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MICROMORPHOLOGY AND ULTRASTRUCTURE OF THE FLORAL NECTARIES OF *VIOLA ODORATA* L. (VIOLACEAE)

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Abstract. In *Viola odorata* two inferior anthers have connective appendages (nectaries) projecting into the corolla spur. Nectaries are approx. 4 mm long, elongate, with the top of the nectary bending to the lateral wall of the spur. In the top part and in the abaxial surface of middle part of the nectary all cells have papillae. Nectar is secreted through the modified stomata distributed mainly in the top of nectary The nectary consists of single-layered epidermis, nectary parenchyma and subnectary parenchyma. Features of the nectary parenchyma cells, like dense cytoplasm containing numerous mitochondria and large nuclei, are connected with high metabolic cell activity. The vascularization includes both phloem and xylem. A slight amount of starch in the nectary cells, the profusion of plasmodesmata connecting secretory cells and the presence of vascular bundles suggest that sugars secreted in the nectar were delivered by the phloem sap.

Key words: *Viola odorata*, nectary, floral nectaries, ultrastructure, micromorphology

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Introduction

Nectaries are secretory structures that occur on the plant surface, being specialized for the secretion of a sweet solution called nectar (Bernardello 2007; Coutinho *et al.* 2012). According to the topography, we can distinguish two different types of nectaries – floral and extrafloral. Nevertheless, their function is to reward animals – pollinators for the pollen disperse and ants for their physical defense (Bernardello 2007).

The genus *Viola* L., belonging to the Violaceae family, contains approx. 550-600 species (including 25 Polish species). The genus is distributed mainly in the Northern hemisphere. Species can be annuals, biennials, deciduous or evergreen perennials, with simple or pinnately lobed leaves and 5-petalled, zygomorphic flowers. Stolons and rhizome-like products may be present, but not compulsory.

Viola odorata L., commonly known as Sweet Violet, is a small perennial with a short rhizome and long, thin stolons. The flowers are aromatic and consist of five sepals, five petals. Two stamens form appendages that are projected into the corolla spur. The stamina appendages are described as nectaries. *V. odorata* prefers fertile, moist soil with neutral pH. It can grow in semi-shade (light woodland) or no shade.

No data were described about the structure or ultrastructure of the floral nectaries of *V. odorata*. Therefore, the aims of present study are to study nectary micromorphology and ultrastructure using scanning and transmission electron microscopy.

Material and methods

The plant material was received from prof. E. Kuta from the Jagiellonian University in Cracow, Department of Plant Cytology and Embryology, than cultivated in plant growth chambers at the University of Gdańsk, Faculty of Biology, Department of Plant Cytology and Embryology. The study material comprised chasmogamous flowers of *V. odorata* (Fig. 1 A, B), sampled at the first day of anthesis.

The nectarines isolated from flowers were fixed in solution of 2.5% glutaraldehyde with 2.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7) at temperature of 4°C for 24 h. When fixed, the samples were washed in cacodylate buffer and postfixed in 1% OsO_4

for 24 h. The plant material was stained with a 1% aqueous solution of uranyl acetate, dehydrated in acetone and embedded in Spurr's low viscosity resin (Spurr 1969).Ultrathin sections (60 nm thick) were treated with 8% solution of uranyl acetate in acetic acid and with lead citrate. The sections were observed and recorded using a Philips CM 100 transmission electron microscopy. Semi-thin control sections for light microscopy were stained with Toluidine Blue O (TBO).

For scanning electron microscopy (SEM) staminal appendages were fixed in 4% glutaraldehyde with 0.1 M phosphate buffer (pH 7.2) at temperature of 4°C for 12 h. Subsequently, the samples were dehydrated in an ethanol series and dried at the critical -point in a Critical Point Dryer Emitech K850 apparatus. Using a CS 100 sputter coater, they were coated with gold. The preparations were observed using a Philips XL 30 SEM.

Results

Light microscopy (LM)

The nectary consists of three layers: a single-layered epidermis, nectary parenchyma and subnectary parenchyma (Fig. 1 C). The nectary epidermis in the top of the nectary has papillae (Fig. 1 D). The nectary parenchyma consists of small cells with densely staining cytoplasm. The subnectary parenchyma is characterized by larger, more loosely packed cells with cytoplasm concentrated near the walls, and the presence of large, centrally located vacuoles (Fig. 1 E). The vascular bundle is present, consisting of both phloem and xylem (Fig. 1 E).

Scanning electron microscopy (SEM)

In *V. odorata* the basal parts of two inferior anthers have connective appendages (nectaries) projecting into the corolla spur. Nectary is elongate, narrower in the top part and distinctly widen in the middle (Fig. 2 A). Top of the nectary is bent to the lateral wall of the spur (Fig. 2 A). The nectary epidermis has papillae and trichomes. In the top part and in the abaxial surface of middle part of the nectary all cells have papillae (Fig. 2 B-D). Trichomes occur below the top of the nectary and in the middle part below nectary bend (Fig. 2 B, D). The papillae have their outer walls covered with a thick striated cuticle, which is noticeably smoother in the trichomes (Fig. 2 D). Both in the middle and in the basal part of the nectary cells do not have papillae and the trichomes are rarely present. Cells here are elongated and polygonal. The cuticle here is smoother and has striations, which are parallel to the cell axis (Fig. 3 A, B).

Anomocytic stomata (Fig. 3 D) are distributed mainly in the top of nectary (Fig. 2 C; Fig. 3 E). Nectar is secreted through the modified stomata (Fig. 3 C, E, F). They are situated in small depressions and surrounded by variable number of epidermal cells (Fig. 2 C; Fig. 3 C, D). A great amount of secretory remnants is visible at the top of nectary (Fig. 3 E, F).

Transmision electron microscopy (TEM)

The epidermal and parenchymal cells of nectary form compact system, without intercellular spaces (Fig. 4 A). At the beginning of anthesis, the nucleus is large, with a conspicuous nucleolus (Fig. 4 A, B). The cells contain a poorly-developed vacuolar system, generally with a single small vacuole or sometimes with lack of it (Fig. 4 A, C). The plastids are numerous (Fig. 4 B, C), with a dense stroma and a well-developed membrane system. The round plastoglobuli and tubular membranes are present (Fig. 4 D, E; Fig. 5 A). Chloroplast-like plastids have been noted (Fig. 4 E). Most plastids contain single starch grain (Fig. 4 F). In the cytoplasm numerous profiles of rough endoplasmic reticulum (rER) (Fig. 4 B; Fig. 5A), numerous mitochondria (Fig. 4 B, C), some microbodies (not illustrated), and few lipid bodies (Fig. 4 E) are observed. Dictyosomes are rarely noted and are not demonstrated any visible signs of activity. The plasmalemma is smooth (Fig. 4 F).The parenchymal cells of the nectary are connected by numerous plasmodesmata that can be involved in symplastic transport of nectar (Fig. 5 B). The autophagic vacuoles, sometimes with plastids inside, in different stages of development are noted in numerous cells.

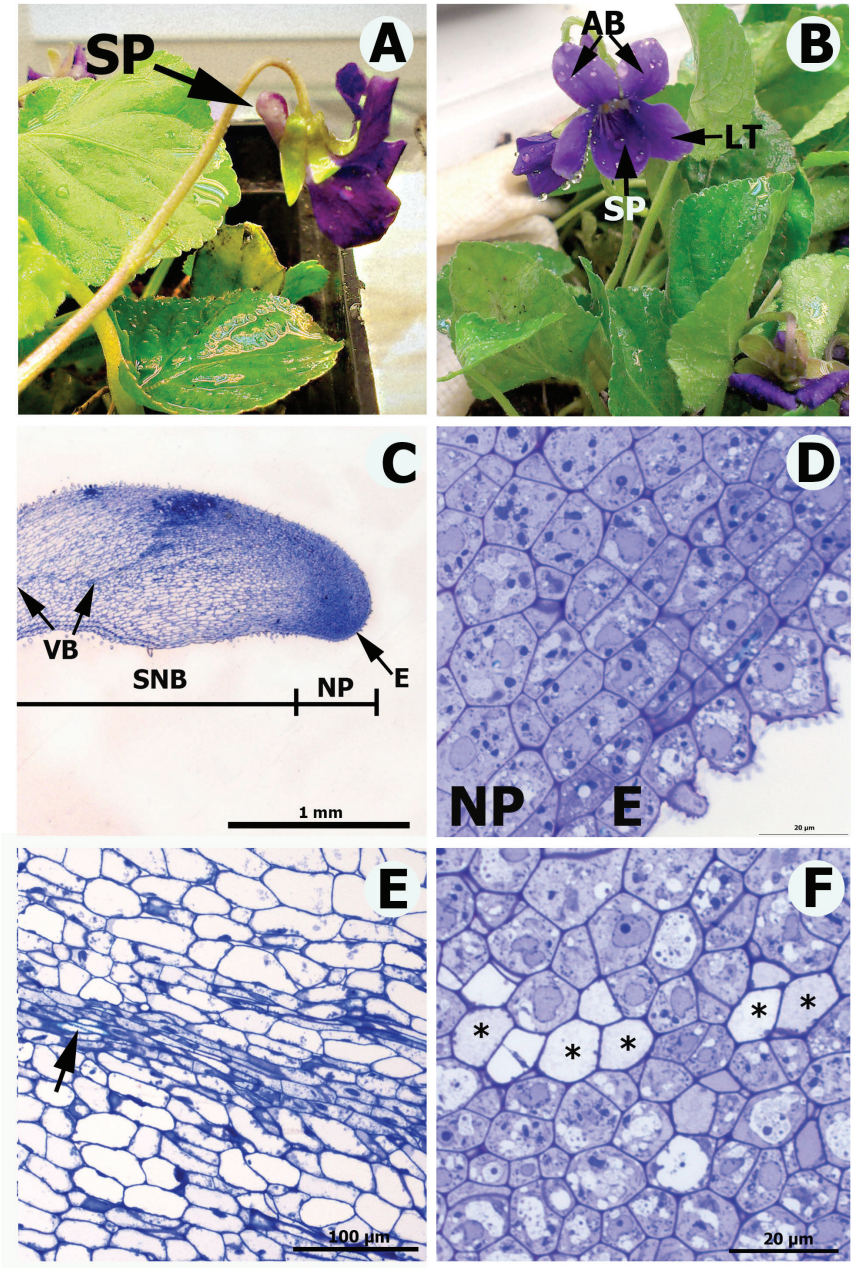

Fig. 1. *Viola odorata*: **A, B** – flowers, each made by five sepals of three kinds in shape, two above alike (**AB**), two on each side alike (**LT**) and one that has a spur (**SP**); **C** – longitudinal section of nectary consisting of epidermis (**E**), nectary parenchyma (**NP**) and subnectary parenchyma (**SBN**) with vascular bundle (**VB**) (LM, TBO); **D** – magnification of **C**, the top part of the nectary including epidermis (**E**) and nectary parenchyma (**NP**), note small cells with densely staining cytoplasm; **E** – subnectary parenchyma with vascular bundle (**VB**), containing xylem indicated by arrow; **F** – nectary parenchyma with phloem cells (**asterisks**).

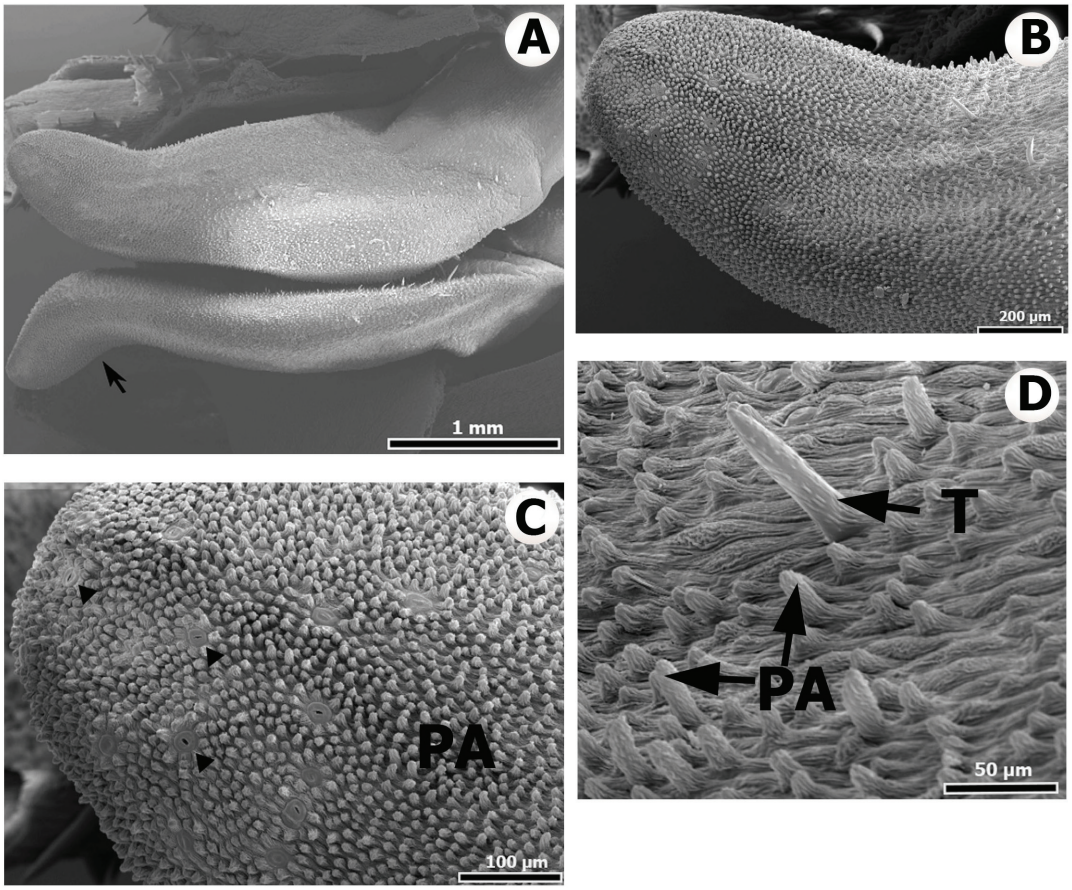

Fig. 2. SEM images illustrating: **A** – nectary with the top bending (**arrow**) to the lateral wall of the spur; **B-C** – magnification of the surface at the apex built by papillae (**PA**) with numerous stomata indicating by triangles; **D** – magnification of **B**, the trichomes in the middle part below nectary bend (**PA** – papilla, **T** – trichome).

Near the top of the nectary phloem cells are noted (Fig. 4 C).

Stomata cells have electron-translucent cytoplasm, large nucleus with nucleolus, lipid bodies, a few mitochondria and large amyloplasts (Fig. 5 F). There is a membrane between stomata cells, with a large intercellular space directly beneath (Fig. 5 E).

There are also small intercellular spaces between the nectary parenchymal cells adjacent to stomata, sometimes filled with darker-stained osmiophilic substance (Fig. 5 C).

With the age, cells become more vacuolated and the cytoplasm is visible as parietal layer (Fig. 5 D).

Discussion

According to NEPI (2007), the nectary is made up of three components: epidermis, nectary parenchyma and subnectary parenchyma. In the nectaries of *V. odorata* all these components of the anatomical structure are recognizable. This model of nectary structure has been reported for many other plant species (DURKEE 1982; FAHN 1979, 1988, 2000; BERNARDELLO 2007; NEPI 2007; PAIVA & MACHADO, 2008; STPICZYŃSKA *et al.* 2012; KOWALKOWSKA *et al.* 2014). Ultrastructural analysis shows that nectary parenchyma comprises a several layers of small cells with dense cytoplasm, small vacuoles, large nuclei, abundant mitochondria

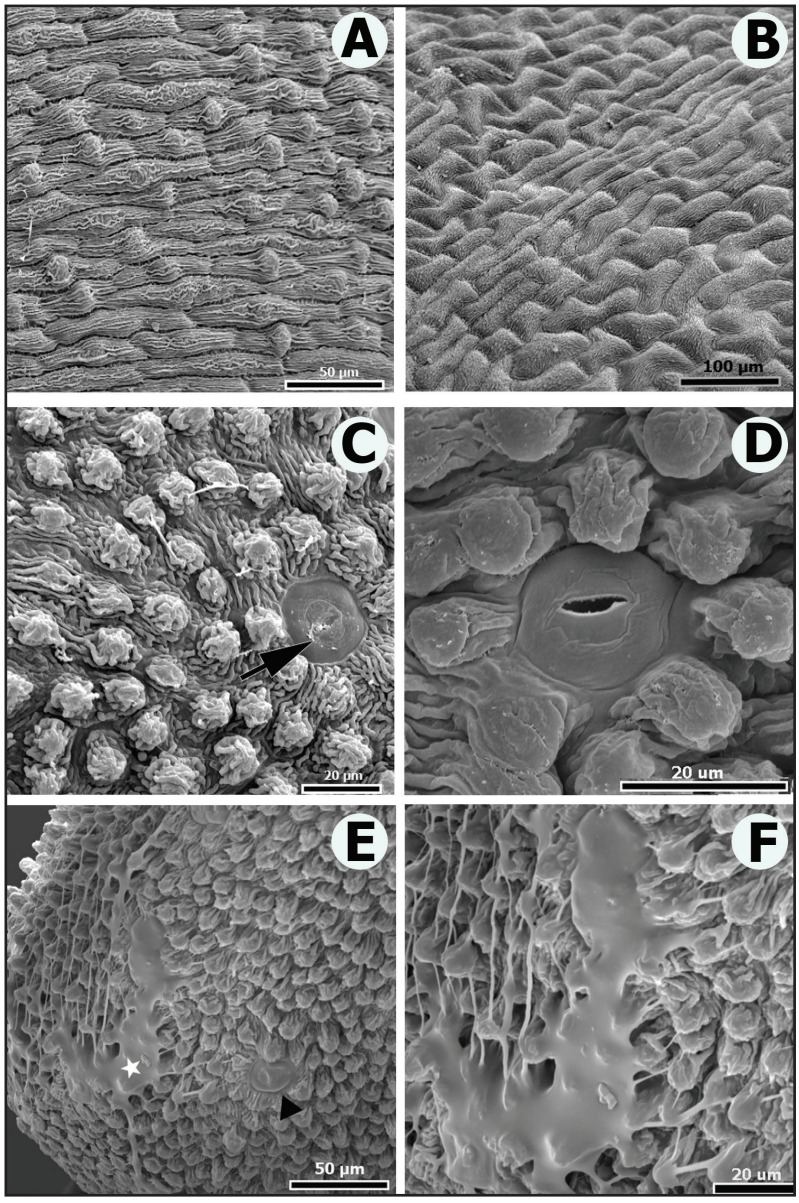

Fig. 3. SEM images illustrating: **A, B** – nectary surface of middle part (**A**) and base (**B**), built by smoother cells with striations, which are parallel to the cell axis of nectary; **C-D** – nectary stomata with secretory remnants (**arrow**); **E** – the apex of the nectary with great amount of secretory remnants (**asterisk**); **F** – magnification of **E**, secretory remnants visible on the top.

and numerous ER. These features have been reported in other plant species and are indicative of high metabolic activities (Fahn 1979, 1988; Durkee 1982, Paiva & Machado 2008; Paiva 2009; Konarska 2011; Kowalkowska *et al.* 2014).

Parenchyma volume is proportional to the quantity of nectar produced by a nectary (Pacini *et al.* 2003). The presence of a well developed rER in nectar-secreting cells and increasing its volume with secretion seems to be associated with the transport of pre-nectar,

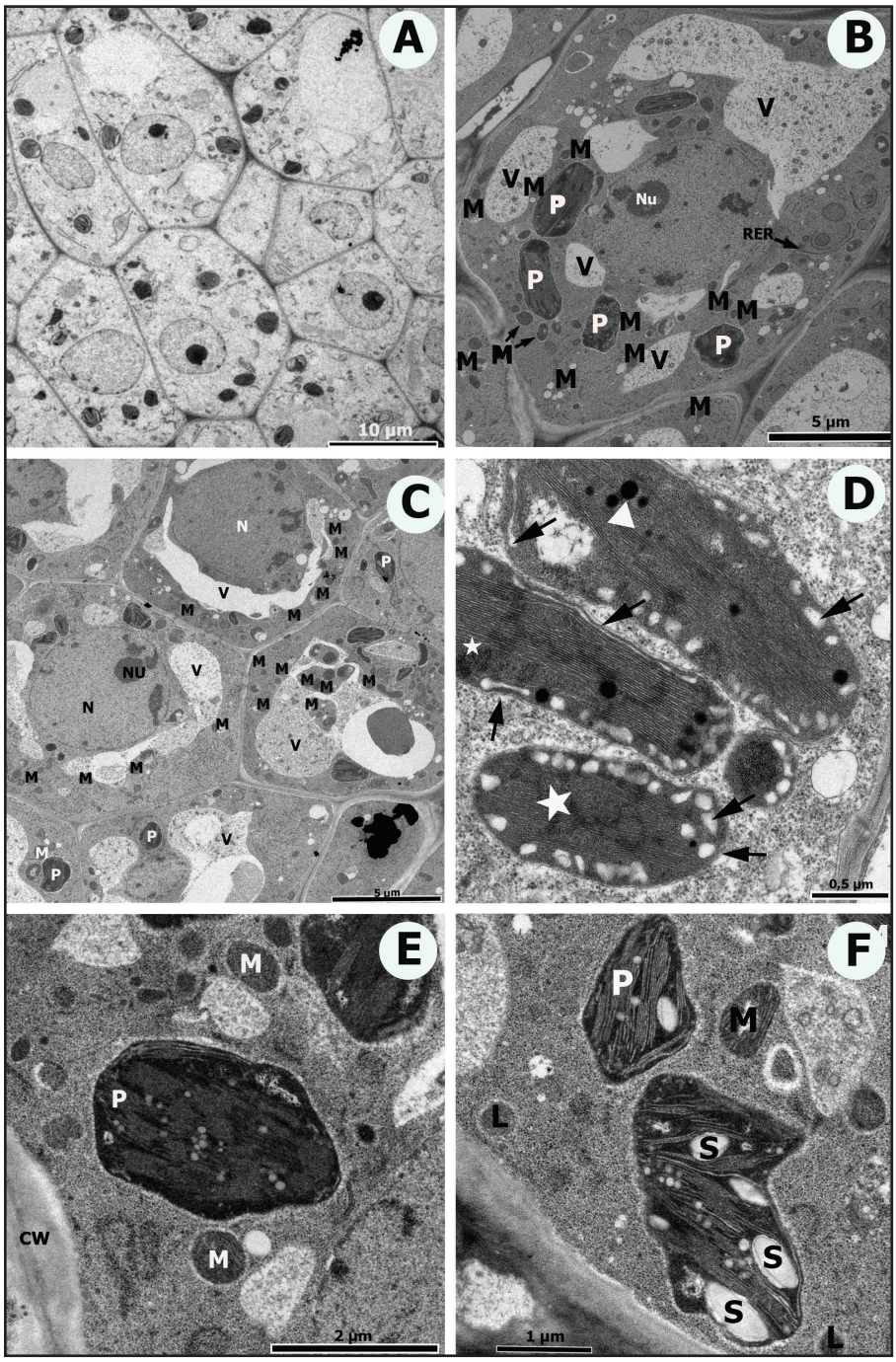

Fig. 4. TEM images: **A** – compact system of parenchymal cells; **B-C** – nectary parenchyma cells, note the autophagic vacuole (**V**) with mitochondria (**M**) inside; **D** – plastids with round plastoglobuli (**white triangle**)and tubular membranes (**arrows**) and phytoferritin indicated by **white asterisk**; **E** – chloroplast-like plastids (**P**); **F** – great amount of rough endoplasmic reticulum (rER) in epidermis cell of nectary in advanced anthesis. Common abbreviations: **CW** – cell wall; **L** – lipid body; **M** – mitochondria; **N** – nucleus; **NU** – nuclei; **P** – plastids; **RER** – endoplasmic reticulum; **S** – starch.

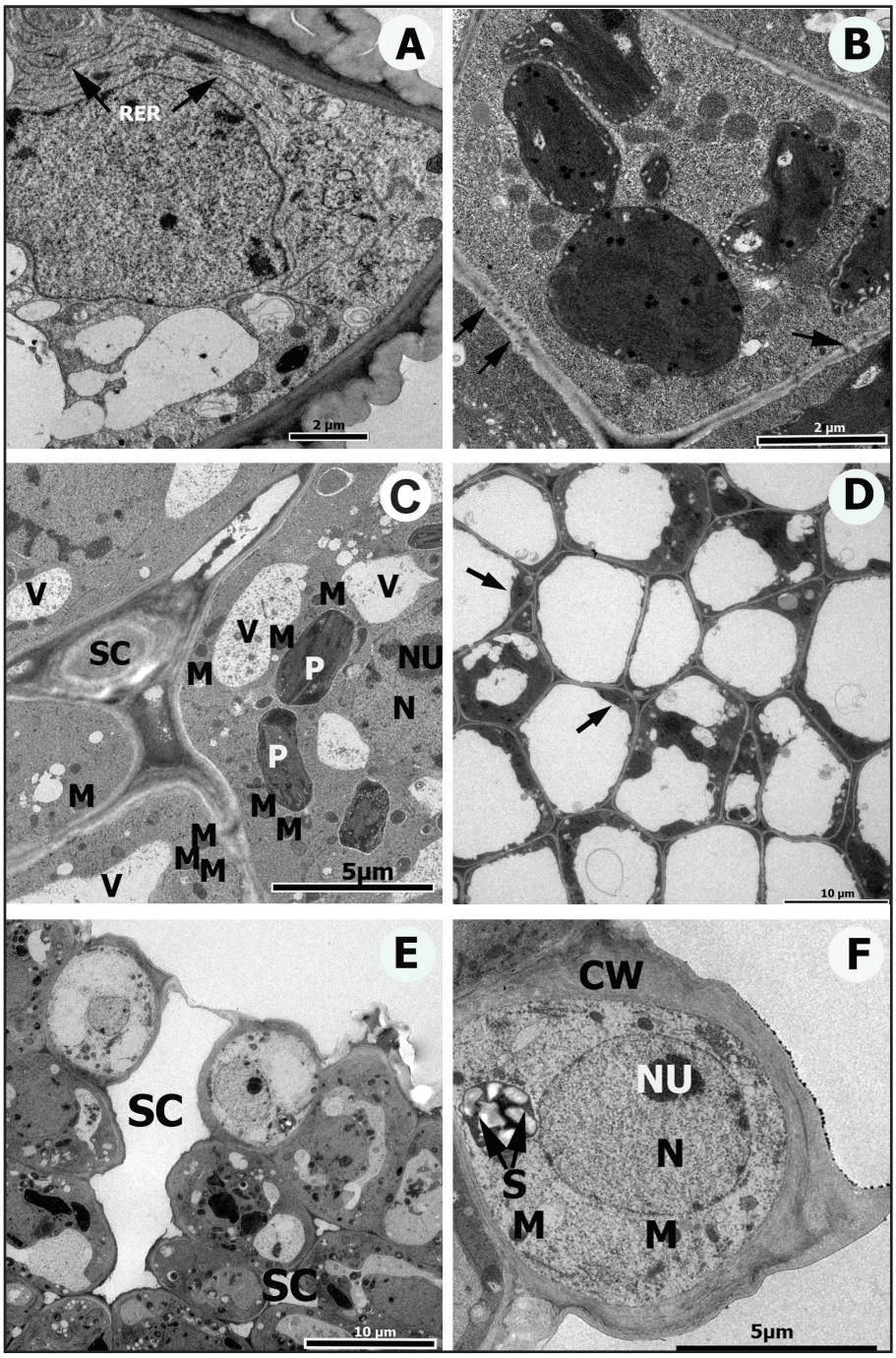

Fig. 5. Ultra-thin sections (TEM) of nectary parenchyma: **A** – plastids with starch grains (**S**); **B** – parenchymal cells connected via numerous plasmodesmata (**arrows**); **C** – substomatal cavity (**SC**) sometimes filled with darker-stained osmiophilic substance; **D** – more vacuolated cells with parietal layer (**arrow**) of cytoplasm at the end of anthesis; **E-F** – nectary stomata with substomatal cavity below, note the amyloplasts with starch grains (**S**) in stomata cells. Common abbreviations: **CW** – cell wall; **L** – lipid body; **M** – mitochondria; **N** – nucleus; **Nu** – nuclei; **P** – plastids; **RER** – endoplasmic reticulum; **S** – starch.

and has been observed in many diverse types of nectaries (Fahn 1988, 2000; Paiva 2009). The presence of different organelles inside autophagic vacuoles seems to be related with programmed cells death (PCD) and autophagic behavior. The plastid degradation mediated by vacuoles during PCD was described in *Digitalis purpurea* L., *Eccremocarpus scaber* Ruiz et Pav., *Arabidopsis thaliana* (L.) Heynh. and *Hymenaea stigonocarpa* Hayne (Paiva & Machando 2008, and references cited therein).

A slight amount of starch in the nectary cells of *V. odorata* and the profusion of plasmodesmata connecting secretory cells suggest that sugars secreted in the nectar were delivered by the phloem sap. Nectariferous tissue, supplied with vascular bundles that ended in the nectary parenchyma, only confirms this model of transport. According to this model, pre-nectar is moved along the symplast within secretory tissue, and then it is transferred to adjacent phloem parenchyma and intercellular spaces, where it is secreted through the stomata. This model of pre-nectar movement were proposed by several researchers (Nepi 2007, and references cited therein; Vassilyev 2010).

Conclusions

In conclusion, results of our studies revealed that in *V. odorata* the nectaries are connective appendages of two inferior anthers. In their anatomy, three layers have been distinguished: epidermis, nectary parenchyma and subnectary parenchyma. This model was previously described in other species. Ultrastructural studies showed that nectary parenchyma cells contain dense cytoplasm, plastids with plastoglobuli, numerous mitochondria and endoplasmic reticulum – these features are indicative of high metabolic activities of parenchymal cells. The presence of vascular bundles, slight amount of starch in the nectary cells and the profusion of plasmodesmata connecting parenchymal cells suggest that sugars secreted in the nectar were delivered by the phloem sap.

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