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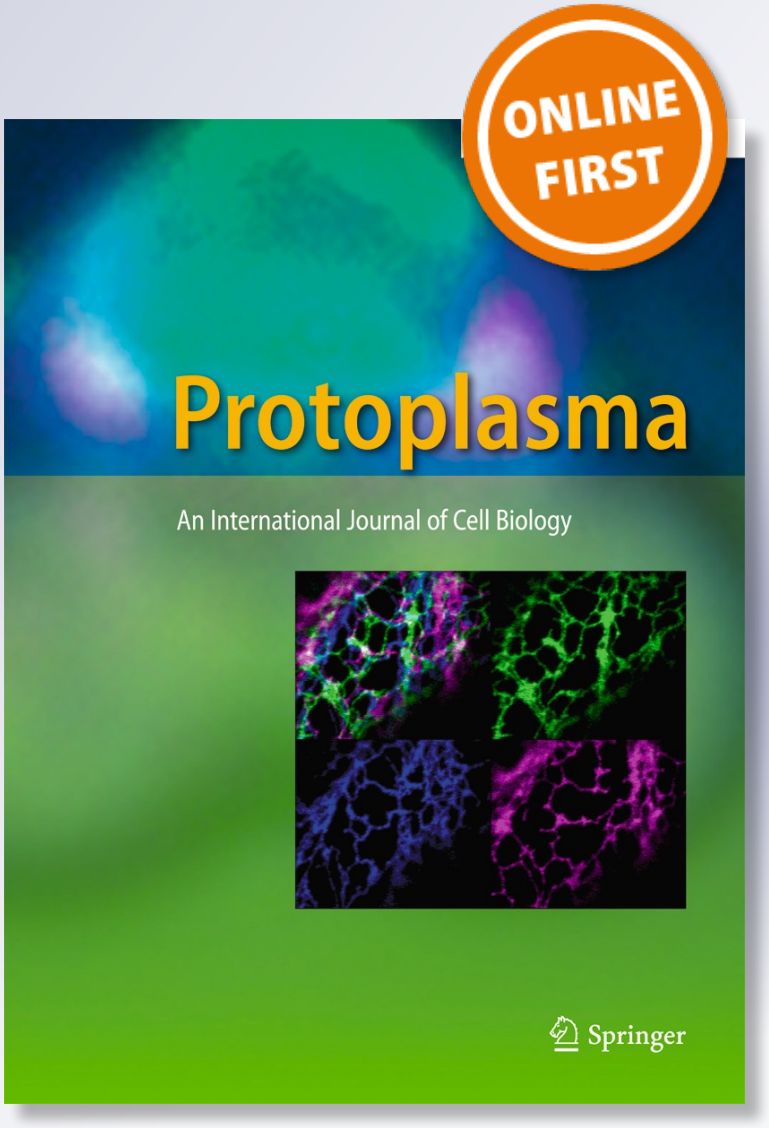

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ORIGINAL ARTICLE

Developmental and ultrastructural characters of the pollen grains and tapetum in species of *Nymphaea* subgenus *Hydrocallis*

Lucía Melisa Zini[1] · **Beatriz Gloria Galati**[2] · **Gabriela Zarlavsky**[2] · **María Silvia Ferrucci**[1]

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Abstract Variations in pollen characters and tapetum behavior were recently acknowledged in the early-divergent family Nymphaeaceae and even within the genus *Nymphaea*, which probably is not monophyletic; some traits such as infratectum and tapetum type are also a matter of different interpretations. In this study, developmental characters of the pollen grains and tapetum in *Nymphaea* subgenus *Hydrocallis* are provided for the first time. Observations were made in *N. amazonum*, *N. gardneriana*, and *N. prolifera* using light, scanning, and transmission electron microscopy. Tapetum is of the secretory type and produces orbicules. At microspore and pollen grain stages, the distal and proximal walls differ considerably. This result supports the operculate condition of the aperture in *Hydrocallis*, and such aperture might be plesiomorphic for Nymphaeoideae. The infratectum is intermediate, composed of inter-columellae granular elements, robust columellae consisting of agglomerated granules, complete columellae, and fused columellae. Narrow microchannels are present and persist until the mature pollen grain stage. The membranous granular layer is often present in the pollen grains of Nymphaeaceae. In *N. gardneriana*, this layer is most probably a component of the intine because it is lost after acetolysis. Orbicules in the Nymphaeaceae are characterized as spherical or subspherical, with a smooth sporopolleninic wall that surrounds an electron-lucent core and with individual orbicules that usually merge to give irregular aggregations. The aperture, pollen wall ultrastructure, and the tapetum of the studied species are discussed in an evolutionary and systematic context, and these characters are also compared with those of other angiosperm lineages.

Keywords Exine · Membranous granular layer · Orbicules · Pollen · Tapetum type

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Introduction

Nymphaeaceae comprises the entirely aquatic order Nymphaeales, together with Cabombaceae and Hydatellaceae. The order is one of the first three earliest lineages that diverged before the main branch, leading to most extant angiosperms (magnoliids, monocots, eudicots) (APG IV 2016). Therefore, this group is important to the understanding of reproductive characters in angiosperm evolution. Nymphaeales includes eight genera, of which *Nymphaea* L., with about 50 species, is the most diverse and widely distributed (Borsch et al. 2011). Research works on molecular phylogenetics of Nymphaeaceae support three clades within *Nymphaea*: subgenera *Anecphya-Brachyceras*, subgenera *Hydrocallis-Lotos*, and the subgenus *Nymphaea* (Löhne et al. 2007; Borsch et al. 2008). However, the monophyly of *Nymphaea* is still controversial; in a topology, the genus is monophyletic with respect to a clade containing the genera *Euryale* Salisb. and *Victoria* Lindl., but an alternative scheme nested the *Euryale-Victoria* clade within *Nymphaea* as sister group to all members of the genus except for the subgenera *Nymphaea*. These three genera are clustered into

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the subfamily Nymphaeoideae (Les et al. 1999; Borsch et al. 2007, 2008; Löhne et al. 2007).

The palynological information on Nymphaeales has been informative for the phylogeny and systematics within this order (Ito 1987; Wiersema 1987; Les et al. 1999; Doyle and Endress 2000; Podoplelova and Ryzhakov 2005; Borsch et al. 2008; Taylor et al. 2015); it has also been helpful to understand the origin and diversification of pollen characters in angiosperms (Walker 1974a, 1974b; Doyle 2005, 2009; Hesse and Zetter 2005; Sampson 2007; Endress and Doyle 2009; Lu et al. 2015). In this field of study, the analysis with transmission electron microscopy acquires crucial importance to reveal data otherwise inaccessible with other techniques, such as details of the infratectum, endexine, and intine (Walker and Doyle 1975). However, although the pollen morphology of *Nymphaea* was described using light or scanning electron microscopes for most Asiatic and European species, as well as for the 14 species of the Neotropical subgenus *Hydrocallis* (Singh et al. 1969; Wiersema 1987; Murthy 2000; Ansari et al. 2005; Volkova and Shipunov 2008; Bhowmik and Datta 2012; Debasis and Mondal 2012), transmission electron microscopic studies were conducted in few species belonging to the subgenera *Anecphya*, *Brachyceras*, and *Nymphaea* (Gabarayeva and Rowley 1994; Meyer-Melikian and Diamondopulu 1996; Gabarayeva and El-Ghazaly 1997; El-Ghazaly and Huysmans 2001; Gabarayeva et al. 2001; Coiro and Barone Lumaga 2013; Taylor et al. 2015).

In *Nymphaea*, the pollen wall architecture is remarkable because of the organization of its components and their features. Recent ultrastructural studies have shown differences in the aperture between the subgenus *Nymphaea* and the clade *Anecphya-Brachyceras*, with these results adding support to the paraphyletic relationship of *Nymphaea* (Coiro and Barone Lumaga 2013). In addition, studies that described the aperture as being of the ring-like type recognize various evolutionary steps based on exine differentiation between proximal and distal walls; hence, the exine structure of *Nymphaea* species can be heterogeneous (subgen. *Nymphaea*; Coiro and Barone Lumaga 2013), intermediate (*N. ondinea* (Hartog) Löhne, Wiersema & Borsch of subgen. *Brachyceras*; Taylor et al. 2015), or entirely homogeneous (*N. caerulea* Savigny of subgen. *Brachyceras*; Coiro and Barone Lumaga 2013).

The infratectum within Nymphaeaceae is another debated issue. Particularly for *Nymphaea*, it is interpreted either as columellate (Gabarayeva and Rowley 1994; Gabarayeva and El-Ghazaly 1997; Gabarayeva et al. 2001; Taylor et al. 2015) or intermediate, with both columellae and granules (Doyle and Endress 2000; Doyle 2005; Borsch et al. 2008; Coiro and Barone Lumaga 2013). The membranous granular layer (MGL) is a little understood layer of the pollen wall in diverse angiosperms and was described in some species of Nymphaeaceae and recognized as a synapomorphic trait (Taylor et al. 2015). Since there are several differences and incongruities in ultrastructural features within *Nymphaea*, further pollen transmission electron microscope analyses are needed to determine the prevailing pollen characters in this genus, to evaluate their systematic implication, and ultimately to conduct evolutionary studies within the Nymphaeaceae.

Variability of tapetum type of *Nymphaea* has also been documented. Secretory tapetum was reported in the subgenera *Brachyceras* and *Lotos* (Gabarayeva et al. 2001; Dai and Zhou 2010), whereas cyclic invasive tapetum was reported in the subgenera *Brachyceras* and *Nymphaea* (Rowley et al. 1992; Gabarayeva and El-Ghazaly 1997). Pollen and tapetum characters are evidently labile in *Nymphaea*; this fact is of particular interest because information is still not available for the subgenus *Hydrocallis*. Therefore, the present study focuses on contributing with developmental and structural characters of the pollen grains and tapetum in *N. amazonum*, *N. gardneriana*, and *N. prolifera*. These data will be crucial to understanding character evolution in *Nymphaea* and to resolve the long-standing homology questions.

Materials and methods

The plant material and information about the samples used, fixation, and embedding methods are presented in Table 1. Voucher specimens are deposited at the Instituto de Botánica del Nordeste herbarium (CTES). For light microscopy (LM), the samples in formalin, acetic acid, ethanol (FAA) were dehydrated in an ethanol series with a rinse using pre-impregnated Biopur® (Gonzalez and Cristóbal 1997). The infiltration in paraffin followed the technique of Johansen (1940). Ten anthers for each stage were embedded in Histoplast® (Biopack, Buenos Aires, Argentina) and were transversely sectioned at 10 μm with a rotary microtome. They were stained with a combination of Astra blue and safranin (Luque et al. 1996) and mounted on slides with synthetic Canada Balsam (Biopur, Buenos Aires, Argentina).

Five anthers of *N. prolifera* pre-fixed in FAA and five of *N. gardneriana* pre-fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 2 h were post-fixed in 1.5% OsO_4 at 2 °C in the same buffer for 3 h. Dehydration was made using ascending graded series of acetone. Samples were embedded in Spurr's resin and sectioned using a Reichert-Jung ultramicrotome. Sections of 1 μm were stained with toluidine blue for LM. Sections of 70 nm were stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM) observations (Zarlavsky 2014). A sample containing pollen grains of *N. gardneriana* was acetolyzed (Erdtman 1960) previous to the processing for TEM to detect acetolysis-resistant structures. Anthers and pollen grains were examined with a JEOL-JEM 1200 Ex II TEM at 85 kV. Three measurements of the ektexine, endexine, and intine were

Table 1 List of the *Nymphaea* species studied and information about the samples used, fixation, and embedding methods

Species	Developmental stages	Fixation (microscopy)	Embedding	Locality and voucher
N. amazonum Mart. & Zucc. subsp. *pedersenii*	MMC—pollen grains	FAA (SEM, LM)	Paraffin	Argentine. Province of Corrientes; Department of San Martín; 28° 33′ 25″ S, 57° 12′ 2″ W, Zini et al.6
N. gardneriana Planch	MMC—pollen grains, and mature anther	FAA (SEM, LM)	Paraffin	Argentine. Province of Corrientes; Department of Empedrado; 27° 49′ 14.3″ S, 58° 46′ 25″ W, Zini et al.9
	MMC to pollen grains	1% glutaraldehyde, 4% formaldehyde in phosphate buffer, pH 7.2, 1.5% OsO_4 in the same buffer (TEM)	Resin	
	Acetolyzed mature pollen grains	1% glutaraldehyde, 4% formaldehyde in phosphate buffer, pH 7.2, 1.5% OsO_4 in the same buffer (TEM)	Resin	
N. prolifera Wiersema	Pollen grains	FAA (SEM) FAA—1.5% OsO_4 in phosphate buffer, pH 7.2 (TEM)	Resin	Argentine. Province of Formosa; Department of Laishi, Di Giacomo 563

obtained from available photomicrographs of unacetolyzed microspores and pollen grains.

Paraffin and resin sections were observed and photographed with a Leica DM LB2 compound light microscope (Leica, Wetzlar, Germany).

Mature anthers and pollen grains of all species were examined under scanning electron microscopy (SEM). The pollen grains of *N. amazonum* were previously acetolyzed. The samples were dehydrated by transferring through an acetone series, critical point dried, and then sputter coated with gold-palladium. The micrographs were obtained with a JEOL 5800 LV at 20 kV. The diameter of 20 orbicules and 20 pollen grains were measured in *N. amazonum* and *N. gardneriana* from available photomicrographs.

Results

The present results integrate LM and SEM observations of *N. amazonum* and LM, TEM, and SEM observations of *N. gardneriana*. TEM and SEM observations of pollen grains of *N. prolifera* are also provided. Four key ontogenetic stages are identified: microspore mother cell, tetrad, free microspore, and pollen grain.

1. Microspore mother cell stage

The anther consists of epidermis, endothecium, two or three middle layers, and tapetal cells disposed in one or two rows (Figs. 1a, b and 2a, b). Ultrastructurally, the cytoplasm of the tapetal cells is more electron-dense than that of the microspore mother cells (Fig. 3a) due to the presence of a great amount of rough endoplasmic reticulum and numerous ribosomes. Mitochondria, dictyosomes, plastids, and vacuoles are also observed. The tapetal cells are binucleated at this stage and in each cell, the inner tangential wall is notably thicker than in the other faces (Fig. 3a, b).

Cytoplasm of the microspore mother cells shows high metabolic activity, with numerous amyloplasts, mitochondria, lipid globules, dictyosomes, cisternae of rough endoplasmic reticulum, and small vacuoles (Fig. 3c). These cells are already rounded, even though the callosic wall is just beginning to form between the plasmalemma and the primary wall. There are cytoplasmic connections between neighboring microspore mother cells (Fig. 3a).

2. Tetrad stage

Simultaneous microsporogenesis is observed in *N. amazonum* and *N. gardneriana* (Figs. 1c, d and 2b). After meiosis completion, the tetrads of microspores are tetrahedral or occasionally isobilateral (Figs. 1e and 2c). The middle layer next to the tapetum starts to degenerate.

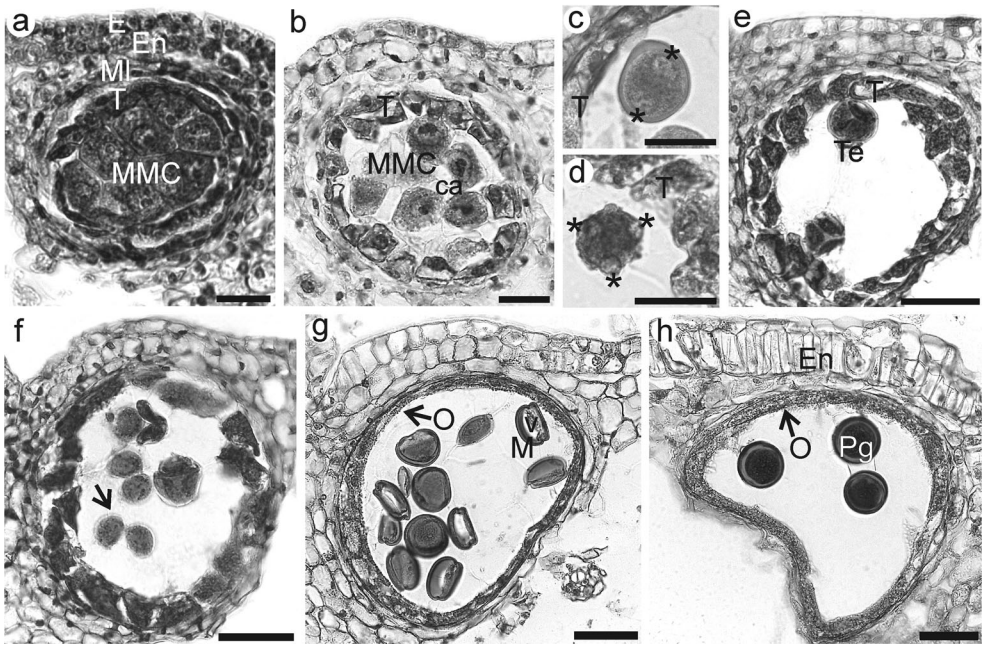

Fig. 1 LM images of the anther development in *Nymphaea amazonum*, with details of anther locules. **a** Anther at microspore mother cell stage showing young microspore mother cells (*MMC*), epidermis (*E*), endothecium (*En*), middle layers (*Ml*), and tapetum (*T*). **b** Mature MMC with callose wall (ca). **c** MMC in meiosis I; two nuclei are observed (*asterisks*). **d** Meiosis II, showing three nuclei of the future tetrad (*asterisks*). **e** Anther locule with tetrad of microspores (*Te*); note irregular boundaries of tapetal cells. **f** Anther locule at the end of the tetrad period, with some microspores partially free (*arrow*) and secretory tapetum. **g** Free microspores (*M*) with vacuole (*v*); note the reduced volume of tapetal cells and the orbicules lining the inner tapetal wall (*O*). **h** Successive stage of anther locule showing young pollen grains (*Pg*), endothecium with thin fibrillar thickenings, and degenerating tapetum with orbicules. *Scale bars* **a**–**d** = 20 μm and **e**–**h** = 40 μm

The tapetal cells are more elongated than in the previous stage; they exhibit irregular contour and in some places are slightly detached from the neighboring cells, but intrusion into the loculus is not observed (Figs. 1e and 2c). The mentioned characteristics of the tapetum are maintained up to the end of this stage (Fig. 1e). TEM images in *N. gardneriana* show that tapetal cells are in a very active phase, with cytoplasm predominantly containing rough endoplasmic reticulum, clusters of ribosomes, mitochondria, dictyosomes with peripheral vesicles, and small vacuoles (Fig. 3d, e). Many portions of cytoplasm are observed surrounded by electron-translucent structures, in some zones bound by two membranes (Fig. 3e). Synthesis of numerous corpuscles of medium electron density recognized as pro-orbicules or pro-Ubisch bodies occurs at this stage. The plasmalemma of tapetal cells has numerous invaginations; many pro-orbicules are already located in the space between the plasmalemma and the cell wall, on the inner tangential and radial faces. The cell wall has a lax appearance (Fig. 3e).

The tetrads are enclosed by a thick callose wall (Fig. 3f). Each microspore exhibits a primexine fibrillar matrix (glycocalyx) between the callose and the plasmalemma, except in the future aperture site, where it is interrupted. The distal primexine layer is much thinner than the proximal one (Fig. 3g, h). Some vesicles and membranous structures are found in the periplasmic space (Fig. 3g, h). The cytoplasm shows numerous active dictyosomes, vesicles, and abundant mitochondria (some of them in division) ribosomes, lipid globules, amyloplasts, and small vacuoles (Fig. 3f–h). The nucleus has undulating contour (Fig. 3g).

3. Free microspore

At the light microscopic level, the most important changes in the anther wall are radial elongation of endothecium cells, collapse of middle layers, decrease in cytoplasmic density and volume of tapetal cells along with increased of vacuoles, and appearance of orbicules lining the inner tangential walls (Figs. 1g and 2d, e). The free microspores become vacuolated; then, they increase cytoplasmic density (Figs. 1g and 2d, e). Ultrastructurally, the tapetal cells of *N. gardneriana* display signs of cell death, i.e., their organelles are quite less distinguishable; the cytoplasm is disorganized, highly vacuolated, and the wall is degraded; and only fibrillar remains can be observed (Fig. 4a). The developing orbicules lie on the inner tangential and radial surfaces of tapetal cells, and they are electron dense (Fig. 4a, b).

In *N. gardneriana*, the exine exhibits clear differences between the proximal and distal poles (Fig. 5a–c). The proximal pole has endexine lamellae, foot layer with some gaps,

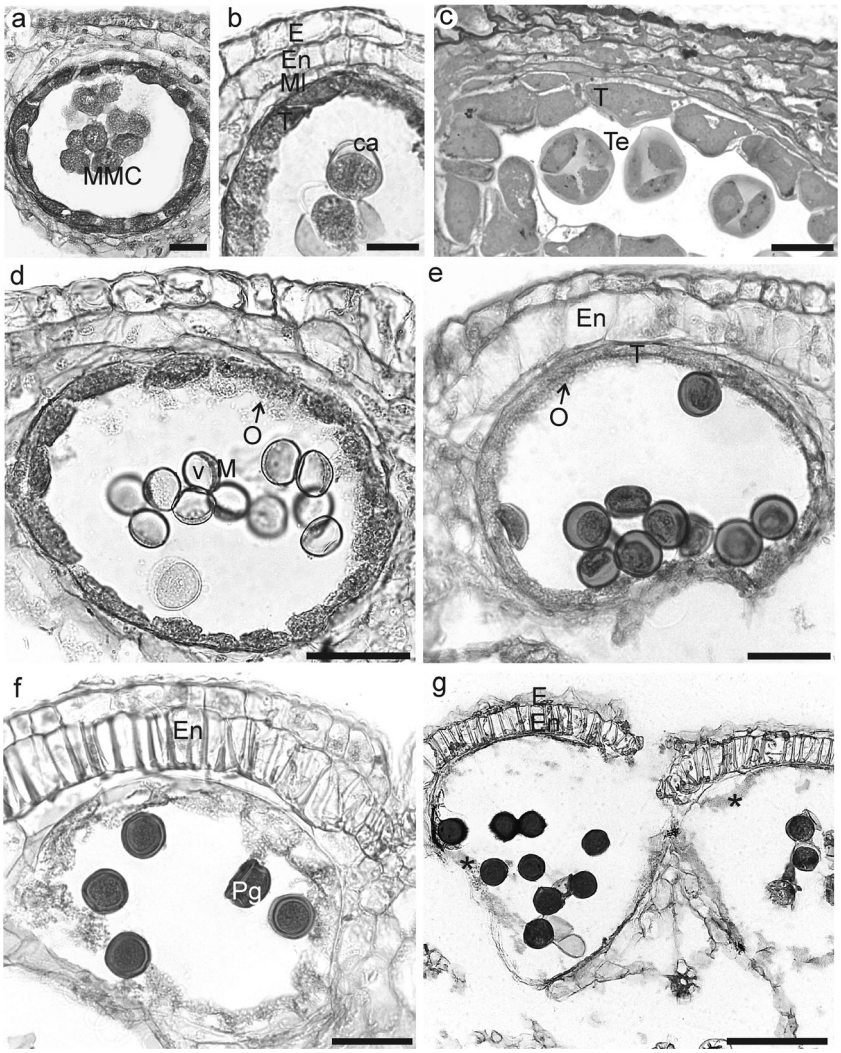

Fig. 2 LM images of the anther development in *Nymphaea gardneriana*, with details of anther locules. **a** Anther at microspore mother cell stage showing rounded microspore mother cells (*MMCs*) with scarce callose. **b** Detail of the epidermis (*E*), endothecium (*En*), middle layers (*Ml*), and tapetum (*T*); MMC with callose wall (ca) is in telophase of meiosis I. **c** Semithin section of anther locule at tetrad stage, showing tetrad of microspores (*Te*) and well-developed secretory tapetum layer. **d** Free microspores (*M*) with vacuole (*v*); note the tapetum with orbicules (*O*). **e** Successive stage of anther locule showing enlarged cells of the endothecium, degenerating tapetum with orbicules. **f** Anther locule with pollen grains (*Pg*), endothecium with fibrillar thickenings, and highly vacuolated tapetum. **g** Detail of dehiscent anther wall with only epidermis and endothecium; note tapetal remnants with orbicules (*asterisks*). *Scale bars* **a**–**c** = 20 μm, **d**–**f** = 40 μm, and **h** = 100 μm

infratectum, and tectum. The endexine presents white-line units intermingled with granules (Fig. 5a). Two zones are distinct in the infratectum. In the lower region, there are very short and thin columellae above the foot layer, whereas in the upper region, there are densely arranged granules (Fig. 5a).

In the distal pole, the lamellar endexine is thicker. Although the endexine looks compacted at its basis, the lamellae can also be observed; the inner end of the endexine is composed of a thin layer with only granular bodies (Fig. 5b). The tectum is at least four times thinner than in the proximal pole. Infratectum and foot layer are indistinct (Fig. 5b). Besides the invaginated plasma membrane, an electron-lucent material is deposited in the periplasmic space and is probably the incipient intine (Fig. 5a, b). The structure of the aperture area is similar to that of the distal pole in, but both regions are separated by a broader portion of ektexine. The cytoplasm of the microspores contains mostly mitochondria, dictyosomes, and endoplasmic reticulum. The nucleus is slightly undulated (Fig. 5c).

4. Pollen grain stage

The anther wall consists of compressed epidermis, endothecium with fibrillar thickenings in a U-shaped pattern, remnants of middle layers, and almost completely degenerated tapetal cells (Figs. 1h and 2f, g). Orbicules are fully formed and fill the tapetal membrane (Fig. 4c, d). These corpuscles are spherical or subspherical, with a smooth surface, and range between 0.2 and 0.7 μm in size. Ultrastructurally, the orbicules have a central core with moderate electron-density and a wall with the same electron density as that of the exine (Fig. 4d). Orbicules may either remain solitary or fuse into aggregates. They are resistant to acetolysis. After this process, the orbicule wall appears more electron-dense and the central core is electron-transparent (Fig. 4e).

Pollen grains of *N. amazonum*, *N. gardneriana*, and *N. prolifera* are heteropolar, medium-sized (polar diameter 22–36 μm, equatorial diameter 35–50 μm), oblate to suboblate, circular in polar view (Fig. 4f–i). The aperture

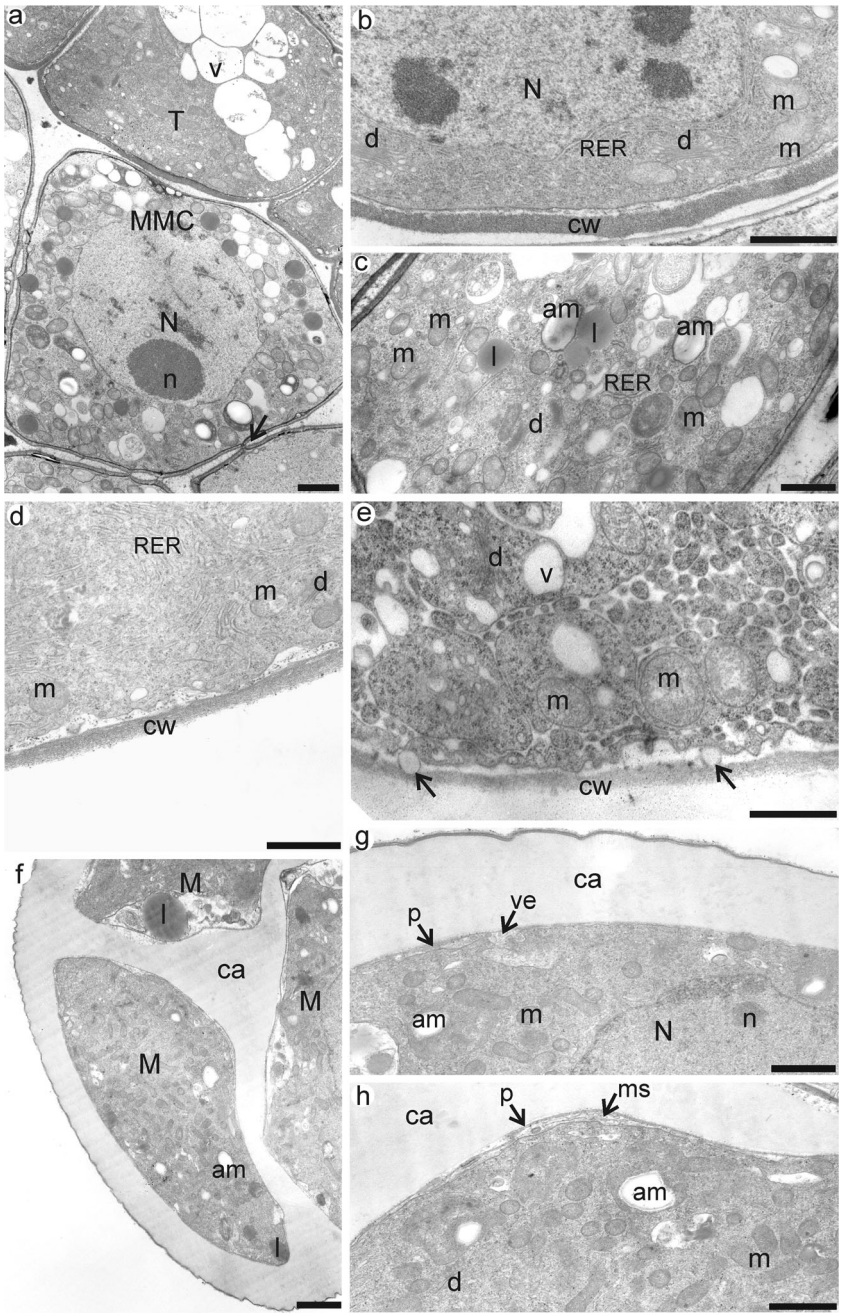

Fig. 3 TEM images of the microspore mother cell (**a**–**c**) and tetrad (**d**–**g**) stages in *N. gardneriana*. **a** General view of a tapetal cell and relatively young MMC; the *arrow* points to cytoplasmic connection between MMCs. **b** Cytoplasm of the tapetal cell in detail. **c** Cytoplasm of the MMC in detail. **d** Detail of the active tapetal cell cytoplasm. **e** Tapetal cell with pro-orbicules in the periplasmic space (*arrows*); note that the cell wall is less electron-dense than in **d**. **f** Detail of a tetraedric tetrad with thick callose wall. **g** Detail of microspore showing cytoplasm and primexine layer at the distal surface. **h** Detail of the cytoplasm and primexine at the proximal surface. Abbreviations: *am* amyloplast, *ca* callose, *cw* cell wall, *d* dictyosome, *l* lipid globule, *M* microspore, *m* mitochondrion, *MMC* microspore mother cell, *ms* membranous structure, *N* nucleus, *n* nucleoli, *p* primexine, *RER* rough endoplasmic reticulum, *T* tapetum, *v* vacuole, *ve* vesicles. *Scale bars* **a**–**e** and **g**–**h** = 1 μm and **f** = 2 μm

resembles the anazonasulculate type (Fig. 4f–i). The proximal pole is always convex, whereas the distal pole may be convex, flattened, or concave according to the hydration degree. The exine is mostly psilate, with the exception of the distal surface which is rugulate (Fig. 4g, i).

N. gardneriana and *N. prolifera* share the main characters of the pollen wall ultrastructure. Thickenings of the wall layers at microspore and pollen grain stages of *N. gardneriana* are compared in Table 2. In the proximal part of the pollen grain, the intine is very thin and the endexine looks homogeneous because individual lamellae are rarely visible. A thin electron-dense layer is observed at the basis of the endexine (Fig. 6a, b). The infratectum at the proximal region is more compact at this stage than at the microspore stage. The infratectal space contains granules of sporopollenin and complete or nearly complete columellae formed by the fusion of granules, or the entire columellae. Sometimes, adjacent columellae seem to merge (Fig. 6a–f).

The intine and endexine of the distal part are substantially thicker than those of the proximal part (Fig. 6c, d). The lamellae of the endexine can be recognized as white lines. The intine has two layers; the upper layer is immediately beneath the endexine and consists of membranous bodies with lumen of low electron density. The inner layer has lower contrast and

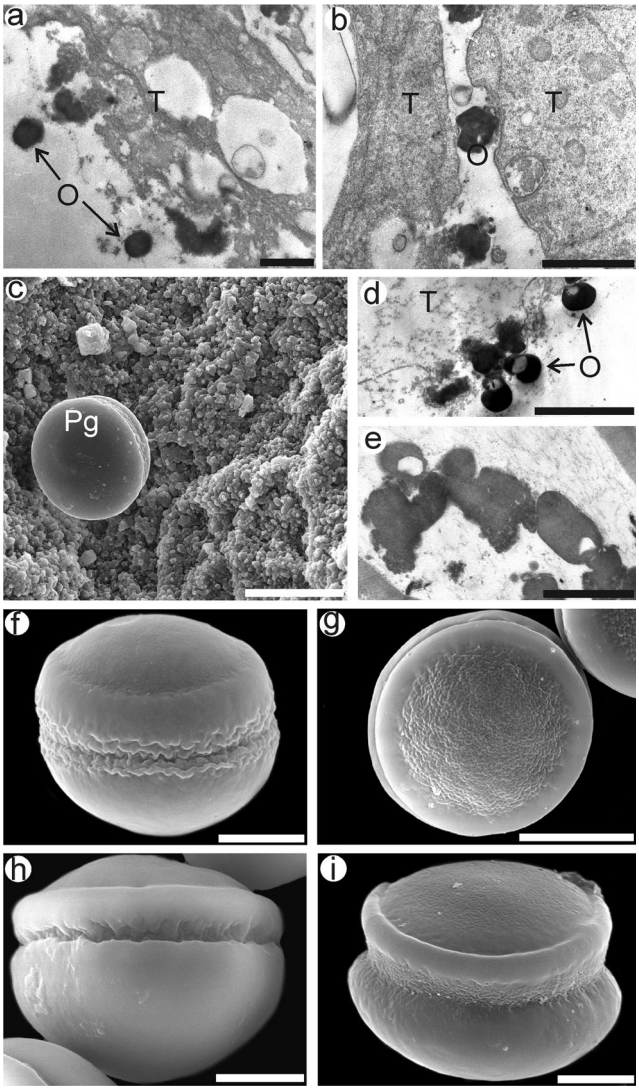

Fig. 4 TEM (**a**, **b**, **d**, **e**) and SEM (**c**, **f**–**i**) images of orbicules and pollen grains. *N. gardneriana* **a** Detail of a tapetal cell (*T*) and orbicules (*O*) at free microspore stage. **b** Detail of orbicules on the radial sides of two tapetal cells. **c** Great amount of orbicules filling tapetal membrane at the pollen grain (*Pg*) stage. **d** Three mature orbicules with central core on the degenerated tapetum at the pollen grain stage. **e** Group of orbicules after acetolysis. **f** Subequatorial view of a pollen grain; note the unequal sized halves and the psilate exine. **g** Distal polar view showing rugulate surface. *N. amazonum* **h** Equatorial view of an acetolyzed pollen grain. *N. prolifera* **i** Subequatorial view of unacetolyzed pollen grain. *Scale bars* **a**, **b**, **d**, **e** = 1 μm; **c** = 30 μm; **g** = 20 μm; **f**, **h**, ***i*** = 10 μm

consists of wavy microfibrils (Fig. 6c). The foot layer is mostly absent or very rudimentary in some regions. In addition to granular elements, the infratectum has an irregularly outlined columellae, which are thick and densely arranged (Fig. 6c, d). The tectum is relatively thin in this part; however, the ektexine is widened at the periphery (Fig. 6e), which is observed as a ridge around the perimeter of this hemisphere under the scanning electron microscope (Fig. 4f–i).

The apertural membrane consists of the reduced ektexine with a discontinuous tectum. The endexine in this region is more compact than the distal pole, and the intine has similar characteristics to the distal pole (Fig. 6e). In the vicinity of the apertural membrane, the electron-dense layer located between the endexine and the intine distends toward the distal region, with both layers of the intine becoming well distinguishable (Fig. 6f). Neither the intine nor the membranous layer is resistant to acetolysis (Fig. 6g, h). The tectum and foot layer of the whole grain are biased by many narrow channels, which are seen as electron-dense lines (Fig. 6a, d).

A generative cell and a vegetative cell are formed in the pollen grain of *N. gardneriana* (Fig. 7a, b). The generative cell has a sinuous wall transparent to electrons and a reduced cytoplasm, with some amyloplasts, plastids, and mitochondria (Fig. 7a, b). The vegetative cell cytoplasm contains amyloplasts, mitochondria, and rough endoplasmic reticulum (Fig. 7a).

Discussion

Pollen aperture

In *N. gardneriana* and *N. prolifera*, the proximal part of the pollen grain has a thick ektexine and relatively thin layers of endexine and intine, whereas the distal part has thin ektexine, very thin and discontinuous foot layer, and well-developed endexine and intine. Furness and Rudall (2003) evaluated operculate pollen in monocots and identified an operculum as an area of reduced exine (i.e., lack of thick foot layer in the non-apertural wall), which covers part of an aperture. Therefore, our transmission electron microscopy observations allow us to interpret the entire distal pole as an operculum in *Hydrocallis* and, therefore, to corroborate the initial assumption of Wiersema (1987) for this subgenus. Furness and Rudall (2003) also emphasize the thickest area of the intine as a key feature to determine the apertural zone in pollen grains. Furthermore, Hesse and Zetter (2005) and Weber and Ulrich (2010) state that in many angiosperms, a thickened endexine is characteristic of apertural regions, whereas in non-apertural parts, the endexine may be discontinuous, reduced, or totally absent. According to the criteria of those authors and own observations, the aperture in *Nymphaea* subgen. *Hydrocallis* can be regarded as a distally broad area that is almost completely covered by an operculum, and the apertural membrane can be considered an annular area in the aperture that is free of operculum. This notion has been proposed by several authors for *Nymphaea* (Wodehouse 1935; Erdtman et al. 1961; Jones and Clarke 1981; Ito 1984). The pollen grain aperture of *Nymphaea* is also interpreted as an encircling ring (zona-aperturate), which has probably been derived from a sulcus covered by an operculum (e.g., Meyer 1964; Walker 1974b; Hesse and Zetter 2005; Borsch et al. 2008; Taylor et al. 2015). The sulcate-operculate aperture

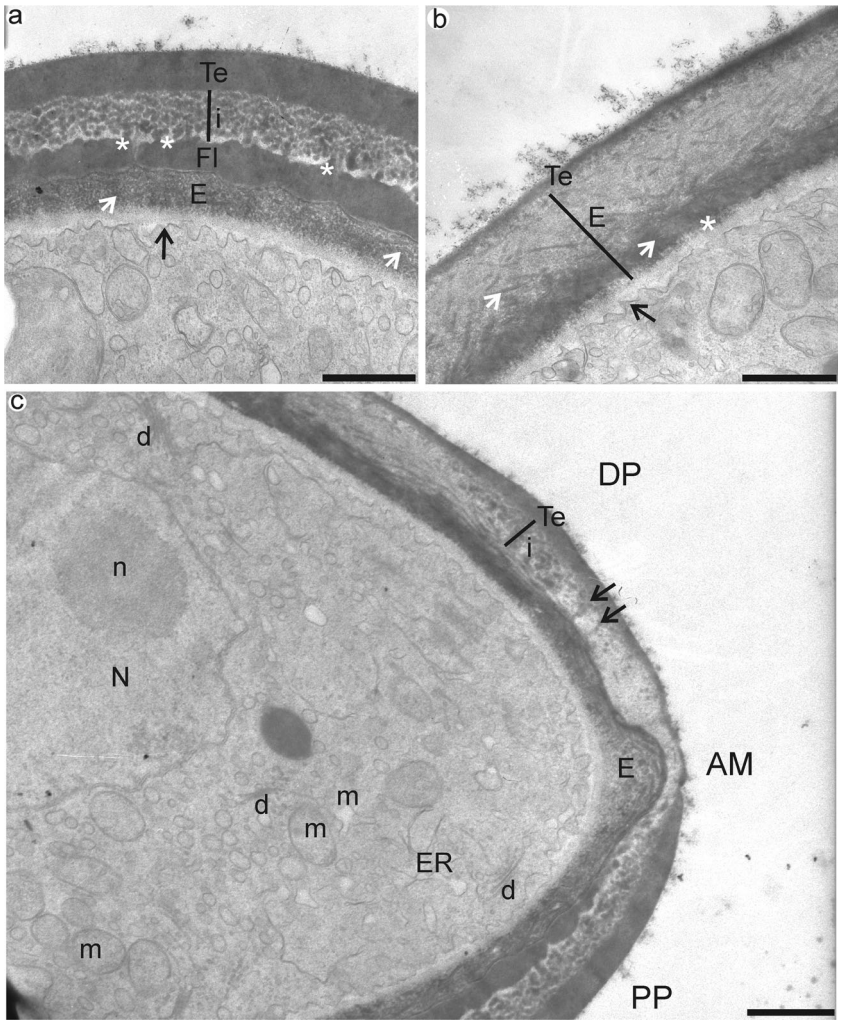

Fig. 5 TEM images of the free microspore stage in *N. gardneriana*. **a** Detail of the proximal pole with developing exine and incipient intine (*black arrow*). Note the infratectum with general granular appearance and some columellae basis (*asterisks*); the endexine has lamellae (*white arrows*). **b** Detail of the distal part of the microspore. Note thick endexine with lamellae (*white arrows*) and the basal-most part with granules only (*asterisk*); the incipient intine is in formation (*black arrow*). **c** Detail of cytoplasmic components and portion of the distal part (*DP*), apertural membrane (*AM*), and proximal part (*PP*) of a microspore. The zone with relatively well-developed infratectum and tectum at the distal part is showed; also, thin columellae are discernible (*double arrows*). *Abbreviations*: *d* dictyosome, *E* endexine, *ER* endoplasmic reticulum, *FL* foot layer, *I* infratectum, *m* mitochondrion, *N* nucleus, *n* nucleoli, *Te* tectum. *Scale bars* = 1 μm

has been recently confirmed for *Nuphar* Sm., with such aperture being supported as the plesiomorphic condition for the family (Coiro and Barone Lumaga 2013).

The considerable structural variation between the distal and proximal walls found in *N. gardneriana* and *N. prolifera* was previously identified in *Barclaya* Wall. (Taylor et al. 2015), *Nymphaea* subgenera *Nymphaea* (*N. candida* C. Presl; Meyer-Melikian and Diamondopulu 1996; *N. mexicana* Zucc.; Gabarayeva and El-Ghazaly 1997; *N. alba* L.; Coiro and Barone Lumaga 2013; *N. odorata* Aiton; Taylor et al. 2015), *Anecphya* (*N. ondinea*; Taylor et al. 2015), and *Brachyceras* (*N. colorata* Peter; Gabarayeva and Rowley 1994). In these species, a thick endexine and intine at the apertural and distal regions resemble the operculate aperture.

Table 2 Mean thickness (in nanometers) of the pollen wall layers in both poles at the microspore and pollen grain stages of *N. gardneriana*

Layers	Distal wall		Proximal wall	
	Free microspore	Pollen grain	Free microspore	Pollen grain
Tectum	102.4 (281.5)	122.6 (500)	406	410
Infratectum	–	100	526	299
Foot	Absent	Absent	432	520
Endexine	1300	600	420	380
Intine	–	1300 (2000)	–	860

The values for the tectum and intine given in brackets indicate the thickness in the flange observed in the distal pole

– indicates that the thickness cannot be measured

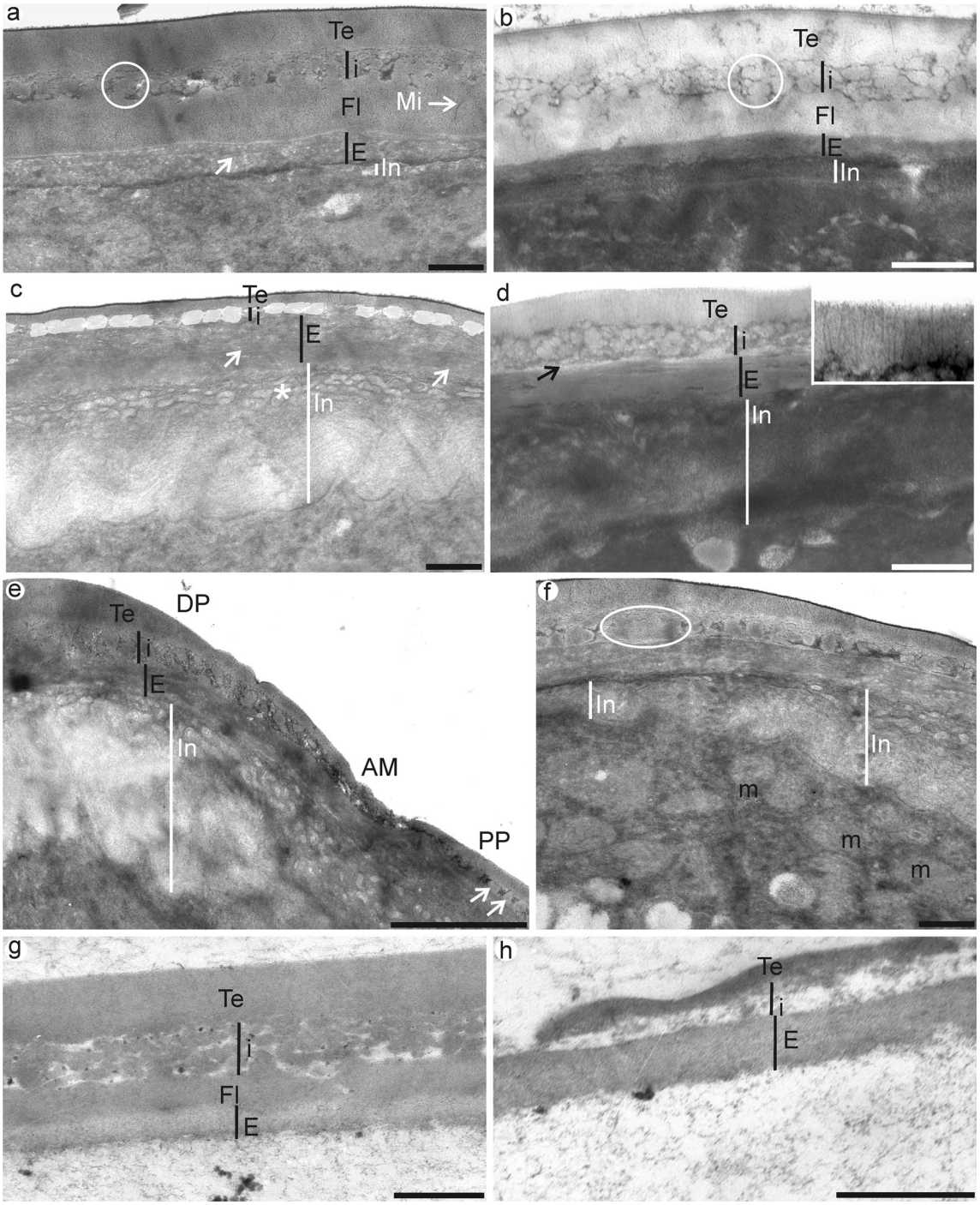

Fig. 6 TEM images of the mature pollen grains of *N. gardneriana* (**a**, **c**, **e**–**j**) and *N. prolifera* (**b**, **d**). **a**, **b** Proximal pollen wall. The endexine is lamellate (*white arrow* in **a**) and no typical columellae are seen. Agglomerated granules forming columellate-like elements are marked with circles. **c**, **d** Distal pollen wall. In **c**, infratectum with complete and robust columellae were *shaded*. The lamellate endexine (*white arrows*) and the intine with membranous granular layer (*asterisk*) above the fibrillar layer are also showed. In **d,** note a thin discontinuous foot layer (*arrow*) and a magnified fragment in the upper right of the image showing the microchannels. **e** Detail of pollen grain showing a zone of the distal part (*DP*), the apertural membrane (*AM*), and a zone of the proximal part (*PP*). Some complete columellae are also indicated (*arrows*). **f** Detail of the pollen wall and cytoplasm in the transitional zone between the proximal part (*left*) and the apertural membrane (*right*). Note the fused sporopollenin elements (*oval*). **g**, **h** Two acetolyzed grains showing the resistance of ektexine and endexine. *Abbreviations*: *am* amyloplasts, *E* endexine, *Fl* foot layer, *I* infratectum, *In* intine, *m* mitochondrion, *Mi* microchannel, *Te* tectum. *Scale bars* **a**–**d** and **f**–**h** = 500 nm; **e** = 2 μm

Unlike in both species studied, there are no remarkable structural wall differences between the distal and proximal hemispheres in *N. caerulea* subgen. *Brachyceras* (Coiro and Barone Lumaga 2013), *Euryale*, and *Victoria* (Meyer-

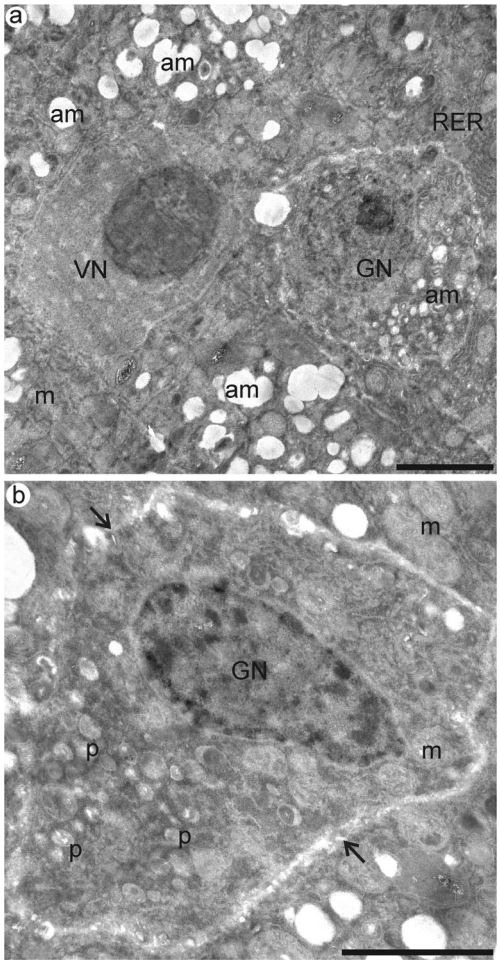

Fig. 7 TEM images of mature pollen grain stage of *N. gardneriana.* **a** Detail of cytoplasm with vegetative nuclei (*VN*) and generative cell with generative nucleus (*GN*). **b** Detail of generative cell; the wall transparent to electrons is indicated (*arrows*). *Abbreviations*: *am* amyloplasts, *m* mitochondrion, *p* plastids, *RER* rough endoplasmic reticulum. *Scale bars* = 2 μm

Melikian and Diamondopulu 1996; Taylor et al. 2015). The presence of a thick intine only under the apertural membrane defines the truly zonasulculate or ring-shaped nature of the aperture, but there are reminiscent characteristics of the plesiomorphic state, such as a thicker endexine at the distal pole. Borsch et al. (2008) assume that the aperture is ring-like in the whole genus *Nymphaea* because the encircling band serves for germinating pollen tube exit and the original operculum has lost its apertural role. This question should be further addressed in all subgenera.

The operculate nature of the distal hemisphere might be plesiomorphic for Nymphaeoideae because there is a structural correspondence of the pollen wall among *Barclaya* (Taylor et al. 2015), the subgenera *Nymphaea* (Meyer-Melikian and Diamondopulu 1996; Gabarayeva and El-Ghazaly 1997; Coiro and Barone Lumaga 2013; Taylor et al. 2015) and *Hydrocallis* (present study). All these taxa share characteristics concerning the thickness of the ektexine, endexine, and intine in the distal wall, and they exhibit similar components of the apertural membrane (i.e., reduced ektexine, endexine, membranous granular layer, intine), which is oriented toward the distal pole. Furthermore, the results support that most Nymphaeaceae conserve the operculate condition. Previous studies have evidenced that this structure was completely lost at least more than once, in the ancestor of *Euryale* + *Victoria* and within the subgenus *Brachyceras* (Borsch et al. 2008; Taylor et al. 2015). The evolutionary shifts are equally parsimonious in both scenarios of the monophyletic and paraphyletic genus *Nymphaea*; hence, the character is not significant for the systematics.

Microchannels of the ektexine

In *N. gardneriana* and *N. prolifera*, the tectum and foot layer of the pollen grains are dissected by many narrow, electron-dense microchannels. Similar channels were reported in mature pollen of *Euryale*, *Nuphar*, some *Nymphaea*, and *Victoria* (revised in Taylor et al. 2015). The microchannels of *N. gardneriana* and *N. prolifera* are morphologically similar to those of *N. mexicana* (subgen. *Nymphaea*), but in the latter species, these channels are indistinct at the pollen grain stage (Gabarayeva and El-Ghazaly 1997). In contrast, in *N. capensis* Thunb. and *N. colorata* (subgen. *Brachyceras*), the channels consist of broader electron-lucent gaps and are persistent (Gabarayeva and Rowley 1994; Gabarayeva et al. 2001). Wide microchannels are also present in close relatives *Cabomba* Aubl. (Cabombaceae) and *Trithuria* Hook. f. (Hydatellaceae) of the order Nymphaeales (Galati 1985; Osborn et al. 1991; Gabarayeva et al. 2003; Remizowa et al. 2008; Taylor et al. 2008). Discontinuous exine was found in many other taxa of distant phylogenetic positions, such as *Betula* L., *Brachypodium* P. Beauv. (Sharma et al. 2015), *Chenopodium* L. (Rowley et al. 1987), *Plantago* L. (Gabarayeva et al. 2016), and *Zea* L. (Tsou et al. 2015). Rowley et al. (1987, 2003) demonstrate that they serve as a route of transport of material (derived from tapetum) from the locular space to developing microspore cytoplasm. For some species with persistent microchannels, these discontinuities could provide an additional advantage by increasing the flexibility of the exine, which therefore relieves the tensions caused by changes in the cytoplasm volume during the harmomegathic mechanism.

Infratectum

The infratectum in Nymphaeaceae is subject to different interpretations. We observed that the mature infratectum of *N. gardneriana* and *N. prolifera* exhibits both columellar and granular elements. In previous studies, the infratectum of *Nymphaea* was characterized as being basically of the columellate type due to the formation of probacula in developing microspores, in which irregular and robust columellae

are later differentiated (Gabarayeva and Rowley 1994; Gabarayeva and El-Ghazaly 1997). According to Gabarayeva and Rowley (1994), the presence of granules in the aperture zone of *N. colorata* results from oblique sections of the sample or cross sections of thin (juvenile) columellae, whereas granules of sporopollenin are observed in the entire pollen wall, including the distal and proximal poles (Fig. 55–59). On the other hand, Gabarayeva and El-Ghazaly (1997) and later El-Ghazaly and Huysmans (2001) recognize granular and rod-like columellae at both poles of the microspores and pollen grains in *N. mexicana*. In addition, TEM images of Taylor et al. (2015) show atypical columellae for mature pollen grains of *Euryale* and some *Nymphaea* species.

Because both granules and columellae have a common developmental basis and the granular exine is actually considered a derived state at the level of angiosperms, Doyle (2009) postulates that the mature appearance of the infratectum has systematic value within angiosperms. Therefore, in cases where granules and columellae coexist, infratectum should be considered an intermediate state. An intermediate infratectum is reconstructed to have originated in the common ancestor of all Nymphaeaceae (Borsch et al. 2008). Disturbed and fragmented columellae were also reported for *C. aquatica* Aubl. (Cabombaceae) (Gabarayeva et al. 2003). Coiro and Barone Lumaga (2013) recognize the intermediate infratectum for *Nuphar lutea* (L.) Sm. and *Victoria cruziana* Orb. Taylor et al. (2013, 2015) explain this enigmatic appearance in *V. cruziana* given by increased thickness of the tectum and foot layer during the microspore stage, which generates compression of the infratectal space, thereby restricting columellae development to short and globular elements. A similar phenomenon probably occurred in the proximal pole of *N. gardneriana*, where it was noticed that infratectal space decreases and the presence of complete columellae was rare. However, granular elements and robust columellae consisting of aggregated granules are also formed in the distal and transitional regions of the pollen grains, where no evidence of compression was noticed.

Membranous granular layer

This component of the pollen wall was reported for all the genera of the family (Taylor et al. 2015). A similar layer is observed in the basal angiosperm *Austrobaileya* C.T. White (Zavada 1984) and was cited in several magnoliids, monocots, and eudicots (Skvarla and Larson 1966; Echlin and Godwin 1969; Kreunen and Osborn 1999; see El-Ghazaly and Huysmans 2001; Galati et al. 2012). Some authors interpret the MGL layer as belonging to the endexine (e.g., Osborn 2000; Gabarayeva and Hemsley 2006; Taylor et al. 2015), whereas others, to the intine (e.g., Gabarayeva 1991, 1996; Xu and Kirchoff 2008; Gabarayeva and Grigorjeva 2012; Galati et al. 2012). El-Ghazaly and Huysmans (2001), however, do not attribute the MGL to either the endexine or the intine. According to these authors, the MGL exhibits distinct ontogeny and staining properties from those of the endexine and intine; this layer has granular electron-dense elements or occasionally has a vesicular appearance. The membranous granular layer resists acetolysis in *Betula*, *Rondeletia* L., *Triticum* L., and *Victoria cruziana* (Roland 1965; El-Ghazaly and Huysmans 2001; El-Ghazaly et al. 2001), but not in *Magnolia* L. (Gabarayeva and Grigorjeva 2012; Galati et al. 2012).

The layer of roundish granules in *V. cruziana* previously described by Roland (1965) as granular endexine is in fact the MGL. The MGL appeared of endexinous nature in the pollen grains of the Nymphaeaceae studied to date (Taylor et al. 2015). In *N. mexicana*, the MGL develops concomitantly with the endexine at the free microspore stage (El-Ghazaly and Huysmans 2001; Figs. 11 and 12) whereas in *N. gardneriana*, the layer is still not formed at such stage. Furthermore, the MGL of the species here analyzed is most probably a component of the intine because it was observed at the pollen grain stage and was lost after acetolysis.

The present study agrees with the concept of El-Ghazaly and Huysmans (2001) in that MGL always occupies the same topographical position (i.e., between intine and endexine). However, the characteristics of this layer, such as thickness, electron density, and persistence during pollen ontogeny, evidently vary among species. In addition, the resistance or lack of resistance to acetolysis is indicating variation in its composition; therefore, the homology of the layer for the Nymphaeaceae as well as for angiosperms is still not resolved. In *Plantago major* L., *Dianthus deltoides* L., *Chamaedorea microspadix* Burret (Arecaceae) and in most Magnoliaceae, the development of an MGL (granular or channeled intine-1), corresponds to a reiteration of mesophase of cylindrical and spherical glycoprotein micelles, respectively, where then sporopollenin accumulates on them (Gabarayeva and Grigorjeva 2010; Gabarayeva et al. 2016; Grigorjeva and Gabarayeva 2015). Therefore, the development and variable characteristics of the MGL in many angiosperms may be better understood in future works focusing on the micellar self-assembly events in the process of sporoderm development.

Tapetum type and orbicules

The tapetum in *N. amazonum* and *N. gardneriana* is of the secretory type. The secretory tapetum consists of a discrete layer around each locule of the anther that retains its integrity during the whole active phase, only degenerating at a relatively late stage of pollen development (Chapman 1987; Pacini and Franchi 1993; Pacini et al. 1985; Pacini 1990). In *N. gardneriana*, there are signs of programmed cell death in the tetrad stage because numerous portions of tapetal cytoplasm are observed surrounded by electron translucent structures, some of them bound by two membranes. According to Papini et al. (2013), these ultrastructural characteristics can be

considered as a classical plant macroautophagy. In numerous clades of angiosperms, the secretory tapetum evolved toward amoeboidal or invasive non-sincitial types, maximizing its contact with developing microspores (Pacini et al. 1985; Furness 2008). Most species of Nymphaeales studied to date exhibit a secretory tapetum (Khanna 1964, 1965, 1967; Galati 1985; Gabarayeva et al. 2001; Taylor and Osborn 2006; Zhou and Fu 2008; Dai and Zhou 2010; Taylor et al. 2012). Therefore, the present study supports the predominance of the secretory tapetum in basal angiosperms and this type is shared with their antophyte ancestors (Furness and Rudall 2001). *N. colorata* (subgen. *Brachyceras*) and *N. mexicana* (subgen. *Nymphaea*) are exceptions to this general concept, since they exhibit a cyclic invasive tapetum at the tetrad and microspore vacuolate periods: tapetal cells elongate strongly, after that, they move toward the loculus, and then these cells retract to the initial parietal position (Rowley et al. 1992; Rowley 1993; Gabarayeva and El-Ghazaly 1997). These events were not observed in the present study. In addition, the tapetum of *Victoria* is viewed as of transitional type between secretory and invasive types, and the tapetum of *C. caroliniana* A. Gray (Cabombaceae) as invasive although retaining some characteristics of the secretory type (Taylor et al. 2008, 2012). However, Galati (1985) described the tapetum of *C. australis* Speg. (= *C. caroliniana*) as exclusively of secretory type. Variations in the secretory tapetal behavior evidently exist for Nymphaeales, but the cases abovementioned should not be considered a new, intermediate tapetum type. By definition, the secretory tapetum maintains its relative position in the boundary of the loculus, but this does not imply that it is a static tissue; changes in shape and volume, such as enlargement of cells that enhance the contact with microspores, radial separation between tapetal cells, and presence of irregular boundaries, are commonly observed and can be considered events related to their basic function followed by the degenerative process. Pacini (1997) mentions the eventual existence of cycles of hyperactivity in certain species with parietal tapetum, in which some tapetum cells penetrate deep into the locular cavity or show bulges toward loculus, increasing and decreasing in volume.

Orbicules or Ubisch bodies attached to the tapetal membrane were observed in *N. amazonum* and *N. gardneriana*. In accordance with previous contributions, these corpuscles develop simultaneously with the formation of the pollen exine and are products of the tapetal cell activity, with the rough endoplasmic reticulum involved in its synthesis (e.g., Pacini 1997; Galati 2003). The resistance of orbicules to acetolysis confirms the sporopolleninic nature of their wall. Whichever the tapetum type, secretory or invasive, orbicules were reported in other taxa of Nymphaeaceae, such as *N. capensis* (Gabarayeva et al. 2001), *N. colorata* (Rowley et al. 1992), *N. mexicana* (Gabarayeva and El-Ghazaly 1997), *N. lotus*, *N. prolifera* (Zini, SEM observations, unpublished), and both species of *Victoria* (Taylor et al. 2012, 2013).

Conclusions

The species studied of the subgenus *Hydrocallis* exhibit plesiomorphic states expected for the subfamily Nymphaeoideae (*Euryale*, *Nymphaea*, and *Victoria*), such as secretory tapetum, exine differentiation associated with the operculate condition, and presence of orbicules. We found that the structural differentiation of the pollen wall between both hemispheres has no implication to resolve the phylogenetic relationships in *Nymphaea*. A complex architecture of the infratectum for the genus is reinforced in this study: *N. gardneriana* and *N. prolifera* have an infratectum of intermediate organization. Exine microchannels persist at the last stage of pollen development. The MGL is a common character in the pollen grains of Nymphaeaceae. However, it has been found to be not only a component of the endexine but also an intine precursor. Therefore, this trait should be carefully analyzed in other species, as well as its phylogenetic implication. Previous studies and new observations on the ultrastructure of orbicules in the family lead to more complete characterization of them as spherical or subspherical, with an outer sporopolleninic wall of smooth surface that surrounds an electron-lucent core and with individual orbicules that usually merge to give irregular aggregations. Further ultrastructural research is now required specially in *Barclaya* and *Nymphaea* to improve our understanding of pollen grains and tapetum diversity in the Nymphaeaceae.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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