



BIOLOGICAL SCIENCES

Pollen and microsporangium development in *Ziziphus jujuba*, *Z. mucronata*, *Paliurus spina-christi* and *Gouania ulmifolia* (Rhamnaceae)

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Abstract: The aim of this paper is to investigate the ultrastructural events that occur during pollen grains development, with emphasis in pollen grain wall and tapetum ontogeny in *Ziziphus jujuba*, *Z. mucronata*, *Paliurus spina-christi* (Paliureae) and *Gouania ulmifolia* (Gouanieae). Anthers at different developmental stages were processed according to classic techniques for transmission electron microscopy. Differences in the number of endothecium layers and in the number of tapetal cell nuclei were found. Tapetal cells present an anastomosing tubular network and large vesicles with fibrillar content in the cytoplasm. Pollen grain development and ontogeny of pollen grain wall are similar in the four species. The number of endothecium layers, the number of nuclei of the tapetal cells and tapetal cells ultrastructure of the four species support the phylogenetic relationships previously published for the Rhamnaceae family. Tapetal vesicles with fibrillar or polysaccharide content seem to be an exclusive characteristic of the tribes Paliureae and Gouanieae. Some ultrastructural characters of the pollen grain wall development are common to other species of Rhamnaceae, such as the primexine matrix present at the microspore mother cell stage, the aperture entirely built up during the tetrad stage, the thick and fibrillar intine, and the granular infractectum.

Key words: male gametophyte development, pollen wall development, Rhamnaceae, tapetal polysaccharide vesicles, tapetum ultrastructure.

INTRODUCTION

Rhamnaceae is a family with a cosmopolitan distribution that includes 55 genera and 900 species (Medan & Schirarend 2004, Perveen & Qaiser 2005). Richardson et al. (2000a, b) proposed a classification with 11 tribes, which is strongly supported in three clades: Rhamnoid, Ziziphoid and Ampeloziziphoid. Hauenschield et al. (2016) claimed that there are some notable uncertainties and that the morphological characters known until now do not support a formal taxonomic description of these three clades as subfamilies. According to Gotelli et al. (2016a), the morphology and the ultrastructure of the nectaries of Rhamnaceae underpin the

plastid DNA-based phylogenetic analysis made by Richardson et al. (2000a). In this family, the anatomic data of the reproductive sporophytic structures show more systematic value than the gametophytic structures (Gotelli et al. 2016b).

Pollen morphology and tapetum studies

Palynology is considered a useful tool to discriminate among closely related taxa. There are some previous studies on pollen morphology in Rhamnaceae (Papagiannes 1974, Schirarend & Köhler 1993, Perveen & Qaiser 2005). Erdtman (1952) studied about 25 species from 13 genera and considered the family to be stenopalynous. Kajale (1944) and Johri et al.

(1992) analyzed pollen and anther development in *Zizyphus mauritiana* Lam. Pollen morphology of 25 Chinese species representing six genera in the tribe Rhamneae was studied by Zhang & Chen (1992). Schirarend & Köhler (1993) carried out an extensive research where they described twelve morphological types of pollen grains. Nasri-Ayachi & Nabli (1995) analyzed pollen wall ultrastructure and ontogeny in *Z. lotus* L. Schirarend (1996) examined pollen morphology of the genus *Paliurus* and considered that although morphological pattern coincides with the descriptions made for the family, general differences can be recognized in relation to pollen size, shape and tectum architecture. Gotelli et al. (2016b) described pollen development and anther morphology in 14 species of Rhamneae (Rhamnoids Clade), Paliureae, Pomaderreae, Colletieae and Gouanieae (Ziziphoids Clade) and concluded that morphological and anatomical studies are necessary to complement molecular information in order to resolve the phylogeny of Rhamnaceae.

Tapetum is a fundamental tissue of the anther for the normal development of pollen grains (Johri et al. 1992, Raghavan 1997). Many authors consider that the tapetum is involved in different aspects of pollen development (Johri et al. 1992). According to Maheshwari (1950), the tapetum is a tissue with a considerable physiological significance. Researchers, while comparing the cytology of tapetal cells between male sterile and their fertile homologous, found that male sterility is linked to abnormalities in the tapetum (Raghavan 1997, Vardar & Ünal 2012). Alterations manifested in the ultrastructure of the cells of this tissue can generate non-viable pollen (Li et al. 2006, Pacini 2010). In Rhamnaceae, the ultrastructure of tapetal cells was studied in *Colletia paradoxa* and *Discaria americana* from the Colletieae tribe (Gotelli et al. 2012), and *Hovenia dulcis* belonging to the tribe

Paliureae (Gotelli et al. 2016c). In tapetal cells of this last species, Gotelli et al. (2016c) found an anastomosing tubular network and large vesicles with fibrillar content that react positively with PAS. They named them polysaccharide vesicles and their function is still unknown.

The aim of this paper is to describe the ultrastructure of pollen grains and microsporangium development with special attention to tapetum and pollen grain wall ontogeny in species from the Paliureae and Gouanieae tribes, in order to broaden the embryological knowledge of family and to evaluate the characters of reproductive sporophytic structures that support the current classification for Rhamnaceae.

MATERIALS AND METHODS

Plant material

Flowers at different developmental stages of *Zizyphus mucronata*, *Z. jujuba*, *Paliurus spinachristi* (Paliureae) and *Gouania ulmifolia* (Gouanieae) were collected from individuals cultivated in the campus of the Facultad de Agronomía, Universidad de Buenos Aires (34° 35' 37" S, 58° 29' 03" O). Voucher specimens of these species were deposited in the Herbarium Gaspar Suarez (BAA).

Transmission electron microscopy (TEM)

Anthers at different stages of development were pre-fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 48 h and then post-fixed in OsO₄ at 2°C in the same buffer for 3 h. The material was embedded in Spurr's resin after dehydration in an ethanol series. Ultrathin sections (750- 900 nm) were made using a Reichert-Jung ultramicrotome and stained with uranyl acetate and lead citrate (Zarlavsky 2014). The sections were observed and photographed with a JEOL-JEM 1200 EX II TEM at 85.0 kV.

RESULTS

The anther is tetrasporangiate and its wall consists of epidermis (ep), endothecium (en), two to three middle layers and a secretory type tapetum (t). Tapetal cells are uninucleate in *G. ulmifolia*, *P. spina-christi*, and binucleate in both species of *Ziziphus*.

Microsporogenesis and microgametogenesis

Stage 1: Sporogenous tissue

At this stage, microspore mother cells start to differentiate. In their cytoplasm a conspicuous

nucleus, rough endoplasmic reticulum and mitochondria are observed (Figure 1a).

Stage 2: Microspore mother cell stage with callose

Microspores mother cells of the four species present many mitochondria, rough endoplasmic reticulum, dictyosomes and vesicles (Figure 1b). Lipid globules and plastids are observed in the cytoplasm of these cells in *P. spina-crhisti* (Figure 1c). A thick callosic wall is formed between the plasmalemma and the primary wall (Figure 1d).

Stage 3: Microspore tetrads

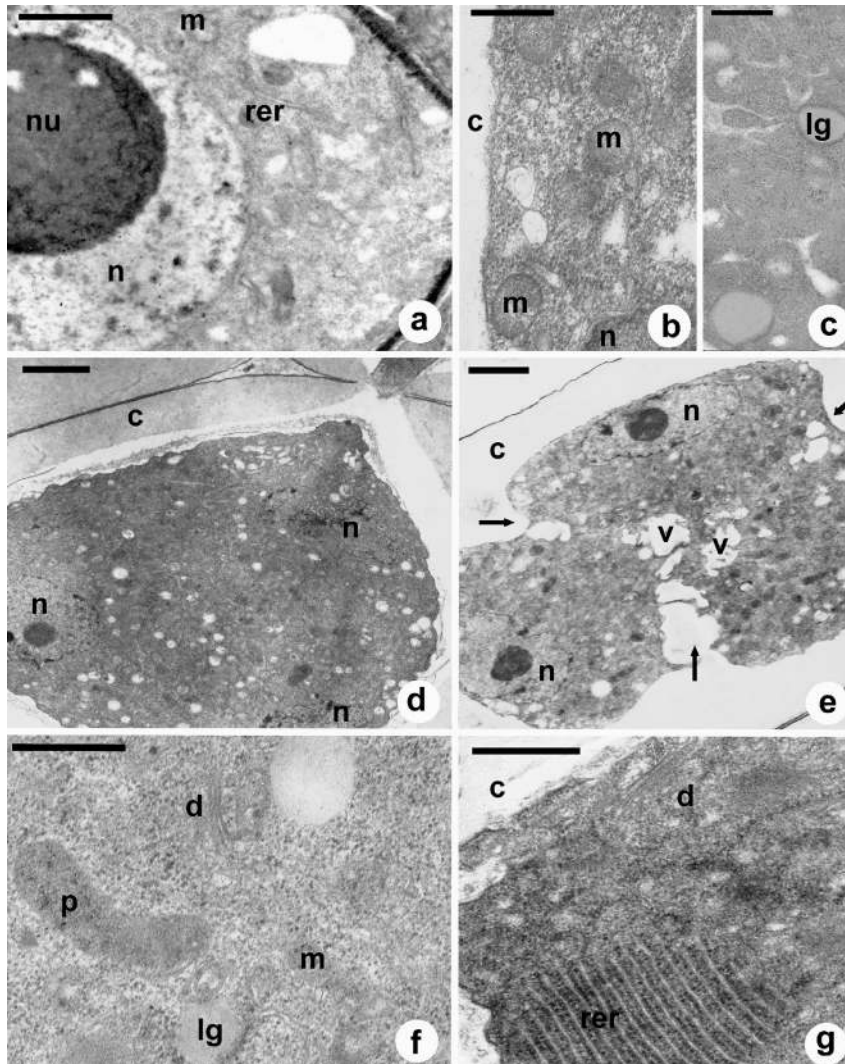


Figure 1. Microsporogenesis. (a) *Gouania ulmifolia*. Microspore mother cell without callose. **(b)** *Ziziphus mucronata*. Microspore mother cell with callose. **(c)** *Paliurus spina-christi*. Microspore mother cell with callose. **(d)** *Z. mucronata*. Karyokinesis of microspore mother cell. **(e)** *Z. jujuba*. Cytokinesis. v: vacuole **(f)** *P. spina-christi*. Microspore tetrad. **(g)** *Z. jujuba*. Microspore tetrad. Scale bars: a: 1 μ m; b, c, f, g: 500 nm; d, e: 2 μ m. n: nucleus, nu: nucleolus, m: mitochondria, rer: rough endoplasmic reticulum, c: callose, d: dictyosome, lg: lipidic globule, p: plastid, arrows: furrows.

Microsporocytes undergo simultaneous meiosis, forming tetrads with a tetrahedral arrangement. Before cytokinesis takes place, the amount of dictyosomes increases and plasmodesmata are observed between former microspore mother cells (Figure 1d). They remain surrounded by a thick callosic wall (Figure 1d, e). Cytokinesis starts by simultaneous centripetal furrows (Figure 1e). The microspore cytoplasm of the four species shows many mitochondria, dictyosomes, endoplasmic reticulum of rough type, plastids and ribosomes (Figure 1f, g).

Stage 4: Free microspores

Microspores have a conspicuous nucleus (Figure 2a). Mitochondria, dictyosomes, rough

endoplasmic reticulum and plastids are observed in their cytoplasm (Figure 2b, c).

Stage 5: Pollen grain

The generative and vegetative cells are formed by a mitotic division of the microspore. The generative cell migrates from a parietal position towards a central position. It appears to be surrounded by many small vesicles present in the cytoplasm of the vegetative cell (Figure 2d). Rough endoplasmic reticulum, lipidic globules, a few dictyosomes and some mitochondria are observed in the cytoplasm of the vegetative cell (Figure 2e). The vegetative nucleus is lobed and surrounded by mitochondria (Figure 2f).

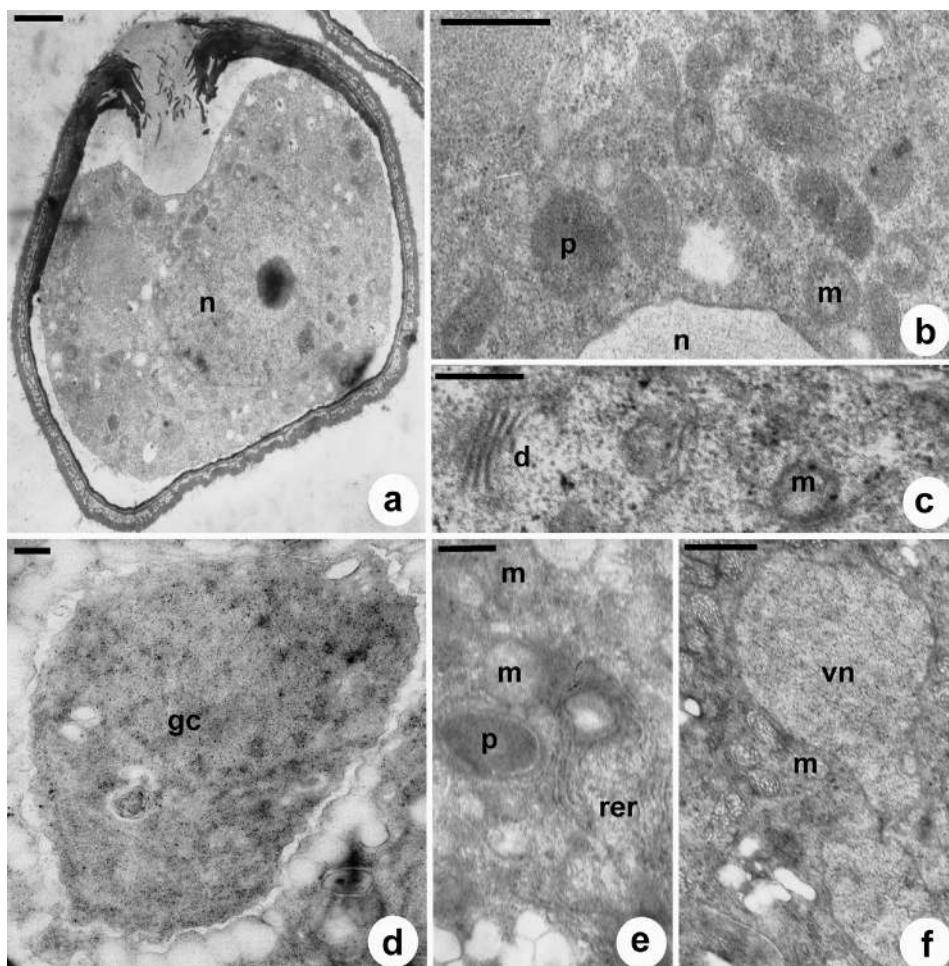


Figure 2. Microgametogenesis. (a) *Z. mucronata*. General aspect of the free microspore. (b, c) *G. ulmifolia*. Detail of the microspore cytoplasm. (d) *Z. jujuba*. Generative cell surrounded by many small vesicles (arrows). (e) *P. spina-chrisi*. Detail of the cytoplasm of the vegetative cell. (f) *Z. mucronata*. Vegetative nucleus. Scale bars: a, f: 1 µm; b-e: 500 nm. n: nucleus, m: mitochondria, rer: rough endoplasmic reticulum, d: dictyosome, p: plastid, gc: generative cell, vn: vegetative nucleus.

Pollen grain wall development

At the microspore mother cell stage, vesicles with a fibrillar and electrondense border are observed between the plasma membrane and the callose (Figure 3a). After karyokinesis, but before cytokinesis, the borders of these vesicles start to coalesce forming an electrondense granular and fibrillar structure that includes circular areas with low electrondense content. This structure is the primexine matrix (Figure 3b).

At the young tetrad stage, the primexine matrix is more fibrillar and a low electrondense protectum starts to differentiate. Electrondense double membrane structures appear to be initiating the basal layer (Figure 3c). At the

more advanced tetrad stage, the protectum is observed more electrondense and the basal layer is more continuous. In the periphery of the circular areas of the primexine matrix a more electrondense substance is deposited. In this way the probacules are delimited. These last are irregular and somewhat bifurcated (Figure 3d). As this stage progresses, branched pro-endexinic lamellae surrounding the oncus of the proto-apertures can be observed (Figure 4a).

At the free microspore stage, in the four species, the thickness of the tectum is increased and a fibrillar and electrondense substance is present on it. An homogeneous endexine and a continuous basal layer can be observed. The basal layer is thinner than the endexine. As this stage progresses, the irregular probacules are

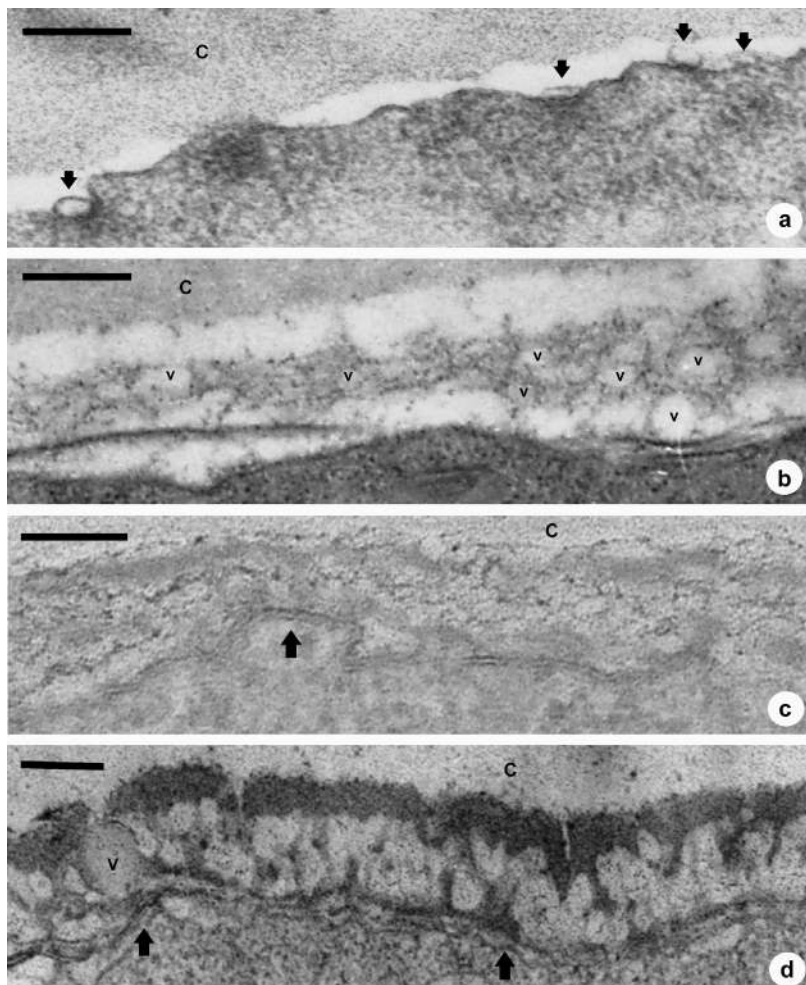


Figure 3. Pollen wall development. (a) Detail of the microspore mother cell wall of *Z. jujuba*. (b) Detail of the wall of the microspore mother cell during karyokinesis of *Z. mucronata*. (c) Detail of the wall of one microspore of a young tetrad of *P. spina-chisti*. (d) Detail of the wall of one microspore of a tetrad in a later stage of *Z. mucronata*. Scale bars: a, b, d: 250 nm; c: 100 nm. c: callose, v: vesicles, arrows looking down: vesicles; arrows looking up: double membrane structure.

compressed by the thickening of the tectum and therefore a granular infratectum is formed (Figure 4b). As the microspore matures, the basal layer thickens and the ectexine presents less electron density. The infratectum reduces its thickness and keeps a granular aspect. Dark-contrasted depositions are observed between the plasmalemma and the endexine (Figure 4c).

The mature pollen grain wall of all species here studied has a thick and fibrillar intine and an endexine more electrondense than the ectexine (Figure 4d).

Tapetal ultrastructure

During the microspore mother cell stage, tapetal cells contain many plastids, dictyosomes, rough endoplasmic reticulum, and mitochondria. Lipid globules are only observed in *P. spina-christi* (Figure 5a). At this stage, dictyosomic vesicles are very abundant in the cytoplasm and some

of them are connected with cisternal stacks of dictyosomes by an anastomosing tubular network (tn). Some of these vesicles are larger and have inside a slightly fibrillar content. Vesicles vary in size in the same tapetal cell (Figure 5b).

At microspore tetrad stage, tapetal cells are filled with fibrillar vesicles, endoplasmic reticulum of rough type, and a few mitochondria and dictyosomes (Figure 5c). Plastids are only observed in tapetal cells of *P. spina-christi* (Figure 5c, d).

Tapetal cells show at the free microspore stage similar characteristics to those of the previous stages, and the most abundant organelles in the cytoplasm are the fibrillar vesicles (Figure 5e, f). Tapetal cells are no longer observed at the mature pollen grain stage.

Orbicules were not observed in any of the species.

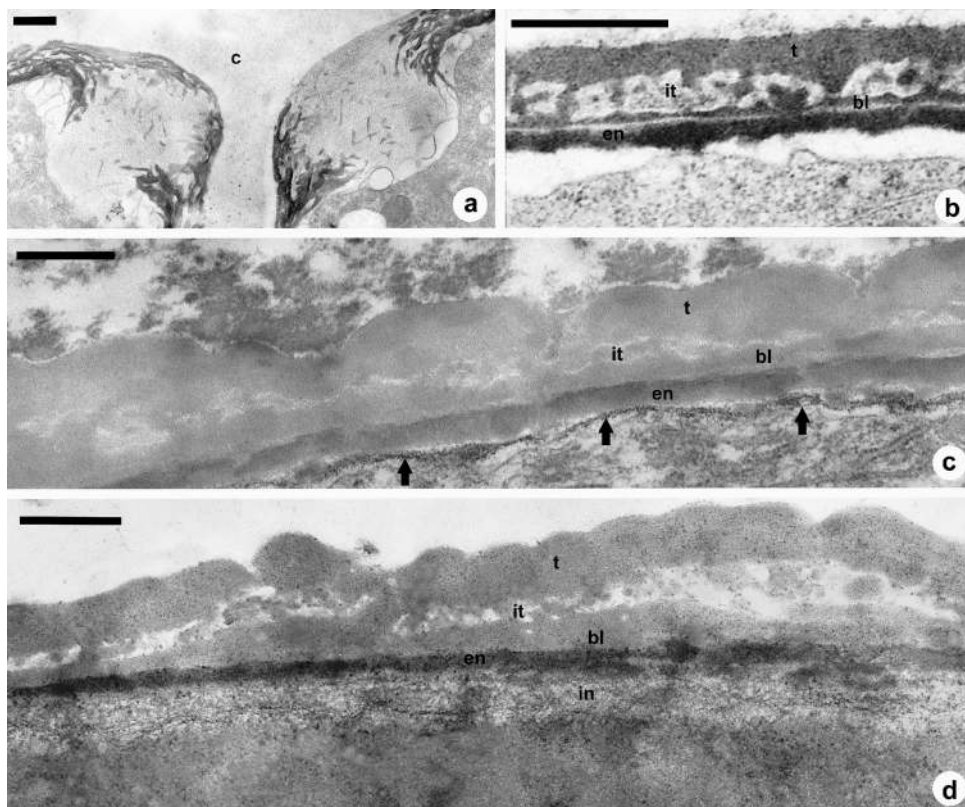


Figure 4. Pollen wall development. (a) Protoaberture (arrows) in a tetrad of *Z. mucronata*. (b) Detail of a young microspores wall of *Z. mucronata*. (c) Detail of a mature microspores wall of *Z. jujuba*. (d) Detail of the pollen grain wall of *Z. mucronata*. Scale bars: 500 nm. c: calose, t: tectum, it: infratectum, en: endexine, in: intine, bl: basal layer, arrows: dark-contrasted depositions.

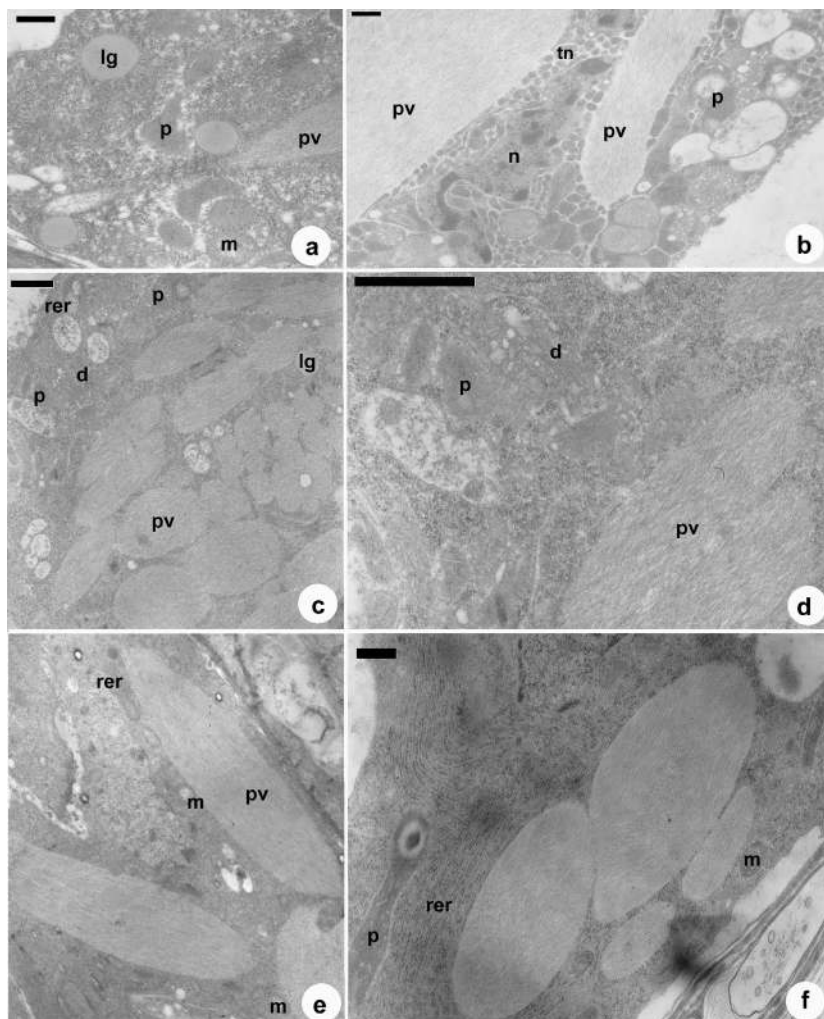


Figure 5. Tapetal ultrastructure. (a) Tapetal cell of *P. spina-christi* at microspore mother cell stage. (b) Tapetal cell of *Z. mucronata* at microspore mother cell stage. (c) Tapetal cell of *P. spina-christi* at tetrad stage. (d) Detail of a tapetal cell of *P. spina-christi* at tetrad stage. (e) Tapetal cell of *G. ulmifolia* at free microspore stage. (f) Tapetal cell of *Z. mucronata* at microspore stage. Scale bars: a, b, f: 500 nm, c, d: 1 μ m, m: mitochondria, lg: lipid globule, p: plastid, n: nucleus, d: dictyosome, rer: rough endoplasmic reticulum, pv: polysaccharide vesicle, tn: tubular network.

DISCUSSION

This study provides new detailed information about the anatomy and the ultrastructure of tapetal cells and pollen development of *Ziziphus jujuba*, *Z. mucronata*, *Paliurus spina-christi* (Paliureae) and *Gouania ulmifolia* (Gouanieae).

The number of endothecium layers and the number of tapetal nuclei are consistent with previous descriptions (Gotelli et al. 2016b). Tapetal cells in *Z. jujuba* and *Z. mucronata* are binucleate as in *Z. mistol*, *Colletia paradoxa*, *C. spinosissima*, *Retanilla patagonica*, *Kentrothamnus weddellianus* (Jafari Marandi & Niknam 2012, Gotelli et al. 2012, 2016b) whereas in

G. ulmifolia and *P. spina-christi* are uninucleate as in *Scutia buxifolia*, *Condalia buxifolia*, *Hovenia dulcis*, *Cryptandra tomentosa*, *Siegfriedia darwinioides* and *Stenanthemum humile* (Gotelli et al. 2012, 2016b) (Table 1). Although Jafari Marandi & Niknam (2012) described the tapetum of *Z. jujuba* as plasmodial, according to our observations and previous researches (Johri et al. 1992, Gotelli et al. 2012, 2016b), the secretory tapetum seems to be a stable character within Rhamnaceae.

According to this and other previous researches (Gotelli et al. 2012, 2016b, c), orbicules are present in all the studied species of the tribes Pomaderreae and Colletieae, and in some

Table I. Summary of anther anatomy features. PO: personal observation, PS: present study.

Clade	Tribe	Species	Tapetum # nuclei	Tapetal vesicles	Endothecium layers	Orbicules	References
Rhamnoids	Rhamneae	<i>Scutia buxifolia</i>	1	-	2-3	-	Gotelli et al. 2016b, PO
		<i>Condalia buxifolia</i>	1	-	2-3	+	Gotelli et al. 2016b, PO
Ziziphoids	Pomaderreae	<i>Cryptandra tomentosa</i>	1	-	1	+	Gotelli et al. 2016b
		<i>Siegfriedia darwinioides</i>	1	-	1	+	Gotelli et al. 2016b
		<i>Stenanthemum humile</i>	1	-	1	+	Gotelli et al. 2016b
	Colletieae	<i>Colletia paradoxa</i>	2	-	2-3	+	Gotelli et al. 2012, 2016b
		<i>C. spinosissima</i>	2	-	2-3	+	Gotelli et al. 2016b, PO
		<i>Discaria americana</i>	2	-	2-3	+	Gotelli et al. 2012, 2016b
		<i>Retanilla patagonica</i>	2	-	2-3	+	Gotelli et al. 2016b, PO
		<i>Kentrothamu sweddellianus</i>	2	-	2-3	+	Gotelli et al. 2016b, PO
	Paliureae	<i>Ziziphus mucronata</i>	2	+	1	-	Gotelli et al. 2016b, PS
		<i>Z. mistol</i>	2	+	1	+	Gotelli et al. 2016b
		<i>Z. jujuba</i>	2	+	1	-	Gotelli et al. 2016b, PS
		<i>Paliurus spina-christi</i>	1	+	1	+	PS
		<i>Hovenia dulcis</i>	1	+	1	-	Gotelli et al. 2015, 2016b
	Gouanieae	<i>Gouania ulmifolia</i>	1	+	2-3	-	Gotelli et al. 2016b, PS

species of the other tribes (Table I). Therefore, the presence or not of these sporopollenin corpuscles coating the anther locule does not seem to be a stable character for all the tribes of Rhamnaceae.

The tapetum is an ephemeral secretory tissue with a nutritional role for pollen grains

and is considered to regulate their development (Chapman 1987, Pacini 2010). The tapetum ultrastructure was analyzed in a few species of the Rhamnaceae family until now (Gotelli et al. 2012, 2016c). The anastomosing tubular network and large vesicles with fibrillar content present in the tapetal cells of the species here studied were

previously described for *Hovenia dulcis* (Gotelli et al. 2016c). These authors found that such vesicles accumulate insoluble polysaccharides and identified them as polysaccharide vesicles. The observations made in different stages of tapetal development of *Ziziphus mucronata*, *Z. jujuba*, *Paliurus spina-christi* and *Gouania ulmifolia* are similar to those made in *Hovenia dulcis* (Gotelli et al. 2016c). The vesicles with fibrillar material inside originate from the dictyosomes and they increase in volume by the transfer of polysaccharides through the anastomosing tubular network. In all species studied, as in *Hovenia dulcis*, once the polysaccharide vesicles reach their maximum size, the network is no longer present. However, in the four species here studied, tapetal cells are degraded at the pollen grain stage while in *H. dulcis* tapetal cells are still present at this stage and show a PAS+ cytoplasm with an homogeneous fibrillar appearance, surrounded by a new structure resembling a lax cell wall (Gotelli et al. 2016c). According to our results, the presence of an anastomosing tubular network and large polysaccharide vesicles in tapetal cells seems to be a characteristic of Paliureae and Gouanieae tribes (Table I) since there are no reports of such structure in tapetal cells of species of other tribes of Rhamnaceae nor for other species of angiosperms in general (Gotelli et al. 2012, 2016c). Gotelli et al. (2016c) hypothesized that the tapetum could be acting as a reservoir of sugar which is possibly translocated to the sweet rachis of the inflorescence while the fruit is formed in *Hovenia dulcis*. In the four species here studied, polysaccharides could be translocated to the fruits, which are known for the large amount of sugar content (Li et al. 2007, Pareek 2013).

The correlation between pollen morphology and the tribal classification of Rhamnaceae seems to be incomplete (Gotelli et al. 2016b).

According to Schirarend & Köhler (1993), each tribe presents several pollen types, and pollen types repeat between tribes. In this research we observe that some ultrastructural characters of the pollen grain wall development are common to other species of Rhamnaceae. For instance, the aperture entirely built up during the tetrad stage was previously described for *Ziziphus lotus* L. (Nasri-Ayachi & Nabli 1995), and the thick and fibrillar intine and a granular infratectum were observed in species of Rhamneae (Zhang & Chen 1992), Paliureae (Lobreau-Callen 1976, Nasri-Ayachi & Nabli 1995, Schirarend 1996, Gotelli et al. 2016b) and Colletieae (Gotelli et al. 2012).

In angiosperms, although there are many differences in the exine morphology of mature pollen grains, the way pollen grain wall develops seems to follow a common pattern (Meier-Melikyan et al. 2003, Grigorjeva & Gabarayeva 2018). However, the moment in which events occur may vary significantly between species (Blackmore et al. 2007, Grigorjeva & Gabarayeva 2018) as well as the morphology and chemical composition of the pollen wall layers (Meier-Melikyan et al. 2003). The microspore tetrad stage is known to be the most important period for the determination of the exine pattern (Dickinson 1970, Heslop-Harrison 1972, Blackmore & Barnes 1987, Gabarayeva 2000, 2014, Gabarayeva & Grigorjeva 2016, Vinckier & Smets 2005, Taylor & Osborn 2006, Taylor et al. 2013, 2015). According to Gabarayeva et al. (2016) the ectexine is organized through physical processes that include self-assembly operating in a very organized glycolyx, which is a cell surface coating formed by glycoproteins and lipopolysaccharides (Rowley 1971, 1973, Pettitt & Jermy 1974, Rowley & Dahl 1977, Pettitt 1979). The exine design depends on the accumulation of sporopollenin on this glycolyx. In this work, we use primexine matrix (Heslop-Harrison

1963, 1972, Dickinson 1970) as a synonym for glycocalyx (Gabarayeva et al. 2016). In *Ziziphus mucronata*, *Z. jujuba*, *Paliurus spina-christi* and *Gouania ulmifolia*, the primexine matrix is an electron-dense granular and fibrillar structure that includes circular areas with low electron-density content. These last areas give place to the “exine template” (Blackmore et al. 2007) defining the probacula structure, since the sporopollenin is deposited in the periphery of the circular areas of the primexine matrix as a more electron-dense substance. That is why, the probacula are observed irregular and sometimes bifurcated defining the primexine.

As the tectum and basal layer thicken throughout the development, the infratectum becomes thin and is visualized as granular. In *Z. lotus*, Nasri-Ayachi & Nabli (1995), observed a columellate infratectal layer in the beginning of pollen wall development that becomes granular later because of the thickening of the tectum. According to Doyle (2009) some authors question the concept of granular structure, based on the occurrence of apparent precursors of probacula early in the development. However, Gabarayeva (1995) recognized that granular and columellar structures have a common basis in their development and one may derive from the other. Our observations reaffirm this last concept.

According to Blackmore et al. (2007), during the free microspore stage tapetal cells are involved in the synthesis of sporopollenin precursors, which incorporate to specific sites in the ectexine and on its surface. At this stage, it is possible to observe in *Ziziphus mucronata*, *Z. jujuba*, *Paliurus spina-christi* and *Gouania ulmifolia*, abundant fibrillar and electron-dense material in the anther loculus and on the exine surface of the microspores. This material can be interpreted as sporopollenin precursors in accordance with the observations of Lattar et

al. (2012). According to Gabarayeva & Grigorjeva (2014) this fibrillar material observed on the free microspores surface may carry out a direct contact between these and tapetal cells.

At the advanced microspore stage, in the species here studied, dark-contrasted depositions are observed between the plasmalemma and the endexine. This may be related to the intine formation as, at this stage the endexine reaches its maximum thickness and the intine is not present yet. At the mature pollen grain stage, the ectexine is observed less electron-dense, since the sporopollenin reaches its maximum grade of polymerization.

Taxonomic considerations

In Rhamnaceae, the anatomy of the reproductive sporophytic structures seems to have more systematic value than the gametophytic structures (Gotelli et al. 2016b). Differences observed in the anther anatomy support those found in previous studies for this same family (Kajale 1944, Johri et al. 1992, Gotelli et al. 2012, 2016b, c). The number of endothecium layers, the number of nuclei of the tapetal cells and tapetal cells ultrastructure of *Ziziphus jujuba*, *Z. mucronata*, *Paliurus spina-christi* and *Gouania ulmifolia* underpin this hypothesis and the phylogenetic relationships published by Richardson et al. (2000a, b) and Hauenschild et al. (2016) (Table I). On the other hand, the presence of polysaccharide vesicles seems to be an exclusive characteristic of the tribes Paliureae and Gouanieae, since it has not been observed for any species belonging to other tribes until now (Table I). Therefore, this character supports one of the optimal trees from the *rbcL* analysis published by Richardson et al. (2000a). This character should be studied in other species of the family, especially from other tribes in order to confirm this hypothesis.

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MG and BG conceived and designed this research. GZ prepared and processed the material for observation. MG, BG and EL analyzed data. MG wrote the manuscript, EL and BG revised and corrected the manuscript. All authors read and approved the manuscript.

