Acta Botanica Brasilica - 34(1): 31-39. January-March 2020.
doi: 10.1590/0102-33062019abb0120

Revisiting hydropotes of Nymphaeaceae: ultrastructural features associated with glandular functions

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Received: April 3, 2019
Accepted: August 12, 2019

ABSTRACT

Hydropotes are specialized epidermal structures involved in water and mineral flux into and out of the plant body. We analyzed hydropote morphology of four species of Nymphaeaceae: *Nymphaea caerulea*, *Nymphaea lotus*, *Nymphaea rubra*, and *Victoria amazonica*. Leaf samples were processed following conventional techniques for plant anatomy and for scanning and transmission electron microscopy. We observed hydropotes comprising an elongated apical sharp-pointed portion with a base composed of two to three short specialized cells. In a later developmental stage the apical sharp-pointed portion was detached and the mature hydropotes comprised an upper lens-shaped cell, a bowl-shaped cell and a large foot cell. Ultrastructural analysis revealed that the lens-shaped cell possesses labyrinthine projections in its outer periclinal wall and abundant plasmodesmata in its inner periclinal wall. Several mitochondria were present in the cytoplasm of both the lens-shaped and the bowl-shaped cells. The cytoplasm of the foot cell was reduced and possessed plastids with starch grains. The ultrastructure of the hydropotes is typical of cells involved in transporting substances and corroborates their role in the flux of substances into and out of the cell. These findings contribute to a better understanding of the functioning of hydropotes and shed light on this little-explored issue.

Keywords: *Nymphaeae*, subcellular features, structure, trichomes, *Victoria*

Introduction

Plants communicate with the environment through specialized epidermal structures, which are involved in the maintenance of their essential physiological and biochemical processes (Carpenter 2006) and in their establishment in the most diverse environments (Dickson 2000; Javelle *et al*. 2011). Hydropotes (water drinkers) are epidermal appendices specialized for life in an aquatic environment (Fahn 1979). They are present on the abaxial surface of floating leaves (Fahn 1979; Wilkinson 1979; Catian & Scremin-Dias 2013) and reproductive floating organs (Zini *et al*. 2017; Coiro & Lumaga 2018) of some species of aquatic plants such as those belonging to Aponogetonaceae, Menyanthaceae, Potamogetonaceae, Nymphaeaceae, Alismataceae (Lavid *et al*. 2001), and Cabombaceae (Borsch *et al*. 2008).

These glandular epidermal structures are involved in water and mineral transport into and out of aquatic plants (Fahn 1979; Mishra & Dubey 2010), and are able to retain more ions than common epidermal cells (Lüttge *et al*. 1971). Due to these functional characteristics, plants bearing hydropotes have shown great potential for phytoremediation (Lavid *et al*. 2001), as is the case with species of *Nymphaea*. It has been shown that when exposed to cadmium, for example, hydropotes are able to trap cadmium-crystals through the activities of peroxidase

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and polyphenol oxidase (Lavid *et al*. 2001). However, despite their ecological importance for plants growing in aquatic environments, the morphological and subcellular features of hydropotes remain poorly-known.

The presence of hydropotes is widespread in Nymphaeales (Carpenter 2006). Lüttge & Krapf (1969) described the hydropotes of species of Nymphaeaceae as comprising four cells: a cap cell, a lens-shaped cell, a cup-like cell and a basal cell. These authors also observed small vacuoles filled by dark electron-dense material and walls forming protuberances in the lens-shaped cell and cup-like cell. Later, Carpenter (2006) described hydropotes with a hair-like portion and, in discussing the distribution and homology of epidermal structures among basal angiosperms, showed the presence of hydropotes in *Nuphar*, *Nymphaea*, *Euryale*, *Victoria* (Nymphaeaceae) and *Brasenia* (Cabombaceae). In the same study, Carpenter (2006) described hydropotes as comprising three cells: a lens-shaped cell, a bowl-shaped cell and a foot cell (basal). For the plants naturally occurring in aquatic environments in Brazil, the literature reports the presence of hydropotes only in the floating organs of *Nymphaea* species (Catian & Scremin-Dias 2013; 2015). For these plants, Catian & Scremin-Dias (2013) reported hydropotes constituted by a basal cell, medial cell, and an apical cell deciduous part. Nevertheless, detailed morphological studies focusing on the hydropotes of Brazilian aquatic plants, mainly involving ultrastructural analysis, are lacking.

In this study we characterized in detail the structure and the ultrastructure of hydropotes found in species of Nymphaeaceae in order to better understand their functioning.

Materials and methods

Plant material

We studied *Nymphaea lotus* L., *N. rubra* Roxb. ex Salisb., *N. caerulea* Savigny and *Victoria amazonica* (Poepp.) J.C. Sowerby. Samples of fully expanded leaves were collected from adult plants (n = 3) living in an artificial lake in the Bauru Botanical Garden (22°20'30"S, 49°00'30"W) in the municipality of Bauru, state of São Paulo, Brazil. Vouchers specimens were deposited in the Bauru Botanical Garden Herbarium (JBMB), under registration numbers 01742 (Oliveira, V.C., n° 94, 23/X/2014), 01729 (Oliveira, V.C., n° 67, 24/IX/2014, 01731 (Oliveira, V.C., n° 69, 24/IX/2014 and 01686 (Oliveira, V.C. & Peixoto, V.A.S., n° 3, 11/IX/2013).

Light microscopy (LM)

Leaf samples were fixed in FAA 50 (formaldehyde, acetic acid and 50 % ethanol; 1:1:18 vol/vol) (Johansen 1940), dehydrated in an ethanol series, and embedded in Leica® historesin. Cross sections (5-µm thick) were stained with 0.05 % toluidine blue, pH 4.3 (O'Brien *et al*. 1964), and mounted in Entellan® on permanent slides. The material was analyzed and the relevant results documented using a photomicroscope Leica® (model DMR, Germany) with a digital camera Leica® (model DFC 425).

Scanning electron microscopy (SEM)

Samples of leaves were fixed in 2.5 % glutaraldehyde with 0.1 M sodium phosphate buffer, pH 7.3, overnight at 4 °C. The samples where then dehydrated in acetone series, critical-point dried using a CPD 030 - Leica (Germany), mounted on aluminum stubs, gold-coated (Robards 1978), and examined with a Quanta 200 - FEI Company (USA) scanning electron microscope.

Transmission electron microscopy (TEM)

For transmission electron microscopy, samples of leaves of *N. lotus* and *V. amazonica* were fixed in 2.5 % Karnovsky in 0.1 M sodium phosphate buffer, pH 7.3, for 24 h; post-fixed with 1 % osmium tetroxide in the same buffer for 1 h; dehydrated in an acetone series; and embedded in Araldite® resin. Ultra-thin sections were obtained with ultramicrotome EM UC7 - Leica (Germany) and stained with uranyl acetate and lead citrate (Reynolds 1963). The samples were examined using a Tecnai Spirit - FEI Company (USA) transmission electron microscope.

Results

In all studied species, hydropotes in two different developmental stages were observed side by side in the expanded leaves (Fig. 1A-B). The young hydropotes were morphologically similar to non-glandular trichomes, being comprised of an elongated sharp-pointed apical portion with 1-4 cells and base composed of two short cells (Fig. 1C). In a later developmental stage the short cell just below the sharp-pointed apical portion divided giving rise to an upper lens-shaped cell (Fig. 2A-B) and a subjacent bowl-shaped cell; the sharp-pointed apical portion then started to senesce and detached from the lens-shaped cell (Fig. 2C-D). Debris of the detached sharp-pointed apical portion were observed on the lens-shaped cell in lesser (Fig. 2D) or greater (Fig. 2E) amounts. Mature hydropotes (Fig. 2E) were short and contained an upper lens-shaped cell, a bowl-shaped cell and a large foot cell (Fig. 2F-I). Although the general structure of the hydropotes was similar among the studied species, the shape and position of the cells varied. In *V. amazonica* the foot cell was oval, and the bowl-shaped cell was very narrow and inserted in a level below the common epidermal cells (Fig. 2F). In the three *Nymphaea* species studied the foot cell was rectangular to square, and the bowl-shaped cell was located above the common epidermal cell level (Fig. 2G-I).

The ultrastructural studies reveal that hydropotes of both studied species, *N. lotus* and *V. amazonica* (Figs. 3, 4),

were similar. Debris of the sharp-pointed apical portion could be seen above the lens-shaped cell (Fig. 3A, B). The lens-shaped cell possessed thick anticlinal and outer periclinal pectin-cellulosic walls. Labyrinthine projections were observed in the outer periclinal wall (Fig. 3C, D); in such cell wall projections, the cellulose microfibrils showed loose arrangement (Fig. 3E). Numerous plasmodesmata occurred in the inner periclinal wall (Fig. 3C, Fig. 4A) connecting the lens-shaped cells to the bowl-shaped cells. The plasma membrane had an irregular contour; and the cytoplasm was dense and abundant (Fig. 3C-D) with several mitochondria with dilated cristae (Fig. 3D). Abundant free ribosomes and

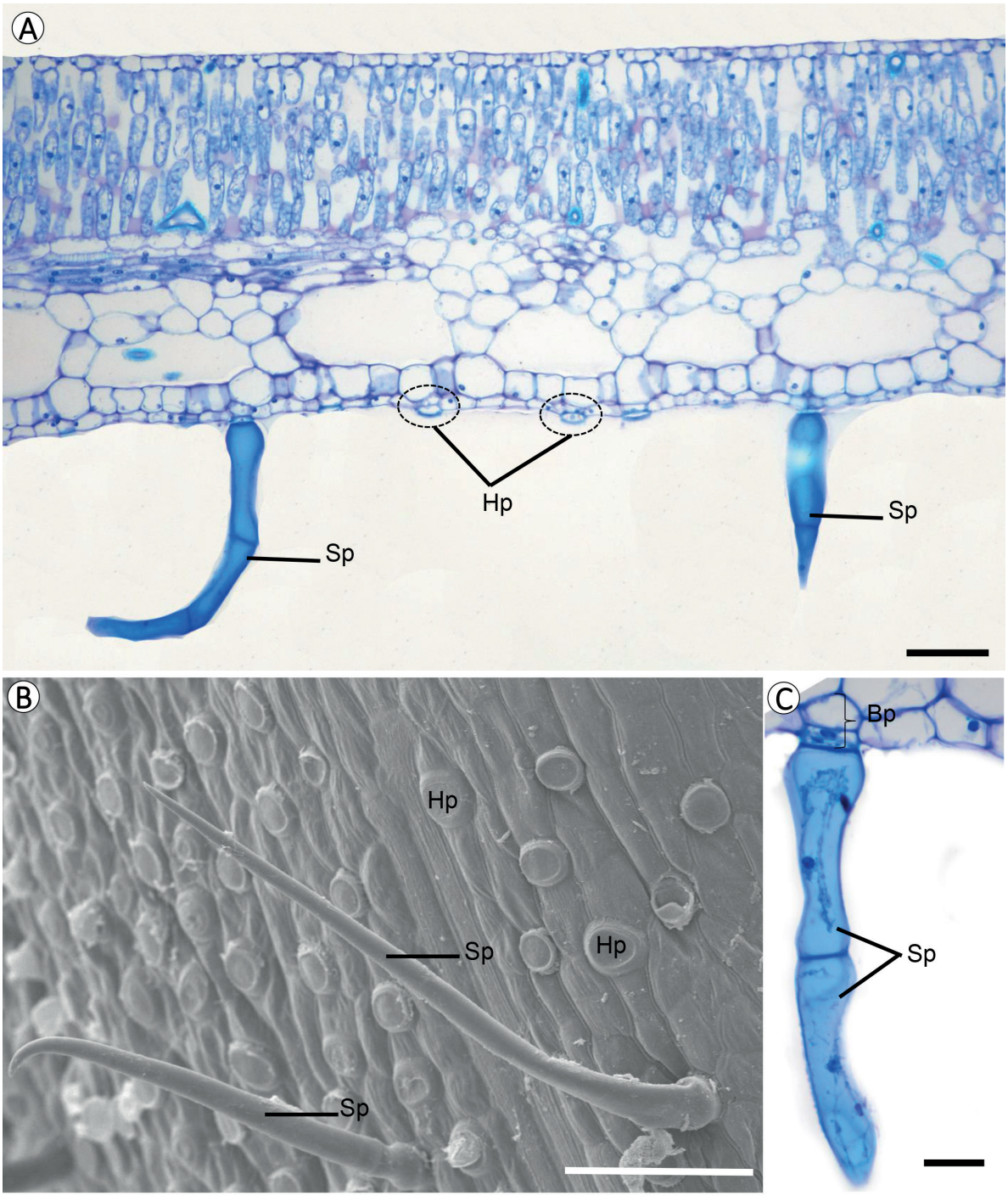

Figure 1. Photomicrographies (**A**, **C**) and scanning electron micrography **(B**) of Nymphaeaceae leaves. **A.** Cross section of *Victoria amazonica* leaf blade showing hydropotes with sharp-pointed apical portion (Sp) and mature hydropotes (Hp) in the abaxial surface. **B.** Abaxial surface of *Nymphaea rubra* leaf showing hydropotes with sharp-pointed apical portion (Sp) and mature hydropotes (Hp). **C.** Detail of hydropote with sharp-pointed apical portion in *V. amazonica* leaf, showing a two-celled apical portion (Ap), and a basal portion (Bp) inserted among the common epidermal cells. Bars: A-B = 100 µm, C = 30 µm.

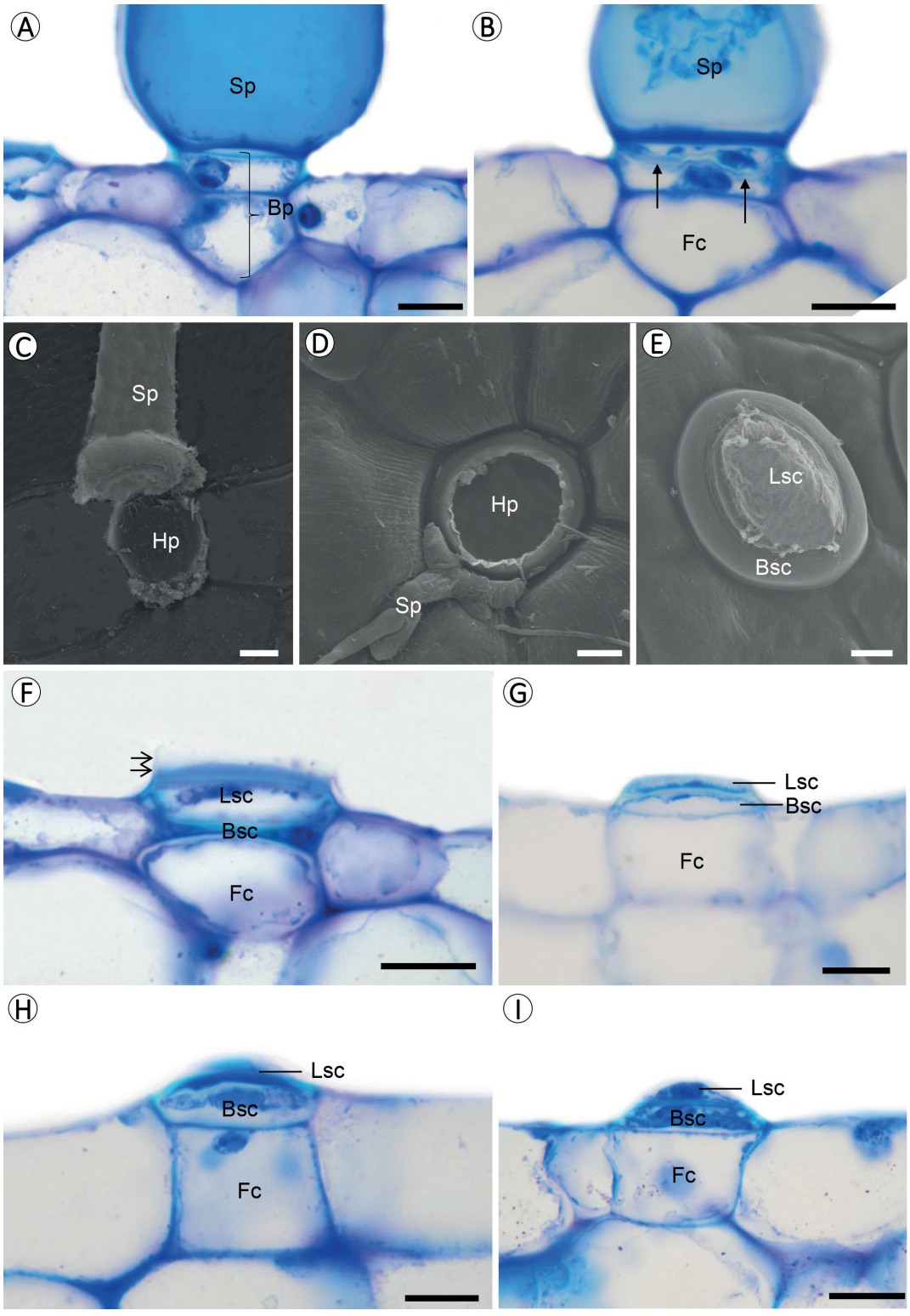

Figure 2. Hydropotes in different developmental stages in Nymphaeaceae species. **A,B, F-I.** Photomicrographs. **C-E.** Scanning electron micrographs. **A.** Hydropote in *Victoria amazonica* showing a sharp-pointed apical portion (Sp) and a basal portion (Bp) with two short cells. **B.** The arrows indicate new wall formed by periclinal division of the upper cell in the basal region of the hydropote in *V. amazonica*. Sp: sharp-pointed apical portion, Fc: Foot cell. **C-D.** Detachment of the sharp-pointed apical portion (Sp) in hydropote of *Nymphaea lotus* **(C)** and *Nymphaeae caerulea* (**D**) giving raise to mature hydropotes (Hp). **E.** Mature hydropote in *Nymphaea rubra* showing a lens-shaped cell (Lsc) and a bowl-shaped cell (Bsc). **F-I.** Mature hydropotes showing a large Foot cell (Fc), a bowl-shaped cell (Bsc) and a lens-shaped cell (Lsc) in *V. amazonica* **(F)**, *N. lotus* **(G)**, *N. caerulea* (**H)** and *N. rubra* **(I)**. Double arrows indicate remaining of the sharp-pointed apical portion. **G** Bars: A-B, F-I = 15 µm, C = 10 µm, D-E = 5 µm.

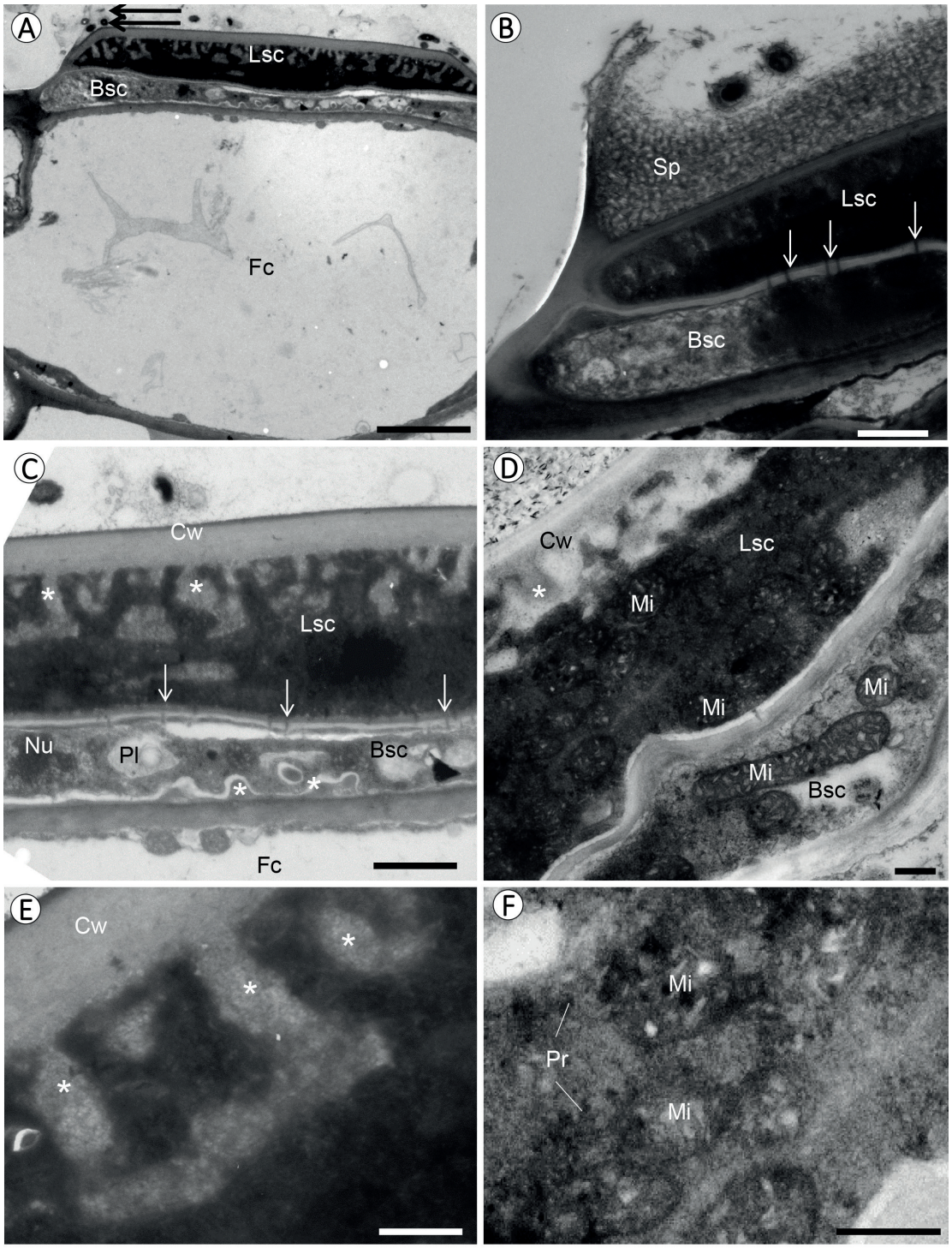

Figure 3. Transmission electron micrographs of hydropotes in leaves of *Nymphaea lotus* (**A**, **C, E**) and *Victoria amazonica* (**B, D, F**). **A.** General feature of hydropote showing lens-shaped cell (Lsc), bowl-shaped cell (Bsc) and foot cell (Fc). Double arrows indicate remaining of the hair-like portion. **B.** Detail showing debris from the detachment of the sharp-pointed apical portion (Sp), lens-shaped cell (Lsc) and bowl-shaped cell (Bsc). Arrows indicate plasmodesmata connecting the lens-shaped cell (Lsc) and the bowl-shaped cell (Bsc). **C.** Lens-shaped cell (Lsc) with labyrinthine projections (*) in the outer periclinal wall, numerous plasmodesmasta (arrows) in the inner periclinal wall, and dense cytoplasm; in the bowl-shaped cell observe labyrinthine projections (*) in the inner periclinal wall, nucleus (Nu) and cytoplasm with plastids (Pl); Cw: cell wall; Fc: foot cell. **D.** Detail showing lens-shaped cell (Lsc) with labyrinthine projections (*) and dense cytoplasm with numerous mitochondria (Mi), and the bowl-shaped cell (Bsc) with numerous mitochondria (Mi) with dilated cristae. **E.** Detail of lens-shaped cell showing the cell wall (Cw) with labyrinthine projections (*) with cellulose microfibrils exhibiting loose arrangement. **F.** Dense cytoplasm showing abundant free ribosomes and polyribosomes (Pr) and mitochondria (Mi). Bars: A = 5 µm, B, C, D, F = 1 µm, E = 0.3 µm.

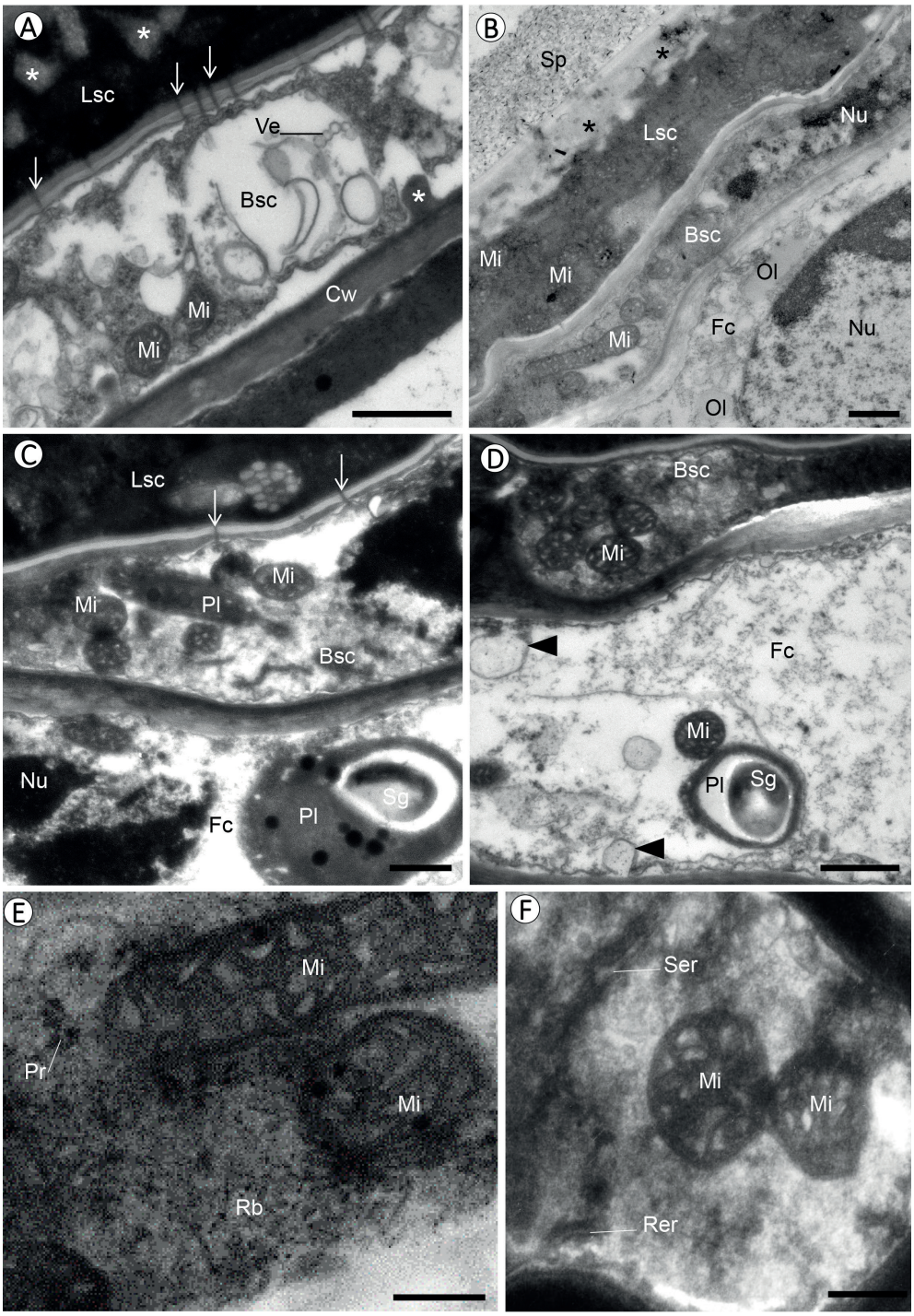

Figure 4. Transmission electron micrographs of the bowl-shaped cell and foot cell of hydropotes in leaves of *Victoria amazonica* (**A**) and *Nymphaea lotus* (**B-F**). **A.** Detail of hydropote showing bowl-shaped cell (Bsc) with numerous plasmodesmata (arrows) in the outer periclinal wall and vesicles (Ve) and mitochondria (Mi) in the cytoplasm. Cw: cell wall; *: labyrinthine projections; Lsc: lens-shaped cell. **B.** Hydropote showing lens-shaped cell (Lsc) with labyrinthine projections (*) in the outer periclinal wall and dense cytoplasm with mitochondria (Mi); bowl-shaped cell (Bsc) with abundant cytoplasm, nucleus (Nu) and mitochondria (Mi) with dilated cristae; foot cell (Fc) with voluminous nucleus (Nu) and oil (Ol) drops in the cytoplasm. Sp: debris of sharp-pointed apical portion. **C.** Detail showing bowl-shaped cell (Bsc) with plasmodesmata (arrow) connecting it to the lens-shaped cell (Lsc); observe mitochondria (Mi) and plastid (Pl) in the cytoplasm; in the foot cell (Fc) observe plastid (Pl) with plastoglobuli and degrading starch grains (Sg). **D.** Bowl-shaped cell (Bsc) with abundant mitochondria with dilated cristae, and foot cell (Fc) with electron lucent cytoplasm with mitochondria (Mi), plastids (Pl) with degrading starch grains (Sg), and vesicles (arrowhead) fusing to the plasma membrane. **E.** Detail of bowl-shaped cell showing cytoplasm with free ribosomes (rb), polyribosomes (Pr) and mitochondria (Mi). **F.** Bowl-shaped cell with smooth (Ser) and rough (Rer) endoplasmic reticulum and mitochondria (Mi). Bars: A-D = 1 µm, E-F = 0.5 µm.

polyribosomes were present in the cytoplasm (Fig. 3F). The bowl-shaped cell was thin-walled; labyrinthine projections were seen in the inner periclinal wall of only *N. lotus* (Fig. 3C). The cytoplasm was abundant and clearer (Fig. 4A-F) than in the lens-shaped cell; the nucleus was elongated with irregular contour (Fig. 4B). In the cytoplasm, the mitochondria were abundant, voluminous with dilated cristae (Fig. 4C-E); electron-dense inclusions were observed in the mitochondria (Fig. 4E). It was remarkable the proximity and apparent connection between mitochondria (Fig. 4C-E, F) in these cells. Polyribosomes and free ribosomes occurred in the cytoplasm (Fig. 4B, E, F). Short profiles of rough and smooth endoplasmic reticulum (Fig. 4F) were visualized in these cells. Small vacuoles with membranous and flocculent inclusions were observed in the cytoplasm of these cells (Fig. 4A). The foot cell possessed thin pectin-cellulosic walls, reduced cytoplasm and developed vacuome (Fig. 4A) The plasma membrane had an irregular contour. The cytoplasm was clear and possessed mitochondria with dilated cristae, plastids with starch grains with signs of degradation (Fig. 4C-D) and plastoglobuli (Fig. 4C); free oil droplets were present in the cytoplasm (Fig. 4B); vesicles were observed fusing to the plasma membrane (Fig. 4D).

Discussion

Our structural and ultrastructural results corroborate the information available in the traditional literature on the hydropotes in Nympheaceae (Lüttge 1964; Lüttge & Krapf 1969; Fahn 1979) and add novelties to this topic. Here, we did not disregard the sharp-pointed apical portion of young hydropotes and we proposed differential functions to hydropotes according to their distinct shape in different developmental stages. The origin of the lens-shaped and the bowl-shaped cells is here demonstrated by the first time. We were able to suggest different routes of substance transport along the hydropote body according to the ultrastructural features of their cells.

We observed hydropotes both with and without a sharp-pointed apical portion situated side-by-side in expanded leaves of *N. lotus*, *N. rubra*, *N. caerulea* and *V. amazonica*. Hydropotes with and without a sharp-pointed apical portion seem to represent different developmental stages of the same glandular structure. In all of these species, we observed the sharp-pointed apical portion of some hydropotes to be senesced and detached, with just the basal portion intact. We considered this basal portion to be a mature hydropote, which represents the most common hydropote structure described in literature (Lüttge 1964; Lüttge & Krapf 1969; Fahn 1979). Hydropotes with and without a sharp-pointed apical portion have also been visualized in tepals of species of Nymphaeaceae (Zini *et al*. 2017). Senescence of the sharp-pointed apical portion was also reported by Carpenter (2006) for four species of Nymphaeaceae. The sharp-pointed apical portion of hydropotes could have an important role in the physical protection of young leaves since, as with non-glandular trichomes (Tozin *et al*. 2016), they could provide a dense covering on the abaxial leaf surface as a defense for herbivory (Levin 1973; Baur *et al*. 1991). They may also facilitate the formation of film of air influencing its fluctuation (Barthlott *et al*. 2009). However, the main function of hydropotes changes later in development.

The general appearance of hydropotes with a sharp-pointed apical portion is similar to the mucilage hairs present in Cabombaceae (Carpenter 2006). However, the histochemical nature of the secretion produced by the hair-like portion of hydropotes of species of Nymphaeaceae has yet to be investigated.

Mature hydropotes of species of *Nymphaea* have been described as containing four cells (Lüttge 1964; Lüttge & Krapf 1969; Fahn 1979). However, the present study found them as comprising only three cells, which is in accordance with Carpenter (2006). We believe that the "cap cell", described by Lüttge (1964) and Lüttge & Krapf (1969) as above the lens-shaped cell, is not a proper cell, but instead debris from the detachment (see Fig. 3A-B) of the sharp-pointed apical portion, since a hair-like portion was not observed by these authors.

We found that the lens-shaped cell and the bowl-shaped cell of the studied hydropotes possess labyrinthine walls and an abundance of plasmodesmata, which are typical characteristics of cells involved in intense transport of substances (Lüttge & Krapf 1969). These features corroborate a role of hydropotes in the transport of water and minerals into and out of the plant (Fahn 1979 and references therein; Mishra & Dubey 2010), and are likely related to the bioremediation importance of hydropotes (Mishra & Dubey 2010). In a study involving light and scanning electron microscopy, Carpenter (2006) suggested that the darker color within the lens-shaped and bowl-shaped cells could be due to the presence of vesicles or crystals. Our analysis using transmission electron microscopy demonstrated that this denser aspect of the lens-shaped cell is likely due to the extensive ingrowths formed in the outer periclinal wall, in addition to the electron-dense cytoplasm. The occurrence of labyrinthine projections in cell walls has been reported for a variety of secretory structures (Bourett *et al*. 1994; Gama *et al*. 2016; Tozin & Rodrigues 2017), including hydropotes (Lüttge & Krapf 1969), and highlights the role of the cells bearing them in the transport of substances. Labyrinthine projections of walls increase the surface area of the plasma membrane supporting exchange among cells (Pate & Gunning 1972). In addition, the bowl-shaped cell possesses numerous mitochondria with dilated cristae, evidencing that this cell plays a role in generating energy (Evert 2006). The close physical juxtaposition between mitochondrial membranes in these cells was a remarkable feature and could optimize the movement of signaling molecules and the exchange of metabolites (Machado *et al*. 2018) involved in the hydropote functioning. The

foot cell possesses plastids with starch grains and signs of degradation, indicating the importance of this cell in the storage of products of photosynthesis and supplying energy for the influx of water and mineral salts (Evert 2006; Taiz *et al*. 2017); the plastoglobuli also present in these plastids may play a key role in regulating photosynthesis regulation, plastid biogenesis and metabolism of various substances (Wijk & Kessles 2017); and a wide central vacuole is typical of cells that are accumulating material (Evert 2006). Thus, we suggest functional compartmentalization among the three cells of mature hydropotes.

The path traveled by a solution, formed by water and mineral salts, seems to vary along the body of the hydropote. Influx from the environment to the lens-shaped cell seems be facilitated by the labyrinthine projections in the cell walls that enlarges the plasmalemma surface favoring the flow of solutions between the apoplast and the symplast (Pate & Gunning 1972). The influx from the lens-shaped cell to the bowl-shaped cell probably occurs via symplast through the numerous plasmodesmata connecting both cells. The abundance of mitochondria with dilated cristae in these cells suggests a high expenditure of energy is involved in this process. From bowl-shaped cell, the influx seems to continue due to, again, the labyrinthine projections in species of *Nymphaea*. In addition, the fusion of vesicles to the plasma membrane in the foot cell (see Fig. 4D) seems to be a pathway for solution influx, in an endocytosis-like process.

Although the general features of the hydropotes of the species studied here are similar, we observed structural peculiarities among them. The position of the bowl-shaped cell and the shape of the basal cell vary between species of *Nymphaea* and *V. amazonica*. In addition, the presence of labyrinthine projections in the inner periclinal wall of the bowl-shaped cell occurred only in *Nymphaea*. It is likely that these features are of taxonomic value.

Considering that hydropotes represent one of the most poorly studied secretory structures in the plant kingdom, this study contributes to a better understanding of the structural features involved in the development and functioning of hydropotes of Nymphaeaceae. Our data may serve to support deeper physiological investigations using experimental approaches to further understand the role of hydropotes in the flux of different salts, and give subsidies to the establishment of bioremediation techniques that are important in the removal of pollutants from aquatic environments.

Acknowledgements

We thank the "Jardim Botânico Municipal de Bauru" for provided the plant samples, and the technical team of the Electron Microscopy Center, IBB UNESP, for assistance in processing the material. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. T.M. Rodrigues receives research grants from Conselho Nacional de Desenvolvimento e Pesquisa, CNPq (Proc 303981/2018-0).

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