

Origin and Ontogeny of the Extrafloral Nectaries Associated to the Inflorescences of *Vigna Candida* and *Vigna Caracalla* (Leguminosae, Phaseoleae)

¹Ojeda, Fabiana Soledad*, ¹Hoc, Patricia Susana, ²Galati Beatriz Gloria, ¹Amela García, María Teresa

¹ Proplame-Prhideb (Conicet); Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencia Exactas y Naturales, Universidad de Buenos Aires Intendente Güiraldes CABA, Argentina

² Cátedra de Botánica, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín CABA, Argentina
ofabisol@gmail.com

Abstract: The morphology, ontogeny and secretion of the extrafloral nectaries (EFNs) in the inflorescences of *Vigna candida* and *Vigna caracalla* were studied. The nodes along the inflorescences were processed with standard techniques for light as well as scanning and transmission electron microscopy. In each node, several EFNs are formed between the flowers. The EFNs originate from flower buds that stop their growth and detach, leaving an orifice below which there are longitudinally enlarged cells (central cells) surrounded by a circular mound (ring). Both zones secrete. Four ontogenetic stages succeed, being the third the secretory one. In each node, different EFNs are in different stages of development simultaneously, so there is always a functional nectary accompanying the flowers buds, the flowers in anthesis or the maturing fruits. Ultrastructure characteristics suggest that secretion follows the granulocrine pathway. The two studied species, together with *Vigna adenantha*, which belong to the same subgenus, share the most important characters, but differ from the corresponding ones of *Vigna luteola*, which is placed in a different subgenus, so the EFN traits support the current classification. This is the first report about taxonomic implications of the EFNs of New World species of *Vigna*.

Keywords: *Vigna candida*, *Vigna caracalla*, extrafloral nectary development, histology, cytology.

Abbreviations: BAFC: Herbario de la Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires); EFNs: Extrafloral nectaries; FAA: solution of Formaldehyde, ethilic alcohol, acetic acid, water; LM: Light microscopy; TEM: Transmission electron microscopy; SEM: Scanning electron microscopy.

1. INTRODUCTION

The inflorescences of the genus *Vigna* Savi consist in double racemes whose node bear a brief secondary globose axis in which commonly two or more flowers and one or more extrafloral nectaries (EFNs) develop. These glands have taxonomic value, at least at generic level, as they allow to distinguish the genera *Vigna* Savi and *Macroptilium* Urban from the closely related genus *Phaseolus* L. (Mc Key, 1989).

EFNs of different families of Angiosperms have been studied concerning morphology, anatomy, ultrastructure and ontogeny (Maheshwari, 1954; Ojehomon, 1968; Durkee et al., Fahn, 1987; 1999; Francino et al., 2006; González and Ocantos, 2006; Sousa Paiva and Rodrigues Machado, 2006; Machado et al., 2008; Latar et al., 2009; Melo et al., 2010), but some aspects, such as if they are irrigated and by what tissues, have not received much attention yet (Ojehomon, 1968; Nepi, 2007).

The EFNs that occur in inflorescences are supposed to originate from aborted floral primordia in Leguminosae (Tucker, 2003) and especially in Papilionoideae (Ojehomon, 1968; Díaz-Castelazo et al., 2005). Ojehomon (1968) described the ontogeny of the EFNs of *Vigna unguiculata* (L.) G. W. Walpers, while, Kuo and Pate (1985) analysed their anatomy during the secretory period.

Recently, Ojeda et al. (2014) studied the ontogeny of the EFNs in *Vigna adenantha* G. F. W. Meyer, the cytology of the secretory stage, the period of secretion and its relation with fruit and seed production as well as the correspondence with pollen and embryo-sac development in the flowers of the same node. This was the first report for a species of *Vigna* belonging to the New World. Other American species which belong to the same subgenus were not studied from this point of view.

Various species from the genus *Vigna* have economic importance. The originally Asiatic and now widely cultivated *V. unguiculata* is used as food (Adam, 1990) or as pasture and manure (Fernandez et al., 1988; Ugborogho and Agomo, 1989; Woolwine and Reagan, 2001; Schinini et al., 2004) and *V. caracalla* (Etcheverry, 2005, 2008) and *V. candida* and *V. adenantha* (Hoc and Ojeda, 2014) as ornamentals.

The understanding of the development and activity of the EFNs associated to their inflorescences is important for fruit production and for the consequent obtention of seeds, as they are patrolled by ants who are supposed to defend the developing fruits from seed predators (Ojeda, 2013).

The aim of this work was to find out the origin and development of the EFNs, the ultrastructure of the secretory stage, as well as the period of secretion in *Vigna candida* (Vell.) Maréchal, Mascherpa and Stainier and *Vigna caracalla* (L.) Verdc., two species that belong to the same subgenus in which *Vigna adenantha* is included, and analyze, consequently, the taxonomic implications of the similarities or differences that may exist.

2. MATERIALS AND METHODS

The studied material was collected from cultivated specimens 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 proceeded from:

V. Candida: ARGENTINA. Salta: Dpto. Capital, Calle Riobamba 655, 27/02/2008, F. Ojeda s/n in Hoc 402 (BAFC). Corrientes: Dpto. Mburucuyá, Estancia Sta. Teresa, A. Burkart 19597 (SI). Misiones: Dpto. San Ignacio, Santa Ana, 13/4/1992, Hoc 236 (BAFC).

V. Caracalla: ARGENTINA. Salta: Dpto. Chicoana, Los Los, A. Krapovickas *et al.* 28312 (SI); Dpto. Gral Güemes, Finca del Desmonte al este de Betania, 24/02/2008, F. Ojeda s/n in Hoc 403 (BAFC). Chaco: Dpto. San Fernando, Resistencia, Boca del Río Negro, A. G. Schulz 1188 (SI). Dpto. La Viña, costado de la ruta, 1/III/1988, Hoc 76, 78 (BAFC).

For observations with light microscopy (LM) the inflorescences were fixed in FAA (formaldehyde, ethanol, acetic acid, water) and preserved in ethanol 70%. From the apex to the base of the inflorescence, each node was sectioned, identified with a code, embedded in paraffin and cut in sections 10 µm thick employing a rotative microtome (Arcano). Histological slides were prepared following the usual technique (D'Ambrogio, 1986): some of them were stained with safranin-fast green and others with cresyl violet. Observations and photographs were performed with an optic Nikon Labophot microscope.

For preparations for scanning electronic microscopy (SEM), each secondary axis was dehydrated in an ascendant series of ethylic alcohol (70, 80, 90, and 100%), submitted to critical point, covered with a gold-palladium alloy and observed and photographed with a Zeiss Supra 40 Scanning Electron Microscope.

The secretory stage was examined with transmission electron microscopy (TEM). For this, the material was fixed in glutaraldehyde 2.5%, soon after it was submerged in buffer phosphate during 24 hours, then fixed in osmium tetroxide (OsO₄) 1.5% at 2°C for 3 hours, dehydrated in an upward series of acetone and embedded in Spurr's resin. For previous observations with light microscopy, sections of 1 µm thick were stained with toluidine blue 0.1 %. Ultrathin sections were stained with uranyl acetate and lead citrate, observed and photographed with a Jeol-Jem 1200 EXII transmission electron microscope.

In the following descriptions, the features common to the two species are enumerated together, without mentioning each species; only the distinct features are pointed out.

Photographic plates and drawings were compounded with Adobe Photoshop software and Paint software, respectively.

3. RESULTS

3.1. General Morphology

The inflorescences of *V. candida* and *V. caracalla* have several nodes: 4 or more (*V. candida*) and 5 or more (*V. caracalla*) along the main axis (Fig. 1, A-B). An ovate (*V. candida*) or spheric (*V. caracalla*) secondary axis originates in each node (Fig. 1, C-D), which bears 2 flowers, occasionally 3 in *V.*

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caracalla, and 5 to 9 (*V. candida*) and 6-8 (*V. caracalla*) EFNs. The nectaries are sessile, tightly disposed. Each one consists of an orifice surrounded by an elevated more or less elliptical (*V. candida*) or circular (*V. caracalla*) ring (Fig. 1, C, D).



Figure1. Inflorescences of *V. candida* and *V. caracalla*. A, C, *V. candida*; B, D, *V. caracalla*. A, B, General aspect of the inflorescences, with the flowers, fruits and the globose secondary axis with the EFNs at each node (arrows); C, D, drawing of the globose secondary axis bearing the EFNs (flowers have been removed).

3.2. Ontogeny

Four stages of development were distinguished in the EFNs. In the following description the development of the first EFN is assessed. The following EFNs of the secondary axis have the same ontogeny.

Stage1. The bud of the EFN is placed in the axil of a bract of the secondary axis (Fig. 2, A-B) which protects the underlying meristem (Fig. 3, A). The bract has simple pluricellular trichomes in its abaxial surface (Fig. 2, B). Around each bud, a ring develops (Fig. 3, A). The bud is irrigated by procambial bands that stain intensely with cresil violet (Fig. 3, A).

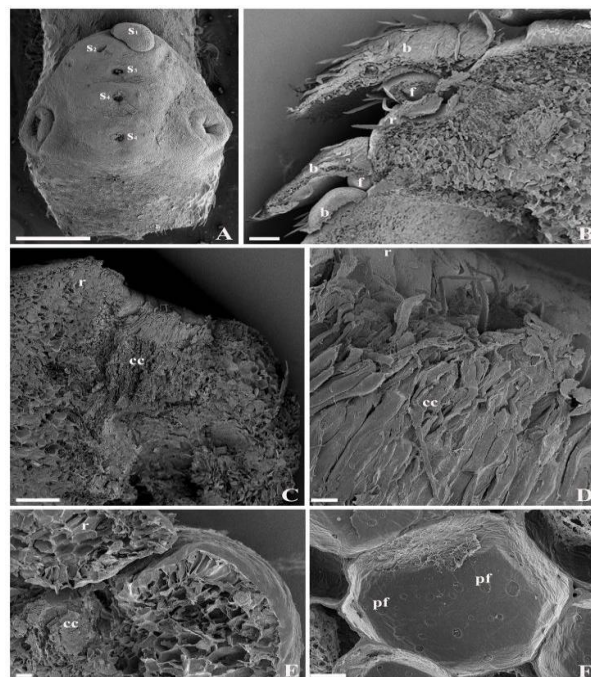


Figure2. SEM of the EFNs ontogeny of *V. candida* and *V. caracalla*. A, secondary axis bearing five more or less aligned EFNs, the one in the top is still covered by the bract (flowers have been removed). B, longitudinal section of a secondary axis showing pilose bracts covering EFN buds. C, longitudinal section of an EFN in the secretory stage showing the ring and the central cells. D, detail of the central cells of the secretory stage. E, longitudinal section of an EFN in stage 4, the central cells have disintegrated and the EFN orifice has narrowed. F, punctuation fields of parenchymatic cells of the ring. Abbreviations: b, bract; cc, central cells; f, flower bud; pf, punctuation field; r, ring; s₁, stage 1; s₂, stage 2; s₃, stage 3; s₄, stage 4. Scales bars: A = 1000 µm; B, C = 100 µm; D, E, F = 10 µm.

Stage2. In the meristematic apex of the bud, sepal primordia appear (Fig. 3, B-C). Two bands of procambial tissue still innervate the floral bud and stain intensely (Fig. 3, B-C). The ring of the future EFN is completely differentiated: it is constituted by an epidermis and a parenchyma without intercellular spaces (Fig. 3, B-C).

Stage3. The bud of the EFN does not continue its development and detaches (Fig. 3, D-E), leaving exposed longitudinally enlarged cells in the center of the now functional nectary (Fig. 2, C-D; Fig. 3, D-E). These cells stain intensely with cresyl violet (Fig. 3, D-E). Below them there is parenchymatic tissue (Fig. 3, D-E). The epidermic and parenchymatic cells of the ring also stain strongly with cresyl violet (Fig. 3, D-E). Vascular bundles below the central cells can be observed (Fig. 3, E). In this stage, secretions occurs.

Stage4. Nectar secretion ceases, the central cells collapsed (Fig. 3, F) and the orifice narrows (Fig. 2, E). The vascular bundles still stain strongly with cresyl violet (Fig. 3, F). The ring becomes highly vacuolated, both in the epidermis and in the underlying parenchyma (Fig. 3, F).

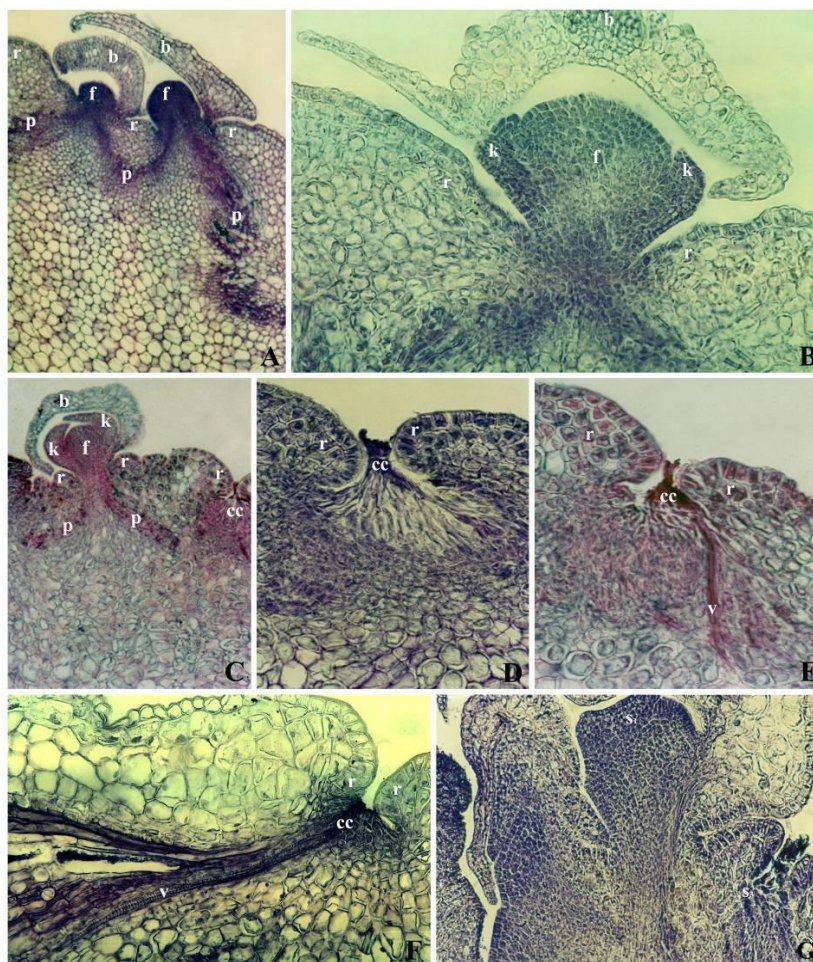


Figure3. LM of the ontogeny of the EFNs of *V. candida* and *V. caracalla*. A, B, D, F, *V. candida*; C, E, G, *V. caracalla*. A, stage 1. Two developing flower buds covered by their corresponding bract and surrounded by elevated parenchymatic tissue protected by epidermis (ring). B, C, stage 2. Developing flower bud with sepal primordia. D, E, stage 3. The flower bud has detached leaving exposed the central cells surrounded by the ring. F, stage 4. The central cells have collapsed and the ring has become completely vacuolated. G, two successive EFNs in a same node in different stages of development: intermediate state between stage 1 to stage 2 (left) and stage 3 (right). Abbreviations: b, bract; cc, central cells; f, flower bud; k, calyx lobes; p, procambium; r, ring; S_1 , stage 1; S_3 , stage 3; v, vascular bundles.

3.3. Nectary Activity Period

During the inflorescence development, once the first EFN of a secondary axis arrives to stage 3, the second EFN begins to differentiate in the next node and so on, in the acropetal sense of the secondary axis (Fig. 2, G). When the flowers of the corresponding node have produced fruits, at least one EFN in stage 4, one in stage 3 and one in stage 1 coexist in the same secondary axis of the inflorescence, so the EFN activity is continuous during maturation of the fruits.

3.4. Ultrastructure of the Secretory Stage

3.4.1. Ring

The epidermal cells of the ring exhibit a thick radial and outer tangential wall of moderate electron density (Fig. 4, A; Fig. 5, A). In *V. candida*, it appears homogeneous (Fig. 4, A); in *V. caracalla*, three different electron dense zones can be observed while the cuticle disintegrates and secretion is liberated (Fig. 5, A). In *V. candida*, the vacuole exhibits high electron dense content, the cytoplasm is plenty of ribosomes, numerous vesicles in exocytosis are placed along the whole length of the plasma membrane facing the radial and the outer tangential wall and particles with the same electron density than the vacuole content cross the radial and outer tangential wall (Fig. 4, A). In *V. caracalla*, mitochondria and dictyosomes are present in the cytoplasm (Fig. 5, A).

The parenchyma cells of *V. candida* have chloroplasts against the walls, dense cytoplasm, vacuole with electron dense content (Fig. 4, C), lipidic globules, mitochondria and vesicles in exocytosis along the plasma membrane to the apoplast (Fig. 4, D).

The parenchyma cells of *V. caracalla* have also lipidic globules and mitochondria, but there are more and smaller vacuoles (Fig. 5, B). Plasmodesmata are evident in *V. caracalla* (Fig. 5, B), in accordance with the punctuation fields observed with SEM (Fig. 2, F).

3.4.2. Central Cells

The outer tangential wall in *V. candida* has wavy invaginations towards the plasma membrane and towards the cuticle; secretion accumulates between it and the cuticle, and is observed liberated outside the cuticle (Fig. 4, E-F). The radial walls have partially degraded middle lamellae (Fig. 4, E).

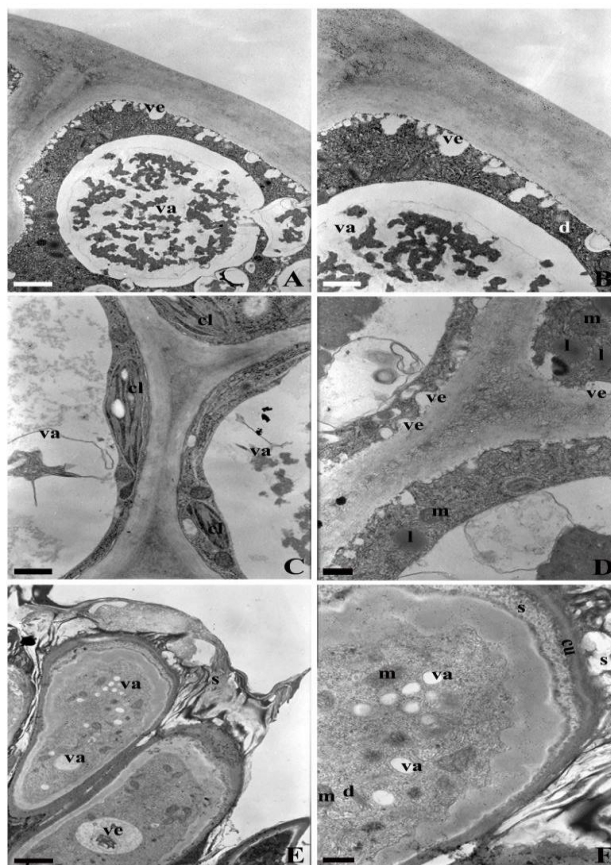


Figure 4. Ultrastructure of the secretory stage of the EFN of *V. candida*. A-D, ring. A, epidermal cells of the ring; B, detail of A. C-D, parenchymatic cells; E, central cells; F, detail of E. Abbreviations: cl = chloroplasts; cu = cuticle; d = dictyosomes; l = lipidic globule; m = mitochondria; s = secretion; va = vacuole, ve = vesicle. Scales bars: A, E = 2 μ m; B, C = 1 μ m; D, F = 0.5 μ m.

In *V. caracalla*, secretion is also observed outside the disintegrating cuticle and rugose endoplasmic reticulum, cisterna parallel to the plasma membrane are observed (Fig. 5, C). In both species, the cytoplasm has numerous ribosomes, abundant mitochondria, dictyosomes and vacuoles (Fig. 4, F; Fig. 5, C-D); in *V. caracalla*, besides, there are lipidic globules and elaioplast (Fig. 5, D)

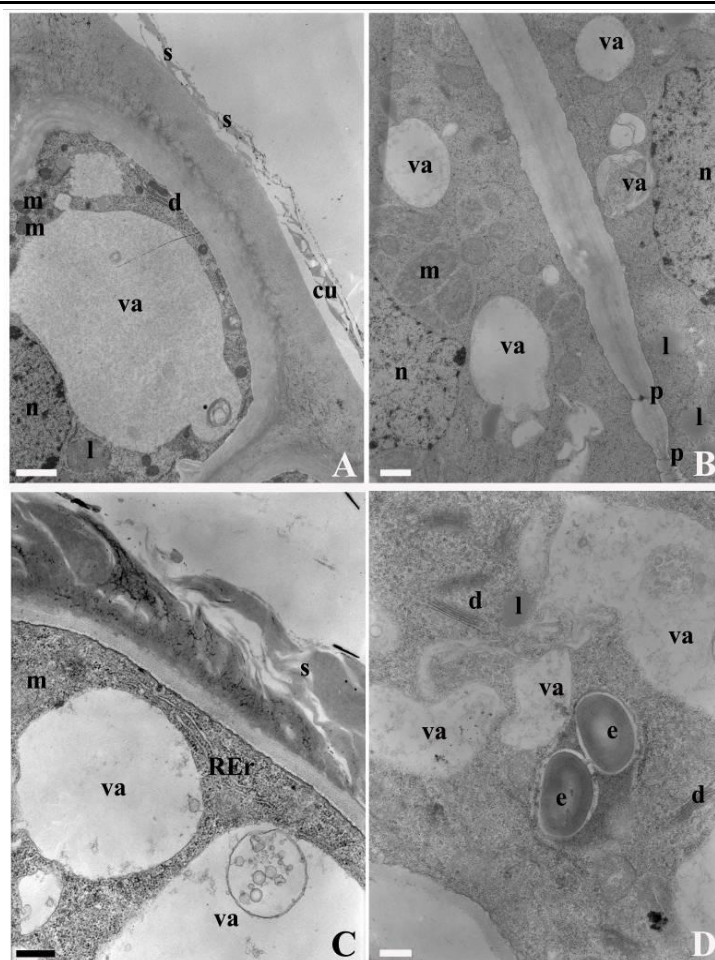


Figure 5. Ultrastructure of the secretory stage of the EFN of *V. caracalla*. A-B, ring. A, epidermal cell; B, parenchymatic cell; C-D, central cell. Abbreviations: cu = cuticle; e = elaiosomes; d = dictyosomes; l = lipidic globules; n = nucleus; p = plasmodesmous; REr = rugose endoplasmic reticulum; s = secretion; va = vacuole. Scales bars: A = 2 μ m; B = 1 μ m; C, D = 0.5 μ m.

4. DISCUSSION

The EFNs of *V. candida* and *V. caracalla* originate from of a floral bud that interrupts its development and detaches. This kind of origin was also observed in *Macroptilium* species (Díaz-Castelazo et al., 2005) in *V. adenantha* (Ojeda et al., 2014), in *V. luteola* (Ojeda, 2013) and has been suggested for other Legumes (Ojehomon, 1968; Tucker, 2003).

Ojehomon (1968) described the glands of *V. unguiculata* as “cushion units” and analyzed their development, inferring that the secretion is the result of the excretion of cellular products and discarding their function as EFNs. The ontogenetic development of the species here studied coincides with the observations of Ojehomon (1968) but not with the role that was suggested. On their way, Kuo and Pate (1985) described the EFNs of *V. unguiculata* as a compound structure constituted by conical subunits, each one formed by secretory parenchyma whose production was discharged simultaneously through an orifice surrounded by a ring formed by epidermis and parenchyma. In *V. candida* and *V. caracalla*, as well as in *V. adenantha* (Ojeda et al., 2014), the EFNs develop independently in each secondary axis and reach the secretory stage at a different moment, following an acropetal sequence, because they are developing floral buds that abort subsequently. So, from the initiation of the floral buds that will become flowers, there is always an active EFN (stage 3) in the same node until ripening of the fruits produced by those flowers. In other Legumes, the EFNs associated to inflorescences are also active until pod maturation (Pate et al., 1985; CAB, 2004; Ojeda et al., 2014).

Kuo and Pate (1985) postulated that the nectar secretion occurs through intercellular spaces underlying the central cells and reaches the surface of the EFN through stomata. In *V. candida* and *V. caracalla*, both the central cells and the cells of the ring secrete, evidenced by their ultrastructure characteristics. The same occurs in *V. adenantha* (Ojeda et al., 2014) but not in *V. luteola* (unpublished data), which belongs to other subgenus.

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In accordance to what happens in *V. adenantha* (Ojeda et al., 2014), the presence of dictyosomes and endoplasmic reticulum in the ring and in the central cells, as well as the vesicles along the plasma membrane, suggest granulocrine secretion. However, Kuo and Pate (1985) interpreted that *V. unguiculata* the nectar would follow an apoplastic route and flow through the orifices of the subconical units.

In *V. candida* and *V. caracalla*, the bract that protects the bud of the EFN has only simple pluricellular trichomes, unlike *V. adenantha* that has also glandular pluricellular trichomes (Ojeda et al., 2014).

In *V. candida* and *V. caracalla* only one bract protects the EFNs bud while in *V. adenantha* besides two bracteoles are present (Ojeda et al., 2014). This feature may be interpreted as an apomorphic character in the evolutive pathway of the Papilionoideae, as Prenner (2004) stated for 30 taxa belonging to 15 tribes. Also, in the studied species, the buds that will develop into flowers have no bracteoles. It should be presumed that the Section *Leptospron*, to which *V. adenantha* belongs, is more primitive than the Sections *Sigmoidotropis* and *Caracallae*, in which *V. candida* and *V. caracalla* are included, respectively (Table 1).

Table1. Species of *Vigna* that grow in Argentina

Subgenus	Section	Specie
<i>Sigmoidotropis</i>	<i>Leptospron</i>	<i>V. adenantha</i>
	<i>Sigmoidotropis</i>	<i>V. candida</i>
	<i>Caracallae</i>	<i>V. caracalla</i>
<i>Vigna</i>	<i>Vigna</i>	<i>V. luteola</i>

From Marèchal et al. (1978)

5. CONCLUSION

The EFNs of *V. candida* and *V. caracalla* originate from floral primordial that interrupt their development and detach. In each inflorescence secondary axis, several EFNs are formed between the floral buds that complete their own development. The EFNs of each secondary axis secrete successively until the maturation of the fruits produced by the flowers; in this way, they offer reward continuously to the ants that walk along the inflorescence axis permanently, which could constitute a defence against florivores, frugivores or seed predators. The secretion proceeds via the granulocrine route. All the studied species of *Vigna* in these aspects differentiate in the shape of the secondary axis and in the number of EFNs but the species included in the same subgenus share characters concerning EFNs development, in contrast to the one placed in another subgenus. This is the first report about taxonomic implications of the New World species of *Vigna* taking into account floral development.

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AUTHORS' BIOGRAPHY



Dr. F. S. Ojeda, has nowadays a postdoctoral fellowship concerning reproductive biology of Ingeae native from Argentina.



Dr. P. S. Hoc, is expertise in Vascular Plants Systematics, specially Leguminosae.



Dr. B. G. Galati, is plant embryologist and anatomist, and has experience in cytology, mainly in Angiosperms.



Dr. M. T. Amela García, studies interactions between plants and animals, in particular, insects.