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Diversity of floral nectary secretions and structure, and implications for their evolution in Anacardiaceae

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The flowers of most Anacardiaceae have a floral nectary disk producing nectar rich in sugars. However, a recent study demonstrated that their nectaries might also produce other substances, including lipids and phenolic compounds. To explore the diversity of floral nectary production and (ultra)structure, and their potential for the systematics of Anacardiaceae, we studied seven genera and 13 species from the two subfamilies. We used spectrophotometry to identify sugars and histochemical tests for other substances, and electron and brightfield microscopy to study nectary (ultra)structure and secretory pathways. The composition of sugars and other substances can vary between closely related species and be more similar in species from different subfamilies, being of limited value for the systematics of the family. The general morphology and structure of the floral nectary and their secretory pathways appear to be conservative in the family, and, like the production of mixed secretions, they might be plesiomorphic. Three morphological types of floral nectaries are defined for the family: nectariferous disk with papillose (1) or smooth epidermis-type (2) and trichomatous-type (3). The secretions may be released both by granulocrine and eccrine mechanisms and exuded through nectarostomata or the cuticle. Further studies are needed to better understand their evolutionary and ecological implications in Anacardiaceae and other sapindalean lineages.

ADDITIONAL KEYWORDS: lipids – mixed secretion – nectar sugar composition – nectariferous disk – phenolic compounds – Sapindales – (ultra)structure.

INTRODUCTION

Floral nectaries are important for the pollination process because they attract and reward the pollinators (Fahn, 1979; Bernardello, 2007). Their secretions are typically referred to as nectar because they mainly contain sugars including the disaccharide sucrose and the monosaccharides fructose and glucose; others such as oligosaccharides and sugar alcohols may be present in minor amounts (Nicolson & Thornburg, 2007). However, other substances also occur, such as amino acids, proteins (also called nectarins), lipids, organic acids, phenolic compounds, alkaloids and terpenoids. In some cases, these substances, especially lipids, may even comprise a large proportion of the solutes from the exudate, which may then be referred to as a mixed secretion (Fahn, 1979; Baker & Baker, 1983a, b; Nicolson & Thornburg, 2007; Machado *et al.*, 2008; Machado, Souza & Guimarães, 2017; Monteiro & Demarco, 2017).

In most Anacardiaceae, flowers have a typical intrastaminal secretory nectary disk (Engler, 1892; Pell *et al.*, 2011) and, as in other members of Sapindales, they are also mainly insect-pollinated, especially

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by small and generalist bees fond of nectar (Jirón & Hedstrom, 1985; Matsuyama, Osawa & Sakimoto, 2009; Pell *et al.*, 2011; Fernandes, Venturieri & Jardim, 2012). However, a recent study revealed that in *Tapirira* Aubl., a dioecious genus of Anacardiaceae, the nectary disk of both male and female flowers not only produces a nectar rich in sucrose, but also synthesizes phenolic compounds and lipids (Tölke *et al.*, 2015). It was also shown that the composition of their mixed secretion is essentially the same in both male and female flowers (i.e. there were no significant differences), and the similar cellular ultrastructure of their nectariferous disk was, in fact, more specialized for the production of oil than for sugars.

Mixed secretions produced by floral nectaries have been demonstrated and well documented in many different lineages of angiosperms (Fahn, 1979; Subramanian, Arumugasamy & Inamdar, 1990; Fahn, 2000; Paiva & Machado, 2008; Possobom, Guimarães & Machado, 2010; Tölke *et al.*, 2015; Mercandante-Simões & Paiva, 2016; Machado *et al.*, 2017). However, this study in *Tapirira* was the first report for the family, and there are only a few scattered studies on the diversity of nectar composition and nectary ultrastructure in Anacardiaceae, and, in fact, in Sapindales in general (Wunnachit, Jenner & Sedgley, 1992; Caris *et al.*, 2006; Giuliani, Bini & Lippi, 2012; Paiva, 2012; Abedini *et al.*, 2013). Anacardiaceae comprise *c.* 82 genera and 800 species circumscribed in two subfamilies, Spondioideae and Anacardioideae (Pell *et al.*, 2011). They all produce a tremendous diversity of substances already well documented in vegetative organs, and of a great potential for the taxonomy and systematics of both subfamilies (Aguilar-Ortigoza & Sosa, 2004; Lacchia & Carmello-Guerreiro, 2009; Pell *et al.*, 2011).

Here we studied 13 species from seven additional genera of Anacardiaceae representing both subfamilies to evaluate the diversity of their nectary secretion, (ultra)structure and secretory pathways

Table 1. Species collected for this study with the respective location and voucher, and number of individuals and flowers tested per sample for each species in the analysis of the sugar composition

Species	Location	Vegetation	Voucher	Number of individuals	Number of flowers per individual
Spondioideae					
Spondias dulcis Parkinson	Brazil, Campinas-SP, UNICAMP	Cerrado	*UEC 119572*	3	10
Spondias macrocarpa Engl.	Brazil, Campinas-SP, UNICAMP	Cerrado	*UEC 119562*	3	10
Spondias mombin L.	Brazil, Matinhas-PB, Cachoeira do Gama	Caatinga	*UEC 119564*	3	50
Spondias purpurea L.	Brazil, Puxinanã-PB, Serra do Maracajá	Caatinga	*UEC 119569*	3	20
Spondias tuberosa L.	Brazil, Puxinanã-PB, Serra do Maracajá	Caatinga	*UEC 119567*	3	20
Anacardioideae					
Astronium graveolens Jacq.	Brazil, Campinas-SP, Mata Ribeirão Cachoeira	Cerrado	*UEC 007856*	6	10
Anacardium humile A.St.-Hil.	Brazil, Mogi Guaçu-SP, Fazenda Campininha	Cerrado	*UEC 119573*	3	6
Anacardium occidentale L.	Brazil, Puxinanã-PB, Serra do Maracajá	Caatinga	*UEC 119566*	3	10
Lithraea molleoides (Vell.) Engl.	Brazil, Rubião Júnior-SP	Cerrado	*UEC 066719*	3	10
Mangifera indica L.	Brazil, Puxinanã-PB, Serra do Maracajá	Caatinga	*UEC 119571*	3	20
Schinus molle L.	Brazil, Campinas-SP, UNICAMP	Cerrado	*UEC 119570*	6	50
Schinus terebinthifolia Raddi	Brazil, Campina Grande-PB, UEPB	Caatinga	*UEC 119572*	6	50
Schinopsis brasiliensis Engl.	Brazil, Gurjão-PB, PB 176	Caatinga	*UEC 119568*	3	50

Acronyms for Brazilian states: PB – Paraíba, SP – São Paulo.

and the potential of these traits for the systematics of the family, whether they are inherited from a common ancestor or are constrained by other biological evolutionary forces.

MATERIAL AND METHODS

Plant material

Six genera and eight species of Anacardioideae and one genus and five species of Spondioideae were studied in areas of the caatinga and cerrado (Brazilian savanna) from January to December 2014, from July to December 2015 and from January to July 2016 (Table 1). Since many Anacardiaceae can have functionally unisexual flowers, we also compared male and female flowers in both species of the dioecious genus *Schinus* L. to confirm that, as in *Tapirira*, there is no significant difference between sexual morphotypes, whereas in all other taxa we used bisexual, male or female flowers (Table 2). Vouchers are deposited in the UEC herbarium (Universidade Estadual de Campinas, Brazil).

Analysis of the sugars produced by the floral nectary

For each species, the composition of sugars was analysed for three to six individuals per species, and the number of sampled flowers per individual ranged from six to 50 (Table 1). Secretions produced by the floral nectary were collected in the field between 5

Table 2. Sugar composition of the nectar for 13 species of Anacardiaceae

Species	Floral morphotype	Sugars (%)		
		Sucrose	Fructose	Glucose
Anacardium humile^{H}	♀♂	12.75 ± 1.8	43.29 ± 0.9	**43.94 ± 0.8**
Anacardium occidentale^{S}	♀♂	**61.95 ± 7.6**	18.10 ± 4.1	19.93 ± 3.5
Astronium graveolens^{H}	♂	6.92 ± 1.4	27.12 ± 1.6	**65.93 ± 2.3**
Lithraea molleoides^{H}	♀	9.90 ± 3.1	27.12 ± 3.5	**62.96 ± 5.4**
Mangifera indica^{H}	♀♂	**38.49 ± 0.1**	30.75 ± 0.2	30.74 ± 0.3
Schinopsis brasiliensis^{H}	♂	27.42 ± 1.3	**37.70 ± 0.4**	34.85 ± 0.8
Schinus molle^{S}	♂	**56.05 ± 4.6**	21.36 ± 1.7	22.56 ± 3.0
	♀	**63.79 ± 3.2**	18,07 ± 1.8	18,32 ± 1.6
Schinus terebinthifolia^{H}	♂	31.36 ± 7.3	**34.98 ± 2.8**	33.64 ± 4.5
	♀	**38.6 ± 6.0**	30.33 ± 3.0	31.05 ± 2.9
Spondias dulcis^{H}	⚥	36.05 ± 1.0	**37.25 ± 1.4**	26.68 ± 0.4
Spondias macrocarpa^{H}	⚥	32.75 ± 7.0	32.51 ± 3.6	**34.72 ± 3.3**
Spondias mombin^{S}	⚥	**54.04 ± 6.4**	22.04 ± 3.4	23.90 ± 3.0
Spondias purpurea^{H}	♀	**38.66 ± 8.0**	29.67 ± 4.2	31.65 ± 3.7
Spondias tuberosa^{H}	⚥	31.26 ± 2.8	**34.44 ± 2.0**	34.28 ± 1.2

Sugar percentages (%) represent the relative abundance of individual sugars (sucrose, fructose and glucose). (H) and (S) indicate whether the nectar is richer in sucrose or in hexoses and bold type highlights the dominant individual sugar. The floral morphotypes are indicated by the symbols ♀ (functionally female), ♂ (functionally male) and ⚥ (bisexual). The tabulated data are expressed as measured means ± standard deviation.

Table 3. Histochemical tests used in the characterization of the substances

Test	Substance detected	Positive chromatic reaction	References
Sudan black B	lipids	dark blue to black	Pearse, 1985
Sudan red IV	lipids	red	Pearse, 1985
Lugol's iodine	starch grains	purple to black	Berlyn & Miksche, 1976
Ferric chloride	phenolic compounds	brown to black	Johansen, 1940
Wagner's reagent	alkaloids	red	Furr & Mahlberg, 1981
Schiff's reagent (PAS)	carbohydrates	magenta	McManus, 1948
Alcian blue	acidic mucilages	blue	Pearse, 1985
Ruthenium red	acidic mucilages	magenta to red	Gregory & Bass, 1989
Tannic acid and ferric chloride	mucilages	gray to black	Pizzolato, 1977

and 8 a.m. from open flowers with glass capillary tubes and stored at low temperature on Whatman® Number 1 paper filter (Sigma-Aldrich Co., LLC, China) for the biochemical analysis following the protocol of Galetto & Bernardello (2005). The stored nectar was dissolved in distilled water before sugar composition analysis by spectrophotometry. For the quantitative analysis reagents kits for glucose, fructose and sucrose (Sigma-Aldrich Co., St. Louis, MI, USA) were used following the methodologies proposed by Bergmeyer & Bernt (1974) and Southgate (1976). The absorbance reading was determined at a wavelength of 340 nm in a spectrophotometer (Metrolab 330, Switzerland). The proportion of sugars was expressed as means ± standard deviation. For comparison of the arithmetic means between male and female individuals belonging to the same species of *Schinus*, analyses were performed in R (R Core Team, 2016) to explore the variance in accordance with the *t*-test

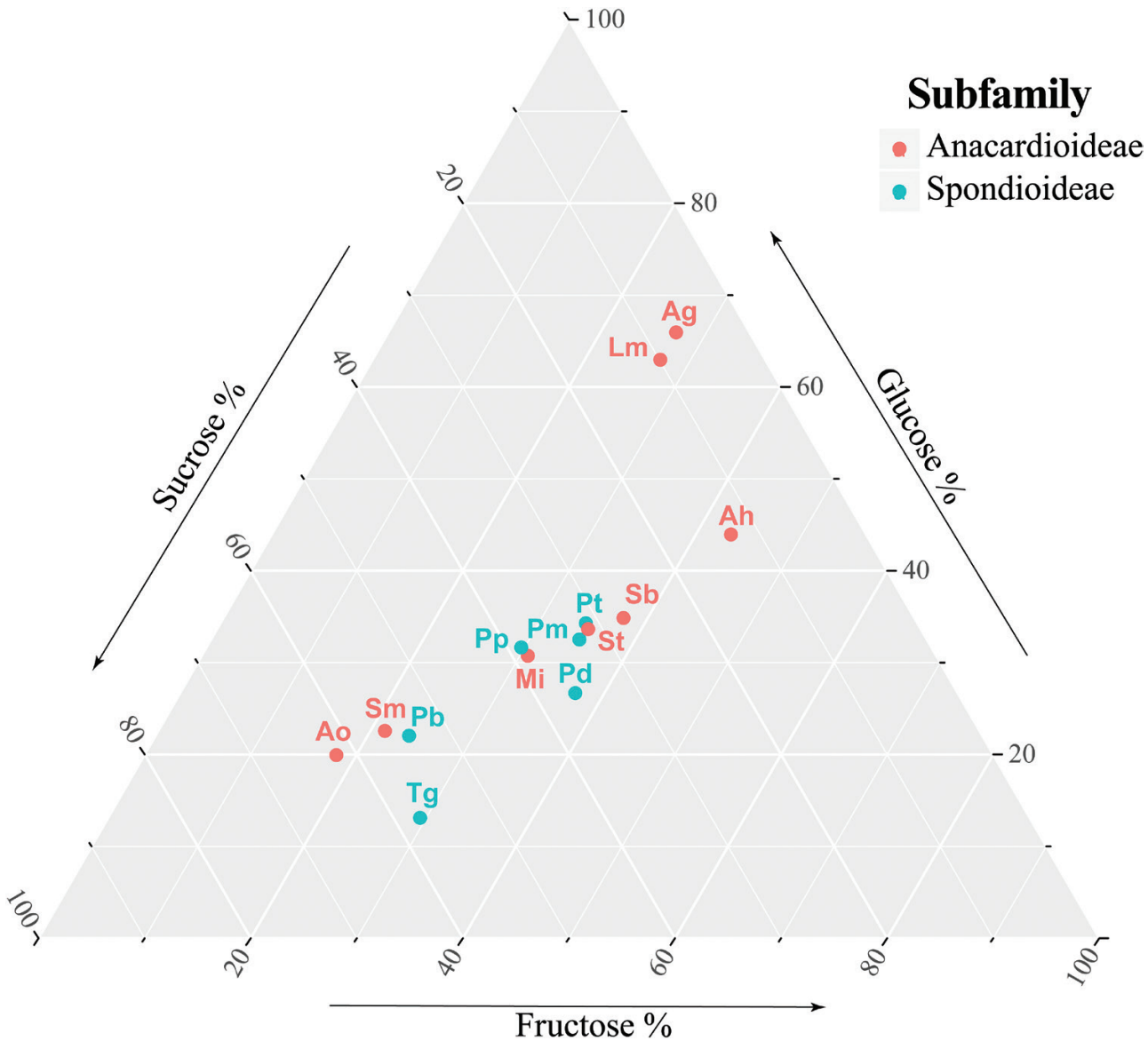

Figure 1. Ternary diagram of sugar composition for the nectar of Anacardiaceae. Abbreviations represent all 13 species studied here (Ah, *Anacardium humile*; Ao, *Anacardium occidentale*; Ag, *Astronium graveolens*; Lm, *Lithraea molleoides*; Mi, *Mangifera indica*; Pd, *Spondias dulcis*; Pm, *Spondias macrocarpa*; Pb, *Spondias mombin*; Pp, *Spondias purpurea*; Pt, *Spondias tuberosa*; Sb, *Schinopsis brasiliensis*; Sm, *Schinus molle*; St, *Schinus terebinthifolia*), plus one from Tölke *et al.* (2015) (Tg, *Tapirira guianensis* Aubl.).

at a significance level of 5% ($P \leq 0.05$). We also constructed ternary diagrams with the package *ggtern* version 2.1.5 (Hamilton, 2016) implemented in R, to represent the possible relationships among the three main sugars: sucrose and the hexoses glucose and fructose.

Histochemical and anatomical analysis

For the histochemical tests, the material was fixed in FAA (formaldehyde, acetic acid, 50% ethanol; 1:1:18 v/v) for 24 h to preserve hydrophilic substances (Johansen, 1940), and in BNF (phosphate buffer solution, formalin; 9:1 v/v) for 48 h to preserve lipophilic and phenolic substances (Lillie, 1965). The material was then dehydrated in a butyl alcohol dilution series and embedded in Paraplast Plus® (Leica Biosystems, Wetzlar, Germany) (Johansen, 1940). Transverse and longitudinal sections 7–9 µm thick were obtained using a Microm HM 340E rotary microtome (ThermoFisher Scientific Inc., Waltham, MA, USA). All sections were tested for carbohydrates, mucilage, phenolic compounds, lipids and alkaloids according to Demarco (2017; Table 3). We also followed Gerlach (1984) for anatomical studies and stained sections with Astra blue (C.I. 48048, Merck KGaA, Darmstadt, Germany) and safranin (C.I. 50240, Merck KGaA, Darmstadt, Germany), and Smith & McCully (1978) to identify vascular tissue in the nectaries with polarized light and aniline blue 0.05%, pH 8 (C.I. 42755, Vetec, Rio de Janeiro, Brazil). All slides were permanently mounted in Entellan® synthetic resin (Merck KGaA, Darmstadt, Germany) and the images were captured with an Olympus DP71 digital camera coupled to an Olympus BX51 microscope (Olympus Optical Co., Ltd, Japan).

Table 4. Histochemical tests on the secretory disk in different phases of development for 13 species of Anacardiaceae

Species/Test	Carbohydrates										Phenolic compounds		Alkaloids		Lipids			
	Lugol		PAS		RutRe		AlcBl		TanAc		FerCh		WagRe		SudBl		SudRe	
	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2	Ph1	Ph2
Anacardium humile	-	+	+	++	-	-	-	-	-	-	++	++	-	-	+	+	++	++
Anacardium occidentale	-	++	++	+	-	-	-	-	-	-	+	+	-	-	+	+	+	+
Astronium graveolens	-	+	+	++	-	-	-	-	-	-	++	++	-	-	+	+	+	+
Lithraea molleoides	-	+	+	++	++	++	-	-	++	++	++	++	-	-	++	++	+	+
Mangifera indica	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Schinopsis brasiliensis	++	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	++	++
Schinus molle	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Schinus terebinthifolia	-	++	++	++	-	-	-	-	-	-	++	++	-	-	-	-	-	-
Spondias dulcis	-	++	++	+	++	++	-	-	++	++	+	+	-	-	++	++	-	-
Spondias macrocarpa	++	+	+	+			-	-	-	-	+	+	-	-	-	-	-	-
Spondias mombin	++	+	+	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-
Spondias purpurea	-	+	+	+	++	++	-	-	-	-	+	+	-	-	+	+	-	-
Spondias tuberosa	-	++	++	++	++	++	-	-	-	-	++	++	-	-	++	++	+	+

Ph1, phase 1 (late floral bud); Ph2, phase 2 (flower in anthesis); Lugol, Lugol's iodine; FerCh, ferric chloride; PAS, Schiff's reagent; AlcBl, Alcian blue; TanAc, tannic acid and ferric chloride; RutRed, Ruthenium red; WagRe, Wagner's reagent; SudBl, Sudan black; SudRe, Sudan red IV; (–) negative reaction; (+) positive reaction, (++) strong positive reaction.

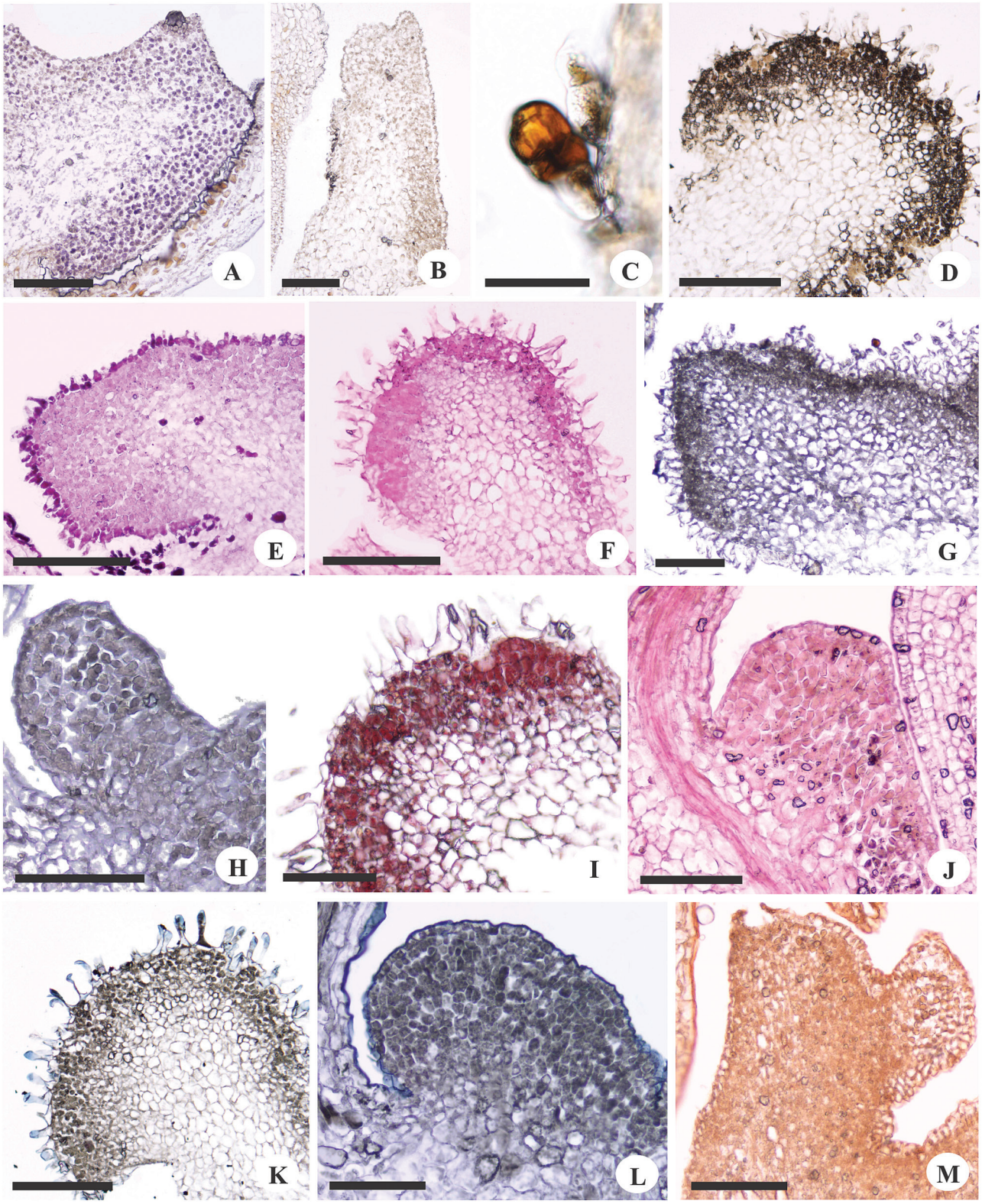

Figure 2. Histochemical tests on longitudinal sections of floral nectaries. (A–B) Reaction in *Spondias macrocarpa* to Lugol's iodine showing starch grains before anthesis (A) and their absence at anthesis (B). (C–D) Positive reaction showing phenolic compounds in *Anacardium humile* (C) and *Spondias tuberosa* (D). (E–F) Positive reaction to Schiff's reagent showing

Scanning electron microscopy

Flowers fixed in FAA were dehydrated in an increasing ethanol dilution series up to 100%, critical point dried with CO_2 and sputter coated with gold (exposure time 200 s on a Balzers SCD-050 sputter coater, ONLINK Technologies GmbH, Germany). All samples were studied and imaged with a JEOL JSM 5800 LV scanning electron microscope (SEM) (GenTech Scientific Inc., New York, USA).

Transmission electron microscopy

For the study with transmission electron microscopy, *Spondias dulcis* Parkinson in Spondioideae and *Lithraea molleoides* (Vell.) Engl., *Schinus molle* L. and *Anacardium humile* A.St.-Hil. in Anacardioideae were selected for comparison based on the preliminary results of histochemical tests and histological analyses. Samples of flowers in pre-anthesis and anthesis were fixed in 2.5% glutaraldehyde (Sigma-Aldrich Co., St. Louis, MI, USA) in 0.1 M phosphate buffer, pH 7.3 for 24 h at 5 °C. They were post-fixed in 1% osmium tetroxide (Sigma-Aldrich Co., St. Louis, MI, USA) in the same buffer for 1 h at 25 °C, dehydrated in an acetone dilution series and embedded in Araldite resin (Machado & Rodrigues, 2004). Ultrathin sections were obtained with a Diatome (Hatfield, USA) diamond knife and stained with 1% methylene blue (M9140, Sigma-Aldrich Co., St. Louis, MI, USA) and contrasted with uranyl acetate (EMS, Germany) (Watson, 1958) and lead citrate (C6522, Sigma-Aldrich Co., St. Louis, MI, USA) (Reynolds, 1963). The material was observed and imaged with a Tecnai G^2 Spirit Bio TWIN TEM (FEI Company, Hillsboro, USA) at 60 kV.

RESULTS

Composition of sugars and histochemical survey

The nectar of all species studied here contains the three types of sugars we tested for (Table 2, Fig. 1). In addition, results showed that nectar sugar composition (1) does not significantly differ between the different floral morphotypes of either *Schinus* spp. ($P > 0.05$); (2) it may vary strongly between closely related species (e.g. *Spondias* L., *Anacardium* L. or *Schinus*) and/or (3) be more similar between species of the two subfamilies (e.g. *Spondias tuberosa* L. and *Schinus terebinthifolia* Raddi, or *Spondias purpurea* L. and *Mangifera indica* L.) (Table 2, Fig. 1). For instance, three out of 13 species have high sucrose nectars (with sucrose > 50%), and ten have high hexose nectars (glucose + fructose > 50%) (Table 2). However, the high sucrose nectars are found in both Spondioideae, in one out of five species of *Spondias*, and in Anacardioideae, in one out of two species of *Schinus* and of *Anacardium*.

Table 5. Nectary characteristics of flowers in anthesis for 13 species of Anacardiaceae

Species	Position	Number of lobes	Epidermis	Stomata	Druses	Vascularization
Anacardium humile	trichomatous	-	-	-	-	-
Anacardium occidentale	trichomatous	-	-	-	-	-
Astronium graveolens	intrastaminal	5	smooth	present	absent	absent
Lithraea molleoides	intrastaminal	10	smooth	present	present	absent
Mangifera indica	extrastaminal	5	irregular	present	present	absent
Schinopsis brasiliensis	intrastaminal	5	smooth	present	present	absent
Schinus molle	intrastaminal	10	smooth	present	absent	phloem
Schinus terebinthifolia	intrastaminal	10	smooth	present	absent	phloem
Spondias dulcis	intrastaminal	10	papillose	present	present	absent
Spondias macrocarpa	intrastaminal	10	smooth	present	present	absent
Spondias mombin	intrastaminal	10	smooth	present	present	absent
Spondias purpurea	intrastaminal	10	papillose	present	present	absent
Spondias tuberosa	intrastaminal	10	papillose	present	absent	absent

insoluble carbohydrates in *Spondias dulcis* (E) and *Spondias tuberosa* (F). (G–H) Positive reaction to tannic acid and ferric chloride test showing the presence of mucilage in *Spondias dulcis* (G) and *Lithraea molleoides* (H). (I–J) Positive reaction to Ruthenium red showing soluble carbohydrates and mucilage in *Spondias tuberosa* (I) and *Lithraea molleoides* (J). (K–L) Positive reaction to Sudan black showing lipids in *Spondias tuberosa* (K) and *Lithraea molleoides* (L). (M) Positive reaction to Sudan red IV showing lipids in *Schinopsis brasiliensis*. Scale bars = 20 μm (C), 50 μm (H–J, L–M), 100 μm (A–B, D–G, K).

In contrast, high hexose nectars are found in all other species of these genera but can still be richer in sucrose than in individual hexoses, like *S. purpurea* and in female flowers of *Schinus terebinthifolia*, or in fructose as in *Spondias dulcis* and *S. purpurea* or male flowers of *Schinus terebinthifolia*, or in glucose as in *Spondias macrocarpa* Engl. and *Anacardium humile* (Fig. 1).

Histochemical tests (Table 4, Fig. 2) reveal that starch grains are present in the parenchyma of the floral nectary of *Spondias macrocarpa* and *S. mombin* L. in Spondioideae, and of *Schinopsis brasiliensis* Engl. in Anacardioideae, even if in *Spondias* they seem to be consumed before anthesis (Fig. 2B). They also reveal that the floral nectary of most studied species typically produces other substances, such as lipids and/or phenolic compounds that, as with sugars, may vary between genera of the same subfamily and be more similar among genera of each subfamily (Table 4, Fig. 2).

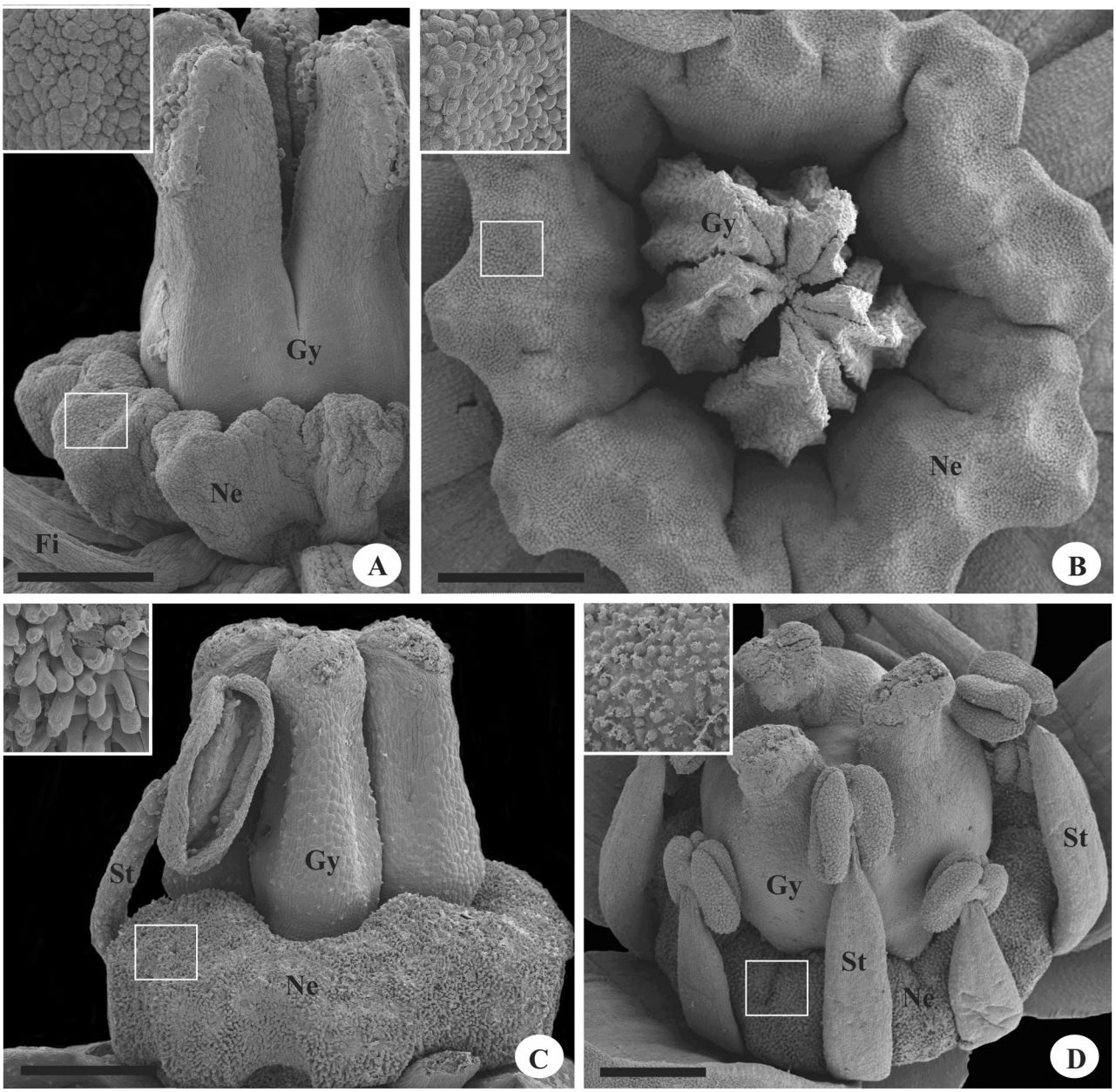

Figure 3. Nectary location and surface in Spondioideae. (A) *Spondias mombin*, smooth epidermis. (B) *Spondias macrocarpa*, smooth epidermis. (C) *Spondias dulcis*, papillose epidermis. (D) *Spondias purpurea*, papillose epidermis. Abbreviations: Fi, filament; Gy, gynoecium; Ne, nectary; St, stamen. Scale bars = 400 μm (D), 500 μm (A–C).

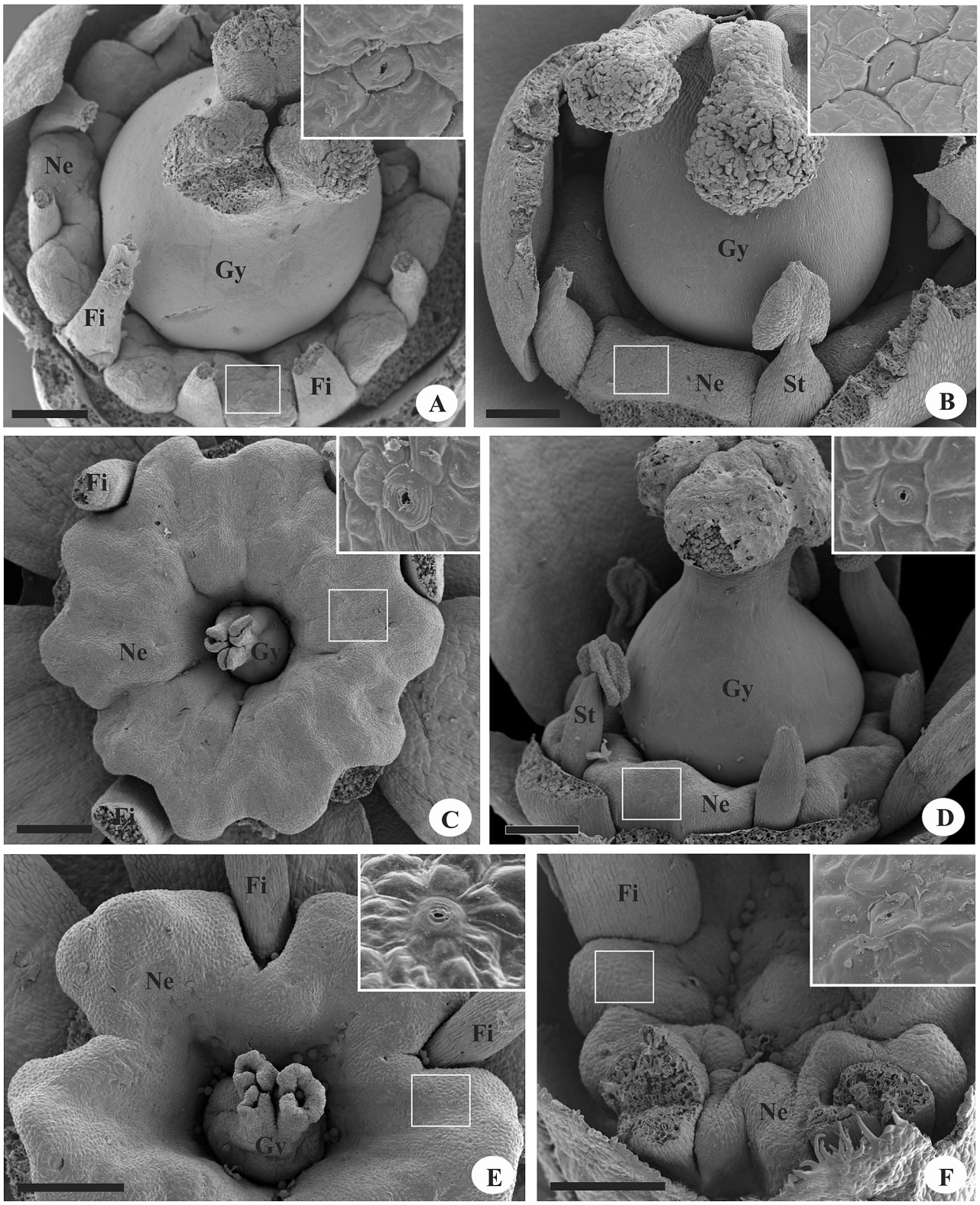

Figure 4. Nectary location and surface in Anacardioideae. Insets show detail of nectarostomata. (A) *Lithraea molleoides*. (B) *Astronium graveolens*. (C–D) *Schinus terebinthifolia*, male (C) and female (D) flowers. (E) *Schinus molle*. (F) *Schinopsis brasiliensis*. Abbreviations: Fi, filament; Gy, gynoecium; Ne, nectary; St, stamen. Scale bars = 200 μm (A–F).

For instance, both lipids and phenols are typically found in nectary cells of *Spondias dulcis*, *S. purpurea* and *S. tuberosa* in Spondioideae, and in both *Anacardium* spp., *Astronium graveolens* Jacq., *Lithraea molleoides* and *Schinopsis brasiliensis* in Anacardioideae. Similarly, only lipids are detected in *Mangifera indica* (Anacardioideae) and only phenols in *Spondias macrocarpa* (Spondioideae) and *Schinus terebinthifolia* (Anacardioideae). However, these tests do not allow quantitative measurements, and in *Schinus molle* (Anacardioideae) the presence of lipids was only revealed with TEM (Table 4).

Nectary structure

The main differences in the nectary structure and anatomy among the species studied here are summarized in Table 5. Our results confirm that most species have a fleshy intrastaminal nectariferous disk (Figs 3 and 4); only in *Mangifera* L. and *Anacardium* it is extrastaminal or absent, respectively (Fig. 5A–C). In all other genera, the nectariferous disk has lobes that alternate with the bases of the filaments, and thus vary in number from ten in diplostemonous taxa like *Spondias* in Spondioideae or *Lithraea molleoides* and *Schinus* in Anacardioideae, to five in haplostemonous taxa such as *Astronium graveolens* and *Schinopsis brasiliensis* in Anacardioideae (Figs 3 and 4).

The surface of the disk is typically covered with nectarostomata and is often smooth, but can be more or less papillose, sometimes in the same genus (e.g. *Spondias*), or irregular with multicellular protrusions as in *Mangifera* (Figs 3–5). However, the anatomy of the nectary disk is similar among the species and genera, including *Mangifera*, and comprises a uniseriate epidermis and a nectariferous and subnectariferous parenchyma (Fig. 6). Only the parenchyma is secretory and both the nectariferous and subnectariferous tissues may contain druses (Table 5; Fig. 6). Most nectaries are not vascularized, but in *Schinus* phloem bundles were detected in the subnectariferous parenchyma of both species (Fig. 6H). In *Anacardium*, in which the disk is absent, the nectar is produced by extrastaminal glandular trichomes that are located at the base of the adaxial surface of petals and of the abaxial surface of the staminal tube, and composed of a uniseriate stalk formed by one or two cells and a biseriate or multiseriate secretory head formed by three or four rows (Fig. 5B–C).

Nectary ultrastructure

Based on previous results, three morphological types of nectary have been distinguished and studied in more detail as potential models for the family: (1) a nectariferous disk with papillose epidermis-type in *Spondias dulcis*; (2) a nectariferous disk with smooth epidermis-type in *Lithraea molleoides* and *Schinus molle* and (3) a trichomatous type in *Anacardium humile*. The second one was studied in two species because of the distinct composition of their products revealed by the histochemical tests.

Nectariferous disk with papillose epidermis-type

Small differences were observed between pre-anthesis (Fig. 7A–H) and anthesis (Fig. 7I–N). Before anthesis, the epidermal cells are covered by a thin cuticle and

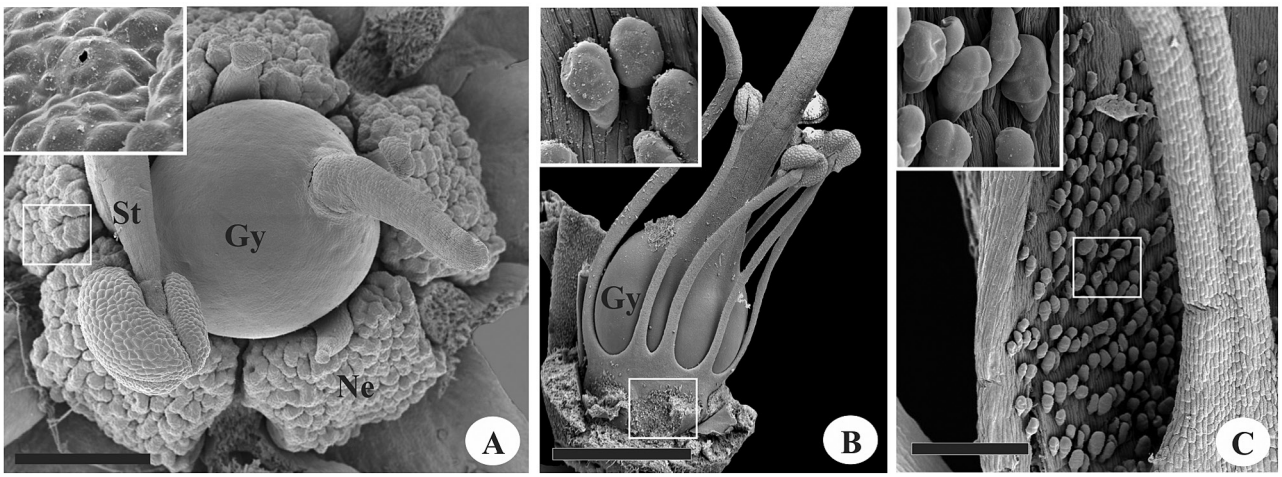

Figure 5. Nectary location and surface in Anacardioideae. (A) *Mangifera indica,* note the irregular surface and the disk in extrastaminal position. (B) *Anacardium occidentale*, glandular trichomes on the abaxial side of staminal tube base. (C) *Anacardium humile*, glandular trichomes on the adaxial surface of petals. Abbreviations: Gy, gynoecium; St, stamen. Scale bars = 200 μm (C), 500 μm (A), 900 μm (C).

contain large vacuoles with osmiophilic content (Fig. 7A–C). The organelles are concentrated at the periphery of the cell (Fig. 7I) and no periplasmic space was observed (Fig. 7B). The nectariferous (Fig. 7D–E) and subnectariferous (Fig. 7F–H) parenchyma are quite similar and contain large vacuoles, polyribosomes,

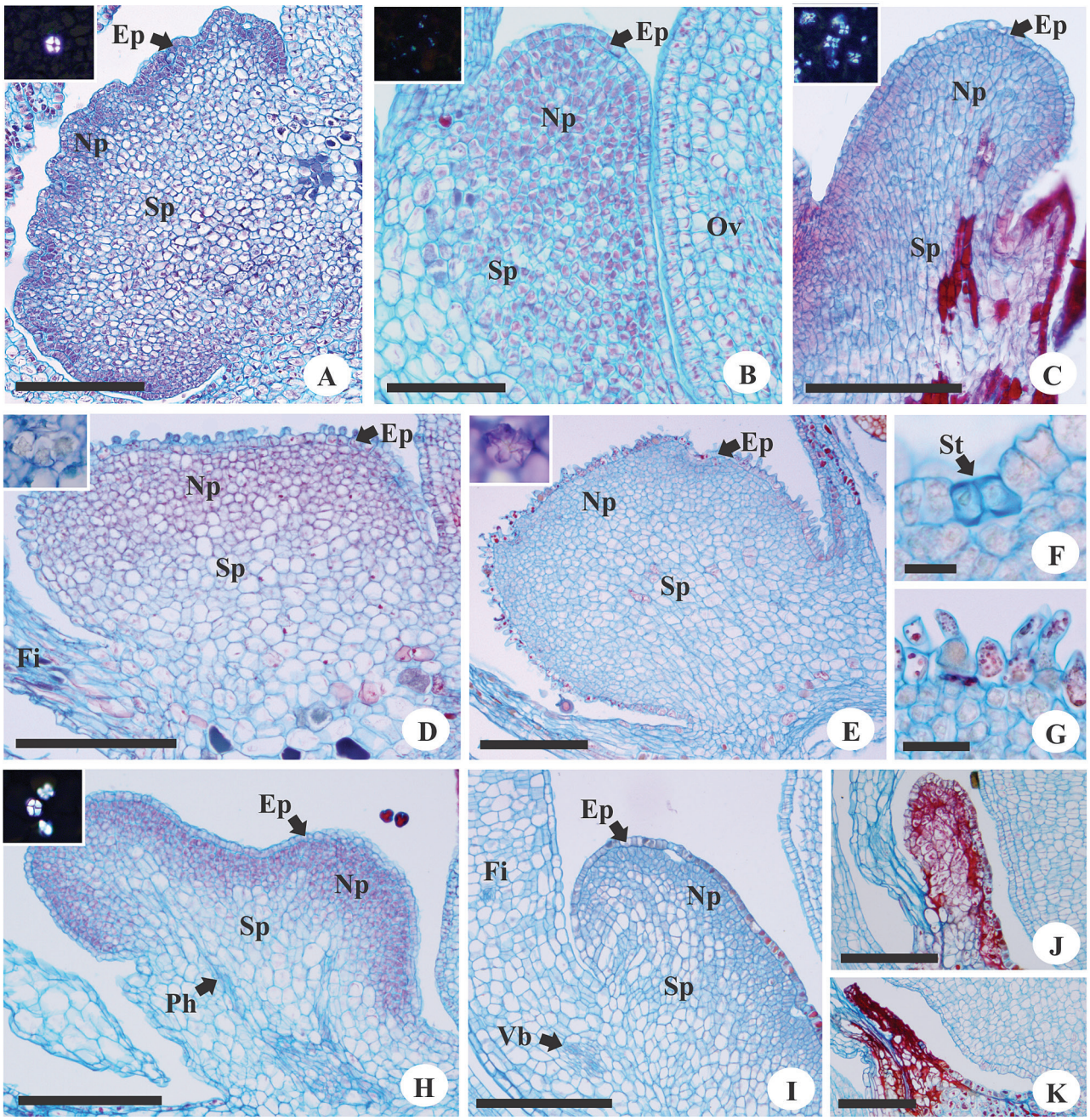

Figure 6. Cross sections of the nectariferous disk in different species of Anacardiaceae. Insets show druse under polarized light. (A) *Mangifera indica*. (B) *Lithraea molleoides*. (C) *Schinopsis brasiliensis*. (D) *Spondias purpurea* and (E) *Spondias dulcis*. Papillose epidermal cells and druses (upper left corner). (F) Nectarostoma in *Spondias dulcis*. (G) Papillose epidermal cells in *Spondias dulcis*. (H) *Schinus terebinthifolia*, vascularization (phloem) and druses (upper left corner). (I–K) *Astronium graveolens*. (I) At anthesis, (J) at the beginning of the degeneration of the nectariferous disk (very young fruit), and in (K) it is completely degenerated and nonfunctional (fruit in an intermediate stage). Abbreviations: Ep, epidermis; Fi, filament; Np, nectariferous parenchyma; Ov, ovary; Ph, phloem; Sp, subnectariferous parenchyma; St, stomata; Vb, vascular bundle. Scale bars = 10 μm (F–G), 50 μm (B), 100 μm (A, C–D, H–K), 200 μm (E).

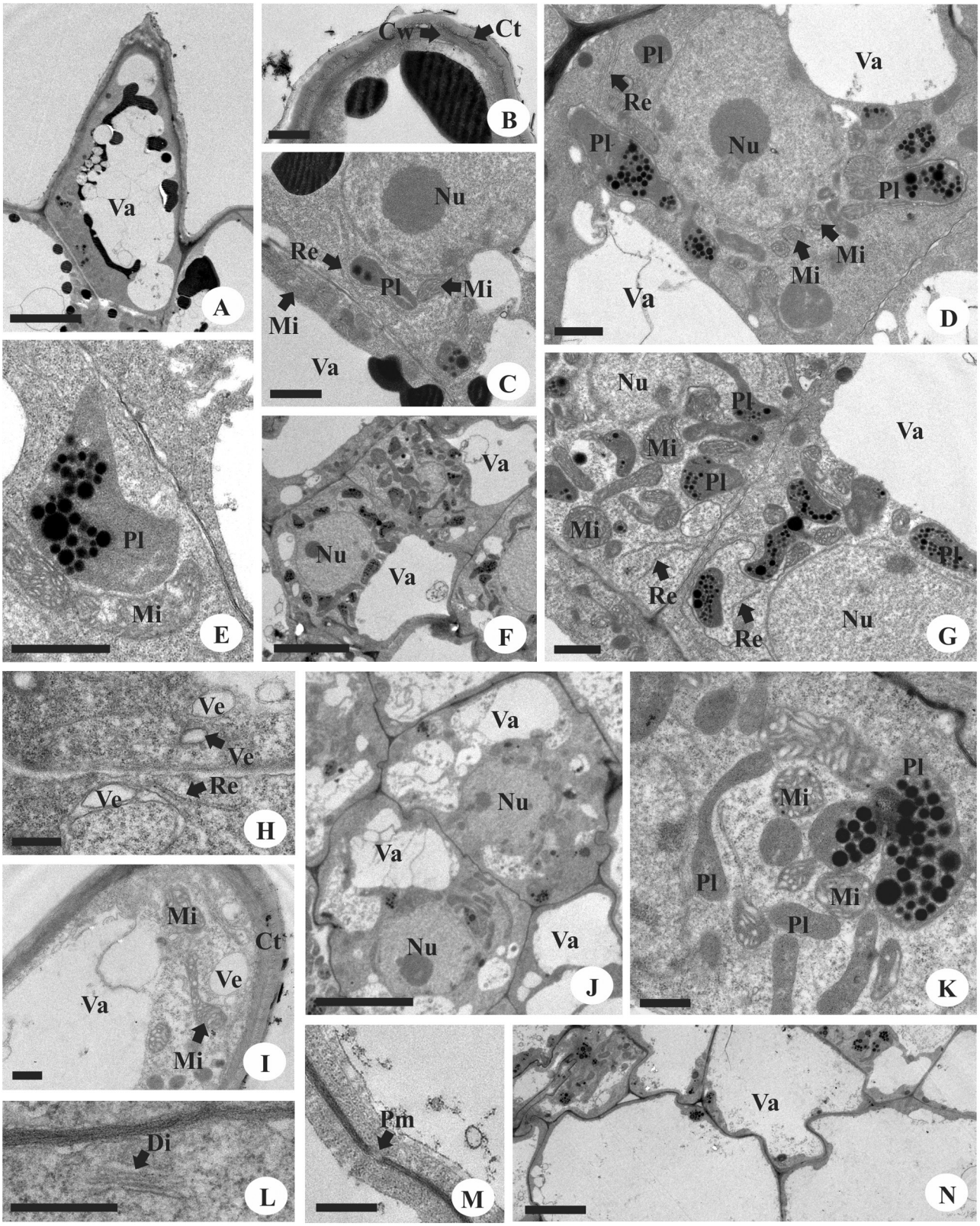

Figure 7. TEM of the nectariferous disk of *Spondias dulcis* (A–H) at pre-anthesis and (I–N) at anthesis. (A) General view of the papillose epidermis. Osmiophilic inclusions inside the vacuoles. (B) Epidermis. (C) Part of cytoplasm of an epidermis

abundant mitochondria, rough endoplasmic reticulum and plastids containing osmiophilic droplets. The vacuoles present membranous material (Fig. 7D, F), and it is possible to observe small vesicles near the plasma membrane, as well as rough endoplasmic reticulum (Fig. 7H). At anthesis, the number of vacuoles increases in the nectariferous parenchyma and small vesicles are now visible close to the membrane of the epidermal cells, and some dictyosomes are observed in the nectariferous parenchyma (Fig. 7I, J, L).

Nectariferous disk with smooth epidermis type

The ultrastructure of the nectary of *Lithraea molleoides* and *Schinus molle* presents a few variations from the previous type and between each other (Figs 8 and 9). In *L. molleoides*, the vesicles containing flocculent or membranous material are close to the plasma membrane in both bud and open-flower stage (Fig. 8A–B, H, L). In pre-anthesis, the dictyosomes are also not well developed (Fig. 8B), but later in opened flowers they are abundant and associated with small vesicles (Fig. 8J). In addition, plastids with plastoglobules (Fig. 8B) and oil droplets close to the membrane are common (Fig. 8E, L–M). In contrast, in *Schinus molle* the dictyosomes start to develop only in anthetic flowers (Fig. 9K–L) and conspicuous oil bodies occur scattered in the cytoplasm and near the membrane (Fig. 9D–E, I, K).

Trichomatous type

At pre-anthesis the cells of the nectariferous trichomes show a uniform aspect with the nucleus in central cell position and vacuoles occupying a large cellular volume (Fig. 10A). Accumulation of osmiophilic material inside the vacuoles is common (Fig. 10A–C). Polyribosomes, globular mitochondria, rough endoplasmic reticulum and plastids containing plastoglobules characterize the cytoplasm of the cells, but dictyosomes are absent (Fig. 10B–C). The cells are connected to each other via many plasmodesmata (Fig. 10D) and commonly surrounded by a periplasmic space (Fig. 10D). The outer periclinal walls of the head cells are covered by a thin cuticle and a narrow periplasmic space with evident paramural bodies (Fig. 10B, E). Vesicles are common at the periphery of the head cells, which also contains scattered electron-lucent droplets (Fig. 10F–G). In addition, the cells show juxtaposed vesicles with myelin-like configuration (Fig. 10H).

At anthesis, the periplasmic space is larger than in the previous phase and numerous paramural bodies are present (Fig. 10I–M), the cuticle between the cells of the secretory head is much thicker (Fig. 10I). The cuticle covering the stalk has irregular ingrowths suggesting that they function as transfer cells (Fig. 10I). The stalk cells also possess a dense cytoplasm and the vacuoles are smaller than in head cells (Fig. 10I). The vacuoles of the head cells contain electron-dense material (Fig. 10I–J), finely flocculent material (Fig. 10I–J) and electron-lucent material (Fig. 10L). Plastids containing plastoglobules (Fig. 10K) are common in both head and stalk cells and plastids with protein bodies (Fig. 10L–M) are found only in the stalk cells. Dictyosomes are present in this phase, but only in the stalk cells (Fig. 10L–M). Cuticle disruption is not observed.

DISCUSSION

Diversity of nectary secretion

Our study confirms that the production of mixed secretions by floral nectaries in Anacardiaceae, first revealed in Spondioideae in *Tapirira* by Tölke *et al.* (2015), may be a common trait in the family. In addition, it confirms that, as in *Tapirira*, monosaccharide hexoses are often the dominant sugars in Anacardiaceae and that sugar composition is not significantly different between sexual morphotypes of the same species of either subfamily (*Tapirira*, Spondioideae, Tölke *et al.*, 2015; *Schinus*, Anacardioideae, this study). However, our study also shows that the composition of sugars and additional substances synthesized in their nectary can vary between closely related species, and they can be more similar in species from different subfamilies. Such variations seem to occur within a species, since a previous study in *Anacardium occidentale* found a high hexose nectar, whereas in our study we found a high sucrose nectar (Wunnachit *et al.*, 1992; this work).

cell showing plastids with plastoglobules, mitochondria and endoplasmic reticulum. (D) Nectariferous parenchyma cell. (E) Plastid with various plastoglobules. (F) General view of subnectariferous parenchyma cells. (G) Subnectariferous parenchyma cells. (H) Subnectariferous parenchyma cell showing small vesicles near to the plasma membrane. (I) Papillose cell of epidermis with vacuole in the central region of the cell and organelles in the peripheral region. (J) General view of nectariferous parenchyma cells. (K) Nectariferous parenchyma cell showing various mitochondria and plastids. (L) Dictyosome. (M) Plasmodesmata between cells of the nectariferous parenchyma. (N) General view of the subnectariferous parenchyma; note vacuole and organelles next to the plasma membrane. Abbreviations: Ct, cuticle; Cw, cell wall; Di, dictyosomes; Mi, mitochondria; Nu, nucleus; Pl, plastid; Pm, plasmodesmata; Re, endoplasmic reticulum; Va, vacuole; Ve, vesicle. Scale bars = 0.5 μm (B, H–I, K–M); 1 μm (C–E, G); 5 μm (A, F, J, N).

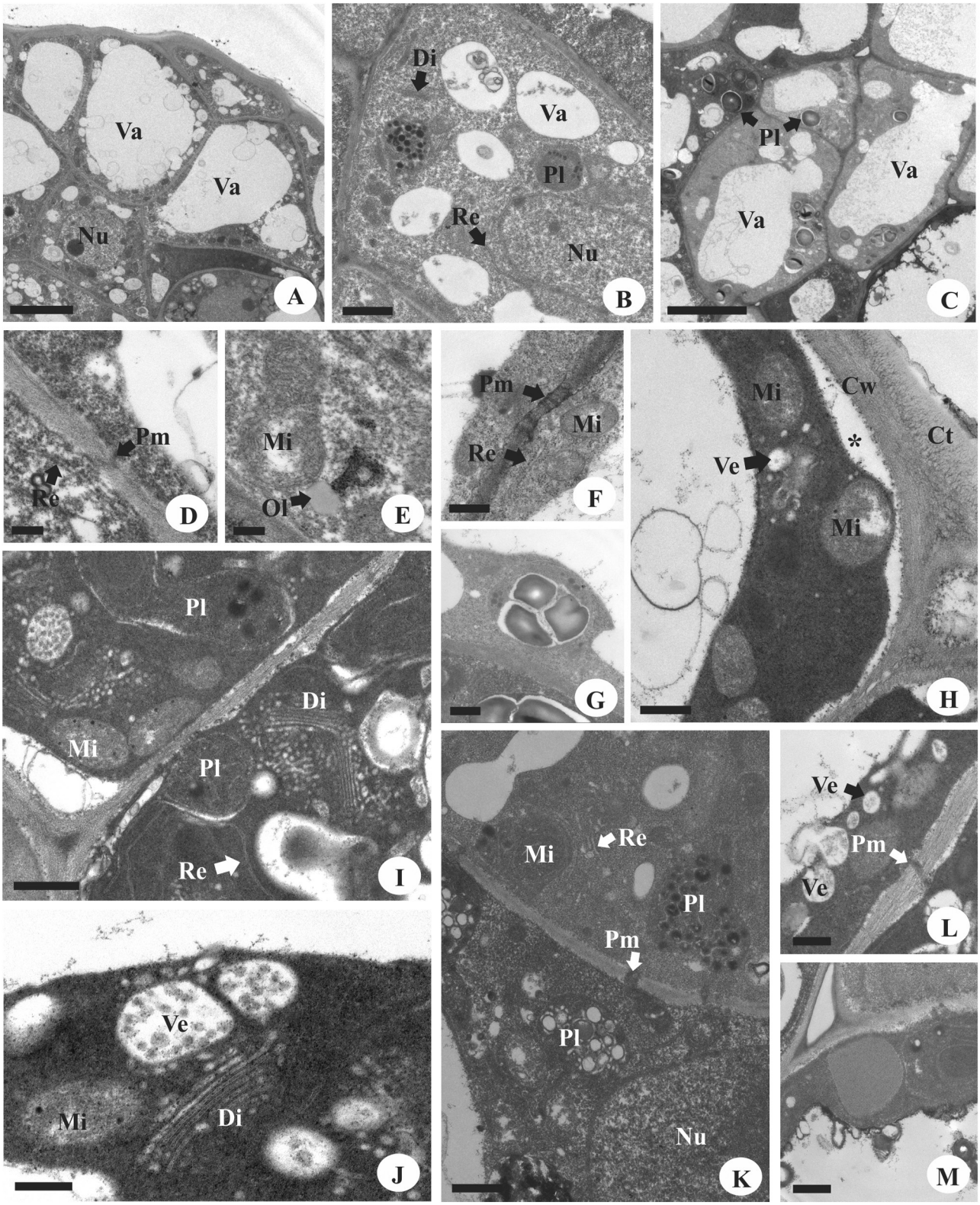

Figure 8. TEM of the nectariferous disk of *Lithraea molleoides* (A–G) at pre-anthesis and (H–M) at anthesis. (A) General view of the epidermis and nectariferous parenchyma. (B) Nectariferous parenchyma cell. (C) General view of subnectariferous parenchyma cells. (D) Plasmodesma and rough endoplasmic reticulum near the plasma membrane. (E) Oil droplet near

Sugar composition may vary even within a day in a flower due to different factors, including the presence of nectar yeasts (Herrera, García & Pérez, 2008).

Phenolic compounds have previously been reported in different secretory structures of vegetative and reproductive organs of various Anacardiaceae of both subfamilies, and these are mainly responsible for the toxic properties exhibited by these taxa (Carmello, Machado & Gregório, 1995; Carmello-Guerreiro & Paoli, 2002, 2005; Aguilar-Ortigoza & Sosa, 2003, 2004; Tölke *et al.*, 2015, 2017). They tend to be less common than lipids in the nectary tissues and, when present, they accumulate in all epidermal cells and random idioblasts scattered in the subnectary and nectary parenchyma, as first reported in *Tapirira* (Tölke *et al.*, 2015). However, their potential role in mixed secretions of Anacardiaceae has not yet been studied.

Together with the presence of lipids in histochemical tests, the presence of oil droplets and plastoglobules in nectary tissues of most species studied here confirmed that, as in *Tapirira*, lipids are synthesized and probably released in the secretions of their nectaries (Tölke *et al.*, 2015). Plastids containing osmiophilic structures and electron-dense droplets, as well as electron-lucent droplets dispersed in parenchyma cells and the proliferation of smooth endoplasmic reticulum, have been described in many lipid-producing structures (Fahn, 1979; Figueiredo & Pais, 1994; Monteiro *et al.*, 1999; Turner *et al.*, 1999; Machado, Gregório & Guimarães, 2006; Possobom, Guimarães & Machado, 2015).

Such a variation in composition of both sugar types and additional compounds had not been reported before in Anacardiaceae or any other families of Sapindales, and the impact on their pollinators is unknown. The presence of lipids in nectariferous tissues is not a novelty since they have been reported in Sapindaceae and other unrelated families of angiosperms, including Apocynaceae, Bignoniaceae and Passifloraceae (Durkee, Baird & Cohen, 1984; Machado *et al.*, 2008, 2017; Giuliani *et al.*, 2012; Abedini *et al.*, 2013; Gama, Aguiar-Dias & Demarco, 2016; Guimarães, Nogueira & Machado, 2016; Avalos *et al.*, 2017; Monteiro & Demarco, 2017). However, the production of mixed secretion in floral nectaries of all species of Anacardiaceae studied here suggests that their production and the synthesis of lipids may be ancestral and/or relatively conserved in the family. In contrast, the relative proportion of sugars and other compounds in floral nectaries do not follow any phylogenetic pattern of inheritance and cannot be used for the systematics of the family at any hierarchical level.

Nectary structure

The structure of the floral nectary disk and its intrastaminal position are essentially similar in all species studied here, and all other insect-pollinated Anacardiaceae (Engler, 1892; Wannan & Quinn, 1991; Bachelier & Endress, 2009; Pell *et al.*, 2011). In fact, it is typically lacking only in a few members of the family, and its extrastaminal position in *Mangifera* and absence in *Anacardium*, in which nectariferous trichomes are present, are thus unusual (this study; see also Bachelier & Endress, 2009). An extrastaminal disk is found elsewhere in the family only in *Swintonia* Griff., which with *Mangifera* forms a robust pair nested in Anacardioideae, in a small clade comprising *Anacardium, Fegimanra* Pierre ex Engl. and *Gluta* L., which all lack a nectariferous disk (Pell *et al.*, 2011).

The surface of the disk is typically covered with nectarostomata, which can vary in density and are smooth in most species studied here, as in most Anacardiaceae (this study; see also Bachelier & Endress, 2009). The presence of a more or less papillose surface in both subfamilies or even in species of the same genus is highlighted for the first time here, and the rugose surface found in *Mangifera* has to date not been described in any other genera. In addition, we confirm that in most species studied here, druses were found in the nectariferous parenchyma as in *Tapirira* (Tölke *et al.*, 2015), and may be common in all Anacardiaceae. In addition, like in *Tapirira* the disk is not vascularized in any species studied here, except for those of *Schinus* (this study; Bachelier & Endress, 2009; Tölke *et al.*, 2015).

The presence of an intrastaminal nectariferous disk is also common in most other families of Sapindales and is frequently mentioned as the plesiomorphic state to the order (Ronse De Craene & Haston, 2006; Solís *et al.*, 2017). However, it can also be in extrastaminal position as in Sapindaceae (see Bachelier & Endress, 2009,

mitochondria. (F) Subnectariferous parenchyma cell showing the plasmodesmata and rough endoplasmic reticulum near the plasma membrane. (G) Plastid containing plastoglobules and starch. (H) Epidermis cell showing small vesicles near to the membrane, some mitochondria and the discreet periplasmic space. (I) Nectariferous parenchyma cell, cytoplasm very dense. (J) Active dictyosomes. (K) Subnectariferous parenchyma cells with the amyloplasts and also plastids containing plastoglobules. (L) Subnectariferous cell showing small vesicles near to the membrane and the plasmodesma. (M) Electron-lucent inclusion. Abbreviations: Ct, cuticle; Cw, cell wall; Di, dictyosomes; Mi, mitochondria; Nu, nucleus; Ol, oil droplet; Pl, plastid; Pm, plasmodesmata; Re, endoplasmic reticulum; Va, vacuole; Ve, vesicle. Scale bars = 0.2 μm (C–D, H); 0.5 μm (F–G, J, M); 1 μm (B, I, K); 5 μm (A, E, L).

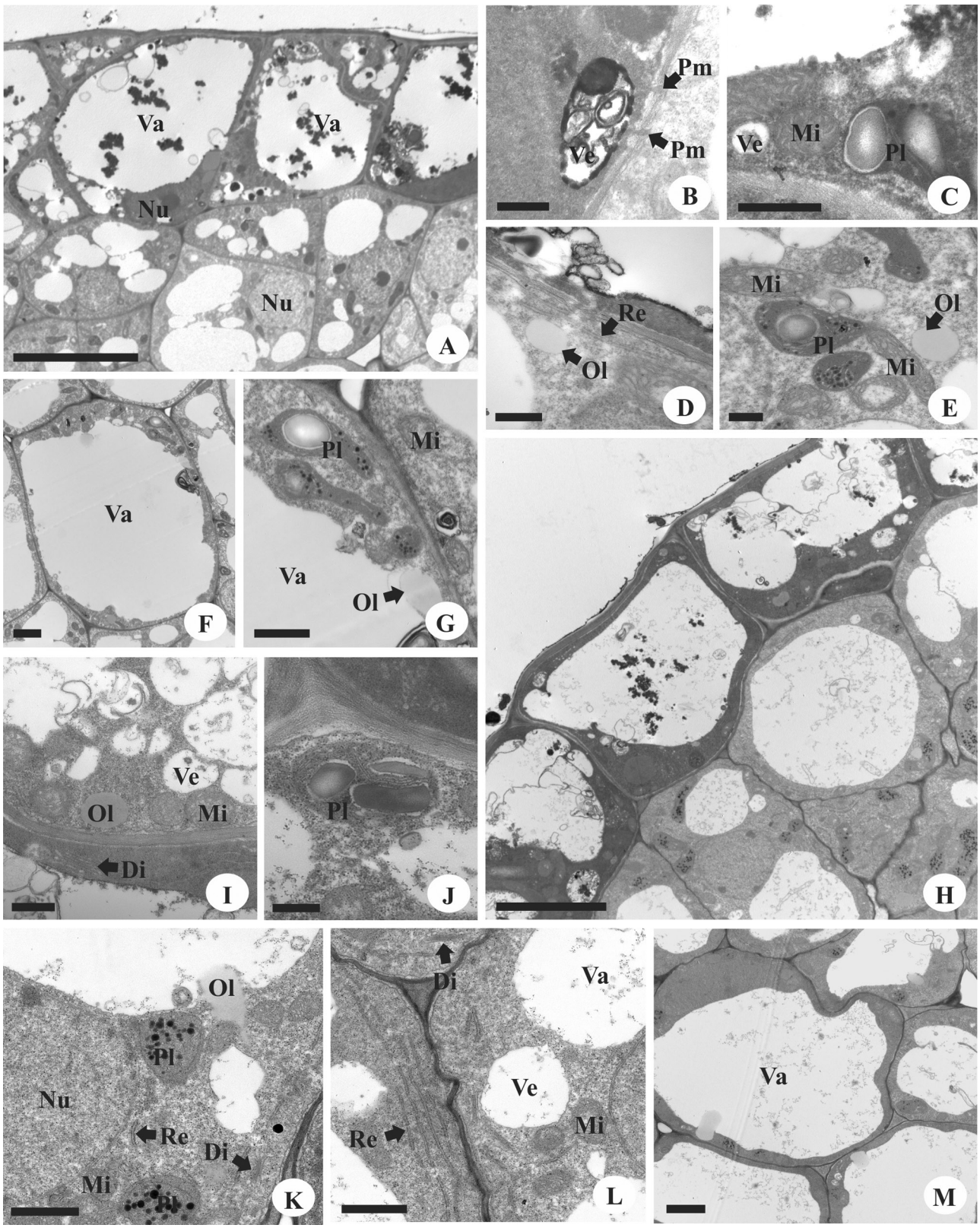

Figure 9. TEM of the nectariferous disk of *Schinus molle* (A–G) at pre-anthesis and (H–M) at anthesis. (A) General view of the epidermis and nectariferous parenchyma. (B) Vesicle containing multivesicular bodies and plasmodesmata. (C)

and references therein), or lacking as in some Meliaceae or Nitrariaceae (Pennington & Styles, 1975; Bachelier, Endress & Ronse De Craene, 2011). In Bierbersteiniaceae (sister to all other Sapindales) the nectariferous disk is absent, but five antisepalous nectary glands are found between the stamens (Muellner, 2011). However, their development, structure and function have not been studied in detail. Little is known about the presence of nectarostomata and the surface of the disk in other sapindalean families. However, when a nectary disk is present, the surface is also covered with nectarostomata and tends to be smooth, as in Burseraceae and Kirkiaceae that with Anacardiaceae form a robust clade in Sapindales (Bachelier & Endress, 2008, 2009; Giuliani *et al.*, 2012). The presence of druses, found in most species studied here, has only been reported to date in members of Sapindaceae and Nitrariaceae (Abedini *et al.*, 2013; Solís *et al.*, 2017). These crystals are inclusions often found in floral and extrafloral nectaries of angiosperms (Davis, Peterson & Shuel, 1988; Horner *et al.*, 2003; Stpiczyńska, Davies & Gregg, 2004). They are related to different functions, such as in sucrose transport, the formation of thin cell walls through calcium sequestration and protection against herbivory (Giaquinta, 1979; Davies, 1999; Horner *et al.*, 2003).

This study is the first report showing floral nectary vascularization in Anacardiaceae, but previous studies identified that trait in Sapindaceae; summarizing, the nectariferous disk may can lack vascularization or be supplied either by both xylem and phloem or only phloem (Ning-Xi & Wu, 2005; Solís & Ferrucci, 2009; Zini, Solís & Ferrucci, 2014; Avalos *et al.*, 2017; Solís *et al.*, 2017). In Nitrariaceae it was reported that only phloem supplies the nectariferous tissue (Abedini *et al.*, 2013). However, little is known to date about the vascularization of floral nectaries in other members of the order, and further studies are necessary to characterize their potential diversity and implications for the composition of their secretions.

Synthesis of the secretions

Considering the nectary ultrastructure of the four species studied in detail here, despite the differences in composition of secretions and structure, little variation was found except for the activity of the dictyosomes, which were more developed in *Lithraea molleoides* and absent in the pre-anthetic flowers of *Schinus molle*.

Mitochondria, rough endoplasmic reticulum, well-developed dictyosomes and abundant vesicles are typical cellular features of nectariferous tissues (Fahn, 1979; Durkee, 1983; Nepi, 2007). In general, it is important to note that some structural changes occur between the pre-anthesis and anthesis phase. In all species studied here during pre-anthesis the dictyosomes are absent or poorly developed, but during anthesis they are abundant and well developed. Similar changes were observed in nectary cells of *Tapirira* (Tölke *et al.*, 2015). With the rough endoplasmic reticulum, the dictyosomes are involved with the production of vesicles (Fahn, 1979, 2000) and are directly related to the production of hydrophilic substances (Durkee, 1983). The paramural bodies act on partial dissolution of the cell wall and are involved in the uptake of enzymes for extracellular synthesis, contributing to the hydrophilic portion of the secretion (Marchant & Robards, 1968; Fahn, 1979; Paiva & Machado, 2006).

Amyloplasts present during the pre-anthetic phase and absent or depleted at anthesis was another important change in the subcellular apparatus. The histochemical tests did not detect any amyloplasts in *Schinus molle* and *Lithraea molleoides*. However, they were observed under TEM in a final stage of depletion at anthesis. This change in amyloplasts indicates that the starch is probably utilized to form the different types of sugars and may also provide energy for the secretory process (Sawidis, Eleftheriou & Tsekos, 1989; Razem & Davis, 1999; Horner *et al.*, 2007; Nepi, 2007; Ren *et al.*, 2007; Paiva & Machado, 2008). In species in which amyloplasts are not present, such as *Spondias dulcis*, the plastids are probably the source of nectar carbohydrates, as has been shown in other angiosperm families (Pacini, Nepi & Vesprini, 2003; Nepi, 2007).

Our results are similar to those presented for *Tapirira* in Anacardiaceae (Tölke *et al.*, 2015) and for Burseraceae (Giuliani *et al.*, 2012), Meliaceae (Paiva, 2012), Nitrariaceae (Abedini *et al.*, 2013) and Sapindaceae (Avalos *et al.*, 2017). These species basically have the same organelles in their nectary cells, especially plastids containing plastoglobules, and oil droplets dispersed in the cytoplasm.

Epidermis cell with amyloplast, mitochondria and small vesicles near to the plasma membrane. (D) Rough endoplasmic reticulum near the plasma membrane and oil droplet in a nectariferous parenchyma cell. (E) Nectariferous parenchyma cell. (F) General view of subnectariferous parenchyma. (G) Subnectariferous parenchyma cell. (H) General view of the epidermis and nectariferous parenchyma in anthesis. (I) Epidermal cell (note the small vesicles). (J) Amyloplast. (K–L) Nectariferous parenchyma cell, the rough endoplasmic reticulum is located near to the membrane. (M) General view of the subnectariferous parenchyma. Abbreviations: Di, dictyosomes; Mi, mitochondria; Nu, nucleus; Ol, oil droplet; Pl, plastid; Pm, plasmodesma; Re, endoplasmic reticulum; Va, vacuole; Ve, vesicle. Scale bars = 10 μm (A); 5 μm (H); 2 μm (F, M); 1 μm (G, K–L); 0.5 μm (B, D–E, I–J); 0.2 μm (C, M).

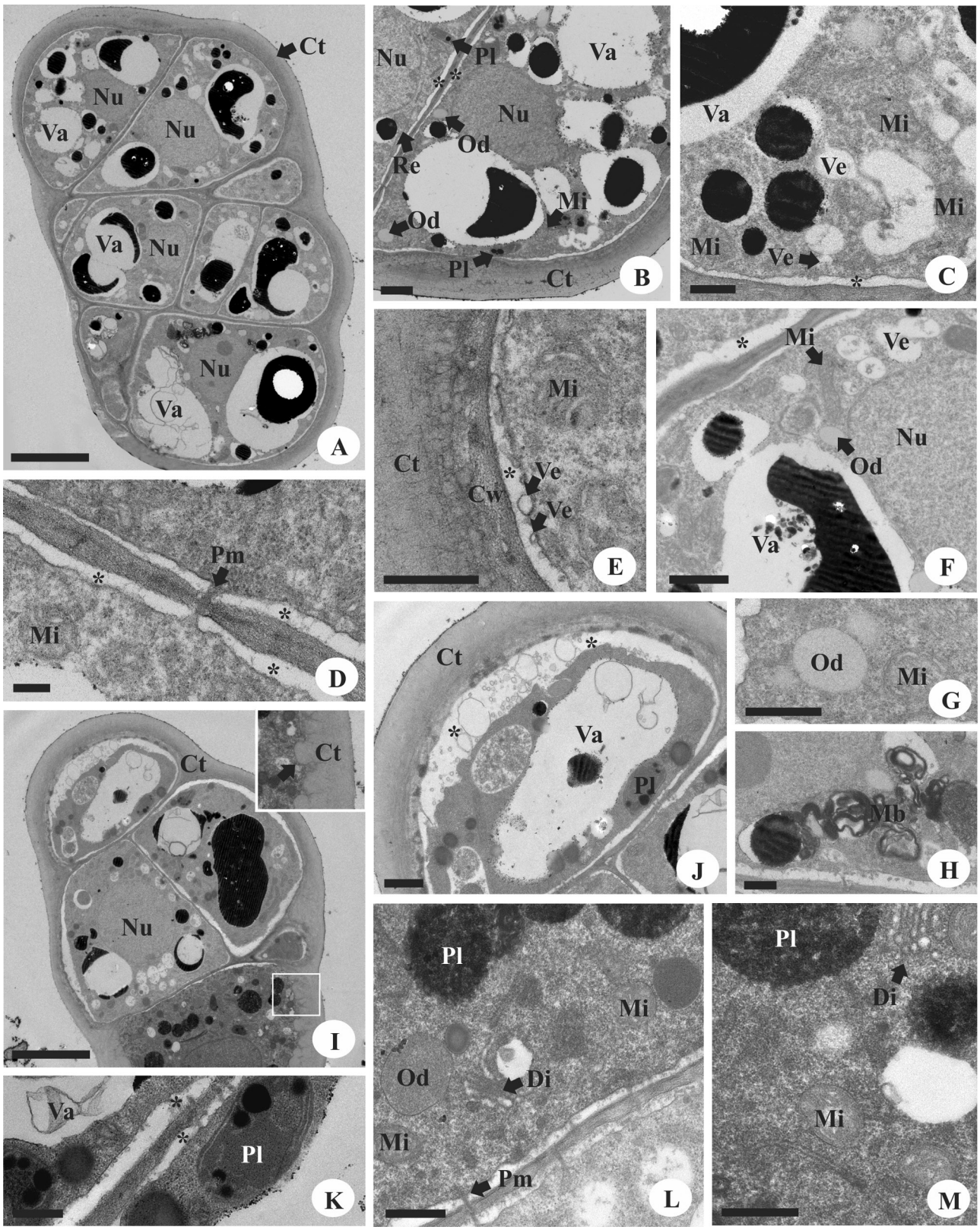

Figure 10. TEM of the nectariferous trichomes of *Anacardium humile* (A–H) at pre-anthesis and (I–M) at anthesis, (*) indicates periplasmic space. (A) General view of the nectariferous trichome. (B) Head cell showing the thick cuticle. The

Transport and exudation pathways

The numerous plasmodesmata found in all studied species indicate that a symplastic transport of nectar is most probably predominant in Anacardiaceae, as evidenced in studies including other angiosperm families (Stpiczyńska *et al.*, 2004). This is also supported by the presence of nectarostomata in all species with a disk, which in symplastic transport typically release nectary secretions transported to the substomatal chambers (Nepi, Ciampolini & Pacini, 1996; Nepi 2007; Vassilyev, 2010). In *Anacardium humile*, the presence of transfer cells, which are common in trichomatous nectariferous tissues (Gunning & Pate 1969; Schnepf & Pross, 1976; Fiordi & Panandri 1982; Stpiczyńska, Nepi & Zych, 2012; Gama *et al.*, 2016), also suggest there is active transport of solutes through the membranes (Maier & Maier, 1972; Gunning, 1977; Joshi *et al.*, 1993).

In all species studied here, the presence of endoplasmic reticulum and numerous mitochondria and the presence of vesicles and dictyosomes suggest that granulocrine secretion is the predominant mode of transport of nectar (Durkee, 1983; Zer & Fahn, 1992; Vassilyev, 2010; Paiva, 2016). However, the presence of numerous starch grains and mitochondria in the parenchymatous cells of nectaries of *Schinus molle* and *Lithraea molleoides*, suggests that, as in *T. guianensis*, an additional mechanism of eccrine secretion is possible in both subfamilies (Rachmilevitz & Fahn, 1973; Fahn, 1979; Heil, 2011; Tölke *et al.*, 2015; Paiva, 2016). In addition, an eccrine secretion mechanism cannot be completely discarded for other species, since they also possess numerous mitochondria in the nectary parenchyma. A combination of both modes of secretion has already been reported in other secretory tissues of other structures of Anacardiaceae and in other families of angiosperms (Durkee, 1983; Davis, Peterson & Shuel, 1986; Zer & Fahn, 1992; Razem & Davis, 1999; Lacchia & Carmello-Guerreiro, 2009; Vassilyev, 2010). In addition, recent works demonstrated that sugars are most probably released via the eccrine pathway, whereas lipids are commonly secreted via the granulocrine mechanism (Gama *et al.*, 2016; Paiva, 2016, 2017).

Nectarostomata are common in Anacardiaceae and in other members of Sapindales, and represent the most likely route of nectary secretions (Bachelier & Endress, 2009; Solís & Ferrucci, 2009; Zini *et al.*, 2014; Tölke *et al.*, 2015; Solís *et al.*, 2017). The exudation of nectar by nectarostomata is widely described in several unrelated taxonomic groups (Fahn, 1979; Durkee, Gaal & Reisner, 1981; Ronse De Craene & Smets, 1991; Galetto & Bernadello, 1992; Fahn & Shimony, 2001; Bernardello, 2007; Zhang, Sawhney & Davis, 2014; Avalos *et al.*, 2017). However, *Anacardium humile* lacks a disk and nectarostomata, and the integrity of cuticle on the nectariferous trichomes indicates that the secretion is probably released through it. This secretion pathway was first suggested by Wunnachit *et al.* (1992) for *A. occidentale*. Indeed, the wax present in the cuticle makes it permeable to fat-soluble substances and might facilitate the release of lipids (Martin & Juniper, 1970; Werker, Ravid & Putievsky, 1985; Ascensão & Pais, 1998; Paiva, 2016, 2017).

CONCLUSIONS

The general morphology and structure of the floral nectary and their secretory pathways appear to be conservative in the family and, like the production of mixed secretions, they might be plesiomorphic in the family. However, variations in sugar composition and in the timing and production of various vesicles suggest some diversity. There are still many genera and species in the family and other members of the order (e.g. in Burseraceae, Meliaceae, Rutaceae and Sapindaceae) that would need to be studied in order to clarify the evolutionary patterns in this clade. Further work is thus needed to determine the ancestral states of the traits related to nectary production and secretion, and to identify the evolutionary and ecological constraints underlying their diversification in Anacardiaceae and other sapindalean lineages.

cytoplasm is dense and presents mitochondria, plastids with plastoglobules, oil droplets, endoplasmic reticulum and plenty of vacuoles with electron-dense material. (C) Part of cytoplasm showing various small vesicles next to the plasma membrane. (D) Plasmodesmata between the cells of the glandular trichome. (E) Head cell. (F) Basal cell showing the cytoplasm with mitochondria, small vesicles, vacuole with electron-dense material and oil droplets. (G) Oil droplet. (H) Vesicles containing myelin-like bodies. (I) General view of the nectariferous trichome in anthesis. The cuticle of the basal cells is thicker than the head cells and possesses irregular ingrowths. (J–K) Head cell showing the plastids with plastoglobules and the periplasmic space. (L–M) Basal cell exhibiting dictyosomes with adjacent vesicles, endoplasmic reticulum profiles, oil droplets and plastids with electron-dense inclusion. Abbreviations: Ct, cuticle; Cw, cell wall; Di, dictyosomes; Mb, myelin-like bodies; Mi, mitochondria; Nu, nucleus; Od, oil droplet; Pl, plastid; Pm, plasmodesma; Re, endoplasmic reticulum; Va, vacuole; Ve, vesicle. Scale bars = 0.2 μm (D); 0.5 μm (C, E, G–H, K–M); 1 μm (B, F, J); 5 μm (A, I).

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