 1983), outlining their taxonomic relevance (Bhattacharya & Maheshwari 1971). In the genus *Vigna* Savi, the EFNs may be present in the inflorescence axis (Bentley 1977b) or in the stipels (McKey 1989), in different quantity and disposition according to the species (Ojeda 2013).

Studies on the development of the EFNs include those of Maheshwari (1954), Ojehomon (1968) and Sousa Paiva & Rodriguez Machado (2010). The EFNs can have varied origins (Diaz Castelazo et al. 2005), depending on their location, as the foliar epidermis (Maheshwari 1954) or abortive flowers (Ojehomon 1968). Ontogenetic studies involving floral development in Leguminosae are abundant (Tucker 1984, Tucker 2003, Gonçalves Leite et al. 2015), especially in some Mimosoideae (Ramirez-Domenéch & Tucker 1988), in several Caesalpinieae (Kantz & Tucker 1994) and in some Papilionoideae (Tucker 1989, Tucker & Stirton 1991, Gonçalves Leite et al. 2015). The EFNs in papilionoid inflorescences studied so far originate due to flower bud abortion (Ojehomon 1968, Tucker 1987, 2003, Ojeda et al. 2014, 2015). Tucker (1987) mentioned the presence of rudimentary flowers in the expanded secondary axis of the inflorescences of *Vigna radiata*, referring that they do not develop into flowers, but in nectaries, though the development process was not described. EFNs development in *Vigna* was studied in *Vigna unguiculata* (L.) G. W. Walpers (Ojehomon 1968, Ahmed et al. 1992, 1993), *Vigna adenantha* (G.F.W. Meyer) Marèchal, Mascherpa & Stainier (Ojeda et al. 2014), *Vigna candida* and *V. caracalla* (Ojeda et al. 2015).

Previous works on secretion of EFNs in three *Vigna* species (*V. adenantha*, *V. caracalla* and *V. candida*) suggest that it is granulocrine (Ojeda et al. 2014, 2015). Granulocrine secretion involves vesicles derived from endoplasmic reticulum or from dictyosomes that fuse with the plasmalemma and release the compounds to the wall area (Durkee 1983, Nepi 2007).

Vigna luteola (Jacq.) Benth. has presumptive African origin, but it is now widely distributed in the Neotropics; in Argentina, it grows along the riverside forests from Misiones to Buenos Aires provinces in the east and occupies the first slopes of the subtropical cloud forests in the north west (Palacios & Hoc 2001). Besides, some populations of *V. luteola* are found in disturbed places from the northwest (in Salta province) to the center (in Córdoba province) (Hoc et al. 2007). This species has value as forage: as it is megathermic and hydrophylous, it can be cultivated in tropical areas, even in flooded lands (Fernández et al. 1988). To cultivate it, it is necessary to understand all the interactions involved with its reproduction, including the presence of EFNs in the inflorescences that keep a constant patrolling of ants who could influence the activity of florivores and/or frugivores. The importance of ants attracted to EFNs as biological control agents of crop pests has been recognized (Bentley 1983) not only for the crop bearing EFNs itself but as for mixed plantations. This interaction could constitute a mutualism in which the ants benefit from the nectar intake and the plants receive protection against consumers of the reproductive organs, thus increasing the fitness of both partners.

Given the variety of quantity, location, morphology and anatomy of the EFNs, it is interesting to know their disposition, ontogeny and activity period, to relate them to their function. The aims of this work were to describe the distribution and morphology of the EFNs of *V. luteola* and to find out their origin and development, as well as to examine the cytology

of the secretory stage, the period of secretion and its relationship with fruit production.

MATERIALS AND METHODS

The *V. luteola* plants used in the study were cultivated at the Campo Experimental of the Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires), situated in the Ciudad Autónoma de Buenos Aires, Argentina. The cultivated specimens were grown from seeds whose collection and herbarium data are as follows: ARGENTINA. Buenos Aires: Pdo. San Isidro, Reserva Ribera Norte, 26 Jan. 2008, F. Ojeda & P. Hoc in Hoc 401 (BAFC); Bajo de San Isidro, 28 April 2002, Hoc 379 (BAFC). Ciudad Autónoma de Buenos Aires**:** Ciudad Universitaria, Rivera del Río de la Plata 27 May 2010, F. Ojeda & P. Hoc in Hoc 427, 428 (BAFC).

For observations with light microscopy (LM), the inflorescences were fixed in FAA (formaldehyde, ethanol, acetic acid, water (100:50:5:35)) and preserved in ethanol 70 %. Later, each of the inflorescences was sectioned from the apex to the base and each of the segments containing a node were identified with a code, included in paraffin and cut into 10 µm thick sections using a rotative microtome Arcano® (India). Histological slides were prepared: some of them were stained with safranin-fast green and others with cresyl violet. Observations and film photographs were performed with a Nikon Labophot optic microscope (Tokio, Japan). The photographs were digitalized with a HP 3470 multifunction printer.

Preparations for scanning electron microscopy (SEM) were performed in the following way: each secondary axis, previously fixed in FAA (formaldehyde, ethanol, acetic acid, water (100:50:5:35)) and preserved in ethanol 70 %, was dehydrated in an ascending series of ethanol (70, 80, 90, and 100 %), CO_2 critical point dried, covered with a gold-palladium alloy and observed and photographed with a Zeiss Supra 40 scanning electron microscope (SEM) (Oberkochen, Germany).

For the examination with transmission electron microscopy (TEM), the material was fixed in 2.5 % glutaraldehyde in phosphate buffer (pH = 7.2) during 24 hr, then fixed in 1.5 % osmium tetroxide (OsO4) at 2 °C for 3 hr, dehydrated in an upward series of acetone and embedded in Spurr's resin. For preceding LM observations, sections 1 µm thick were stained with 0.1 % toluidine blue. Ultrafine sections were stained with uranyl acetate and lead citrate, observed and photographed with a JEOL-JEM 1200 EXII transmission electron microscope (TEM) (Akishima, Tokio, Japan).

Photographic figures were assembled and prepared with Adobe Photoshop software.

According to the considerations exposed in the introduction in relation to EFNs definition, the one of Delpino (1875) is followed in this work.

RESULTS

Morphology, functionality and secretory periods of the nectaries

The inflorescences of *V. luteola* (Fig. 1a) have at least six nodes on the main axis (Fig. 1b); a globose secondary axis originates at each node, which bears two opposite flowers and one to three pedunculate and linearly arranged EFNs between the flowers (Fig. 1c, d). Irrespective of the number of EFNs, only one is functional, and only during the anthesis of the flowers of the same node.

Ontogeny

Four stages can be distinguished during the development of the EFNs.

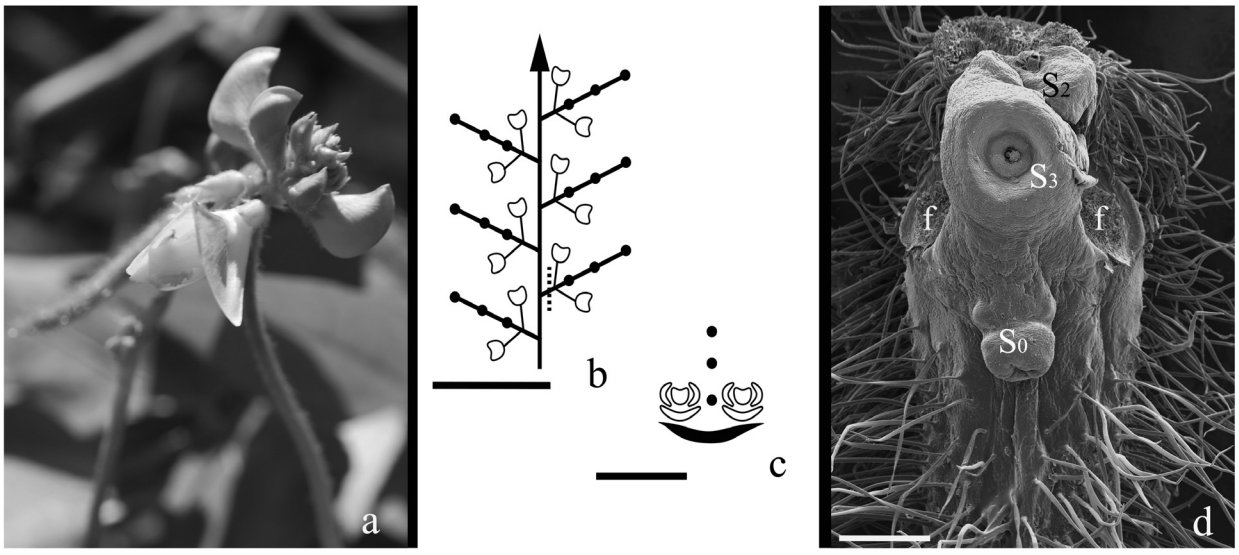

Figure. 1. Inflorescence of *Vigna luteola*. a., Iin situ vivo, on 14 February 2011; b & c., Sschematic representation of an inflorescence (Wwhite semi-circles, flowers; black circles, Eextrafloral nectaries (EFNs):; b., Vvertical diagram of inflorescence., c., Ttransverse diagram showing structures at a secondary axis.; d., SEM of a secondary axis with 3 linearly arranged EFNs extrafloral nectaries (S_0, S_3, S_2) at different stages and lateral flowers (f) removed. sScale bars = b: 15 cm,; c: 3 cm; d: 500 µm.

Stage 1

A flower bud initiates from the axil of a bract in the secondary axis; it is protected by two bracteoles. This bud consists of a tunica layer covering the underlaying meristematic tissue (Fig. 2a). Around the bud, a ring begins to develop (Fig. 2a).

Stage 2

Beneath the bract, the two bracteoles continue growing and sepal primordia are observed (Fig. 2b). At the base of the bud, an abscission zone is delimited (Fig. 2c). Below this, there are densely stained and longitudinally enlarged cells, with thin primary walls and large nuclei, surrounded by vascular bundles (Fig. 2c). At this stage, the ring of the future EFN completes its development; it consists of an epidermis and a tightly packed parenchyma with highly vacuolated cells and conspicuous nuclei (Figs. 2b, c).

Stage 3

The flower bud does not continue its development; it detaches, together with the bract and the bracteoles, from the abscission zone (Fig. 2d). In the center of the now exposed nectary, there are longitudinally enlarged papillose cells (Figs. 3a, c). These cells, as well as the parenchymatic ones below, dye densely with cresyl violet and are tightly disposed, without intercellular spaces (Fig. 2d). In contrast, the epidermal and the parenchymatic cells of the ring do not stain heavily (Fig. 2d). On the surface of the ring, as well as on the central cells, reticulated material is observed (Figs. 3b, c). Around and at the base of the group of central cells, vascular bundles are observed (Figs. 2c, e).

Stage 4

The ring contracts its aperture (Fig. 2f) and the central cells disintegrate (Fig. 3d).

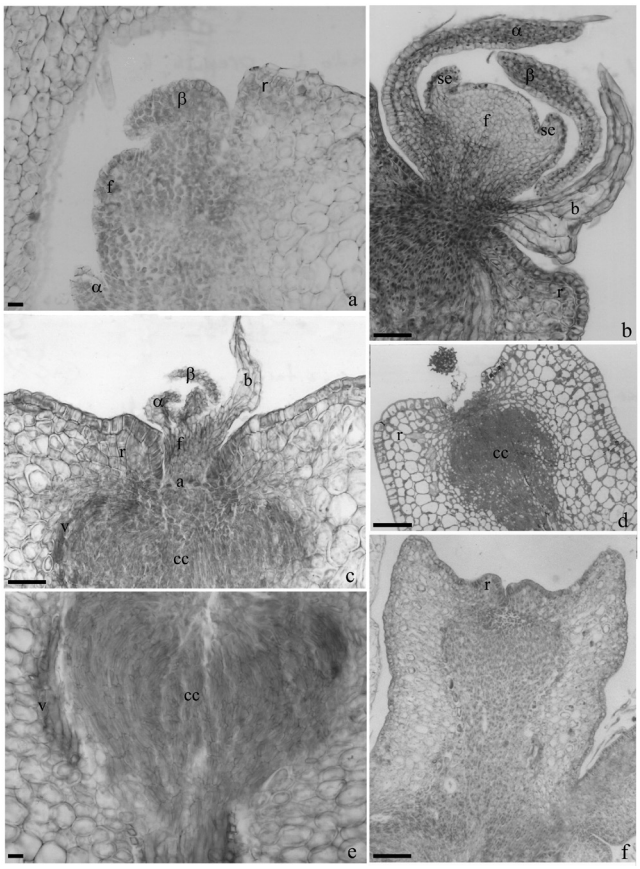

Figure. 2. LM micrographs of longitudinal sections of ontogenetic stages of extrafloral nectary of *V. luteola*;. a., sStage 1: flower bud and bracteoles, surrounded by developing ring;. b-c., Sstage 2: b., Rring completely developed, bract covering bracteoles and the flower bud, flower bud with sepal primordia.; c., Rring, bract protecting bracteoles and flower bud under which abscission zone is visible; (below this, central cells irrigated by vascular bundles).; d-e., Sstage 3: d., Ccentral cells with dense content, in contrast to highly vacuolated cells of ring.; e., Ddetail of vascular supply and central cells.; f., Sstage 4: Ggeneral appearance of degenerated EFN extrafloral nectary with centere narrowed. Abbreviations: α, β, bracteoles; a, abscission zone; b, bract; cc, central cells; f, flower bud; r, ring; se, sepal; v, vascular bundles;. sScale bars = b-d, f: 10 µm,; a, e: 4 µm.

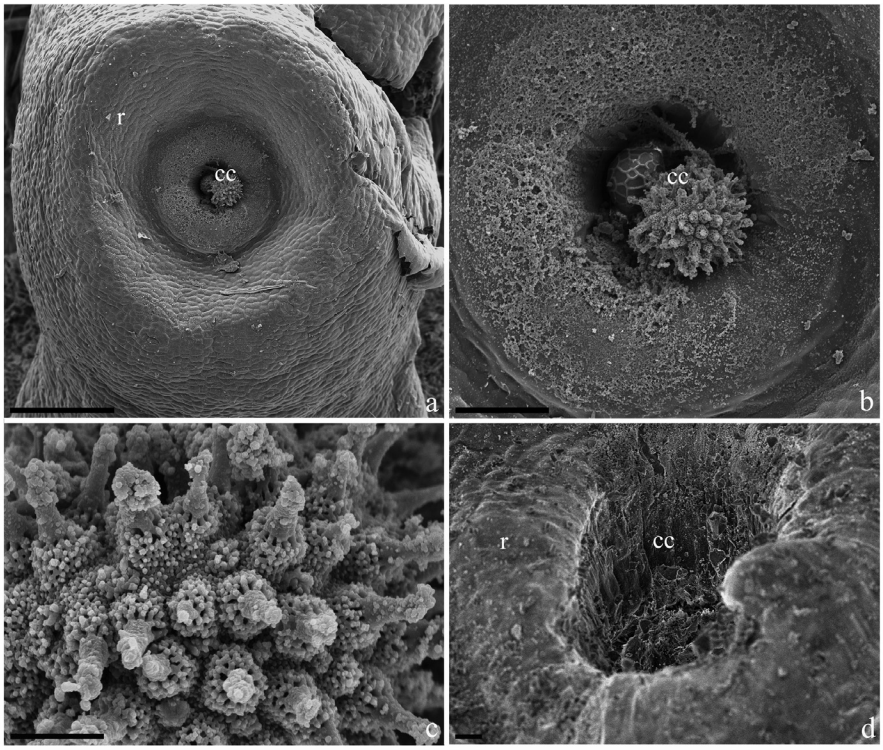

Figure. 3. SEM micrographs of secretory and postsecretory stages of extrafloral nectary of *V. luteola*;. a-c., Sstage 3 (secretory).; d., Sstage 4 (postsecretory).; a., Ccentral cells surrounded by a completely developed ring.; b., Eelongated papillose central cells.; c., Ddetail of papillose central cells.; d., Ddesintegration of papillose central cells while the ring remains. Abbreviations: cc, central cells; r, ring;. sScale bars = a: 200 µm,; b: 50 µm,; c, d: 10 µm.

Ultrastructure of the EFNs at the secretory stage (stage 3)

Ring

From inside to outside, the epidermal cells of the ring have each a large central vacuole with fibrillar content, a peripheral cytoplasm with rugose endoplasmic reticulum (rER) whose cisternae lay parallel to the plasma membrane, abundant free ribosomes, electron-dense corpuscles between the plasmalemma and the outer tangential wall (Fig. 4a), mitochondria and lipidic globules (Fig. 4b, right bottom and top, respectively). The underlying parenchyma cells have conspicuous vacuoles with fibrillar content of varying electron density, rough endoplasmic reticulum, numerous free ribosomes, mitochondria (Figs. 4b left, 4c), dictyosomes, chloroplasts and vesicles fused with the plasmalemma (Fig. 4d); plasmodesmata connect these cells (Fig. 4d, arrows).

Central cells

The cytoplasm of the elongated and papillose cells has amyloplasts, lipidic globules (Fig. 5a top and bottom, respectively), numerous free ribosomes (Fig. 5b), a large central vacuole with fibrillar content that displaces the cytoplasm to the plasmalemma, rugose endoplasmic reticulum (rER) and dictyosomes with many vesicles (Fig. 5c). The cuticle is electron-dense

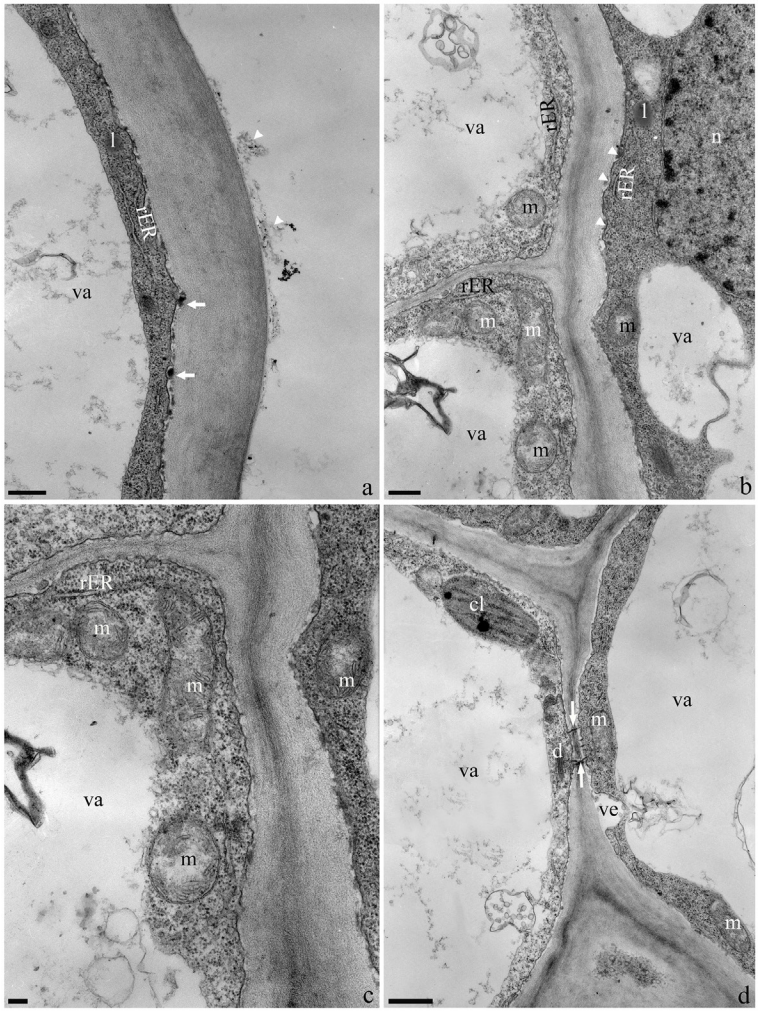

Figure. 4. TEM micrographs of ring cells at secretory stage of an extrafloral nectary of *V. luteola*;. a. Detail of an epidermal cell with rough endoplasmic reticulum and vacuole, electron-dense corpuscules (arrows) between plasma membrane and external tangential wall, as well as on wall, where also secretion is observed (arrow heads).; b., Ddetail of an epidermal cell (right) and two underlying parenchyma cells (left): lipid globules, mitochondria, rough endoplasmic reticulum, vacuoles and secretion from underlying parenchyma (arrows heads).; c., Ddetail of underlying parenchyma cells with free ribosomes, mitochondria, rough endoplasmic reticulum and vacuole.; d., Pplasmodesmata (arrows) connecting parenchyma cells, which exhibit chloroplast, dictyosomes, mitochondria and vacuoles. Note vesicle in exocytosis close to fibrillar and electron-dense content of vacuole (right, bottom). Abbreviations: cl, chloroplast; d, dictyosome; l, lipid globule; m, mitochondria; rER, rough endoplasmic reticulum; va, vacuole; ve, vesicle;. sScale bars = a, b: 0.5 µm,; c: 0.2 µm,; d: 1 µm.

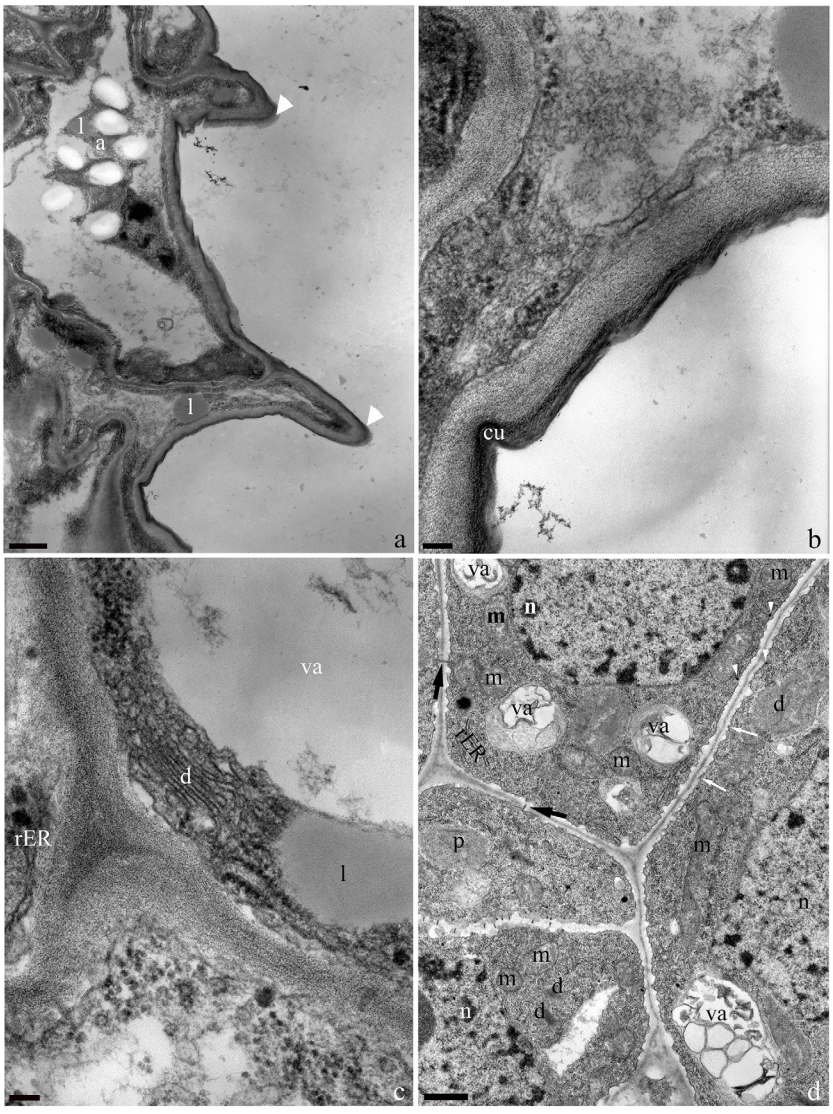

Figure. 5. TEM micrographs of central cells at secretory stage of an extrafloral nectary of *V. luteola*;. a., Llongitudinal section of apical portion of an elongated central cell showing amyloplasts, lipid globules and secretion outside wall (arrow heads).; b., Ddetail of wall of a papillose cell with electron-dense cuticle.; c., Ddetail of a papillose cell with, vacuole, rER, dictyosome and lipid globule.; d., Uunderlying parenchyma cells with big nucleous, rough endoplasmic reticulum, plastid, mitochondria, dictyosomes, small vacuoles, invaginations along plasmalemma (white arrows), electron-dense corpuscules associated with plasmalemma (arrow heads) and plasmodesmata (black arrows). Abbreviations: a, amyloplast; cu, cuticle; d, dictyosome; l, lipidic globule; m, mitochondria; n, nucleous; p, plastid; rER, rough endoplasmic reticulum; va, vacuole;. sScale bars = a, d: 1 µm,; b, c: 0.1 µm.

and thin (Fig. 5b). The underlying parenchymatic cells exhibit a large nucleous, small vacuoles with fibrillar and electron-dense content, mitochondria, free ribosomes, rER (Fig 5d, top), plastids and dictyosomes (Fig. 5d, bottom left); the plasma membrane of these cells is invaginated along the entire perimeter (Fig. 5d, white arrows). There are zones with electron-dense corpuscles that are associated with the invaginations (Fig. 5d, arrow heads). Intercellular cytoplasmic connections can be observed (Fig. 5d, black arrows).

DISCUSSION

The EFN of *V. luteola* originates from the abscission of a floral bud that stops its development. It consists of an elevation around the flower bud abscission scar, and corresponds to the "Hochnektarien" (elevated nectaries) type according to the classification proposed by Zimmerman (1932), coinciding with what was observed in *V. unguiculata* (Ojehomon 1968), *V. adenantha* (Ojeda et al. 2014), *V. candida* and *V. caracalla* (Ojeda et al. 2015).

Díaz-Castelazo et al. (2005) denominated "transformed nectaries" the EFNs that originate from transformation of an organ, including its abscission. In the case of flower bud abortion, a swollen scar with a central cell depression is left; these corresponding respectively, to the ring and the central cells described in this study. In other Legumes, the cessation of meristematic activity also occurs at the stage when sepal primordia have appeared (Ramírez-Domenech & Tucker 1988, Tucker 1987, 2003). The detachment of the developing flower was observed in other Leguminosae, too, for example, in *Macroptilium* (Díaz-Castelazo 2005), and in two other species of *Vigna*, *V. radiata* and *V. luteola* (Tucker 1987), where similarly only two flowers complete their organogenesis.

In *V. luteola*, the EFNs originate independently and at different times, as they develop from floral buds that are subsequently suppressed. The fall of the developing flower, bracteoles and bract occur simultaneously, which differs from *V. adenantha* (Ojeda et al. 2014), *V. candida* and *V. caracalla* (Ojeda et al. 2015) where they occur sequentially. The EFN of *V. luteola* passes through four stages, of which the first and the second are pre-secretory, the third is the secretory and the forth is post-secretory. Contrarily, Kuo & Pate (1985) interpreted the EFNs in the secondary axis of the inflorescences of *Vigna unguiculata* as a compound structure formed by various conical secretory subunits, without clarifying if they are simultaneously active.

The function of the extrafloral glands present in the inflorescences of *Vigna*, except for the interpretation of Ojehomon (1968) as being excretory in *V. unguiculata*, has been interpreted as secretory nectaries, both in *V. unguiculata* as well as in additional species studied by other authors (Kuo & Pate 1985, Ojeda 2013).

In contrast to three other species of *Vigna* (*V. adenantha*, *V. candida* and *V. caracalla*) (Ojeda et al. 2014, 2015), in *V. luteola*, the bulk of the secretion originates mainly in the central cells and in less proportion from the ring, as was also seen in *V. unguiculata* (Kuo & Pate 1985); this is suggested by the weaker staining observed with optical microscopy, though both regions exhibit secretory features (vesicles in exocytosis, plasma membrane invaginations, dictyosomes and rER) when observed with TEM.

The presence and abundance of dictyosomes and endoplasmic reticulum in both the ring and the central cells, the invaginations of the plasmalemma of the parenchyma underlying the central cells as well as the vesicles in exocytosis observed in the ring cells, suggest granulocrine secretion. The cells with invaginations act as transfer cells (Gunning & Pate 1969, Offler et al. 2003, Zheng & Wang 2010), from where the nectar moves across the wall towards the subcuticular spaces. This way of secretion was also suggested for *V. adenantha* (Ojeda et al. 2014), *V. candida* and *V. caracalla* (Ojeda et al. 2015).

Unlike other species of legumes (Marginson et al. 1985, Sousa Paiva & Machado 2006, 2010), even including the rest of the *Vigna* species studied (Kuo & Pate 1985, Ojeda 2013, Ojeda et al. 2014, 2015), in *V. luteola* the functional EFN is active only during anthesis and ceases its activity before fruit development. The brief period of activity of the EFNs would serve as reward for the patrolling ants that would defend flowers only against pollen and/or nectar robbers or thieves during floral anthesis, but not against frugivores during fruit set.

Acknowledgments

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