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Ultrastructural study of the female gametophyte and the epistase in Cabombaceae and Nymphaeaceae

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ABSTRACT

Ultrastructural studies on the female gametophyte are restricted to species at relatively derived positions in the angiosperm phylogenetic tree. Therefore, this topic remains mostly unknown for the early-divergent lineages, in which a four-celled megagametophyte is common. Here, ultrastructure of the megagametophyte and micropylar nucellar epidermis was investigated in *Cabomba caroliniana* A. Gray (Cabombaceae), *Nymphaea gardneriana* Planch. and *Victoria cruziana* Orb. (Nymphaeaceae). The micropylar nucellar epidermis of the studied species differentiates into an epistase. These cells have metabolically active cytoplasm and thickened inner tangential walls. Epistase ultrastructure is compatible with a transfer cell specialization. This tissue may play an adaptive role in the secretion of chemotropic substances to direct the pollen tube growth toward the female gametophyte. The cytological characteristics of the female germ unit in members of Cabombaceae and Nymphaeaceae are generally similar to other angiosperms that develop a typical seven-celled, eight-nucleate female gametophyte; however, they differ in some specific points. In *V. cruziana* and *N. gardneriana*, the micropylar end of the synergids develops a rudimentary filiform apparatus with slight inward projections. By contrast, the synergids lack a filiform apparatus in *C. caroliniana.* Unlike most studied angiosperms, the filiform apparatus in the clade Cabombaceae-Nymphaeaceae is underdeveloped or absent, therefore character state transformations have occurred within basal angiosperms. The potential evolutionary shifts of this reproductive feature are highlighted.

1. Introduction

Ultrastructural studies on the female gametophyte have been conducted among species occupying relatively high positions in the phylogenetic tree of extant angiosperms, i.e., Malvaceae (Jensen, 1965), Asteraceae (Newcomb, 1973), Solanaceae (Mogensen and Suthar, 1979), Fabaceae (Folsom and Peterson, 1984; Folsom and Cass, 1989; Galati et al., 2006), Passifloraceae (Amela García et al., 2003), Poaceae (Jane, 1997; Lovisolo and Galati, 2007) and Amaranthaceae (Coimbra and Salema, 1999). Those studies contributed with highly valuable knowledge, providing clues into the crucial aspects of the female gametophyte at all stages of the reproductive process. The female germ unit, defined as the minimum number of cells required for double fertilization, is composed of the egg cell, the central cell, and the synergids (Huang and Russell, 1992). The components of the female germ unit have distinctive structures that are closely related to their crucial functions. For example, the ultrastructural organization of the synergids, cells that were described as metabolically active, among other features, were experimentally demonstrated in recent years to be linked with the secretion of chemicals for the attraction of pollen tubes (Higashiyama et al., 2001, 2003). Moreover, the cell wall of the two synergid cells is typically thickened at the micropylar end to form the filiform apparatus, which enables the transport of metabolites or chemo-attractants into the female gametophyte (Jensen, 1965; Jensen, 1973; Tilton and Lersten, 1981; Huang and Russell, 1992; Higashiyama, 2002; Higashiyama et al., 2003; Punwani and Drews, 2008).

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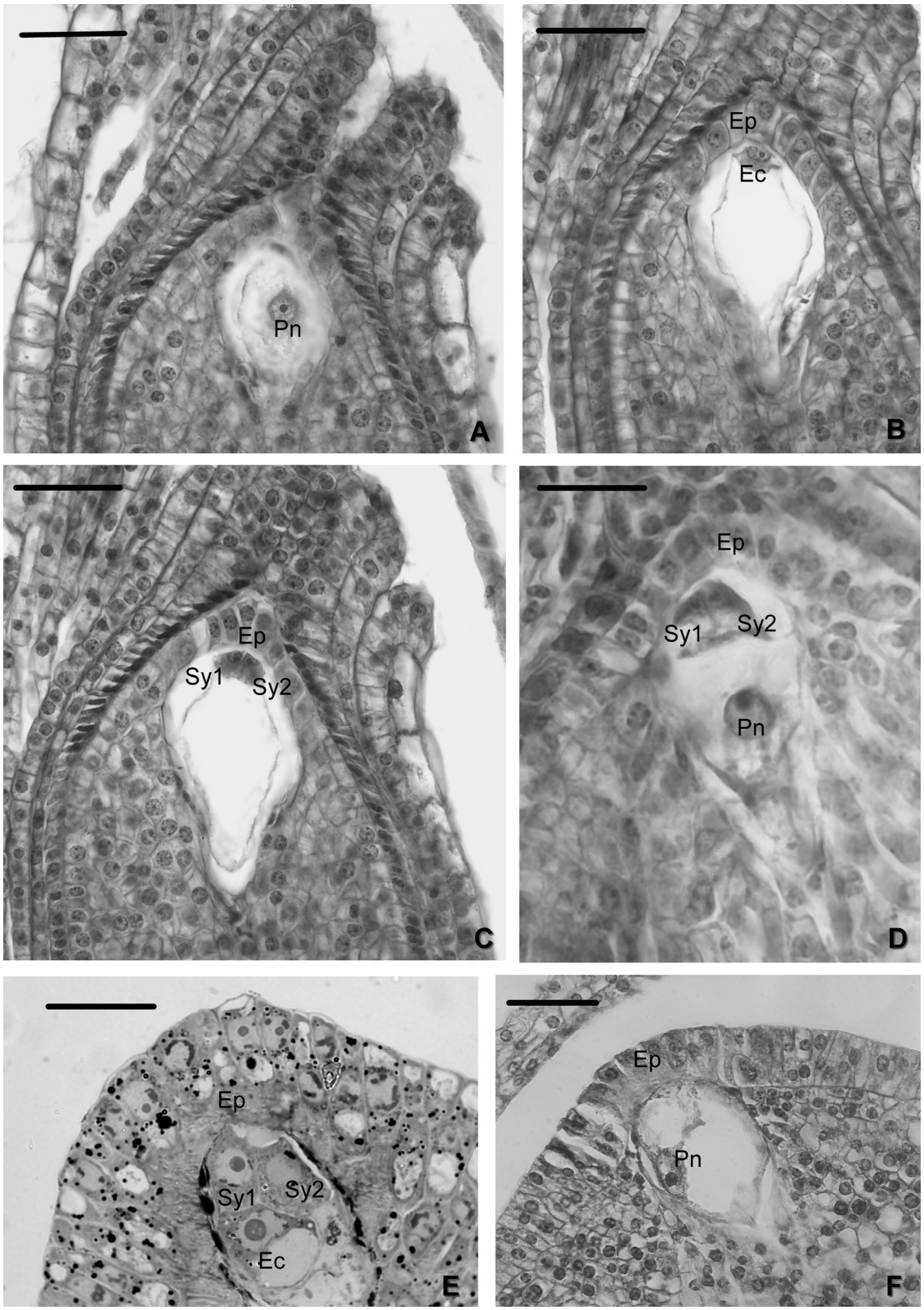

Fig. 1. Structure of the female gametophyte in Cabombaceae and Nymphaeaceae with bright field microscopy. *Cabomba caroliniana.* (A–C) Three serial sections showing the central cell with a polar nucleus (Pn), egg cell (Ec), the two synergids (Sy1 and Sy2), and also the cells forming the epistase (Ep). *Nymphaea gardneriana.* (D) Detail of the micropylar apex of the ovule, showing the epistase (Ep), the two synergids (Sy1 and Sy2) and the central cell with a polar nucleus (Pn). *Victoria cruziana.* (E) Section showing detail of the epistase (Ep) and the egg apparatus with the synergids (Sy1 and Sy2) and the egg cell (Ec). (F) Detail of the polar nucleus (Pn). Scale bars: (A–C) 30 μm, (D, E) 25 μm, (F) 50 μm.

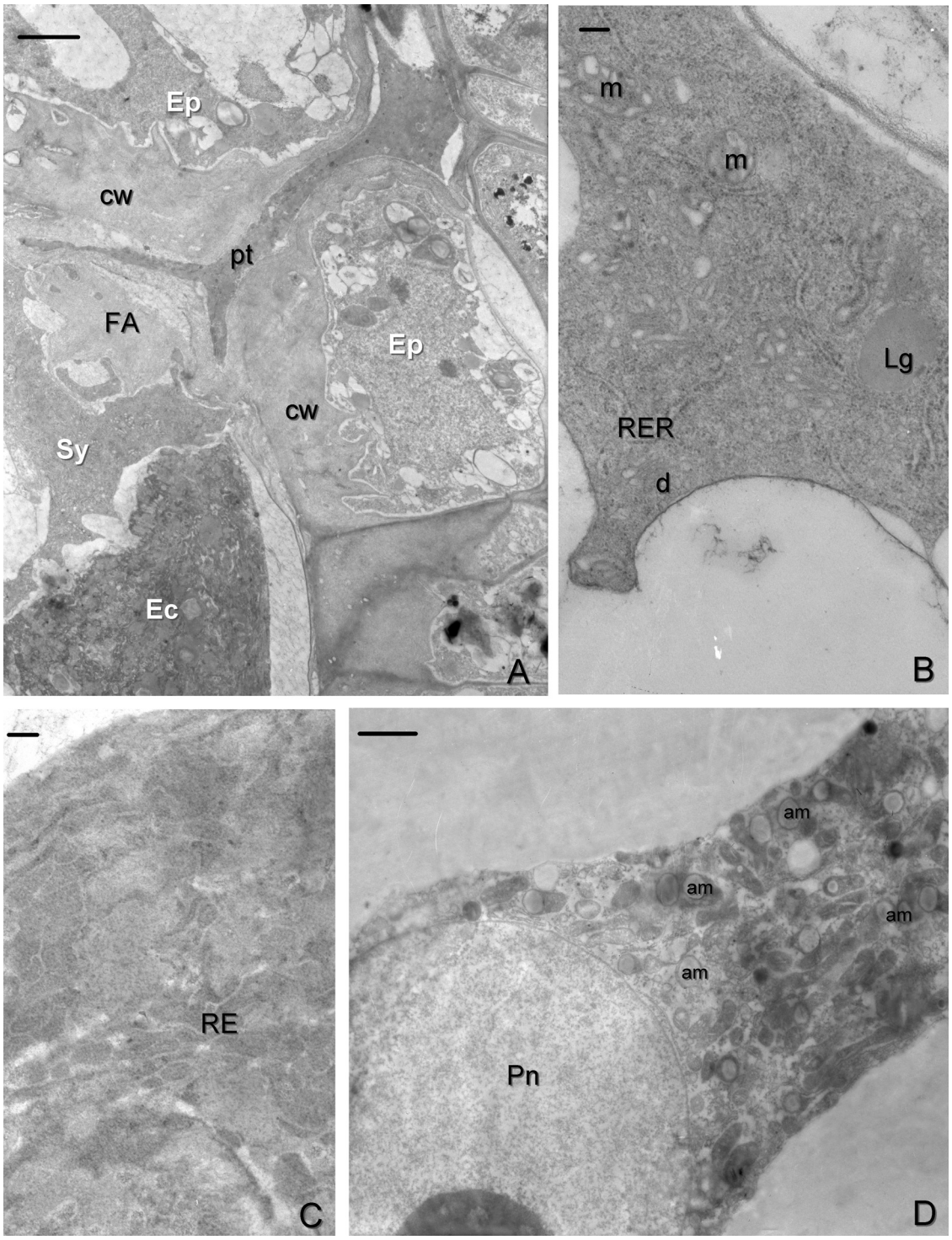

Fig. 2. *Nymphaea gardneriana*, ultrastructure of epistase and female gametophyte. (A) Details of the epistase with conspicuous ingrowths of the cell walls (cw); pollen tube (pt) growing between nucellus cells and reaching the tip of filiform apparatus (Fa) of a synergid (Sy); the egg cell (Ec). (B) Detail of the cytoplasm of a synergid. (C) Cytoplasm of the egg cell appears disorganized and organelles are almost indiscernible. (D) Detail of the central cell with polar nucleus (Pn) and cytoplasm full of amyloplasts (am). Abbreviations: d: Dyctiosome, Lg: lipid globules, m: mitochondria, RER: rough endoplasmic reticulum. Scale bars: (A, D) 2 μm, (B, C) 0.2 μm.

The order Nymphaeales, along with Amborellales and Austrobaileyales, is one of the three lineages that have diverged prior origin of main groups of other extant angiosperms (monocots, magnoliids and eudicots) (APG III, 2009). Nymphaeales comprises the families Nymphaeaceae (*Nuphar* Sm., *Barclaya* Wall., *Nymphaea* L., *Euryale* Salisb., and *Victoria* Buc'hoz), Cabombaceae (*Cabomba* Aubl. and *Brasenia* Schreb.) (Ito, 1987; Les et al., 1999; Podoplelova and Ryzhakov, 2005) and Hydatellaceae, which was recently placed as the sister group of the clade Cabombaceae-Nymphaeaceae by molecular phylogenetic analyzes (Saarela et al., 2007). The early-divergent angiosperms are of great interest to the study of ancestral states and diversification of reproductive traits in extant flowering plants (Friedman, 2001). Developmental and structural studies of the female gametophyte in these clades of angiosperms are

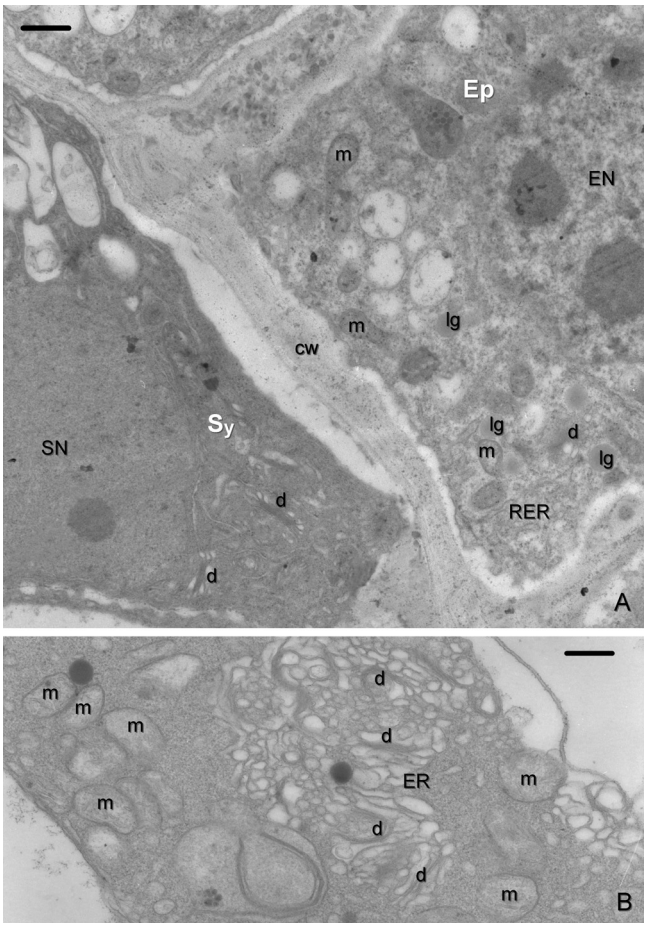

Fig. 3. *Cabomba caroliniana*, ultrastructure of epistase and female gametophyte. (A) Detail of a cell of the epistase (Ep) and a synergid (Sy). (B) Detail of cytoplasm of egg cell, showing dyctiosomes (d), endoplasmic reticulum (ER), and mitochondria (m). Abbreviations: cw: cell wall, EN: epistase nucleus, lg: lipid globules, RER: rough endoplasmic reticulum, SN: synergid nucleus. Scale bars: (A) 1 μm, (B) 0.5 μm.

confined to descriptions based on light microscopy, and very few transmission electron microscopy investigations were conducted (Friedman, 2006; Friedman and Ryerson, 2009).

Species of the Austrobaileyales and Nymphaeales have a four-celled and four-nucleate female gametophyte consisting of only the female germ unit (Galati, 1985; Battaglia, 1986; Winter and Shamrov, 1991; Orban and Bouharmont, 1998; Friedman et al., 2003; Williams and Friedman, 2004; Tobe et al., 2007; Friedman, 2008; Rudall et al., 2008; Zini et al., 2015; Povilus et al., 2015). The female gametophyte of these orders follows a common ontogenetic pattern that receives the denomination of Schisandra type because it was described for first time in species of Schisandra (Schisandraceae, Austrobaileyales) by Batygina et al. (1980) (Zini et al., 2015). The same pattern of development was referred as variant of Oenothera type in Cabombaceae (Galati, 1985), and as variant of Polygonum type (Orban and Bouharmont, 1998) or Nuphar type in species of Nymphaeaceae (Friedman and Williams, 2003). However, according to the priority, the denomination of Schisandra type of female gametophyte should be validated to both Nymphaeales and Austrobaileyales (Tobe et al., 2007; Zini et al., 2015), in the same way that the Polygonum type is applied to numerous orders and families of angiosperms.

Although modern embryological studies have been documented on basal angiosperms, there is no ultrastructural knowledge of female germ unit in the Nymphaeales. *Amborella* Baill., the only early-divergent taxon that has been examined at the ultrastructure level, produces a unique female gametophyte, eight-celled, nine-nucleate (Friedman, 2006; Friedman and Ryerson, 2009). The evolutionary analyzes of the female gametophyte reveals that the ancestral condition for angiosperms has not been resolved because any of the eight-celled (Amborella type), seven-celled (Polygonum type) or four-celled (Schisandra type) female gametophyte could be considered plesiomorphic. However, strong arguments are presented besides the grounds of parsimony to suggest that the four-celled and four-nucleate female gametophyte should be interpreted as the ancestral state for angiosperms (Friedman and Williams, 2004; Williams and Friedman, 2004; Friedman and Ryerson, 2009).

Ultrastructural aspects of the four-celled female gametophyte may provide valuable information about its functional aspects and new bases for tracing the evolution of reproductive traits within the angiosperm clade. Hence, a detailed structural study of the female gametophyte in Cabombaceae and Nymphaeaceae was conducted using transmission electron microscopy. Since the nucellar epidermal cells at the micropylar region differentiate in the epistase, an additional aim of this study was to provide an ultrastructural characterization of this tissue.

2. Materials and methods

Three species of the Nymphaeales were studied: *Cabomba caroliniana* A. Gray, Galati 27847 (BAA), *Nymphaea gardneriana* Planch., Zini et al., 10 (CTES), and *Victoria cruziana* Orb., Zini et al., 15 (CTES). Flowers of *C. caroliniana* and *N. gardneriana* derived from individuals growing in the botanical garden of the Facultad de Agronomía, Universidad de Buenos Aires (Argentina), whereas those of *V.*

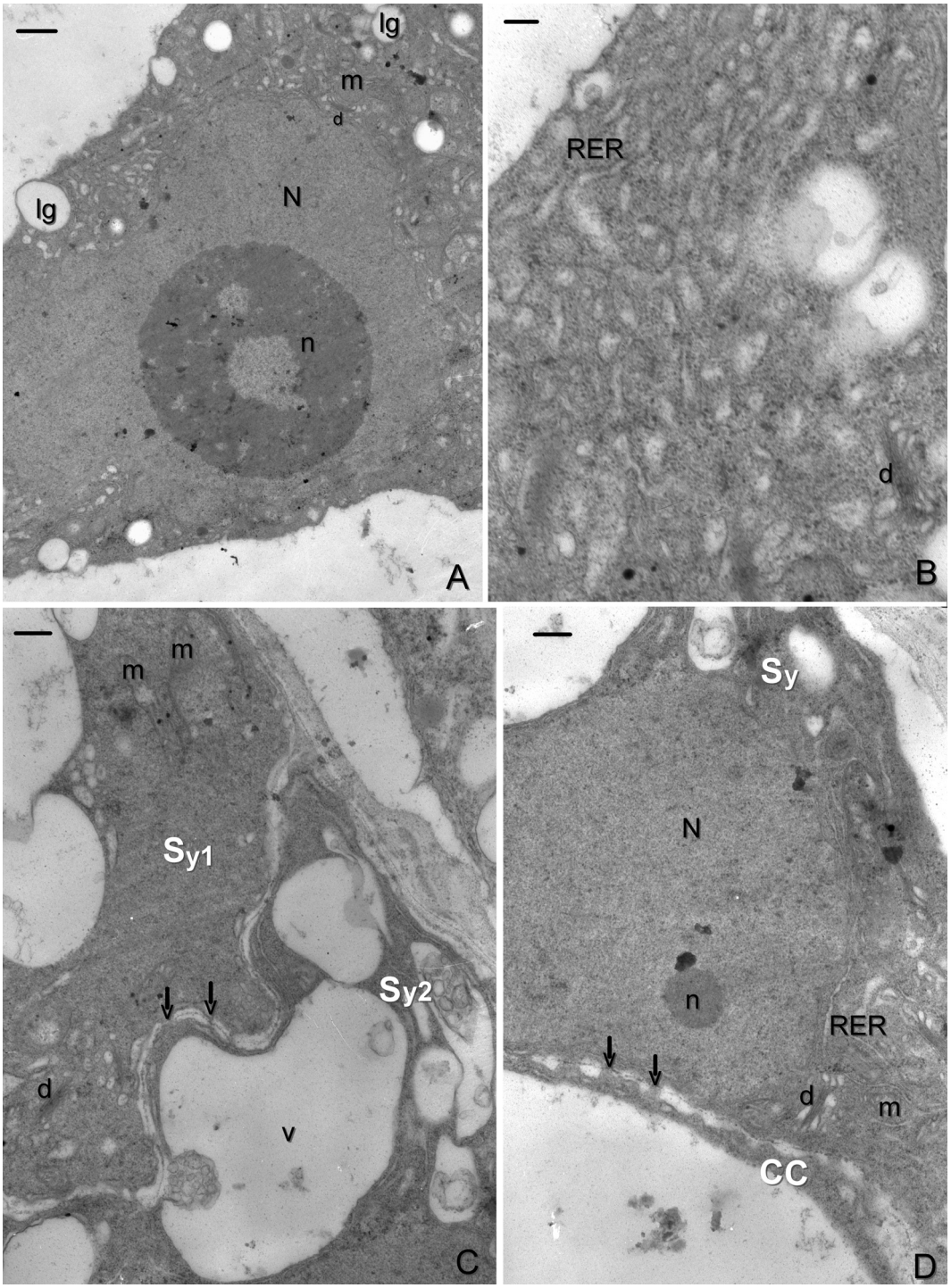

Fig. 4. *Cabomba caroliniana*, ultrastructure of female gametophyte. (A) Polar nucleus and part of the cytoplasm. (B) Detail of cytoplasm of central cell with rough endoplasmic reticulum (RER) and dyctiosome (d). (C) Micropylar region of synergids (Sy1, Sy2) without filiform apparatus; both cells are connected by plasmodesmata (arrows). (D) portion of a synergid showing cytoplasmic connections with the central cell (CC) (arrows). Abbreviations: lg: lipid globules, m: mitochondria, N: nuclei, n: nucleoli. Scale bars: (A) 1 μm, (C, D) 0.5 μm, (B) 0.2 μm.

cruziana were collected from a natural population that grows at the province of Chaco (Argentina).

For transmission electron microscopy, flowers at second-day stage of anthesis were collected. Numerous ovules were pre-fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 2 h. The fixation of ovules of *N. gardneriana* was made from two individuals cultivated, isolated from each other, and in two different moments because they have differed in the anthesis time. The ovules were then post-fixed in 1.5% OsO4 at 2 °C in the same buffer for 3 h. They were dehydrated using an ascending graded series of acetone and embedded in Spurr's resin. Sections 1 μm thick were made on a Reichert-Jung ultramicrotome, stained with toluidine blue, and photographed with a Motic digital bright-field microscope. Ultrathin sections (750–900 nm) were made and stained with uranyl acetate and lead citrate (Zarlavsky, 2014). These sec-

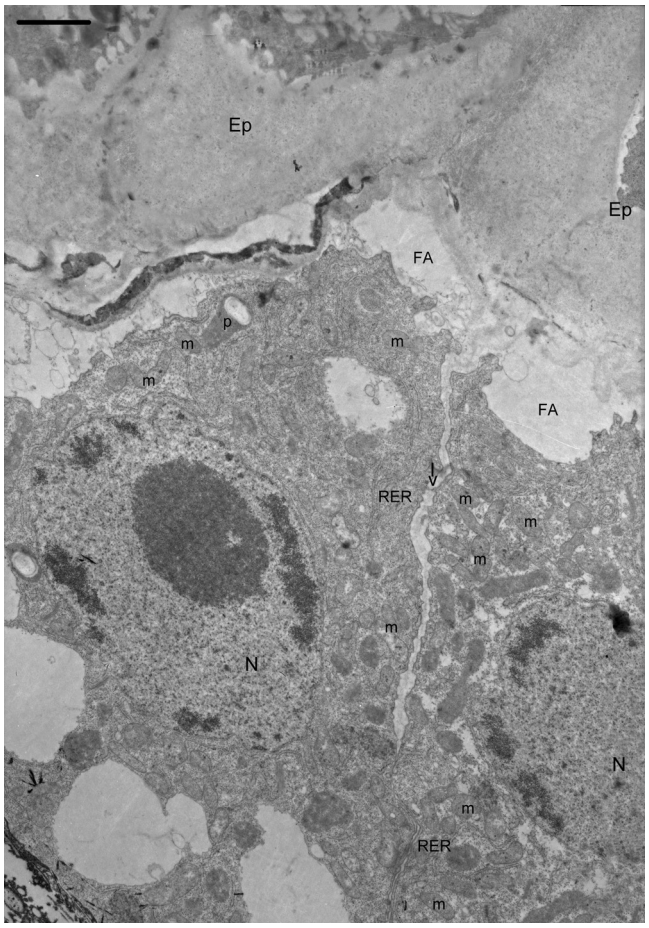

Fig. 5. *Victoria cruziana*, general view of a synergid, a portion of the other synergid and two epistase cells (Ep). Note the plasmodesmata (arrow) and distribution of cytoplasmic organelles in the synergids. Abbreviations: FA: filiform apparatus, m: mitochondria, N: nuclei, p: plastids, RER: rough endoplasmic reticulum. Scale bar: 2 μm.

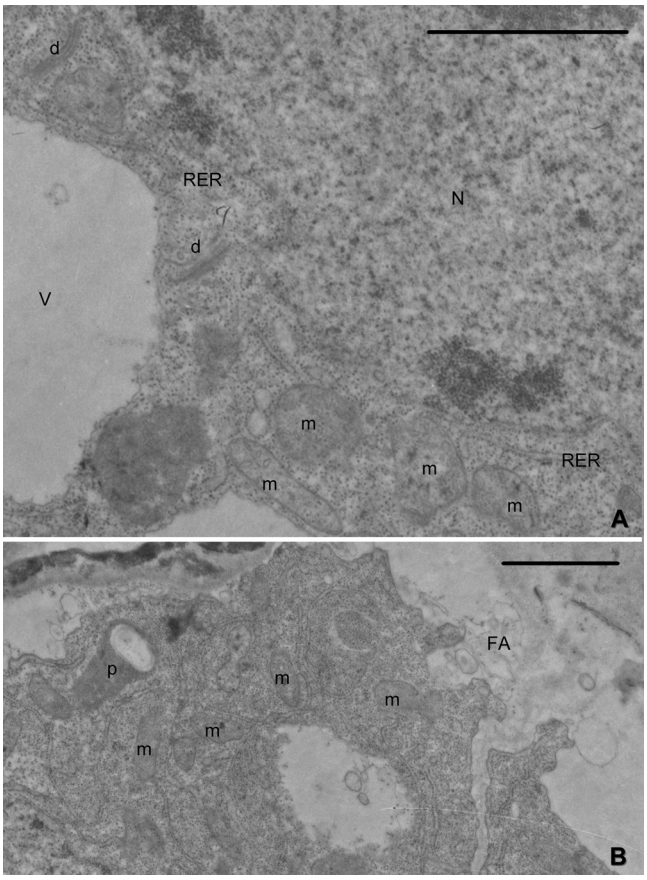

Fig. 6. *Victoria cruziana,* ultrastructure of synergids. (A) Detail of the cytoplasm at the chalazal region. (B) Detail of the micropylar region with a rudimentary filiform appatarus (FA). Abbreviations: d: Dyctiosomes, m: mitochondria, N: nucleus, p: plastid, RER: rough endoplasmic reticulum, V: vacuoles. Scale bars: 1 μm.

tions were examined using a JEOL 1200 Ex II transmission electron microscope.

For observations of mature female gametophytes in paraffin sections, the samples were fixed in formalin–acetic acid–alcohol 1:1:3 (FAA). The material was dehydrated and embedded in paraffin following standard methods, using the technique of Johansen (1940). Longitudinal sections 12 μm thick were made with a rotary microtome, then stained with Astra blue-safranin (Luque et al., 1996), and mounted with synthetic Canada balsam. Slides were observed and photographed with a Motic digital bright-field microscope. To detect callose the sections were stained with 0.05% aniline blue, which imparts a yellow fluorescence (O'Brien and McCully, 1981). Samples were observed with a Zeiss Axioplan epifluorescence microscope (excitation wavelength of 395 nm).

For the analysis of character evolution using parsimony, three unordered character states of the filiform apparatus were determined from the embryological reports. The ancestral state was determined using TNT 1.1 software (Goloboff et al., 2003). Character was mapped onto a synthetic molecular phylogeny for angiosperms as circumscribed APG III (2009). All images were processed with PhotoStyler 5.5.

3. Results

In *C. caroliniana*, *N. gardneriana* and *V. cruziana*, the mature female gametophyte contains a three-celled egg apparatus with two synergids and one egg cell, and the central cell with a single polar nucleus at the chalazal end of the female gametophyte (Fig. 1A–F). This cell is homologous with the central cell of gametophytes of the Polygonum type.

Epidermal cells at the apex of the nucellus and close to the egg apparatus form a tissue called epistase. These cells are sometimes elongated radially and exhibit thickened walls predominantly on the internal tangential face of the cells (Fig. 1A, B, D–F). The cells of the epistase at the mature stage of the female gametophyte did not show fluorescence when stained with aniline blue, indicating that there is no callose in their walls.

3.1. Ultrastructure of the epistase

The cells of the epistase have conspicuous wall thickening and invaginations on their inner tangential side in *N. gardneriana* (Fig. 2A) and in *V. cruziana* (Fig. 5), notably increasing the surface of the plasma membrane, but in *C. caroliniana* the thickening of these cells is less developed and the wall ingrowths are absent (Fig. 3A). In all species, the cytoplasm of the epistase cells shows organelles in a perinuclear position. There are abundant mitochondria, endoplasmic reticulum, lipid globules, vacuoles, dictyosomes and plastids with starch grains (Figs. 2A, and 3A). Many ovules examined of *N. gardneriana* exhibit pollen tubes entering the micropyle and, interestingly, the pollen tube grows between the cells of the epistase, subsequently branching and reaching the tip of the filiform apparatus of a synergid (Fig. 2A).

3.2. Ultrastructure of the four-celled female gametophyte

3.2.1. Synergids

Each synergid contains a large nucleus located near the middle or at the micropylar region of the cell, and some vacuoles distributed at the chalazal end (Fig. 5). The ultrastructure of the cytoplasm is similar among the species studied; the cytoplasm is rich in organelles that are randomly distributed, such as mitochondria with well-defined cristae, rough endoplasmic reticulum, dictyosomes and free ribosomes. Plastids with starch grains and lipid globules are relatively less abundant (Figs. 2B, 3A and 4C–D, 6A–B). Synergids are connected with each other and with the egg cell through plasmodesmata (Figs. 4C and D, 5, 7B). The micropylar end of the synergids of *V. cruziana* and *N. gardneriana* has a thickened cell wall exhibiting slight inward projections that form a filiform apparatus (Figs. 2A, 5 and 6B). By contrast, the synergids of *C. caroliniana* do not have a thickened wall with inward projections at the micropylar end (Fig. 4C). The synergid wall of all the studied species has a fibrillar structure and a low electron-density. This wall thins slightly to the chalazal region, stretching considerably at some points or disappearing and leaving the plasma membranes of the synergid and the central cell in close contact (Fig. 4D).

3.2.2. Egg cell

In *V. cruziana*, the egg cell has a vacuole located at the chalazal end, and a large nucleus slightly nearer the micropylar end. The cell walls of the egg and of the synergid have a similar structure. The cell wall gradually thins and becomes incomplete at its extreme chalazal side (Fig. 7A and B). Its cytoplasm is rich in plastids, but it rarely contains starch grains. Mitochondria and rough endoplasmic reticulum are scanty (Fig. 7A and B). In contrast, the cytoplasm of the egg cell in *C. caroliniana* has numerous mitochondria, smooth endoplasmic reticulum with dilated cisternae, and dictyosomes (Fig. 3B).

In *N. gardneriana*, the egg cell from all ovules analyzed differs noticeably because it appears more electron-dense than the synergid cells, and scarce membrane structures can be observed. The key morphological difference between the egg cell and synergids is the presence of a filiform apparatus in the second, thus potential existence of degenerating synergids was ruled out. Most organelles of the egg cell are disintegrated and only the remains of endoplasmic reticulum can be recognized. The phenomenon described above coincides with the arrival of pollen tubes derived from the same flower. Although pollen tubes properly penetrated the micropylar region, the ovules examined remain unfertilized (Fig. 2A and C). The processing of ovules derived from the single flower at anthesis of an individual which was isolated, under controlled conditions, ensures that the only pollen present belong that same flower.

3.2.3. Central cell

This cell is mainly occupied by large vacuoles, hence, its cytoplasm is confined to a small region around the polar nucleus (Fig. 2D) and to a parietal position. In *V. cruziana*, the cell with polar nucleus has diverse cytoplasmic contents such as mitochondria with expanded cristae, rough endoplasmic reticulum sometimes with dilated cisternae, amyloplasts, lipid globules, and dictyosomes with associated vesicles (Fig. 8A–D), whereas in *N. gardneriana* this cell contains principally amyloplasts (Fig. 2D). In *C. caroliniana* the most abundant organelles observed are mitochondria and rough endoplasmic reticulum with dilated cisternae; lipid globules are also present (Fig. 4A and B). There are plasmodesmata connecting the cell containing the polar nucleus with the synergids (Fig. 4D).

4. Discussion

This is the first ultrastructural analysis of the female gametophyte in Cabombaceae and Nymphaeaceae. The present findings about cytological characteristics of the female germ unit in these two families is generally in accordance with the knowledge from other angiosperms that develop, for example, a typical seven-celled, eight-nucleate female gametophyte. However, they differ at some specific points which will be treated in the following discussion.

The micropylar nucellar epidermis forms the epistase in the three species examined. Maheshwari (1950) defined the epistase as a special modification of the nucellar epidermis, consisting of radially elongated cells with thickened or suberized walls; however, there is still little or no information about its function among the few plant groups where it was reported, within the families Commelinaceae (Johri et al., 1992), Nymphaeaceae (Cook, 1902, 1906; Khanna, 1964, 1967; Winter and Shamrov, 1991; Povilus et al., 2015; Zini et al., 2015) and Zingiberaceae (Mangaly and Sworrupanandan, 1977). In *N. gardneriana* and *V. cruziana*, ultrastructural characteristics of the epistase correspond to those of the transfer cell. Transfer cells are distinguished to have secondary wall ingrowths in association with a cytoplasm reflecting high metabolic activity, given by the prevalence of well-developed mitochondria and endoplasmatic reticulum (Gunning and Pate, 1969). Transfer cells occurrence is widespread in plant tissues, including ovular tissues such as integuments and nucellar cells that surround the female gametophyte and embryo (Jensen, 1965; Schulz and Jensen, 1968; Gunning and Pate, 1969; Tilton, 1980; Cass et al., 1986; Sumner and van Caeseele, 1989; Olesen and Bruun, 1990; Johansson and Walles, 1994; Galati et al., 2006). In *N. gardneriana* and *V. cruziana*, the epistase showed thickened cell walls with numerous ingrowths and a metabolically active cytoplasm, therefore, it can be recognized as transfer cells. The same patterning was already reported for *V. cruziana* in previous studies with transmission electron microscope (Zini et al., 2015) and for *Nymphaea thermarum* Eb. Fisch. with bright field microscope observations (Povilus et al., 2015). In *N. gardneriana* and *V. cruziana* callose is not present in the inner tangential cell walls of the epistase at the mature stage of the female gametophyte. This is a notable difference with *N. thermarum* in which a layer of callose accumulates in that region up to anthesis time (Povilus et al., 2015).

In the literature, transfer cells are considered to play a central role in substances absorption or secretion by increasing enormously the surface area of the plasma membrane, hence facilitating high rates of material transport (Gunning and Pate, 1969; Offler et al., 2003). Previously, some authors associated transfer cells of the nucellar cap with a secretory activity responsible for the guidance of pollen tubes toward the female gametophyte (Tilton, 1980; Tilton and Lersten, 1981; Bruun and Olesen, 1989). In line with the above hypothesis, and given that transfer cells in *N. gardneriana* and *V. cruziana* are in close proximity with the mature egg apparatus, a participation of the epistase in secretion of chemotropic substances for pollen tube attraction may be suggested. Although there are no cell wall ingrowths in the epistase of *C. caroliniana*, the ultrastructure of these cells also suggests high metabolic activity, so the function could be similar to that of the other two species. The epistase can be considered as synapomorphic for the clade Cabombaceae-Nymphaeaceae.

The egg cell ultrastructure differs among the studied species. *V. cruziana* showed several plastids with starch and few mitochondria, whereas no endoplasmic reticulum or dictyosomes were observed as in *C. caroliniana*. Variations in the organelles composing the cytoplasm of the egg cell and in organelle frequency were commonly described among other plants, but few studies reported the usual occurrence of dictyosomes and smooth endo-

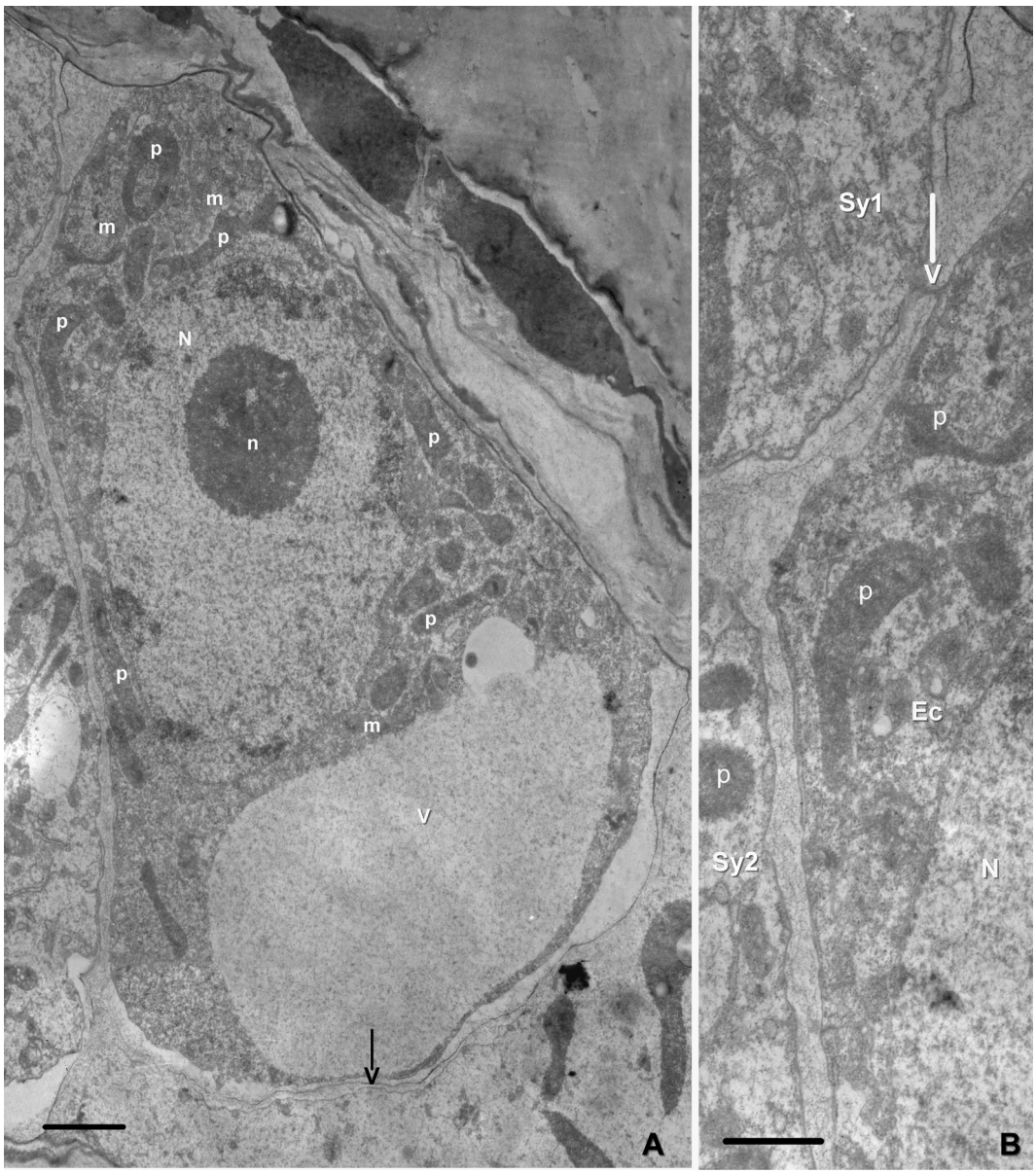

Fig. 7. *Victoria cruziana*, ultrastructure of egg cell. (A) General aspect and cytoplasmic organelles. Note the thinning of the wall in the area of contact with the cell with polar nucleus (black arrow). (B) Detail of cell walls of the egg (Ec) and synergids (Sy1, Sy2) with plasmodesmata (white arrow). Abbreviations: m: Mitochondria, N: nucleus, n: nucleolus, p: plastids. Scale bars: (A) 2 μm, (B) 1 μm.

plasmic reticulum (reviewed in Huang and Russell, 1992; Russell, 1993), as observed in *C. caroliniana*. For example, abundant dictyosomes were found in the mature egg cell of *Lotus glaber* Mill. (Galati et al., 2006), and a great amount of both dictyosomes and smooth endoplasmic reticulum were only noted in the zygote of *Quercus gambellii* Liebm. in correlation with formation of its cell wall (Mogensen, 1972).

The egg cell of *N. gardneriana* revealed signs of cell death, concomitantly with the entrance of self-pollen tubes into the micropyle. In Cabombaceae and Nymphaeaceae, the temporal separation of sexes through protogyny appears as a common phenomenon to promote allogamy (Schneider and Jeter, 1982; Thien et al., 2009). However, the observations above mentioned suggest that in *N. gardneriana* the stigma remains receptive at second day of anthesis and the pollen tubes of the same flower may reach the ovules, although they had no signs of fertilization. Mechanisms of self-sterility are not well documented in the Nymphaeales (Allen and Hiscock, 2008). It is known that *Nuphar* may have a post-zygotic system of self-sterility because species of this genus produces deformed fruits after self-pollination (Schneider and Moore, 1977). In contrast, Wiersema (1987) found via crosses that *N. gardneriana* lacks autogamous seed production. Thus, self-pollination is capable in that species, but a mechanism of self-incompatibility may be acting at a relative late stage, to prevent fertilization.

In the three species studied here, the central cell is rich in endoplasmic reticulum, plastids, amyloplastids, mitochondria, dictyosomes and ribosomes, suggesting that this cell is quite active. Similarly, this characteristic is shared with the central cell of numerous angiosperms (reviewed in Raghavan, 1997). In this respect, Huang and Russell (1992) assume that the central cell of gametophytes is well prepared to initiate endosperm after fertilization and acquire the nutritional requirements of the embryo. Nevertheless, it is known that the central cell in *Cabomba*, *Nymphaea* and *Victoria* gives rise to a small amount of endosperm after fertilization process (whose function is not yet understood), but instead copious perisperm is the embryo-nourishing tissue

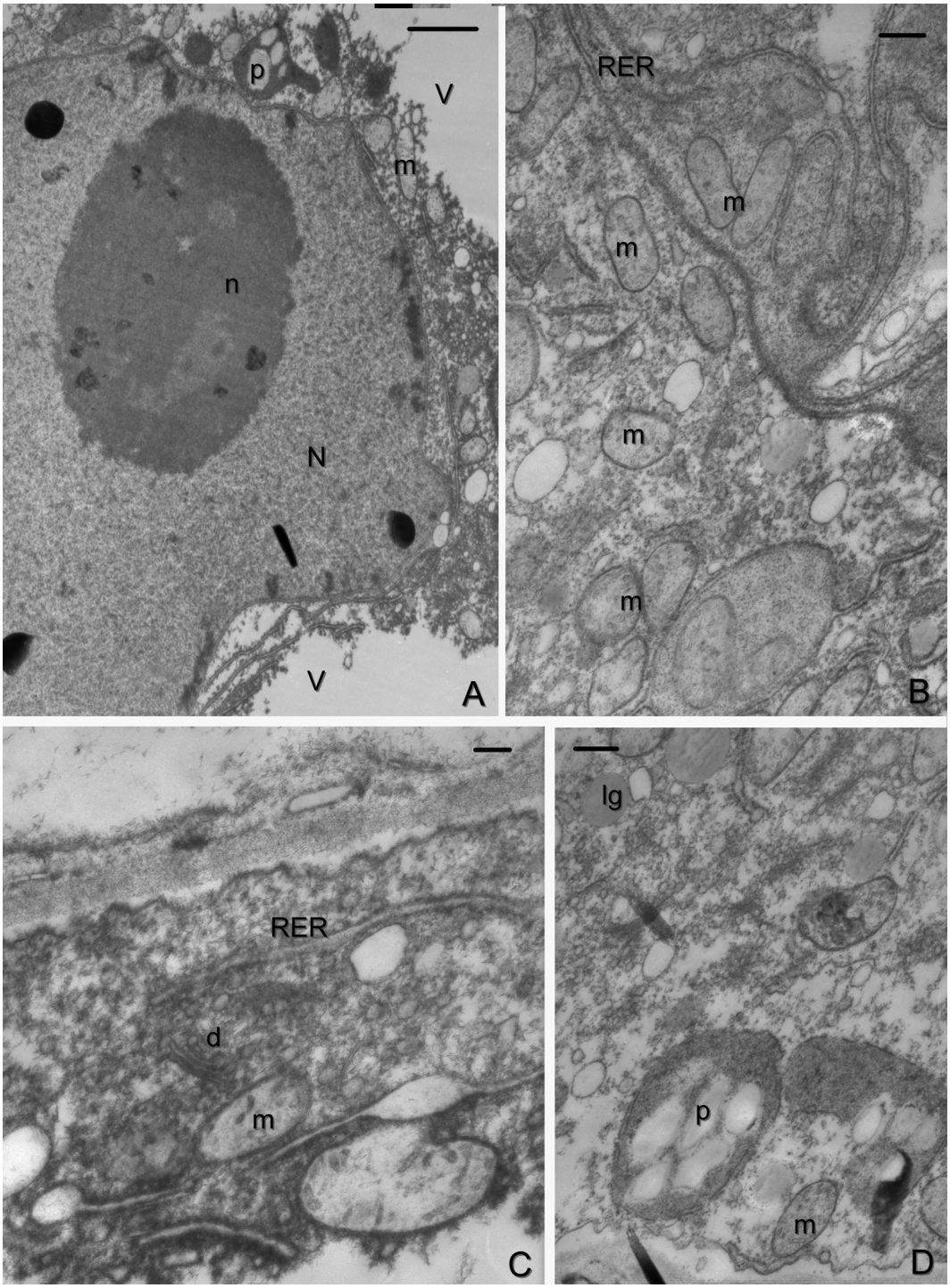

Fig. 8. *Victoria cruziana*, ultrastructure of the central cell. (A) Polar nucleus. (B-D) Details of cytoplasm showing diverse components. Abbreviations: d: Dyctiosome, lg: lipid globule, m: mitochondria, N: nucleus, n: nucleolus, p: plastids, RER: rough endoplasmic reticulum, V: vacuole. Scale bars: (A) 2 μm, (B) 0.5 μm, (C) 0.2 μm, (D) 0.5 μm.

within the seed in those genera (Cook, 1906; Khanna, 1967; Galati, 1987; Floyd and Friedman, 2000; Floyd and Friedman, 2001; Povilus et al., 2015).

In *C. caroliniana*, *N. gardneriana* and *V. cruziana*, the cytoplasmic components of the mature synergids reflecting high degree of metabolic activity (i.e., abundant mitochondria with well developed cristae, rough endoplasmic reticulum, and dictyosomes) were similar to those of many other angiosperms described to date (reviewed in Willemse and van Went, 1984; Huang and Russell, 1992; Higashiyama, 2002; Punwani and Drews, 2008). The typical feature of synergids is the filiform apparatus, located at the micropylar end of both cells. This structure displays variable morphologies among different angiosperms, but in most studied species the filiform apparatus is an elaborated labyrinth of ingrowths or well-developed finger-like forms (Maheshwari, 1950; Jane, 1997; Huang and Russell, 1992). Nevertheless, the exception to this generalization is in the clade Cabombaceae-Nymphaeaceae, because transmission electron microscope observations provide direct evidence for a relatively simple filiform apparatus with very scarce finger-like projections of the cell wall in *N. gardneriana* and

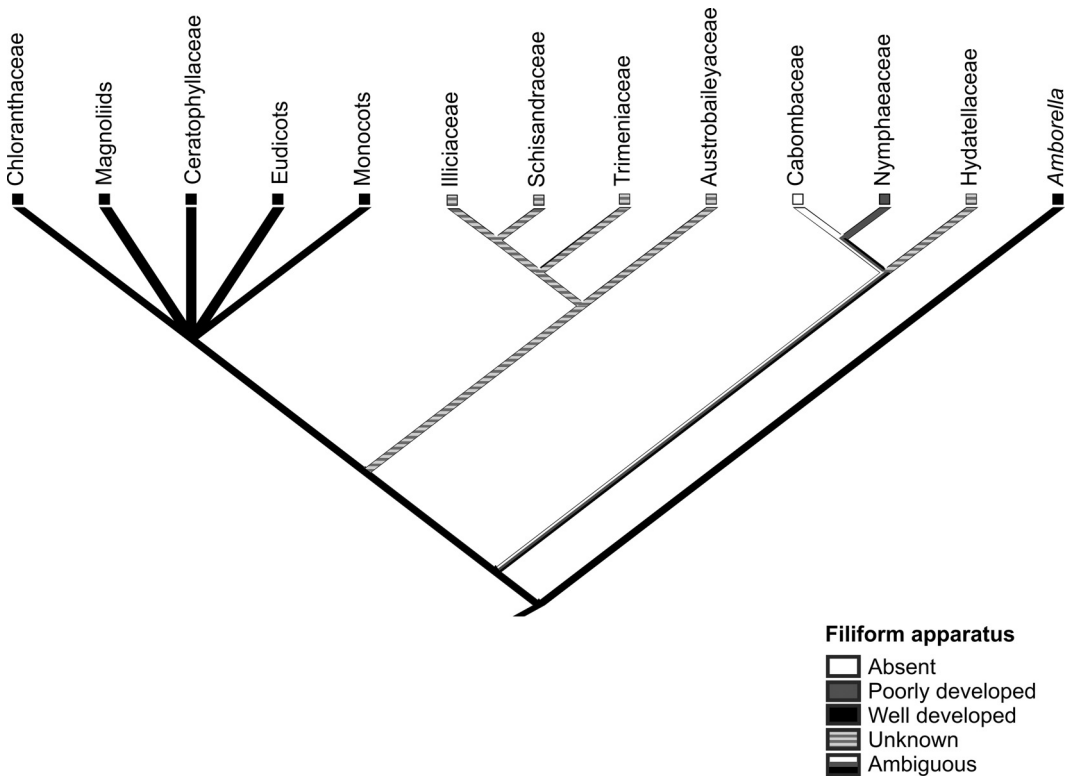

Fig. 9. Distribution of the filiform apparatus states onto a simplified angiosperm phylogenetic tree. The reconstruction based on parsimony suggests that the ancestral female gametophyte is likely to have had synergids with a well-developed filiform apparatus, but this result is preliminary due to the absence of comparative data in Hydatellaceae and in Austrobaileyales.

V. cruziana, and demonstrate the absence of wall ingrowths in *C. caroliniana*.

The structure of the filiform apparatus differs notably among the angiosperms studied (Huang and Russel, 1992). The filiform apparatus of *Nymphaea* and *Victoria* is electron-translucent and homogeneous in structure. A similar characteristic was observed in *Helianthus* L. and *Cynara* L. (Newcomb, 1973; Figueiredo et al., 2006). In *Nicotiana* L. (Mogensen and Suthar, 1979) and *Taraxacum* F.H. Wigg. (Płachno et al., 2014) there are a single phase containing scattered electron-dense inclusions, but these components were not observed in Nymphaeaceae. In contrast, the filiform apparatus consists of both low electron-dense and medium electron-dense phases in *Gossypium* L. (Jensen, 1965), *Amaranthus* L. (Coimbra and Salema, 1999), *Eleusine* Gaertn. (Lovisolo and Galati, 2007), *Genlisea* A.St.-Hil., *Lotus* L. (Galati et al., 2006), *Passiflora* L. (Amela García et al., 2003), and *Utricularia* L. (Plachno, 2011).

The elaborations of the synergid cell wall, whose universal function appears to be increasing the amount of secreted attractants, are in general consistent with a transfer cell specialization (Gunning and Pate, 1969; Willemse and van Went, 1984; Huang and Russell, 1992; Higashiyama, 2002). Kasahara et al. (2005) provide support for the hypothesis mentioned above; they have identified a mutation of MYB98 gene in *Arabidopsis thaliana* (L.) Heynh. affects notably the existence of wall-ingrowths in the filiform apparatus. In *myb98*, the filiform apparatus consist of a relatively homogenous mass of cell wall at the micropylar pole. Despite the pollen tube guidance is also affected, the cytoplasm of the synergids function normally and the female gametophyte mutants can be fertilized. These authors conclude that a functional filiform apparatus (i.e., with intricate wall-ingrowths) is necessary for pollen tube guidance, but is not required for pollen tube entry into the synergid cell. Huang and Russel (1992) noted that the proportion and complexity of the filiform apparatus seems to be consistent within the families of angiosperms. Within the eudicots clade, trends to reduce the filiform apparatus (absence of it or without intricate wall-ingrowths) were documented only among some members of the families Lentibulariaceae (Plachno, 2011) and Asteraceae, in the last family in association with an apomictic reproductive system (Newcomb, 1973; Kuroiwa, 1989; Figueiredo et al., 2006). It is important to note that in *Nymphaea* and *Victoria*, the filiform apparatus is developed only at the apical part of synergid cells, but in *Genlisea*, *Utricularia* (Lentibulariaceae), *Taraxacum* and *Cynara* (Asteraceae), the thickening also continues toward the lateral sides of both synergids (Figueiredo et al., 2006; Plachno 2011; Płachno et al., 2014). There is no apparent adaptive explanation for the simplistic morphology of the filiform apparatus in *N. gardneriana* and *V. cruziana*. In these cases, the function of the filiform apparatus in the pollen tube guidance may be compensated by the specialized cells in the epistase, because they would contribute with the secretion of chemotropic substances. On the other hand, the absence of filiform apparatus in the synergid cells of *C. caroliniana* could be offset by the presence of an egg cell with a more active cytoplasm that would contribute to attracting pollen tube.

In the present work, a preliminary evolution of the filiform apparatus was assessed (Fig. 9). In this reconstruction, a well-developed filiform apparatus seems to be an early specialization for the synergid cells of angiosperms, and could represent the plesiomorphic condition. The female gametophyte of *Amborella* has three synergids, and each of these cells have thickened walls forming the filiform apparatus (Friedman and Ryerson, 2009). This research on *Amborella* exhibits transmission electron microscope image of a filiform apparatus confined to the micropylar side of each synergid cell, but consisting of numerous finger-like projections, which is in contrast with the observations made in this work on the species of Cabombaceae and Nymphaeaceae.

It is hypothesized that there was a reduction of the filiform apparatus in the ancestor of the Nymphaeales, and then a complete loss in *Cabomba*. However, the character in the ancestor of the order is ambiguous because there is no data about the filiform apparatus in Hydatellaceae. Similarly, the data is coded as unknown for the clade Austrobaileyales. Although there are some recent studies of the female gametophyte in the order (Friedman et al., 2003; Williams and Friedman, 2004) neither of these works show in fine detail the synergid cells micropylar end. We consider that additional electron microscopic studies of female germ unit in early-divergent lineages will help to clarify the plesiomorphic condition of the filiform apparatus for extant angiosperms. Finally, morphological comparison of the filiform apparatus indicates that remarkable transformations have occurred among three early-divergent families of angiosperms ultrastructurally examined to date, and this is in part consistent with previous studies that documented high degree of structural lability and developmental experimentation for many embryological and floral traits in basal angiosperms (Endress, 2001; De Craene et al., 2003; Zanis et al., 2003; Soltis et al., 2005; Friedman, 2006; Rudall et al., 2008; Taylor et al., 2012).

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