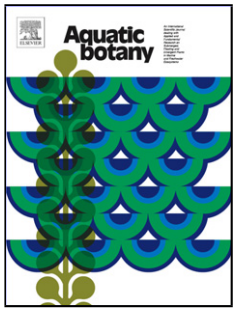

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Original article

Nectary ultrastructure of *Cabomba caroliniana* Gray (Cabombaceae)

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Highlights

- The nectary is active during the two days of the anthesis.
- Nectar is released mainly by four-celled trichomes
- The sugar content is equivalent in both anthesis days
- Cycles of contraction-expansion of the trichome cells protoplast are present
- Nectar is released through the cell wall and the cuticle of the trichome apical cell

Cabomba Aubl. is a genus considered as a potential genetic model for studies of early angiosperm evolution, accordingly, it is important to expand the knowledge of it. This paper reports the study of the anatomy and the ultrastructure of the nectary of *Cabomba caroliniana* Gray using bright-field microscope, scanning and transmission electron microscope in order to understand its secretion mechanism. *C. caroliniana* has protoginous flowers and the anthesis lasts two days. Nectaries of *C. caroliniana* are located in two basal lobes or yellow auricles of each white petal. Most nectar is observed in the area above the pronounced auricles. The secretion is released mainly by the four-cellular trichomes or hydropotens present in both nectary epidermis. The cellular ultrastructure indicates that the nectary is active during the two days of the anthesis. This agrees with the fact that in both anthesis days the fertile structures of the flower (first the stigmata and then the anthers) are disposed above the nectaries. The nectar secretion mechanism is discussed in relation to the present knowledge. The results of this study are related to what has been described for other basal angiosperms.

Keywords: *Cabomba*; nectary; hydropotens; ultrastructure

1. Introduction

Nectaries are organs or parts of them that secrete nectar. Nectar is an aqueous solution that contains sugars (sucrose, glucose, and fructose), carbohydrates in small amounts, amino acids, proteins and many other compounds, such as inorganic ions, organic acids, vitamins, antioxidants, phenolics, alkaloids, lipids, and terpenoids in minor concentrations (Lüttge, 1961, 1962; Baker and Baker, 1983; Nicolson and Thornburg, 2007). This solution is a

reward for animals that consume it and transport pollen in return. Pollen was considered the main reward for pollinators in the earliest phase of angiosperm evolution. However, in the Early Cretaceous, flowers may also have produced nectar as additional reward (Friis et al., 2011).

In the extant basal angiosperm groups (ANA grade (Amborellales, Nymphaeales, Austrobaileyales) and magnoliids), nectaries are diverse in structure and relatively rare. According to Erbar (2014), this can be interpreted as indicative of convergent evolution. In the ANA grade and in the magnoliids, pollen is the main reward, and when there is nectar secretion, it is in small amounts (Erbar, 2014). In basal angiosperms, nectar secretion is found in species of half of the families. Within Nymphaeales, only Cabombaceae and Nymphaeaceae present nectaries. In the first family, nectar secreting cells are located in the perianth, and in the last one in staminodes. In turn, within Cabombaceae only *Cabomba* presents nectaries, because *Brasenia* is wind pollinated (Osborn and Schneider, 1988), while *Cabomba* is pollinated by bees (Apidae and Halictidae), wasps (Vespidae) and small flies (Diptera) (Schneider and Jeter, 1982; Taylor and Williams, 2009; Bezerra da Silva and de Lima Leite, 2011).

In nectaries of basal angiosperm, which secrete only a limited amount of nectar, it is considered that nectar secretion is through the epidermis (Erbar, 2014). *Cabomba* presents nectaries located in two more or less distinct basal lobes of the petals. These lobes are minute appendages in *C. palaeformis* and distinct auricles in *C. caroliniana* (Erbar, 2014). The histology of the nectary was described exhibiting protoplasm-rich epidermal and mesophyllary cells and multicellular trichomes (Vogel, 1998; Endress, 2008). However, the ultrastructure of these cells, and the mechanism of nectar secretion were not explored.

The aim of this study is to describe the cellular ultrastructure of the nectary of *C. caroliniana* in order to understand its secretion mechanism and to contribute to the knowledge of nectaries evolution in angiosperms, since this species is considered as a genetic model for studies of early angiosperm evolution (Vialette-Guiraud et al., 2011).

2. Material and methods

Nectaries of *C. caroliniana* in pre-anthesis, on the first and second day of anthesis, and in post-anthesis were collected during midday in the Botanical Garden of the Facultad de Agronomía, Buenos Aires, Argentina, where this species is cultivated. A voucher specimen was deposited in the Herbarium Gaspar Xuarez (27847 BAA). The material was pre-fixed in 2.5 % glutaraldehyde in phosphate buffer (pH 7.2) for 2 h and post-fixed in 1.5 % OsO_4 at 2 °C in the same buffer for 3 h. Then, it was dehydrated in ascending acetone series and embedded in Spurr's resin. Sections 1 μm thick were made on a Reichert-Jung ultramicrotome and stained with toluidine blue, observed and photographed with a Motic digital bright-field microscope. Ultrathin sections were stained with uranyl acetate and lead citrate (Zarlavsky, 2014) and photographed in a JEOL 1200 EX II.

Fresh flowers on both anthesis days were photographed with a Wild M5 stereomicroscope and an Arcano digital camera.

Nectar was collected *in situ* in the afternoon, between 12:00 and 15:00, on 20 fresh flowers from each anthesis day. Due to the small nectar production, it was not possible to collect it by traditional methods. However, by rubbing the nectaries with a small brush it was possible to obtain the volume necessary to measure the dissolved solids with a manual refractometer (Eclipse, E-Line ATC model, 0-50 ºBx). Sugar content was expressed as soluble solids.

3. Results

Each white petal of *C. caroliniana* has two basal lobes or yellow auricles where the nectaries are located. Most nectar is observed in the area above the pronounced auricles. This area was considered mainly for the histological and ultrastructural study of the nectary at pre-anthesis, anthesis and post-anthesis stages (Fig. 1A-C).

The transversal section of the nectary presents two to three cellular layers of nectariferous parenchyma between the adaxial and abaxial epidermis (Fig. 2A-E). Epidermal and parenquimatic cells have reduced cytoplasm, plastids with osmiophilic globules and conspicuous vacuoles. Scarce vascular bands with only phloem can be observed (Fig. 2E).

The epidermis presents four-cellular trichomes or hydropotens (Fig. 2F). They are more abundant in the adaxial epidermis (Fig. 2A-F).

This species has protogynous flowers. Flowers on the first-day of anthesis have indehiscent stamens and pollen receptive stigmata (Fig. 1A, C) and on the second day, the stamens elongate to the level of the stigmata, and extrorse dehiscense occurs above the nectaries (Fig. 1B). The anatomy and ultrastructure of the nectary at both anthesis days is coincident (Fig. 2B, C). The cells of all strata of the nectary are less vacuolated than in pre anthesis and present a dense cytoplasm. The ultrastructure of the epidermal and sub-epidermal cells shows abundant dictyosomes, rough endoplasmic reticulum and numerous mitochondria. Conspicuous plastids with numerous osmiophilic globules inside can be observed, both in epidermal and sub-epidermal cells. Some of these plastids present also conspicuous starch grains (Fig. 3A-D).

Trichomes have a large basal cell (BC) at the same level as the other epidermal cells, two thin cells in the middle (MC1 and MC2) and one apical convex cell (AC) (Fig. 4). The outer tangential cell wall of the epidermal cells and the apical cell of the trichomes are covered by a thin striate cuticle. The four cells of the trichomes show the same ultrastructure in pre-anthesis and anthesis stages. The cytoplasm of these cells has several small vacuoles, abundant mitochondria, RER and dictyosomes. Vesicles are present in the cytoplasm and between the plasmalemma and the cell wall of the trichome cells. Numerous plasmodesmata can be observed connecting all the cells of the trichome between them, although some of them are broken (Fig. 5A-D). Fibrillar content can be observed between the cell wall and the plasmalemma of cells of the trichome. When this content is present, the cytoplasm of the cell is contracted, and when it is not observed, the cytoplasm is distended (Figs. 4, 5A). On the second anthesis day, some trichomes have the apical cell contracted, but the ultrastructure of the trichome cells is the same as on the first anthesis day.

In post-anthesis, the nectary cells have a reduced and degraded cytoplasm. The trichome cells present the cytoplasm with signs of cell death. The apical cell is dehydrated or contracted, so it is observed concave and its cell wall is wavy (Fig. 6B, C).

Sugar content in nectar

Sugar content expressed as soluble solids was 2.025 °Bx (SD: 0.54, n=20) in the first day of anthesis and 2.005 °Bx (SD: 0.50, n=20) in the second day of anthesis.

4. Discussion

In basal angiosperms mesophyllary, trichomatic and most commonly, epithelial nectaries were described (Erbar, 2014). However, there are no ultrastructural studies in order to compare our findings in this group. Therefore, this is the first report at ultrastructural level of nectaries in basal angiosperms.

According to Erbar (2014), nectar is not abundant and pollen is the principal reward for pollinators in basal angiosperms. However, in *Cabomba*, the number of pollen grains per anther is low (personal observation). This suggests that the low amount of nectar present during both days of anthesis could be enough for the small pollinators observed by Bezerra da Silva and de Lima Leite (2011). Therefore, nectar would be the main reward for pollinators in this species.

The results indicate that the nectary is active during the two days of the anthesis. In this stage the nectariferous cells have a dense cytoplasm, with numerous dictyosomes, RER and mitochondria. Abundant dictyosomic vesicles between the plasmalemma and the cell wall can be observed. An increased dictyosome activity during this stage was observed in nectaries of other species (Mosti et al., 2013). According to Benner and Schnepf (1975) dictyosomes are the main organelles involved in sugar transport. All these characteristics are related to secretory cells. At pre-anthesis, only the trichomes show these features and they were not observed in any of the nectariferous cells at post-anthesis stage.

The apical cell of the hydropotens is contracted after the secretion ends. This fact seems to indicate that the nectar is released mainly by these trichomes. Great concentration of dictyosomes is observed in trichome cells, and numerous vesicles are present between the plasmalemma and the cell wall of these cells at pre-anthesis stage. Plasmodesmata connecting the trichome cells between them are abundant suggesting an exchange of

substances (Mosti et al., 2001). All these characteristics observed in the trichome cells of *Cabomba* indicate that the activity of the trichomes begins at pre-anthesis stage. The fibrillar substance present between the plasmalemma and the cell wall of the trichome cells of the nectary at anthesis stage can be related with the secretion, since nectar is observed at this stage. According to Nepi (2007) a well-developed endoplasmic reticulum, numerous dictyosomes and vesicles are characteristic of granulocrine secretion. However, Vassilyev (2010) considers that the concept of granulocrine secretion of nectar should be discarded. According to this author, sugars cross the plasma membrane by active transport ('eccrine secretion'). Recently, Paiva (2016) proposed a new hypothesis involving a mechanical action of the protoplast. According to this author, successive cycles of contraction and expansion cause the material accumulated between the plasmalemma and the cell wall to pass through the latter and the cuticle. These contraction and expansion cycles are produced by the accumulation of Golgi vesicles that release their contents in the periplasmic space. Thus, the secreted substances can be released, without a requirement of energy, even against a concentration gradient, as pointed-out by Lüttge and Schnepf (1976). On the other hand, according to Nepi et al. (2011), the pressure is not necessarily generated by the mechanical action of the protoplast, but instead by an osmotic process.

The model of mechanical action of the protoplast proposed by Paiva (2016) could occur in *C. caroliniana*, since the trichome cells present numerous dictyosomes with abundant vesicles in pre-anthesis and anthesis stages. These vesicles carry the nectar and their expansion, as they fill with nectar, could generate the expansion of the protoplast. This theory is reinforced by the fact that the protoplast of some trichome cells is observed contracted, and in others expanded. When the cytoplasm is expanded, numerous vacuoles are also present. These vacuoles could be the result of fusion of dictyosome vesicles containing nectar. Moreover, the rupture of some plasmodesmata that connect the trichome cells to each other is a consequence of the pressure generated by the substances accumulated in the periplasmic space. All of this could be indicating cycles of contraction and expansion. In addition, the ultrastructure of the cell wall of the trichome apical cell seems to facilitate the nectar passage, since it presents a lax fibrillar structure and the cuticle is very thin.

The epidermal and sub-epidermal cells of the nectary have numerous conspicuous plastids with osmiophilic globules. These plastids would be responsible for the yellow colour of the nectary. According to Whitney et al. (2013) the colour contrast of a flower with its background has a greater influence on bee preference. In the same way, the yellow auricles where the nectaries are located contrast with the white background of the petal. This would conduct or guide to the pollinator directly to the nectar secretion.

C. caroliniana has protogynous flowers. According to Erbar and Leins (2013) nectar production is limited mainly to the female phase of the protogynous flower. By the nectar presentation in the female phase of anthesis, the attractiveness of the flower would be also assured in the non-pollen presenting phase of anthesis (Erbar, 2014). However, in *C. caroliniana* the nectar secretion is present in the two days that anthesis lasts, or what is the same, in both phases (``female´´ and ``male´´) of the anthesis. However, the secretion seems to be more reduced on the second day, because it is observed merely as a bright pellicle on the nectary, while at the first day of anthesis, small drops of nectar are present on the petal auricles. Coincidentally, numerous trichomes are observed with the apical cell contracted on the second day of anthesis. This would indicate that several trichomes have ceased to secrete and for this reason the amount of nectar is more reduced. Despite this, the sugar content is equivalent in both anthesis days. According to Schneider and Jeter (1982) at the first-day of anthesis, flowers of *C. caroliniana* have short, indehiscent stamens and longer pollen-receptive stigmata which arch outward over the nectaries. In second day of anthesis, the stamens elongate to the level of the stigmata and extrorse dehiscence occurs above the nectaries. Therefore, the fertile structures of the flower are disposed above the nectaries during the two days that anthesis lasts. This is in agreement with the activity of the nectary detected in this research, which develops during both anthesis days.

Conclusions

The nectary of *C. caroliniana* is active during the two anthesis days. The secretion is released mainly by the four-celled trichomes. The secretion crosses through the cell wall and the cuticle. Cycles of contraction and expansion of the protoplast of the trichome cells seem to be generating the passage of the nectar from one cell to another and finally its

release through the cell wall and the cuticle of the apical cell. These results contribute to the general knowledge of this genus, in order to be used as a genetic model for the studies of the early evolution of the angiosperm.

Conflict of interest

The authors declare that they have no conflict of interest.

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Contributors

BG conducted the research. BG and MG wrote the manuscript, revised and completed the information. SR revised and corrected the manuscript. LF measured the sugar content. GZ prepared and processed all the materials for observation. All authors have approved the final article.**References**

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Fig. 1. General aspect of the *C. caroliniana* flowers. (A) Flower in first anthesis day. (B) Flower in second anthesis day. (C) Detail of nectar drops in flower in first anthesis day. Scale bars: (A) 0.53 mm, (B) 0.52 mm, (C) 0.26 mm.

Fig. 2. Nectary photomicrographs with bright field microscope. (A) Transversal section of the nectary in pre-anthesis stage. (B) Transversal section of the nectary in first day of anthesis. (C) Transversal section of the nectary in second day of anthesis. (D-F) Transversal section of the nectary in post-anthesis stage; vb: vascular band. (F) Detail of a trichome or hydropoten (h). Scale bars: (A-F) 50 µm.

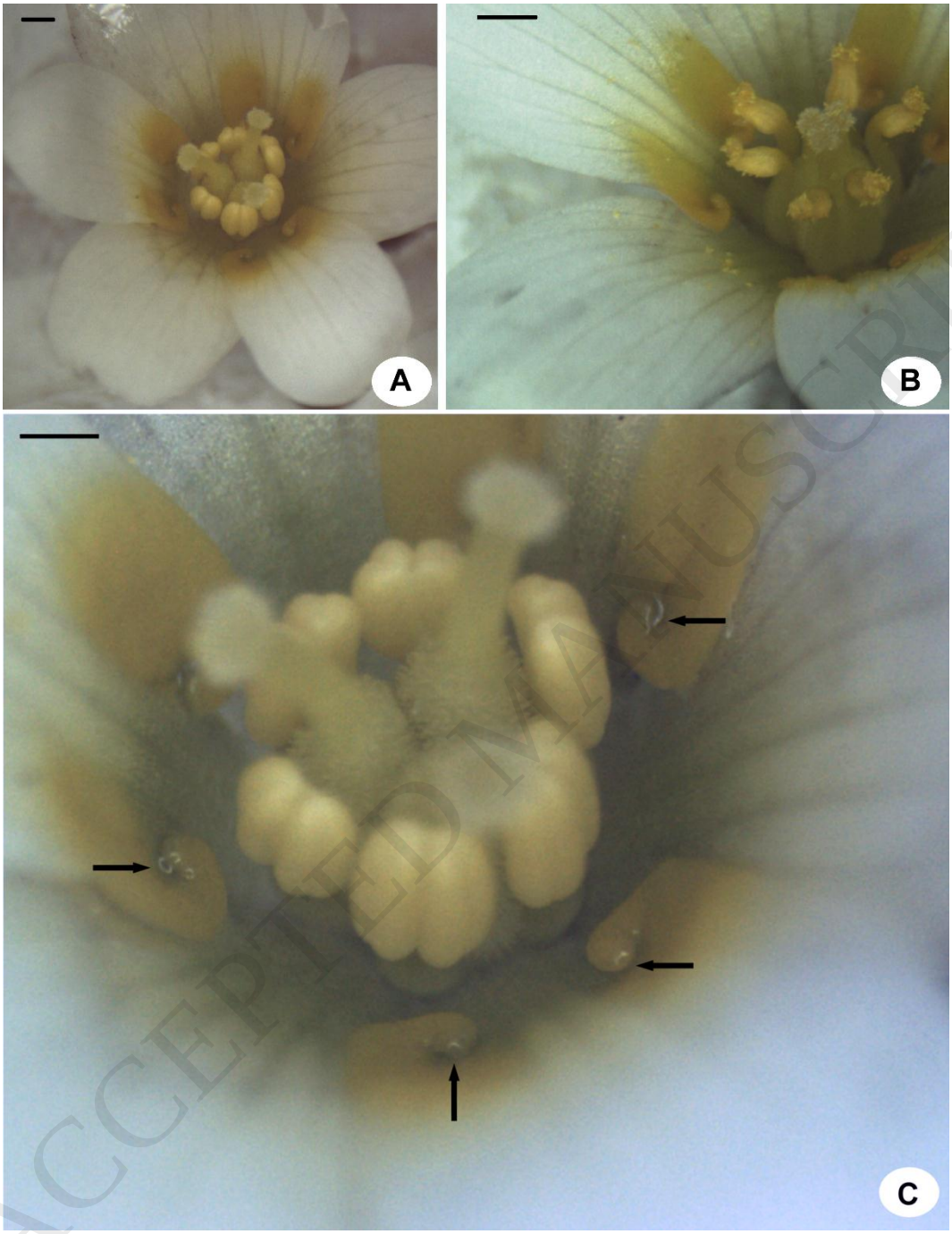
Fig. 3. Nectary ultrastructure. (A-C) Detail of epidermal cell of the nectar. (D) Detail of cytoplasm of sub-epidermal cell of the nectary. p, plastid; s, starch; m, mitochondria; d, dictyosome. Scale bars: (A) 1 µm, (B-C) 0.5 µm.

Fig. 4. Longitudinal section of the trichome in first day of anthesis observed with TEM. BC: basal cell. MC1: middle cell 1. MC2: middle cell 2. AC: apical cell. Scale bar: 1 µm.

Fig. 5. Trichome ultrastructure. (A-B) Detail of basal cell (BC), middle cell 1 (MC1) and middle cell 2 (MC2), showing abundant plasmodesmata (arrows), mitochondria (m), plastids (p) and dictyosomes (d). (C) Detail of apical cell (AC) and middle cell 2 (MC2) showing nucleus (N), mitochondria (m), rough endoplasmic reticulum (RER) and plasmodesmata connecting both cells. (D) Detail of apical cell cytoplasm showing mitochondria (m), nucleus (N), rough endoplasmic reticulum (RER) and dictyosomes (d). Scale bars: (A-C) 0.5 µm.

Fig. 6. (A) General aspect of the trichomes observed with SEM. (B) Detail of longitudinal section of the trichome at post-anthesis stage observed with TEM. Scale bars: (A) 50 µm, (B) 0.5 µm.

Figr-1



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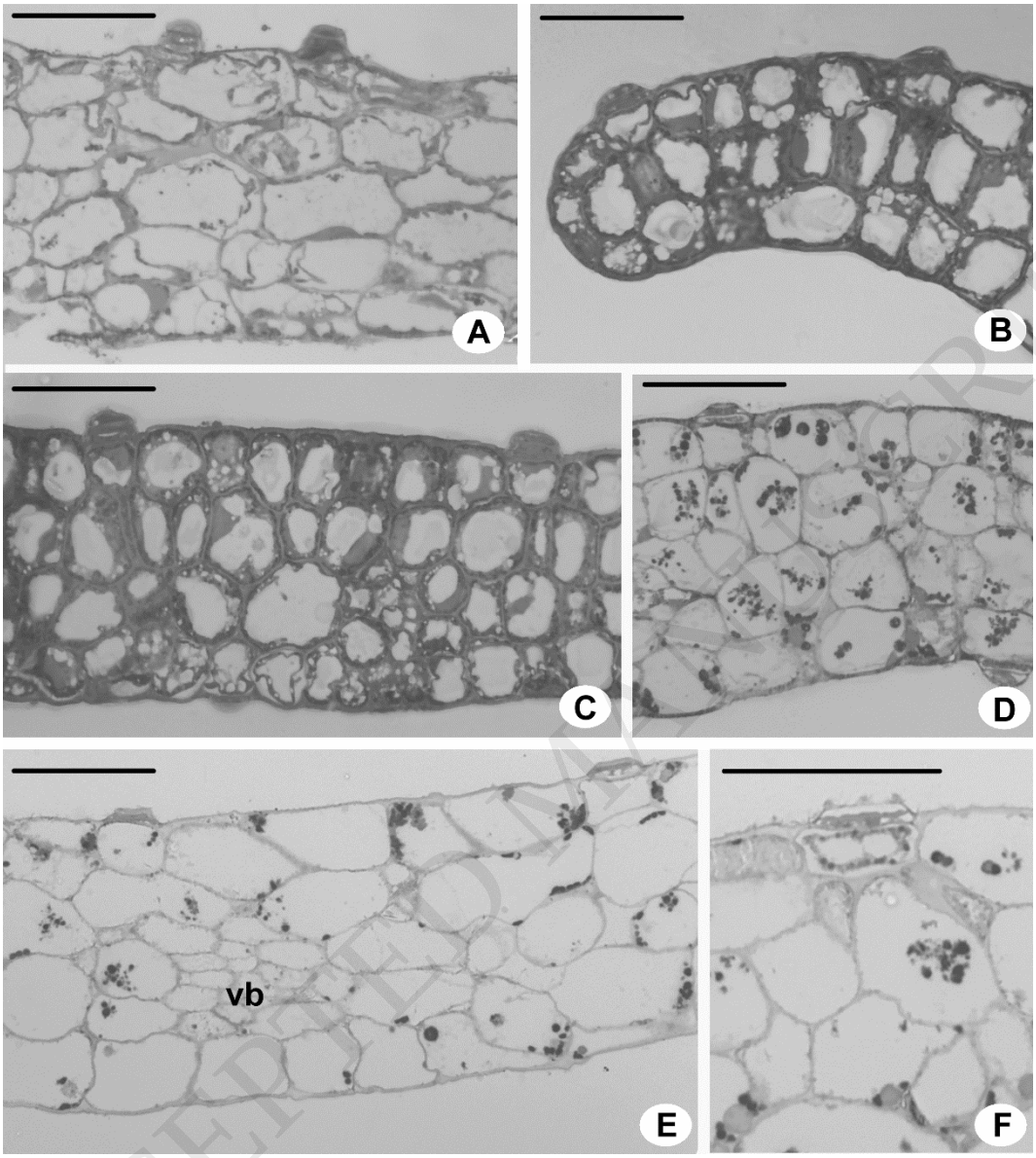

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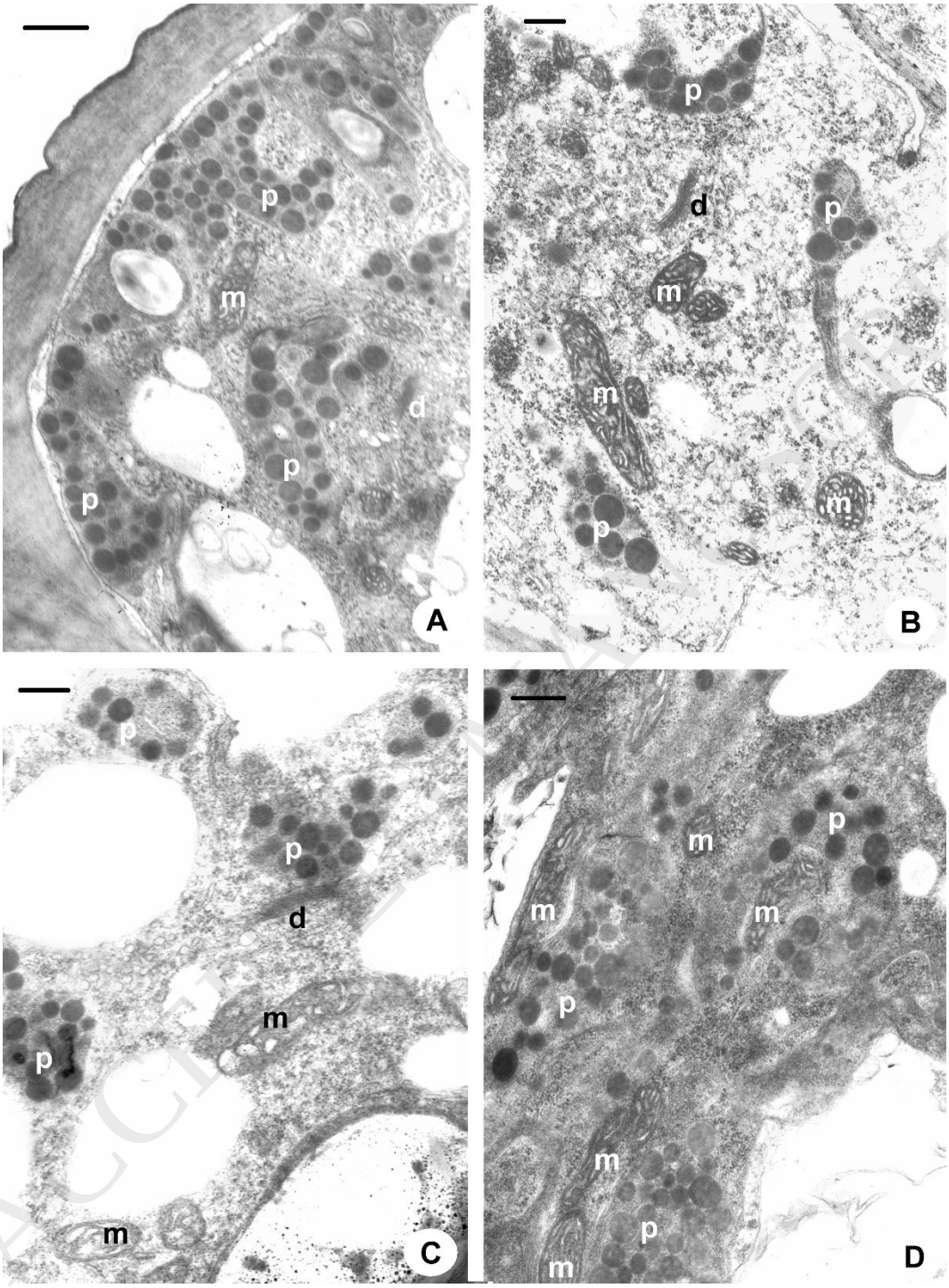

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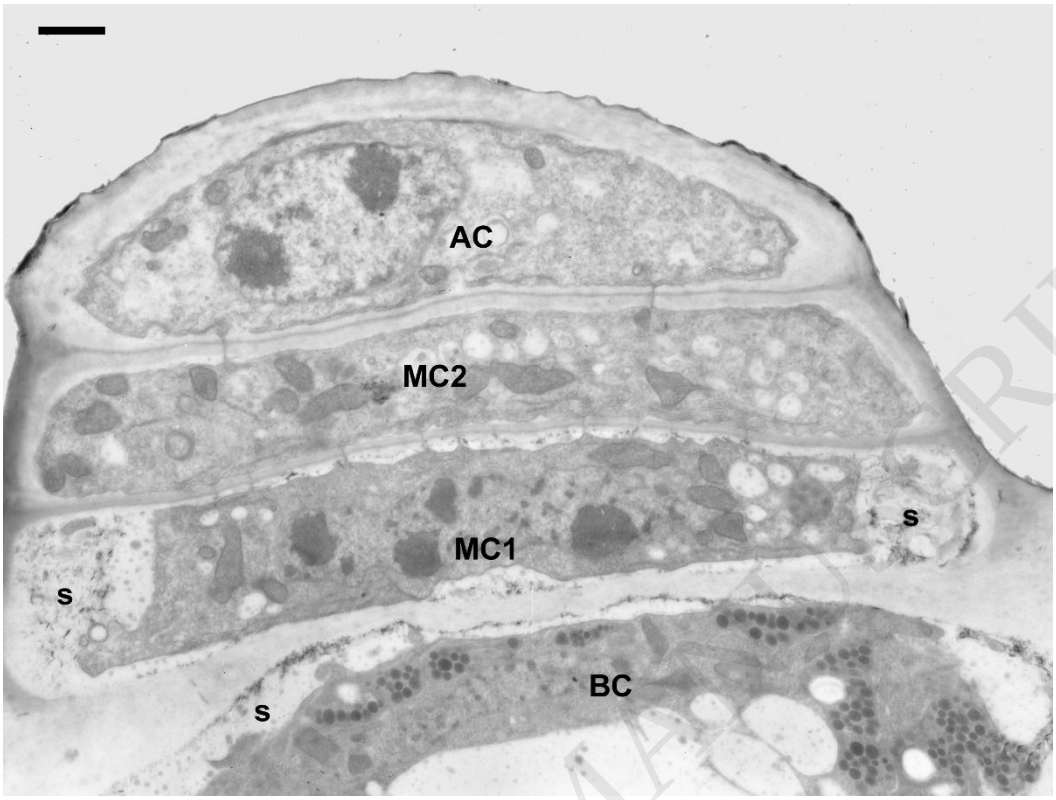

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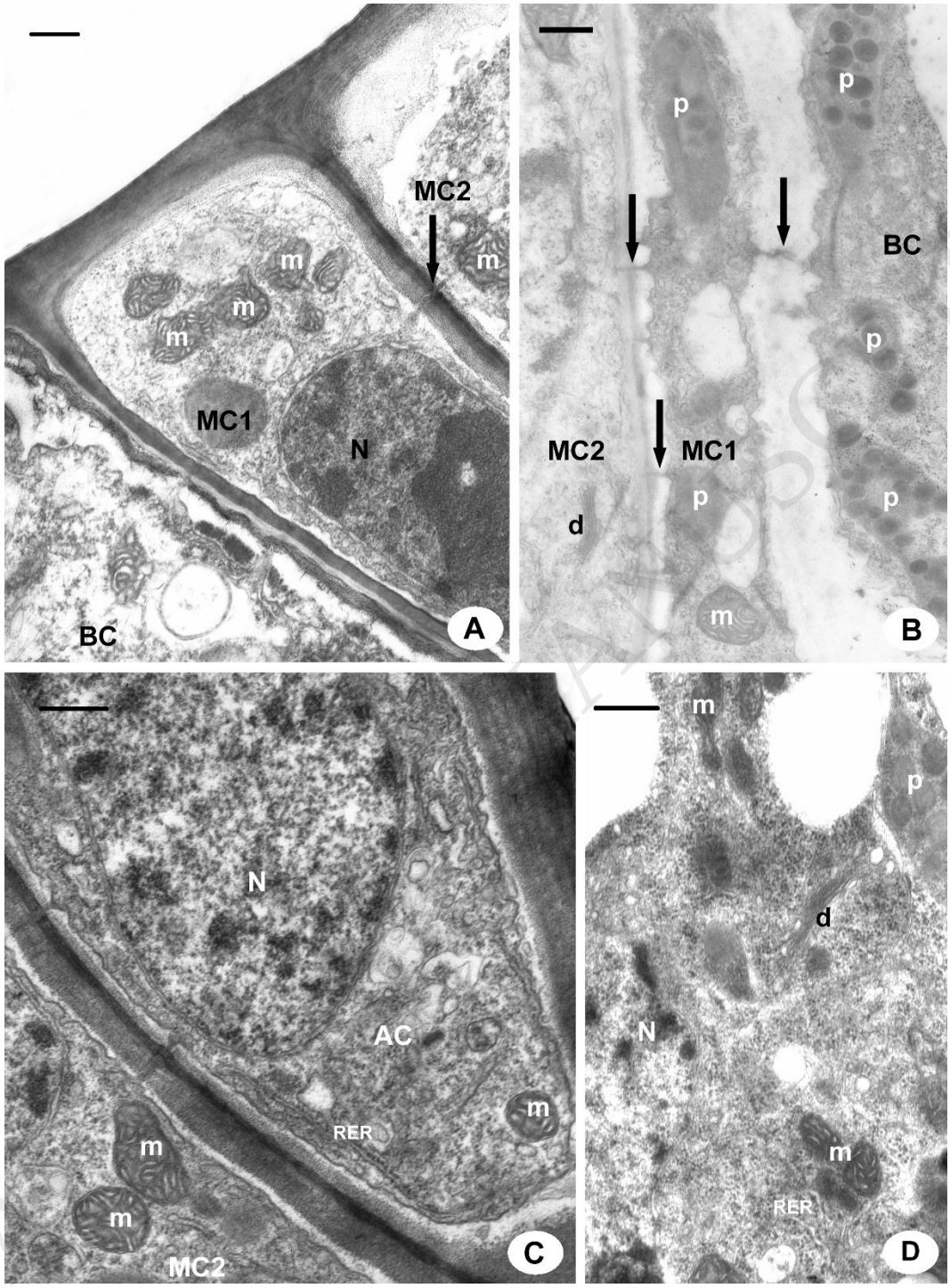

Figr-6