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Floral nectaries of *Heliocarpus popayanensis* and *Luehea divaricata* (Malvaceae-Grewioideae): structure and ultrastructure

Elsa C. Lattar[A,B,F], *Beatriz G. Galati*[C], *Constanza S. Carrera*[D,E] *and María S. Ferrucci*[A,B,D]

[A]Instituto de Botánica del Nordeste (IBONE-UNNE-CONICET).
[B]Cátedra de Morfología de Plantas Vasculares, Facultad de Ciencias Agrarias, Universidad del Nacional del Nordeste, Corrientes, Argentina.
[C]Universidad de Buenos Aires, Facultad de Agronomía, Depto. de Recursos Naturales y Ambiente, Cátedra de Botánica General, Buenos Aires, Argentina.
[D]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
[E]Instituto de Fisiología y Recursos Genéticos Vegetales, CIAP-INTA, Córdoba, Argentina.
[F]Corresponding author. Email: elsilattar@gmail.com

Abstract. The structure and ultrastructure on floral nectaries of *Heliocarpus popayanensis* Kunth. and *Luehea divaricata* Mart (Malvaceae-Grewioideae) were investigated for the first time. The floral nectaries of the analysed species are structured (can be recognised macroscopically and microscopically) and of trichomatic type. Significant morphological differences were observed between the nectaries of perfect and pistillate flowers of *H. popayanensis*, as well as between nectaries of this species and those of the related species, *Triumfetta rhomboidea* Jacq. The volume of nectar produced in the perfect and pistillate flowers of *H. popayanensis* and in the perfect flowers of *L. divaricata* presents significant differences that could be related to the rewards offered to flower visitors. No differences were observed in ultrastructure features of the glandular trichomes between *H. popayanensis* and *L. divaricata*. Nectar accumulation occurs between the wall and the cuticle. The predominant floral visitors in perfect and pistillate flowers of *H. popayanensis* were bees, and less frequently flies; whereas for *L. divaricata* were wasps, bumblebees, butterflies and beetles. Our results support the inclusion of each genus in different tribes according to the recent tribal classification based on molecular and morphological data of the subfamily Grewioideae.

Additional keywords: floral visitors, Grewioideae, multivariate analysis, nectar, nectary structure, secretory trichomes.

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Introduction

Malvaceae Juss. is a cosmopolitan family comprising 243 genera and probably more than 4300 species (Bayer and Kubitzki 2003). Recent studies based on morphological, molecular and biogeographic data have demonstrated that Malvaceae *s.l.* consists of nine subfamilies (Bayer *et al*. 1999; Judd *et al*. 1999; Bayer and Kubitzki 2003), including Byttnerioideae Burnett and Grewioideae Dippel (Bayer and Kubitzki 2003). The latter subfamily is considered monophyletic along with its sister group Byttnerioideae, which includes many of the genera that were previously included in Sterculiaceae (Bayer *et al*. 1999; Nyffeler and Baum 2000; Bayer and Kubitzki 2003). Recently, Brunken and Muellner (2012) proposed a new subdivision within Grewioideae into two tribes, Apeibeae and Grewieae, based on morphological and molecular phylogenetic evidence.

Nectary in Malvaceae

Vogel (2000) indicates that one of the features that characterise Malvaceae *s.l.* is the presence of floral nectariferous trichomes (Judd and Manchester 1997; Vogel 2000). However, Vogel (2000) also highlights the lack of nectaries in some taxa of the basal subfamilies within Malvaceae; such is the case of the species of *Corchorus* L. (Bayer and Kubitzki 2003) present in the American continent. According to Vogel (2000), the absence of nectaries in these basal groups could be interpreted as a plesiomorphy. The subfamily Grewioideae is characterised by the presence of nectarial glands at the base of petals or on the androgynophore. Anatomical and ultrastructural aspects of floral nectaries have been poorly studied, as well as nectar production in the genera of Grewioideae. Leitão *et al*. (2005) focussed on the anatomy of floral, bracteal and foliar nectary in *Triumfetta semitriloba* Jacq., and Lattar *et al*. (2009) studied floral and extrafloral nectaries of *T. rhomboidea* morpho-anatomically.

Pollination syndromes in Malvaceae

Pollination syndromes differ within Malvaceae. In Byttnerioideae, pollination in *Herrania* L. is by phorid flies (Diptera: Phoridae).

In *Theobroma* L., the prevalent pollinators are midges (Diptera: Ceratogonoidea, particularly from the genus *Forcipomya*) (Young *et al*. 1984, 1987; Venturieri and Silva 1997). In Tilioideae Arn., the pollination occurs mainly by insects (Bayer and Kubitzki 2003), whereas in Helicteroideae (Schott & Endl.) Meisn., especially in *Helicteres isora* L., pollination is ornithophilous type (Santharam 1996), as in *H. ovata* Lam. (Sazima and Sazima 1988). In *Dombeya burgessiae* Gerrard ex Harv. & Sond., (subfamily Dombeyoideae Beilschm.) the pollination is melitophilous type (Yeo 1993). However, some species of Bombacoideae Burnett are pollinated by vertebrates and occasionally by bees (Vogel 1969, 2000; Toledo 1975; Eguiarte *et al*. 1987; Oliveira *et al*. 1992). In contrast, in the subfamily Malvoideae Burnett, the species are pollinated mainly by birds. Gottsberger (1972) observed that pollination may be ornithophilous or entomophilous in species of the following genera: *Abutilon* Mill., *Hibiscus* L. and *Pavonia* Cav. This author indicated that ornithophilous pollination occurs exclusively in the members of Malvoideae that inhabit the Neotropic. Finally, in the subfamily Brownlowioideae Burrett the type of pollination is unknown (Bayer and Kubitzki 2003). In Grewioideae, studies demonstrated that pollination by bees is possible (e.g. in *Grewia occidentalis* L.) or by vertebrates also, including trap-lining phyllostomid bats (Sazima *et al.* 1982; Zietsman 1991).

Heliocarpus L., *Luehea* Willd. and *Triumfetta* L. are the three genera of Grewioideae found in South American that present nectaries. The latter was the only one studied so far (Leitão *et al*. 2005; Lattar *et al*. 2009). Considering this, our research aims to: (1) characterise the morpho-anatomy and ultrastructure of the floral nectaries present in *Heliocarpus popayanensis* Kunth and *Luehea divaricata* Mart.; (2) determine morphological and anatomical differences in floral nectaries between perfect and pistillate flowers in *H. popayanensis*, and between different floral morphs of *H. popayanensis* and *T. rhomboidea*; (3) analyse nectar volume in relation to different floral morphs in both species; and (4) verify the frequency of floral visits in the studied species.

Materials and methods

Plant material

Sampling of nectar and collection of flowers from both floral morphs (monoclinous and pistillate flowers) of *H. popayanensis* species were conducted between 14 and 21 July 2012, in a single population of port city of Eldorado, Misiones, Argentina. In the study area, *H. popayanensis* blooms from late June to early September. Flowering of individual plants was asynchronous. Sampling of nectar and collection of monoclinous flowers of *L. divaricata* were preformed between 10 and 16 February 2014 in the city of Corrientes, Argentina. Flowering of this species occurs from October to April.

Anatomical analysis

Flowers at different developmental stages, pre-anthesis, anthesis and post-anthesis, were fixed in formalin, acetic acid, alcohol (FAA) for 24 h (Johansen 1940). Samples of flowers were dehydrated with histological dehydrating BIOPUR SRL (Gonzalez and Cristóbal 1997) and infiltrated in paraffin Histoplast (Biopack, Buenos Aires, Argentina), according to Johansen (1940). The material was sectioned transversely and longitudinally (10–12 µm thickness) using a rotary microtome (Microm, Walldorf, Germany). Sections were stained in a safranin-astra blue combination (Luque *et al*. 1996) and mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). The serial sections were examined under a Leica DMLB2 (Leica, Wetzlar, Germany) brightfield microscope equipped with a digital camera (Canon Power Shot S50 AIAF, Tokyo, Japan).

Scanning electron microscopy (SEM)

Dissected flowers in anthesis stage and fixed previously in FAA were dehydrated using a graded series of ethanol solutions. The material was then critical-point dried with solvent-substituted liquid carbon dioxide and coated with gold-palladium. Micrographs were obtained with a JEOL 5800 LV (JOEL USA Inc., Peabody, MA, USA) scanning electron microscope operating at 20 kV.

Transmission electron microscopy (TEM)

Nectar glands in pre-anthesis, anthesis and post-anthesis stage were fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 2 h and post-fixed in 1.5% OsO4 at 2°C in the same buffer for 3 h. The materials were dehydrated using ascending graded series of acetone, and then embedded in Spurr resin. Sections of 1 µm thick were made on a Reichert-Jung ultramicrotome and stained with toluidine blue. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate (O'Brien and McCully 1981). The sections were examined using a JEOL 1200 EX II (JOEL USA Inc.).

Analysis of nectar and floral visitors

Nectar was extracted using glass capillaries from flowers at first day of anthesis, which were previously covered with cloth bags to prevent visitors from having access to it. For *H. popayanensis* 40 flowers of each morph (monoclinous and pistillate) were used for this analysis. Nectar volume of each morph was measured during the following time intervals: 0900–1200 hours; 1300–1600 hours and 1600–1900 hours. A total of 240 samples (120 samples of each floral morph, and each sample containing nectar gathered from only one flower) were collected for this analysis.

In the case of *L. divaricata*, nectar volume was measured during the same time intervals as those used for *H. popayanensis.* A total of 60 samples (20 flowers in three time intervals each) were collected for this analysis. The nectar volume was calculated with capillary tube of 75 mm length and of 80 µL.

Floral visitors

The frequency of floral visits was recorded during focal observations over five non-consecutive days with cloudless, calm weather to avoid limitations in pollinator activity. Floral visitors were studied on 20 individuals of *H. popayanensis*, 10 with monoclinous flowers and 10 with pistillate flowers, during three periods: 0900–1000 hours; 1200–1300 hours, and 1600–1700 hours (June and July 2014). Observations on *L. divaricata* were made on 10 individuals during the following

periods: 0830–0930 hours; 1230–1330 hours and 1630–1730 hours (February and March 2014). We recorded the number of visitors and identified them to the order taxonomic level.

Statistical analyses

A total of 50 samples of transverse serial sections of floral nectaries in anthesis stage of *H. popayanensis* and *T. rhomboidea* (previously published by Lattar *et al*. 2009) were used to analyse the following variables: head length (HL), head diameter (HD), stalk length (SL), stalk diameter (SD), trichome length (TL), length of anticlinal basal cell wall (LABCW), length of radial basal cell wall (LRBCW), largest diameter of crystalliferous idioblast (LDCI) and smallest diameter of crystalliferous idioblast (SDCI).

We performed a principal components analysis (PCA) to identify the anatomical variables that best contribute to the differentiation and characterisation of the floral nectaries of monoclinous flowers (HPF) and pistillate flowers (HPIF) of *H. popayanensis* and monoclinous flowers of *T. rhomboidea* (TR). The results of this analysis are observed in a Biplot graph (Gabriel 1971) built from the two first principal components (PC1 and PC2) derived from the PCA.

The statistical differences corresponding to nectar volume of pistillate and monoclinous flowers of *H. popayanensis* and of monoclinous flowers of *L. divaricata* (LPF) at the same time of the day (morning and early and late afternoon) and at different moments within each time of the day were estimated using an analysis of variance (ANOVA), at a significance level of 5% ($P \leq 0.05$). Whenever the ANOVA test indicated a significant difference, a pair wise comparison of means by Fisher's least significant different (l.s.d.) (Sokal and Rohlf 1995) was performed. From censuses of visit frequencies, we estimated total visit frequency distributions of only insect visitors actually or potentially performing pollination (i.e. those contacting anther and/or stigma). All statistical analyses were performed using the software Infostat (Di Rienzo *et al*. 2009).

Herbarium material of *H. popayanensis* and *L. divaricata*, as well as floral visitors were deposited at the Instituto de Botánica del Nordeste herbarium (CTES), Corrientes, Argentina.

Examined material

Heliocarpus popayanensis. ARGENTINA. Prov. Misiones. Dpto. Eldorado. 05.VIII.2009, Lattar E. and H. Keller 8 (CTES); idem., Lattar E. and H. Keller 9 (CTES); idem., Lattar E. and H. Keller 10 (CTES); idem., Lattar E. and H. Keller 11 (CTES); idem., 11.IX.09, Lattar E. and L. Ritter 12 (CTES); Dpto. San Ignacio. 11.VI.2011, Lattar E. and H. Keller 13 (CTES).

Luehea divaricata. ARGENTINA. Prov. Corrientes. Dpto. Corrientes. 05.III.2009, Lattar E. and M. S. Ferrucci 5 (CTES). Prov. Misiones. Dpto. Apóstoles. Camino a Azara, 13.XII.2011, Miguel *et al*. 25 (CTES).

Results

Floral nectary morphology and anatomy of Heliocarpus popayanensis

The trichomatic floral nectaries consist of four subrectangular nectariferous glands, located on a short androgynophore in monoclinous flowers (Fig. 1*a*, *c*), and of four subcircular nectariferous glands located on a small gynophore in pistillate flowers (Fig. 1*b*, *d*). The floral nectary consists of an epidermis with secretory trichomes and a subepidermal secretory parenchyma (Fig. 2*a*–*f*).

Epidermis

In surface view, SEM observations show heads of glandular trichomes with smooth cuticles (Fig. 1*e*, *f*). In transverse section the secretory trichomes are capitate-shaped, with a unicellular stalk and a basal cell in both types of flowers. The secretory head is multicellular and biseriate, composed of 4–6 cells in monoclinous flowers, and of 6–8 cells in pistillate flowers (Fig. 2*a*–*f*). The basal cell has dense cytoplasm and conspicuous nucleus. The stalk cell is highly vacuolated and the head cells are stained intensely and the nucleus is frequently observed in parietal position (Fig. 2*d*, *e*).

Secretory parenchyma

It consists of isodiametric cells with small intercellular spaces. These cells have dense cytoplasm and conspicuous nuclei located in parietal position. Small crystalliferous idioblasts are observed in monoclinous flowers, whereas in pistillate flowers, these idioblasts are slightly larger; and small cavities are also observed (Fig. 2*a*, *b*).

Floral nectary morphology and anatomy of Luehea divaricata

The trichomatic floral nectaries are located at the base of adaxial surface of petals. These nectariferous glands are subcircular to circular (Fig. 3*a*). The floral nectary consists of an epidermis with glandular trichomes and a subepidermal secretory parenchyma (Fig. 4*a*, *f*).

Epidermis

In surface view, SEM observations show trichome head cells with smooth cuticle (Fig. 3*b*).

In transverse section, capitate-shaped multicellular glandular trichomes are observed. These trichomes consist of a basal cell, a unicellular stalk and a biseriate head, composed by 4–6 cells (Fig. 4*a*–*c*). The basal cell presents dense cytoplasm and conspicuous nucleus in parietal position; the stalk and head cells of trichomes are stained in the same way and present conspicuous nuclei (Fig. 4*c*).

Secretory parenchyma

It consists of isodiametric small cells, with dense cytoplasm, apparently without intercellular spaces. Idioblasts are present (Fig. 4*a*–*c*, Fig. 4*e*, *f*). Elements of the xylem were observed in the secretory parenchyma (Fig. 4*d*).

Floral nectary ultrastructure of Heliocarpus popayanensis

At pre-anthesis stage, the head cells of secretory trichomes have a very dense cytoplasm with numerous mitochondria, rough endoplasmic reticulum and plastids (Fig. 5*a*). The cuticle that covers these cells and the cell wall are evenly thick. At anthesis, the head cells have numerous small vacuoles and free ribosomes. Cytoplasmic connections (plasmodesmata) between

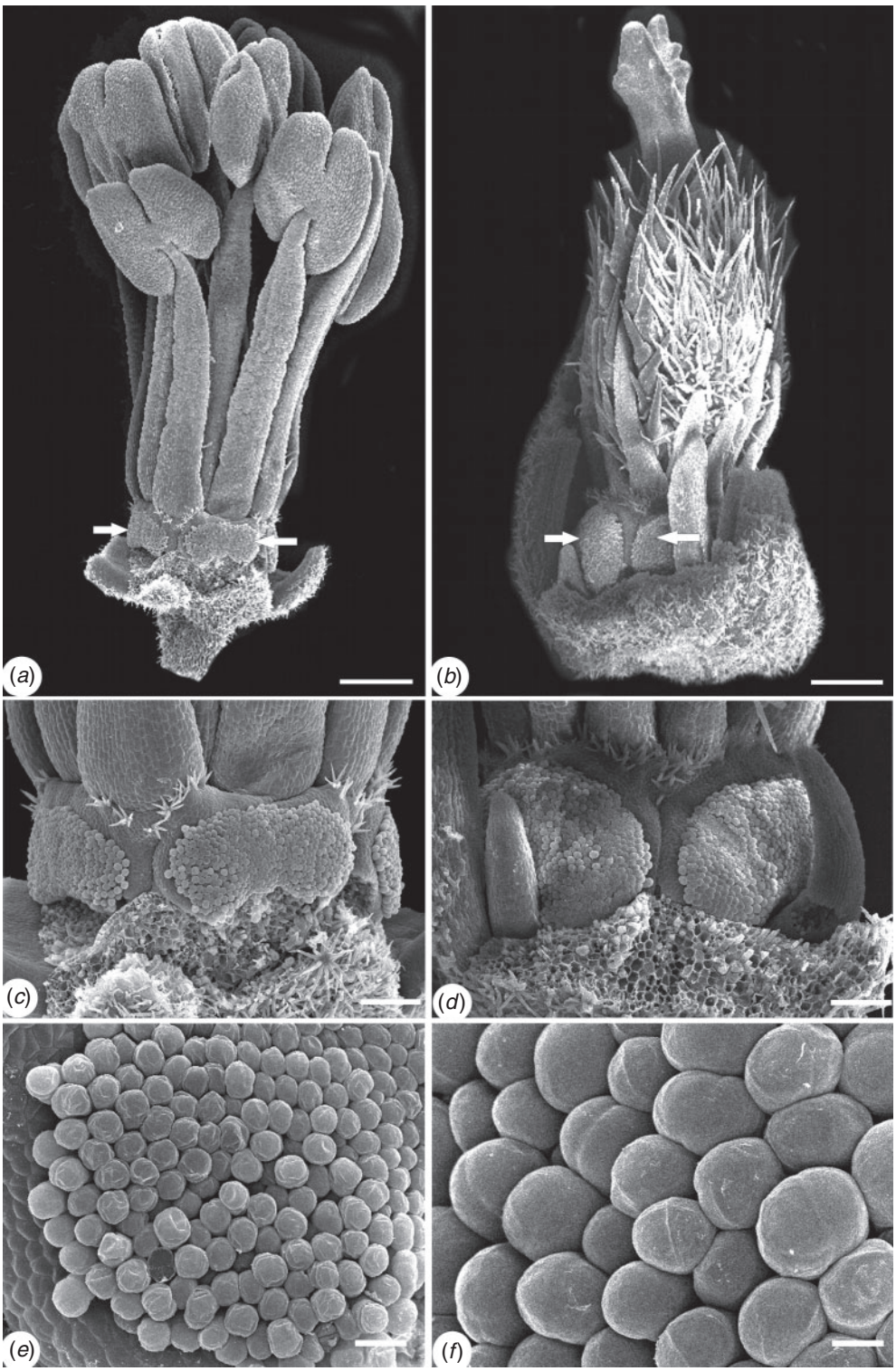

Fig. 1. Scanning electron photomicrographs of *Heliocarpus popayanensis*. (*a*) Monoclinous flower devoid of sepals and petals showing subrectangular nectariferous glands located on a short androgynophore (arrows). Scale bar = 20 µm. (*b*) Pistillate flower devoid of sepals showing subcircular nectariferous glands located on a small gynophore (arrows). Scale bar = 20 µm. (*c*) Details of nectariferous glands in monoclinous flowers. Scale bar = 100 µm. (*d*) Details of nectariferous glands in pistillate flowers. Scale bar = 100 µm. (*e*) Heads of glandular trichomes in monoclinous flowers. Scale bar = 50 µm. (*f*) Heads of glandular trichomes in pistillate flowers. Scale bar = 20 µm.

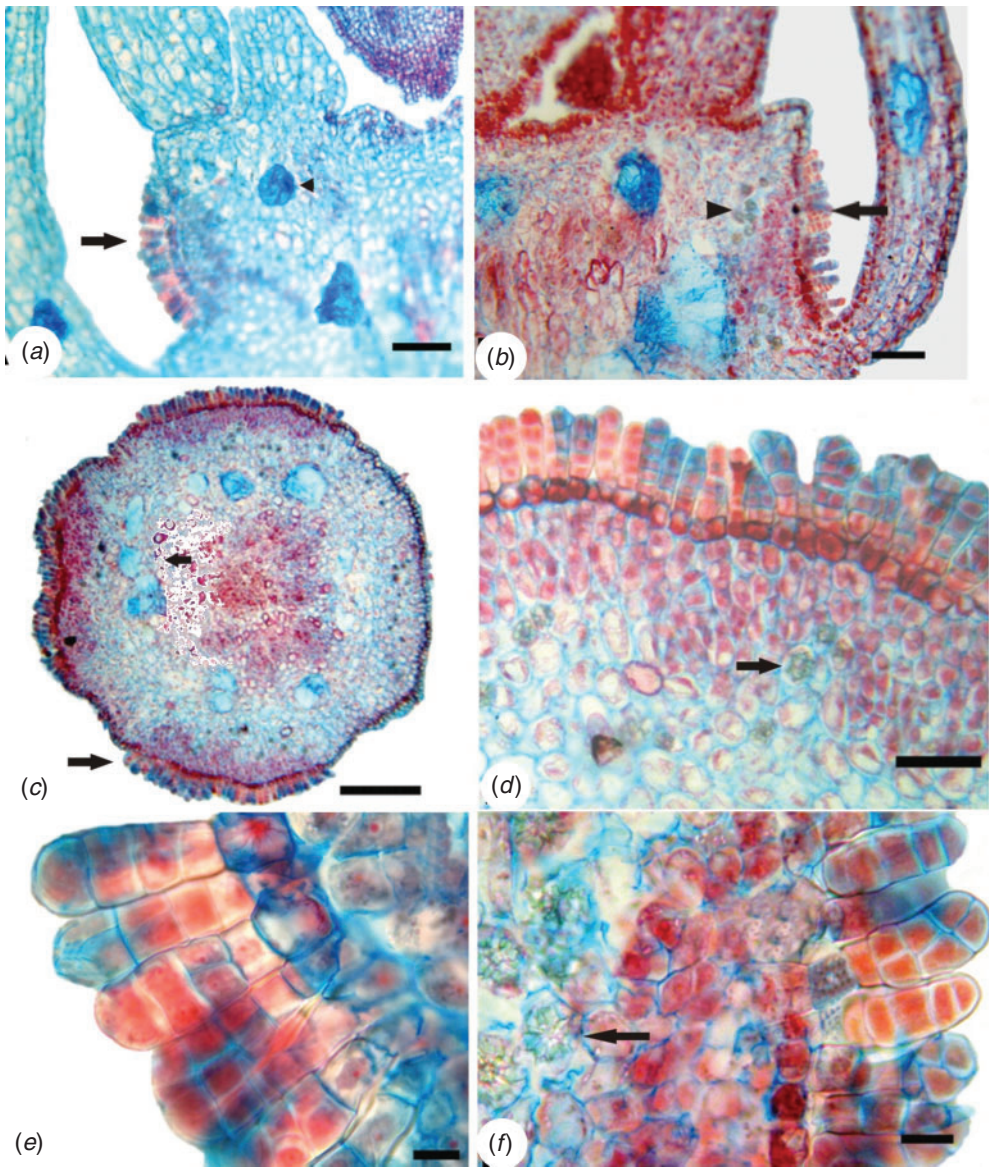

Fig. 2. Photomicrographs of histological sections of floral nectaries of *Heliocarpus popayanensis*. (*a*) Longitudinal section of monoclinous flower showing the floral nectary (arrow) and mucilaginous cavities (arrowhead). Scale bar = 200 µm. (*b*) Longitudinal section of pistillate flower; notice the floral nectar (arrow) and druses in the secretory parenchyma (arrowhead). Scale bar = 200 µm. (*c*) Transverse section of androgynophore of the monoclinous flower showing four nectar glands, mucilaginous cavities and druses (arrows). Scale bar = 100 µm. (*d*) Transverse section of gynophore with glandular trichomes of the pistillate flower; note the druses in the secretory parenchyma (arrows). Scale bar = 50 µm. (*e*) Details of glandular trichomes in monoclinous flowers. Scale bar = 50 µm. (*f*) Details of the glandular trichomes in pistillate flower showing druses in the secretory parenchyma (arrow). Scale bar = 50 µm.

the secretory cells of the trichome head and numerous vesicles between the plasmalemma and the cell wall are observed (Fig. 5*b*). A large number of mitochondria, numerous plastids and dictyosomes in the cytoplasm are also observed (Fig. 5*b–e*). Moreover, the rough endoplasmic reticulum is well developed, showing cisterns arranged in parallel (Fig. 5*e*). Multilamellar bodies within vacuoles can be observed (Fig. 5*d*). Nectar accumulates between the wall and the cuticle, which are noticeably separated. At post-anthesis, the cuticle is more distended and there is no secretion between it and the cell wall. Cuticular pores are not observed (Fig. 5*f*). No differences in ultrastructural features of the secretory trichomes are observed between perfect and pistillate flowers.

Floral nectary ultrastructure of Luehea divaricata

At pre-anthesis and anthesis stages, the head cells of the secretory trichomes have dense cytoplasm with numerous vacuoles and mitochondria, and abundant cytoplasmic connections (plasmodesmata) between the secretory cells (Fig. 6*a*). The cuticle is more electron-dense than the cell wall. The mitochondria are abundant and numerous dictyosomes and

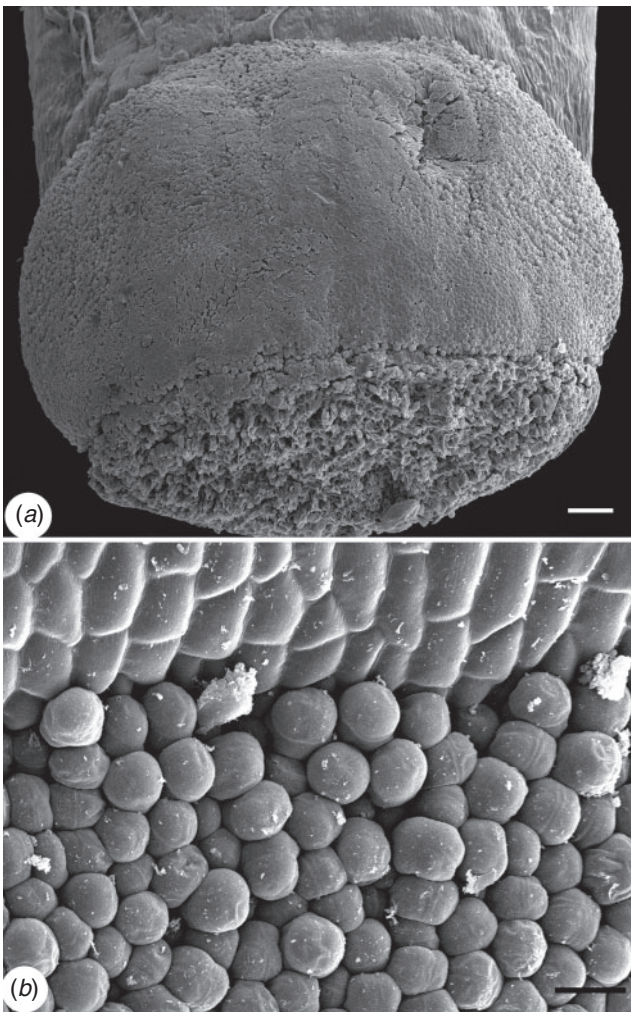

Fig. 3. Scanning electron photomicrographs of *Luehea divaricata*. (*a*) Detail of nectar gland located on the adaxial surface of petals base. Scale bar = 100 µm. (*b*) Detail of the heads of glandular trichomes. Scale bar = 20 µm.

dictyosomic vesicles are observed (Fig. 6*b*, *c*). Small vacuoles with osmiphilic contents are present (Fig. 6*a*, *c*). The rough endoplasmic reticulum is not organised in parallel cisterns (Fig. 6*c*). Nectar secretion is observed between the plasmalemma and the cell wall and between the cuticle and the cell wall (Fig. 6*c*). The cuticle begins to separate from the wall, and at later stages, it becomes distended, although less noticeably than in the previously described species. There is no presence of pores in the cuticle.

Multivariate Analysis

The first two principal components of PCA (Fig. 7) explained 83.1% and 16.9% (PC1 and PC2 respectively) of the total variation in the data, as observed in the biplot. The biplot reduced adequately the multidimensional matrix of the database, with the two first components explaining 100% of the total variation observed in the analysed variables in different individuals. PC1 separated the two analysed species, whereas TR was more strongly associated and positively correlated with the variables ALBCW, HD, SDCI and RLBCW. HPF and HPIF were associated and positively correlated with HL, TL and SL (Fig. 7); accordingly, the greatest variability between species was explained with these variables. A high and negative correlation between the groups of discriminating variables (ALBCW, HD, SDCI, RLBCW HL, TL, SL) was observed.

Total nectar volume

The flowers of *H. popayanensis* open early in the morning, with the presence of sunlight. Anthesis of a single flower lasted 5 days in monoclinous flowers and 3.5 days in pistillate flowers. Total average nectar volume (µL) produced by flower at the first day of anthesis was 1.23 µL in monoclinous flowers and 1.89 µL in pistillate flowers.

The flowers of *L. divaricata*, as those of *H. popayanensis*, open in the early morning, with the presence of sunlight. Anthesis of a single flower lasted 2 days. Total average nectar volume produced per flower at the first day of anthesis was 0.386 µL.

The analysis of variance corresponding to total nectar volume in HPF, HPIF, and LPF at different times of the day indicated a significant increase of nectar production ($P < 0.0001$) during the day for both species, reaching maximum values at noon (Fig. 8*a–c*). The difference between the maximum values (noon) and minimum (late afternoon) of total nectar volume was 303% for HPF, 151% for HPIF and 400% for LPF (Fig. 8*b*, *c*). The ANOVA also showed that, at different times throughout the studied days, HPF produced greater volume of nectar than HPIF, and that *H. popayanensis* produced greater nectar volume than LPF (Fig. 8*a–c*). Average nectar values produced at noon were 3.75 µL in HPF, 1.88 µL in HPIF and 0.85 µL in LPF.

Floral visitors

Floral visitors were observed from the moment that flowers opened, between 0800–0900 hours, until the end of anthesis. The most abundant and active visitors in pistillate and monoclinous flowers of *H. popayanensis* were the honeybees *Apis* sp. Some individuals visited 10 flowers of the same plant for 1 min. Butterflies also visited this species, but less frequently. Monoclinous flowers were also frequently visited by flies (Diptera), which remained on the same plant during long periods of 15 to 25 min. In *L. divaricata*, monoclinous flowers were visited by bumblebees (Hymenoptera), wasps (Hymenoptera), beetles (Coleoptera) and butterflies (Lepidoptera) (Figs 9–11).

Discussion

Morpho-anatomy of the floral nectary

The structure of floral nectaries significantly supports the recent tribal classification of the subfamily Grewioideae (Brunken and Muellner 2012), based on molecular and morphological data.

The floral nectaries of *H. popayanensis* and *L. divaricata* belong to the structured type according to Nepi (2007). The nectary area is well defined, and can be recognised macroscopically and microscopically. It produces nectar regularly. These findings are in agreement with characteristics of other species of Grewioideae, such as *T. semitriloba* and *T. rhomboidea* (Leitão *et al*. 2005; Lattar *et al*. 2009). According to Vogel (2000), one important character of Malvaceae *s.l.* is the presence of

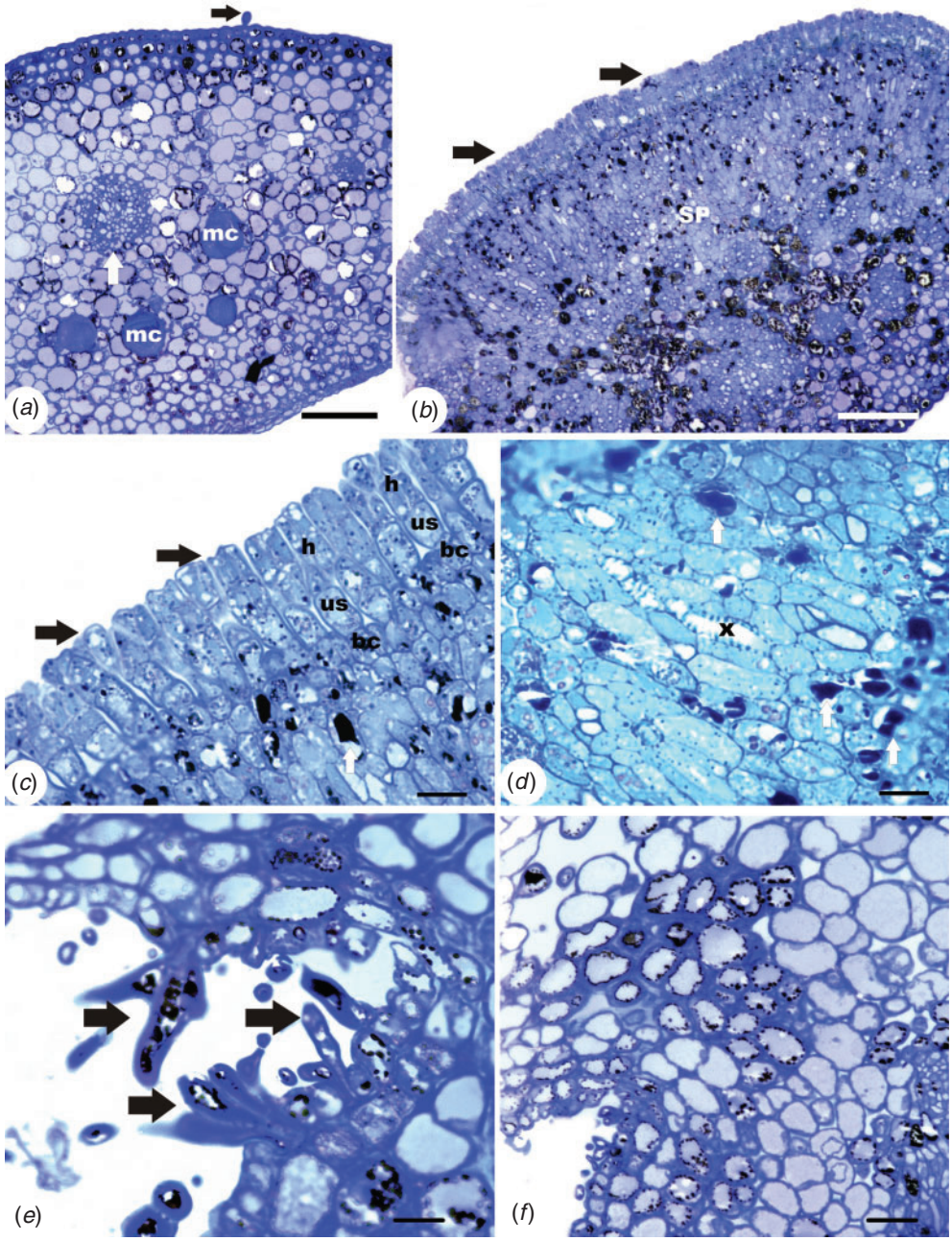

Fig. 4. Light photomicrographs section of floral nectary of *Luehea divaricata*. (*a*) Pre-anthesis stage. General aspect of the secretory parenchyma, showing a secretory trichome (arrow) and mucilaginous cavities. Scale bar = 100 µm. (*b–d*) Anthesis stage. (*b*) General aspect of the floral nectary; notice the glandular trichomes (arrows) and the secretory parenchyma (sp). Scale bar = 200 µm. (*c*) Detail of the glandular trichomes (arrows), showing the head (h), unicellular stalk (us) and basal cell (bc). Scale bar = 50 µm. (*d*) Detail of the vascularisation; note the xylem (x) in the secretory parenchyma. Scale bar = 50 µm. (*e*, *f*) *Post-anthesis stage*. (*e*) Detail of glandular trichomes. Scale bar = 50 µm. (*f*) Disappearance of the glandular trichomes showing the parenchyma being degraded. Scale bar = 50 µm.

trichomatic nectaries, a type absent in the families close to 'core' Malvales. In the studied species, the nectaries correspond to this type; however, in the floral nectaries of some species of Malvaceae belonging to the subfamily Byttnerioideae, secretion is released through stomata (Young *et al.* 1984). Trichomatic nectary is considered an apomorphic character for the family.

H. popayanensis and *L. divaricata* possess persistent floral nectaries according to the classification proposed by Smets (1986). According to the position of floral nectaries, *H. popayanensis* has thalamic nectaries, which coincides with observations reported for *T. rhomboidea* (Lattar *et al*. 2009), whereas *L. divaricata* exhibits perigonal nectaries (Fahn 1982). Regarding vascularisation, according to Frey-Wyssling (1955),

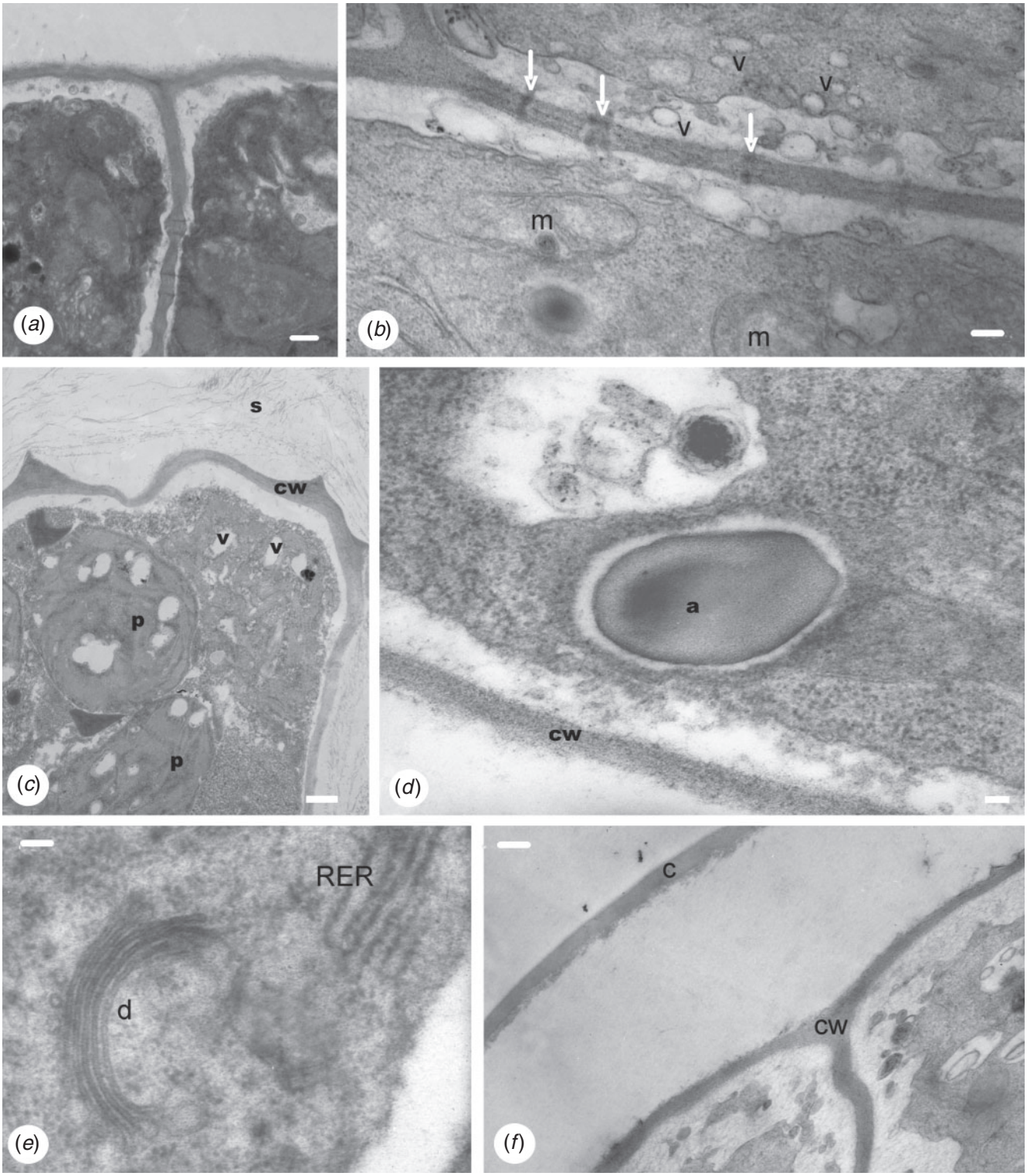

Fig. 5. Transmission electron microscopy (TEM) photomicrographs of the trichome head cells in floral nectary of *Heliocarpus popayanensis*. (*a*) *Pre-anthesis*. Detail of two cells in monoclinous flower. Scale bar = 1.2 µm. *Anthesis stage.* (*b*) Detail of two cells showing several plasmodesmata (white arrows), mitochondria (m) and numerous vesicles (v), some of them between the plasmalemma and the cell wall. Scale bar = 400 nm. (*c*) Detail of a cell with numerous plastids (p) and small vacuoles (v). Secretion (s) can be observed outside the cell wall (cw). Scale bar = 1.2 µm. (*d*) Detail of an amyloplast (a) in the cytoplasm of a trichome head cell in monoclinous flower. Scale bar = 300 nm. (*e*) Detail of a dictyosome (d) and rough endoplasmatic reticulum (RER) in a trichome cell of pistillate flower. Scale bar = 500 nm. (*f*) Detail of two cells showing the cell wall (cw), and the cuticle (c) distended in monoclinous flower. Scale bar = 1.2 µm.

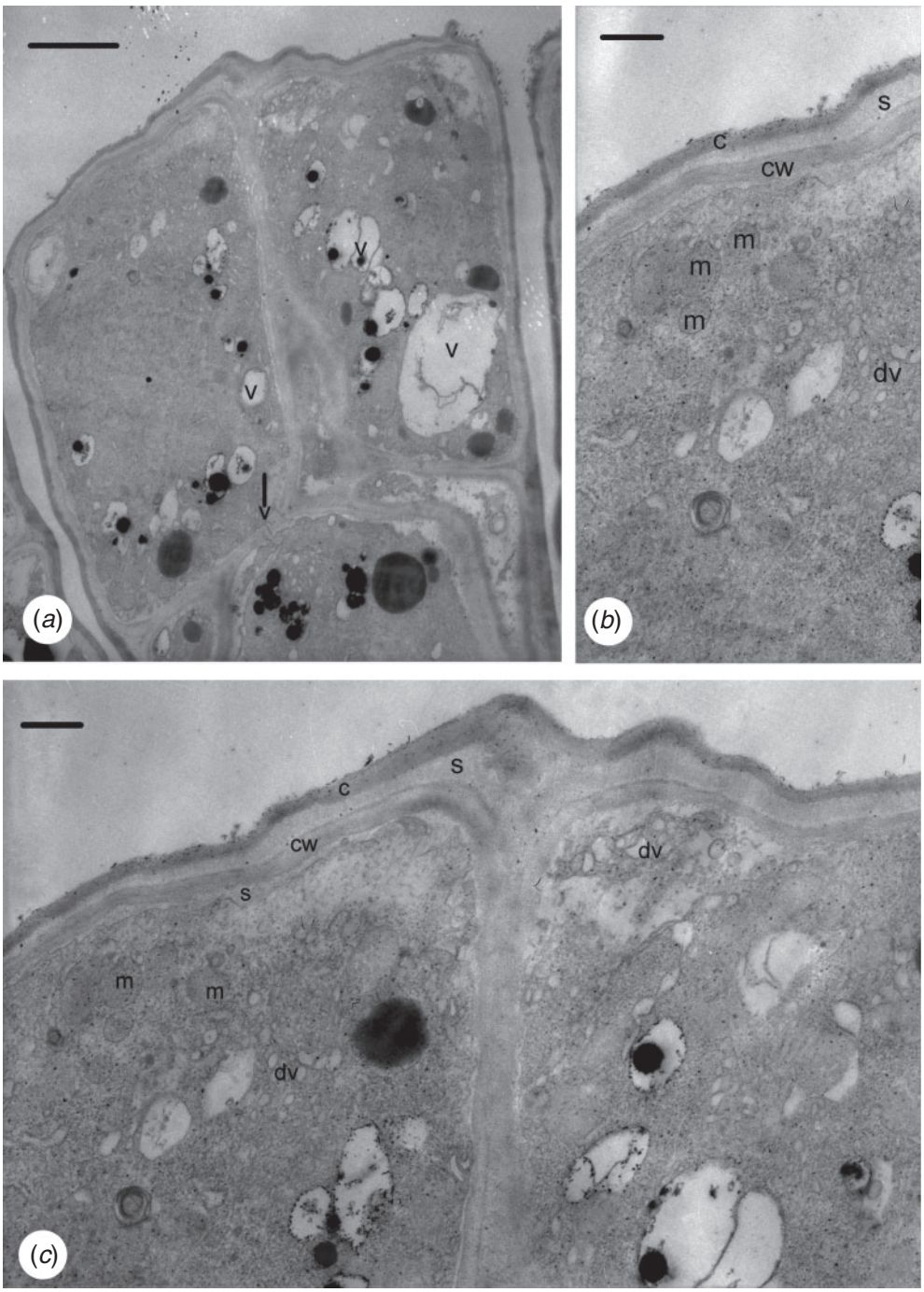

Fig. 6. Transmission electron microscopy (TEM) phtomicrographs of the trichome head cells of *L. divaricata*. (*a–c*) *Pre-anthesis stage.* (*a*) Detail of secretory trichome cells, showing a dense cytoplasm, presence of numerous vacuoles (v) and plasmodesmata (arrow). Scale bar = 1µm. (*b*) Detail of cytoplasm of a cell with numerous mitochondria and dictyosomic vesicles (dv), cell wall (cw) and cuticle (c). Scale bar = 500 nm. (*c*) Detail of two cells showing the secretion (s) between the plasmalemma and the cell wall (cw), and this last one and the cuticle (c). There are numerous dictyosomic vesicles (dv) and mitochondria (m). Scale bar = 500 nm.

the nectaries present in *H. popayanensis* and *L. divaricata* would be an evolved type, because they possess their own vascular tissue. This character is shared with other species of the subfamily Grewioideae, such as *T. semitriloba* and *T. rhomboidea* (Leitão *et al*. 2005; Lattar *et al*. 2009), and with *Dombeya wallichii* and *D. natalensis* (Dombeyoideae) (Rocha *et al*. 2010), and *Abutilon striatum* and *Hibiscus rosa-sinensis* (Malvoideae) (Findlay and Mercer 1971*b*; Sawidis *et al*. 1987; Robards and Stark 1988). In *L. divaricata* the floral nectaries are innervated by phloem and xylem, like

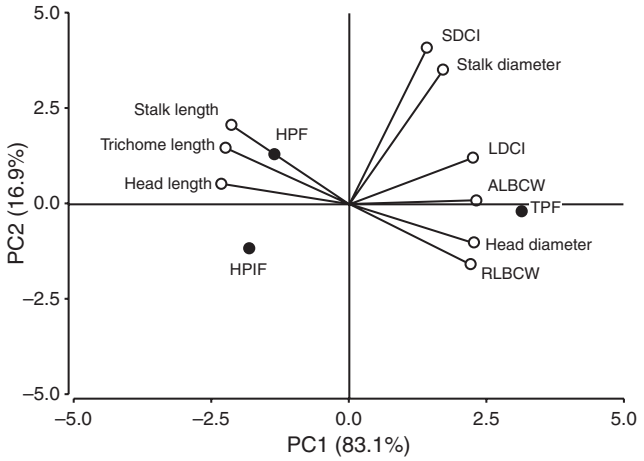

Fig. 7. Biplot showing relationships between morpho-anatomical variables (white circles) and the two taxa analysed (black circles): perfect and pistillate flowers of *Heliocarpus popayanensis* (HPF and HPIF) and perfect flowers of *Triumfetta rhomboidea* (TPF).

T. semitriloba (Leitão *et al*. 2005). The presence of vascular bundles composed by both vascular tissues was also observed in *D. wallichii* and *D. natalensis* (Rocha *et al*. 2010). Xylem was not observed in the nectariferous parenchyma of *H. popayanensis*, the presence of only phloem traces in this tissue is a character shared with *A. striatum*, *H. rosa-sinensis* (Findlay and Mercer 1971*a*, 1971*b*; Sawidis *et al*. 1987; Robards and Stark 1988) and *T. rhomboidea* (Lattar *et al*. 2009).

Ultrastructure of floral nectary

The ultrastructure of floral nectaries was studied in different families of Angiosperms (Fahn 1979; Durkee 1983; Radice and Galati 2003; Weryszko-Chmielewska and Bożek 2008; Bartoli *et al*. 2011; Konarska 2011; Mosti *et al*. 2001, 2013; Antoń and Kamińska 2015); however, there is very little information available for Malvaceae *s.l.* The floral nectaries of *A. striatum* and *H. rosa-sinensis* were described as being represented by secretory trichomes, with cells exhibiting a dense cytoplasm, numerous mitochondria and rough endoplasmic reticulum, and small vacuoles that fuse during nectar production (Findlay and Mercer 1971*a*, 1971*b*; Findlay *et al*. 1971; Sawidis *et al*. 1987; Robards and Stark 1988). Although the morphology and structure of secretory trichomes in *H. popayanensis* and *L. divaricata* are similar to those of the above mentioned species, their ultrastructure showed some differences. The cytoplasm of the secretory trichomes of *H. popayanensis* is very dense and has numerous mitochondria, RER, amyloplasts, dictyosomic vesicles and few vacuoles. However, in *L. divaricata*, the cytoplasm is less dense than in *H. popayanensis* and presents numerous dictyosomic vesicles, abundant mitochondria and small vacuoles. Fahn (1979), Wist and Davis (2006), Stipczyńska *et al*. (2011), and Antoń and Kamińska (2015) reported that the presence of a dense cytoplasm with abundant RER and vesicles in secretory cells would be related to nectar production. This is in agreement with the characteristics observed in this study. On the other hand, the secretory cells with high metabolic activity possess many

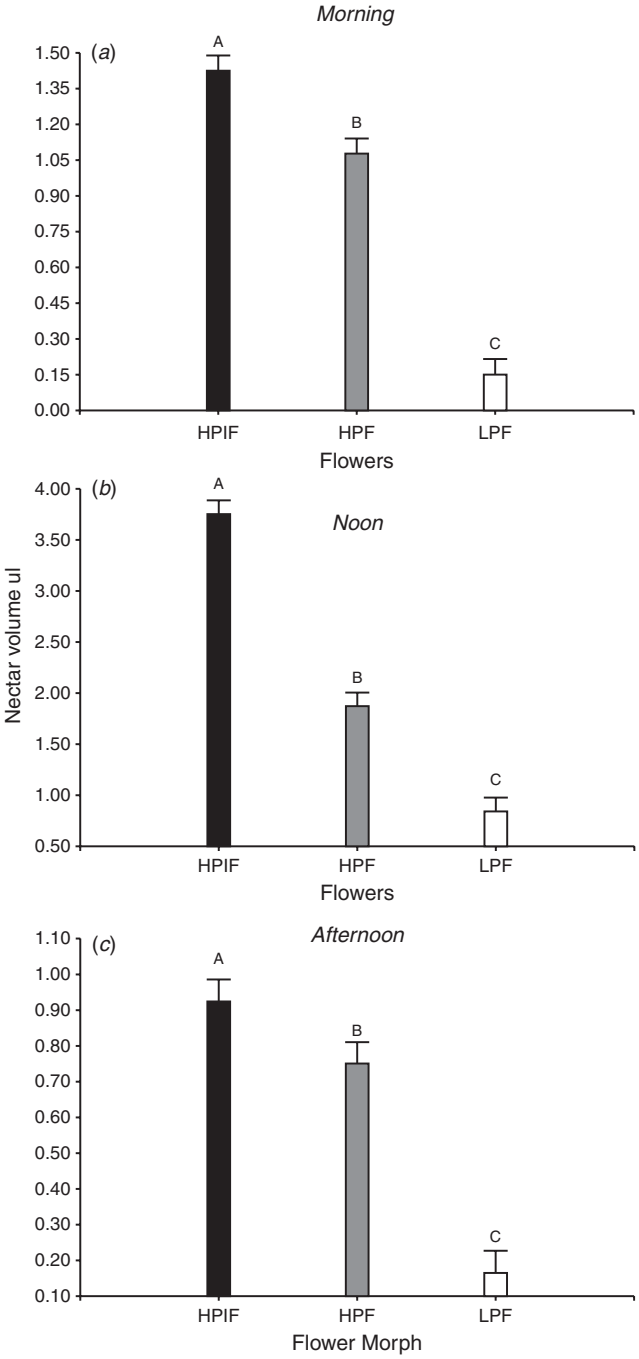

Fig. 8. Bar graphs corresponding to total nectar volume (in µL) produced at different times of the day in the pistillate and perfect flowers of *Heliocarpus popayanensis* (HPF and HPIF) and perfect flowers of *Luehea divaricata* (LPF). (*a*) Morning (0900–1200 hours). (*b*) Noon (1300–1600 hours). (*c*) Afternoon (1600–1900 hours). Different letters indicate statistically significant differences ($P \leq 0.05$).

mitochondria and plastids; these organelles interact and are important during the formation of nectar in the secretory tissue (Wist and Davis 2006; Antoń and Kamińska 2015). The cytoplasm of the secretory trichomes of *H. popayanensis* and *L. divaricata* has numerous mitochondria but plastids were only

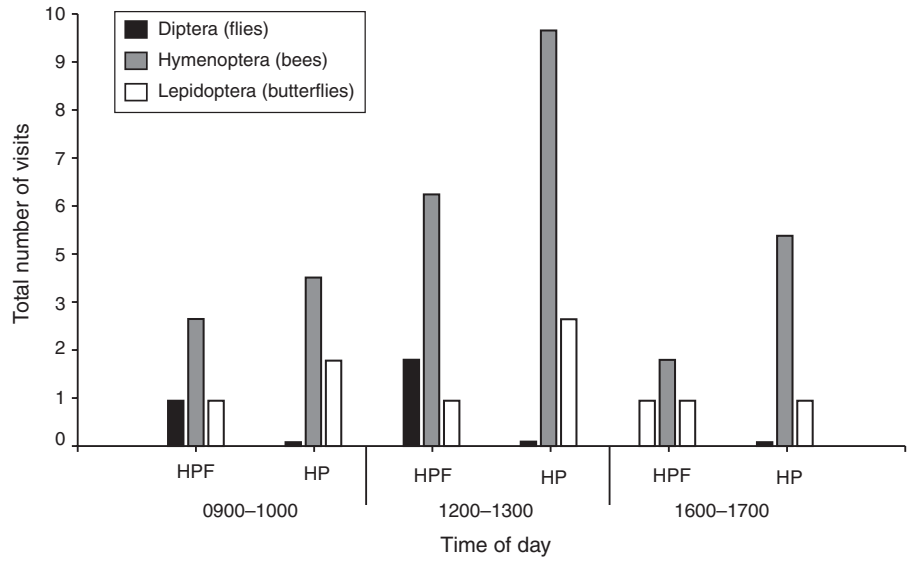

Fig. 9. Frequency of floral visits in monoclinous flowers (HPF) and pistillate flowers (HP) of *Heliocarpus popayanensis* during five non-consecutive days between June and July 2014 at Eldorado (Misiones, Argentina).

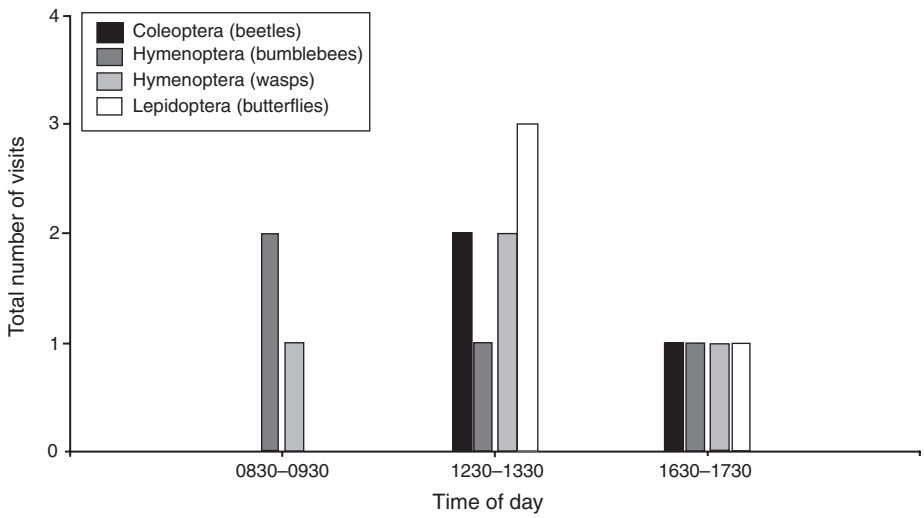

Fig. 10. Frequency of floral visits in monoclinous flowers of *Luehea divaricata* during five non-consecutive days between February and March 2014 at Corrientes (Corrientes, Argentina).

observed in *H. popayanensis*. The presence of numerous amyloplasts and vascular bundles with only phloem in the nectariferous tissue suggests that the secretory trichomes of this species receive and accumulate sugars originated from other green tissues; which could be located in the flower or in other plant organs. It has been observed in nectaries of different species that the starch stored in the parenchyma is quickly hydrolysed immediately before nectar secretion (Durkee *et al.* 1981; Zer and Fahn 1992; Sawidis 1998). Therefore, the presence of amyloplasts suggests that the floral nectary of *H. popayanensis* produces more nectar than that of *L. divaricata.* This fact was confirmed via the analysis of variance of nectar volumes measured in both species. On the other hand, vacuoles with osmiophilic contents were only observed in cells of secretory trichomes of *L. divaricata.* A similar electrondense material along the inner side of the tonoplast that tends to form a continuous layer adhering to the tonoplast was observed in *Selenicereus grandiflorus*. The authors related it with lipidic material that could also be constituent of the secretion (Mosti *et al.* 2001).

The presence of numerous plasmodesmata between cells of the secretory parenchyma and the basal cell of the trichome, and between this last one and the cells of the trichome head, suggests that nectar transport is via the symplastic pathway. In *H. popayanensis* and *L. divaricata*, abundant dictyosomes and numerous vesicles between the plasmalemma and the cell wall of the trichome head cells were observed. According to Nepi (2007) the presence of a well-developed endoplasmic reticulum,

Fig. 11. Floral visitors present in *Heliocarpus popayanensis*. (*a*) Detail of floriferous branch with perfect flowers visited by a butterfly. (*b*) Perfect flowers visited by *Apis mellifera* (arrow). (*c*) Detail of a fly visiting the flowers. Floral visitors present in *Luehea divaricata*. (*d*) Detail of a bumblebee (Hymenoptera) visiting a flower. (*e*) Detail of a young flower visited by wasp. (*f*) Detail of a butterfly (Lepidoptera) visiting a flower. Photographs by E. Lattar.

numerous Golgi bodies and vesicles is considered to be the distinctive feature of granulocrine secretion of nectar. However, Vassilyev (2010) considers that the concept of granulocrine secretion of nectar should be discarded. According to this author, nectar moves by a pressure-driven mass flow in the nectary apoplasm whereas pre-nectar sugars diffuse from the sieve tubes to the secretory cells via symplast, where nectar is formed and sugars cross the plasma membrane by active transport ('eccrine secretion'). Recently, Paiva (2016) proposed a new hypothesis involving cyclic mechanical actions of the protoplast. This author indicates that the mechanical action of the protoplast, in the form of successive cycles of contraction and expansion, causes the material accumulated between the plasmalema and the cell wall to pass through the latter and the cuticle. This cell-cycle model could occur in *H. popayanensis* and *L. divaricata*, where the secretory trichomes present numerous vesicles in anthesis stage which are related with carrying the nectar. The presence of vacuoles and vesicles observed in the species studied could indicate the expansion of the protoplast as in *L. grandiflora* (Paiva 2016).

Nectar accumulation in the studied species occurs between the cell wall and the cuticle, as observed in other species of Malvaceae, such as *A. striatum*, *H. rosa-sinensis* and *L. grandiflora* (Sawidis *et al*. 1987; Robards and Stark 1988; Paiva 2016). In *A. striatum*, nectar is secreted through pores located in the cuticle (Robards and Stark 1988). In *H. popayanensis* and *L. divaricata* the presence of cuticular pores was not detected, but the cuticle appeared highly distended, and the secretion between it and the cell wall is no longer present at post-anthesis stage. This last fact suggests that the cuticle must be permeable allowing the nectar secretion.

Finally, Nepi (2007) indicates that the secretory parenchyma can have three possible fates: being involved in nectar reabsorption; differentiating into other tissue types or degenerating. In the studied species, the secretory parenchyma is totally degraded at the post-anthesis stage. Presence of multilamellar structures within vacuoles in secretory cells of the studied species suggests autophagy. This fact could be related to the programmed cell death of these cells (van Doorn and Papini 2013; Papini *et al*. 2014; Papini and van Doorn 2016).

Relationship between nectar and floral visitors

The floral nectar plays an essential role in plant-pollinator interactions and reflects a mechanism for direct coevolution, since it is not part of the reproductive system itself, but a reward offered to an external agent (Dafni 1992). The flowers of *H. popayanensis* exhibit variations in relation to nectar volume. In monoclinous flowers, the average was 1.23 µL and in pistillate flowers, it was 1.89 µL. Moreover, in *L. divaricata* flowers, the average was 0.39 µL. Although floral visitors were observed in both species, each would offer different floral rewards. The flowers of *L. divaricata* would offer pollen as the main reward, and pistillate flowers of *H. popayanensis* would offer only nectar, whereas monoclinous flowers both rewards. Nectar production involves a physiological cost to the plant, which is offset when animals arrive to collect nectar and inadvertently transfer pollen (Koptur 1994). Nectar volume would also be related to flower size or biomass (Baker and Baker 1983; Galetto and Bernardello 2004). Small flowers produce less than 3 µL day^{-1} of nectar, whereas large flowers produce 15 µL day^{-1} of nectar (Baker and Baker 1983). Pollinator size would also be involved in nectar volume, because a high amount of nectar is needed to attract bats, moths and large birds (Baker and Baker 1983). The flowers of *H. popayanensis* are small and produce less than 2 µL day^{-1} of nectar (1.23–1.89). This amount of nectar would be related to the pollinators visiting both types of flowers of the analysed species, which are mostly honeybees, *Apis* sp. The flowers of *L. divaricata* are medium-sized, but produce smaller volume of daily nectar (0.39 µL day^{-1}); this finding supports the hypothesis that pollen would be the main reward in *L. divaricata* flowers.

Statistical analysis

The biplot of morphological characters reveals the separation of *H. popayanensis* from *T. rhomboidea* by the following quantitative variables: ALBCW, HD, SDCI, RLBCW, HL, TL and SL. Lattar *et al*. (2009) previously analysed the morpho-anatomy of floral and extrafloral nectaries of *T. rhomboidea*, and differentiated the three types of nectaries present in this species based on the trichome head length and length of periclinal cell wall of the trichome basal cell. The variables head diameter, stalk length and stalk width, distinguish floral nectaries from extrafloral nectaries. These results are similar to those reported by Leitão *et al*. (2005), who found significant differences between the floral nectary and extrafloral nectaries of *T. semitriloba*, according to the length and width of the head of glandular trichomes. However, those authors did not make a further comparison of means of the variables to discriminate between types of nectaries. The ANOVA indicated that in *H. popayanensis*, pistillate flowers produced a higher amount of nectar volume than monoclinous flowers and that nectar production of both flower types exceeded that of *L. divaricata* flowers.

General conclusions

The position of floral nectaries is an important character. In *L. divaricata*, the nectariferous glands are located at the base of the adaxial surface of petals; while in *H. popayanensis*, the nectariferous glands are located on an androgynophore. Moreover, morphological differences between the nectaries of these species were supported statistically. These results would support the inclusion of each genus in different tribes, *Luehea* in Grewieae and *Heliocarpus* in the tribe Apeibeae. Within Grewoideae, the nectariferous glands located in the androgynophore represent an advanced character with respect to its position at the base of the petals. Finally, nectar secretion and its relationship with floral visitors is an interesting aspect that should be explored within the family Malvaceae to contribute to the understanding of the ecological relationships between plant species and pollinators, which are likely to have an important role in the evolution of this family.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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