Morphological and anatomical characterization of extrafloral nectaries of *Opuntia streptacantha* and *Ferocactus recurvus* (Cactaceae)

Caracterización morfológica y anatómica de los nectarios extraflorales de *Opuntia streptacantha* y *Ferocactus recurvus* (Cactaceae)

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Abstract

Background: The Cactaceae family displays remarkable diversity in the morphology of extrafloral nectaries (EFNs). Despite their taxonomic, ecological, and evolutionary significance, their anatomy and morphology are poorly understood.

Questions: How are the morphological and anatomical attributes of extrafloral nectaries in *Opuntia streptacantha* and *Ferocactus recurvus*?

Studied species: *Opuntia streptacantha* Lem. and *Ferocactus recurvus* (Mill.) Borg.

Study site and dates: Helia Bravo Hollis Botanical Garden, Zapotitlan Salinas, State of Puebla, México, during 2017.

Methods: EFNs samples were collected from the plants, fixed in glutaraldehyde, and processed for analysis using scanning electron microscopy and light microscopy.

Results: In both species, EFNs are modified spines adapted for nectar secretion. In *F. recurvus*, they are elongated and blunt, and epidermal cells are wrinkled, forming a lump at the tip. In *O. streptacantha*, EFNs possess an apical secretory cone where nectar is stored and exuded. This region has globular and imbricated, bag-shaped epidermal cells without stomata. We distinguished three regions in these nectaries: an apical secretory cone, a middle elongation section, and a basal meristematic region. The apical secretory cone has globular epidermal cells that surround a lignified region of the spine. We could not detect vascularization in the extrafloral nectaries of *O. streptacantha*.

Conclusions: This study reports, for the first time, the existence of EFNs in O. streptacantha and sheds light on the histological and morphological characteristics of EFNs in F. recurvus.

Keywords: microscopy, nectar secretion, nectaries, EFNs, secretory spines.

Resumen:

Antecedentes: La familia Cactaceae muestra una notable diversidad en la morfología de los nectarios extraflorales (EFNs). A pesar de su importancia taxonómica, ecológica y evolutiva, su anatomía y morfología son poco conocidas.

Preguntas: ¿Cómo son los atributos morfológicos y anatómicos de los nectarios extraflorales en *Opuntia streptacantha* y *Ferocactus recurvus*?

Especies de estudio: *Opuntia streptacantha* Lem. and *Ferocactus recurvus* (Mill.) Borg.

Sitio y años de estudio: Jardín Botánico Helia Bravo Hollis, Zapotitlán Salinas, Estado de Puebla, México, durante 2017.

Métodos: Se recolectaron muestras de EFNs de las plantas, se fijaron en glutaraldehído y se procesaron para el análisis utilizando microscopía electrónica de barrido y microscopía de luz.

Resultados: En ambas especies, las EFNs son espinas modificadas adaptadas para la secreción de néctar. En *F. recurvus*, son alargadas y romas, y las células epidérmicas están arrugadas, formando un bulto en la punta. En *O. streptacantha*, las EFNs poseen un cono secretor apical donde se almacena y exuda el néctar. Esta región tiene células epidérmicas globulares e imbricadas, con forma de bolsa, sin estomas. Distinguimos tres regiones en estos nectarios: un cono secretor apical, una sección de elongación media y una región meristemática basal. El cono secretor apical tiene células epidérmicas globulares que rodean una región lignificada de la espina. No pudimos detectar vascularización en los nectarios extraflorales de *O. streptacantha*.

Conclusiones: Este estudio informa, por primera vez, la existencia de EFNs en *O. streptacantha* y arroja luz sobre las características histológicas y morfológicas de las EFNs en *F. recurvus*.

Palabras clave: microscopía, secreción de néctar, néctar, EFNs, espinas secretoras.

Introduction

Extrafloral nectaries (hereafter EFNs) are secretory structures present in different plant structures such as leaves, petioles, stipules, young stems, and in adult vegetative structures (Elias 1983). Given their position on plants, these glands are not related with pollination, but they have a function in rewarding arthropods, mainly ants, with sweet drops of nectar, in exchange for protection against herbivore insects (Del-Claro et al. 1996, Ness 2003, Oliveira Freitas 2004). EFNs also function to distract ants away from flowers and prevent them from attacking pollinators, reducing plant fitness (Assunção et al. 2014, Ness 2006, Villamil et al. 2019, Wagner Kay 2002). EFNs are widely distributed across vascular plants, and several studies have shown that they differ among plant taxa in their anatomy, morphology, and position in the vegetative or reproductive structures of the plants (Bentley 1977, Elias 1983, Weber Keeler 2013, Zimmermann 1932). Despite the ubiquity of EFNs, knowledge about their anatomy and morphology is still scarce, particularly in the Cactaceae family.

Only about 96 species among 1866 species in Cactaceae family have EFNs (Weber et al. 2015). This number could be higher, but more research and field observations are needed to increase it. *Opuntia* is the richest genus within Cactaceae, with nearly 200 described species, while the genus *Ferocactus* has about 30 species (Anderson 2001). Despite this diversity, the literature on EFNs in these two genera is limited. For example, in *Ferocactus wislizeni* (Engelm.) Britton & Rose, EFNs were small modified spines that exude nectar and are located on the top of the plant near the flowers (Morris et al. 2005). In studies on *F. gracilis* and *F. acanthodes* subsp. *lecontei* (Engelm.) G.E. Linds., secretory spines were placed on the areoles surrounding the top of the plant; ants were observed feeding on their sweet secretions (Blom Clark 1980, Ruffner Clark 1986). Similarly, in *O. acanthocarpa* (Engelm. & J.M.Bigelow) F.M.Knuth and *O. engelmanii* (Salm-Dyck) Engelmann, the EFNs were embedded in areolae of young vegetative and

reproductive buds (Chamberlain et al. 2010, Pickett Clark 1979). In *Opuntia robusta* Wendl. ex Pfeiff., young cladodes and flower buds developed areoles with modified secretory spines acting as EFNs, active only during the early growth phase, suggesting ants' participation in the indirect defense (Sandoval-Molina et al. 2018). In *O. stricta* (Haw.) Haw. EFNs are located in the areoles of the developing vegetative cladodes (Diaz-Castelazo et al. 2005, Oliveira et al. 1999).

Although the morphology and position of EFNs in the Cactaceae family have taxonomic importance, there is a lack of information regarding their cytological structure and morphology (Mauseth et al. 2016). The presence of droplets on young spines of growing tissues in plants from the *Opuntia* genus and the continuous secretion of nectar in plants from the *Ferocactus* genus, suggests a complex structure and morphology of tissues forming EFNs, rather than just a hard mass of lignified tissues. Although Zimmermann (1932) reported the presence of EFNs in *F. recurvus*, he did not provide detailed descriptions on the morphology and anatomy of these secretory glands. Additionally, no previous study has reported the presence of EFNs in *O. streptacantha*, and as far as we know, no previous studies have examined the morphology and anatomy of EFNs in either *O. streptacantha* or *F. recurvus*; therefore, their cytological characteristics have not been examined so far.

The aim of this study was to characterize the morphology and anatomy of the EFNs of *O. streptacantha* and *F. recurvus* using light microscopy and scanning electron microscopy. We aimed to classify EFNs following the structural–topographical classification proposed by Zimmermann (1932) and modified by Elias (1983). This study was motivated by the lack of information about EFNs in cacti and aims to contribute to the understanding of these structures, their morphology and anatomy.

Materials and methods

Study species. Ferocactus recurvus is an endemic plant distributed in the semiarid region in the states of Puebla and Oaxaca. The height of the plants of this species ranges between 10 to 50 cm. Similarly to other species of *Ferocactus,* they present a spiral arrangement of ribs and curved red spines, and have extrafloral nectaries located at the top of the plant, surrounding floral meristems, and attract ants (Marazzi et al. 2013, Mauseth et al. 2016). Their flowers are self-incompatible and xenogamous and have diurnal anthesis between 11 to 18 hr, remaining opened for 2-5 days (Córdova-Acosta et al. 2017). *Opuntia streptacantha* is an endemic plant from Mexican semiarid zones, distributed in the states of Guanajuato, Hidalgo, México, Oaxaca, Puebla, Querétaro, San Luis Potosí, Tlaxcala, and Zacatecas. Individuals of this plant are arborescent their height ranges from 2 to 4 m. Their flowers are yellow to orange. Their blossoming period extend from March to June and their fructification occurs from June to September (Arias et al., 2012). Previously during our field studies, EFNs were observed within the areolae of young cladodes and at the basal section of their flower buds (M. A. Sandoval-Molina, personal observation).

Study area. Samples were collected at the Helia Bravo Hollis Botanical Garden (18° 19´ 54'' N, 97° 27´ 21'' W) located in the municipality of Zapotitlan Salinas, State of Puebla, México, within the Tehuacán-Cuicatlán biosphere reserve. Rainfall in this place averages 376.4 mm per year. There are two well-defined seasons with high interannual predictability: the rainy season (June to September) and the dry season (October to May). The average annual temperature is of 20.7 ºC (Valiente 1991). The vegetation type is mainly to crassicaule scrub, dominated by *Neobuxbaumia tetetzo* and the spiny shrubs *Prosopis laevigata, Mimosa luisiana, Mamillaria collina* (Zavala-Hurtado 1982).

Scanning Electron Microscopy (SEM). For scanning electron microscopy, we collected the areoles of plants with active EFNs, where ants were foraging on them, using a razor blade. We followed the method used by Sandoval-Molina et al. 2017a, to prepare the samples. We cross-sectioned each areole collected and fixed them in glutaraldehyde solution (2.5 % glutaraldehyde in 0.1 M phosphate buffer Sorensen's at pH = 7.2). The areoles were then postfixed in 1% osmium tetraoxide in water at 22 ° C for two hours. After two washes (30 min each) with deionized water, we dehydrated the tissues in an ethanol series and dried them to critical point using a Samdri-7801 (TOUSIMIS Research Corporation, Rockville, USA). We coated the samples with gold-palladium (80 %:20 %) in a JFC-1100 (Fine coat ion sputter JFC-1100, JEOL Ltd., Tokyo, Japan) and observed them with a SEM microscope (JSM 6390 JEOL, Japan) working at 15 kv at WD 10 mm.

Light microscopy. Each areole containing EFNs was fixed in glutaraldehyde solution (2.5 % glutaraldehyde in 0.1 M phosphate buffer Sorensen's at pH7.2) for twelve hours under vacuum. Then, they were washed twice with phosphate buffer. We post-fixed the fragments in 1% osmium tetroxide in water at 22 °C for two hours. Afterwards, we washed the samples twice with deionized water and dehydrated them in an ethanol series. Then, we embedded the dehydrated tissues in medium hardness Spurr's resin (Polysciences Inc., PA, USA) according to manufacturer's instructions.

We obtained semi-thin sections (1 μm) with a glass knife and an ultramicrotome (Om U3, Reichert-Jung) and stained them with a 1:1 mixture 1:1 of 1 % methylene blue in 1 % borax: 1 % azure II in water and 1 % toluidine blue according to Sandoval-Molina et al. (2017b). We conducted microscopic analysis and obtained the images under an Axiostar Plus light microscope

(Carl Zeiss, Germany) and recorded the images with a Moticam 5MP camera (Motic Asia, Hong Kong).

Results

EFNs of *F. recurvus* are located on the areoles of the apex of the stem below the large spines and surrounded by dense non-secretory trichomes (Figure 1A). Each areole has two or more EFNs located around the flower meristems, but they are absent on flowers or fruits. In *O. streptacantha,* EFNs are modified spines within the areolae on young cladodes and flower buds that secrete sweet drops of nectar. We found one or two glands per areola surrounded by non-secretory trichomes and glochids (Figure 1B).

According to the external morphology of EFNs, in *F. recurvus* we found they are elongated and blunt glands. The epidermal cells were wrinkled, forming a lump at the tip (Figure 2A, B). In all examined EFNs, the epidermis had neither stomata nor trichomes, but we observed the presence of an apperture at the top of the gland (Figure 2C). In *O. streptacantha,* EFNs were young spines and had an apical secretory cone at the tip where nectar was stored and exuded (Figure 2D, E). This region has globular and imbricated epidermal cells, bag-shaped without stomata (Figure 2F).

Anatomical observations of EFNs of *O. streptacantha* showed the presence of three sections (Figure 3): the apical secretory cone, the middle elongation section, and the basal meristematic section. The apical secretory cone had globular epidermal cells with large vacuoles, surrounding a lignified region of the spine that grows inside (Figure 3A). The middle section of the nectary presented smaller epidermal cells than in the apical cone and has elongated lignified cells inside (Figure 3B). The basal meristematic section is characterized by a compact group of highly vacuolated diving cells with dense cytoplasm and large nuclei (Figure 3C, D). We did not

observe direct vascularization of the EFNs (Figure 3D). The apical secretory cone consisted of small, lignified cells, surrounded by larger epidermal cells (Figure 3E). In the middle of the secretory cone, we found cells with thick walls, separated from the epidermal cells by a large intercellular space, probably where nectar accumulates prior to secretion (Figure 3F).

Discussion

Plants from the Cactaceae family display an impressive morphological diversity of EFNs (Almeida et al. 2012, Ávila-Argáez et al. 2019, de Melo Silva et al. 2020, Marazzi et al. 2013, Mauseth et al. 2016, Sandoval-Molina et al. 2018). This trait is useful for taxonomic, ecological, and evolutionary studies. However, their anatomy, and morphology are still poorly understood. In this work, we described the morphology and anatomy of EFNs in *F. recurvus* and *O. streptacantha* and reported, for the first time, the existence of EFNs in *O. streptacantha*, which was previously unknown in the literature. Our work aims to contribute to the knowledge of these secretory structures, which are widely distributed in plants.

Structure and morphology of EFNs. According to the classification of Zimmermann (1932) and modified by (Elias 1983) the EFNs of *F. recurvus* are elevated nectaries, structures elevated from the surrounding tissues in the areole. Similarly, in *O. streptacantha*, EFNs are transformed nectaries, modified spines that secrete sweet drops of nectar. However, the morphology of EFNs from both species also fits the most recent classification proposed by Mauseth et al. (2016) for cacti: in *F. recurvus* they are highly modified spines that are short, broad, and blunt, whereas in *O. streptacantha* they resemble ordinary spines with an apical secretory cone, likely acting as reservoir for nectar secretion.

Studies carried out on several species of Cactaceae family have revealed that nectar secretion involves highly modified spines acting as EFNs (Diaz-Castelazo et al. 2005, Mauseth et al. 2016, Sandoval-Molina et al. 2018). Interestingly, the EFNs of both species studied here derived from spines, suggesting that even in phylogenetically distant species (Hernández-Hernández et al. 2011), the transformation of spines into secretory structures resulted in a successful adaptation that improved plant fitness, probably because of their association with defensive ants. Such pattern suggests that EFNs exhibit high evolutionary convergence and are influenced by natural selection promoting the evolution of these structures (Nogueira et al. 2012, Weber Keeler 2013). The presence of young cells with a high metabolic rate in growing spines is a trait that promotes a switch of the metabolic pathways of such non-specialized cells to the secreting functions. This trait could explain why EFNs have appeared in different and taxonomically unrelated species.

Based on our morphological and histological observations of EFNs in *O. streptacantha*, we inferred that secretions occur in modified spines capable of performing nectar production, transport, accumulation, and secretion. EFNs in this species had similar anatomical and morphological characteristics as the EFNs of *O. robusta* (Sandoval-Molina et al. 2018). Similar to the results of Sandoval-Molina et al. (2018) for *O. robusta*, we propose here that extrafloral nectar is produced by internal and subepidermal cells, such as those located at the base of EFNs, similar to those described for nectariferous tissues: a compact group of cells, with dense cytoplasm, and relatively large nuclei, indicating an intense metabolism (Fahn 1979, Nepi 2007). Then, extrafloral nectar is transported to the intercellular spaces and to the epidermal cells of the apical secretory cone, which act as a nectar reservoir, before the nectar can be released through epidermis break caused by pressure or caused by ants biting. According to de Melo Silva et al. (2020) in *Nopalea cochenillifera* (L.) Salm-Dyck and *Brasiliopuntia brasiliensis* (Willd.) A.

228 Berger, glochids are involved in nectar secretion; however, based on our observations in *O.*
229 *streptacantha*, we could not detect their secretory activity. In *F. recurvus*, our morphological
230 characterization suggests that nectar is produced elsewhere, probably in the subnectary
231 parenchyma, then it is transported to the nectary tissues and intercellular spaces, where it is stored
232 and released from the tip of the nectary.

233 The mechanism, dynamics, and selective benefits associated with the vascularization of EFNs
234 in the Cactaceae family are still poorly understood. For example, in *Cylindropuntia imbricata*
235 and in *O. stricta*, vascularized EFNs have been reported (Ávila-Argáez et al. 2019, Diaz-
236 Castelazo et al. 2005), while in other species such as *O. robusta*, *N. cochenillifera* and *B.*
237 *brasiliensis*, their EFNs are not directly vascularized, but traces of vascular tissues reach only the
238 base of the EFNs (de Melo Silva et al. 2020, Sandoval-Molina et al. 2018). The vascularization of
239 EFNs in other species from the Opuntioideae and Cactoideae subfamilies is unknown. Our
240 observations did not allow us to detect vascular tissues of the nectaries in either of the two
241 species studied here. As stated by de Melo Silva et al. (2020) the vascularization of EFNs is not
242 well understood due to the methodological challenges in accessing the basal region of the EFNs,
243 as analyzing a large number of samples is necessary to reach the vascular tissues.

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Data availability statement

Not applicable.

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Figure. 1. Ants foraging in the extrafloral nectaries of A) *Ferocactus recurvus* and B) *Opuntia streptacantha*. In both species, EFNs are modified spines adapted for nectar secretion (arrows). In *F. recurvus*, EFNs are yellowish glands surrounded by glochids. In *O. streptacantha*, EFNs are young globose spines behind a bract leaf surrounded by glochids.

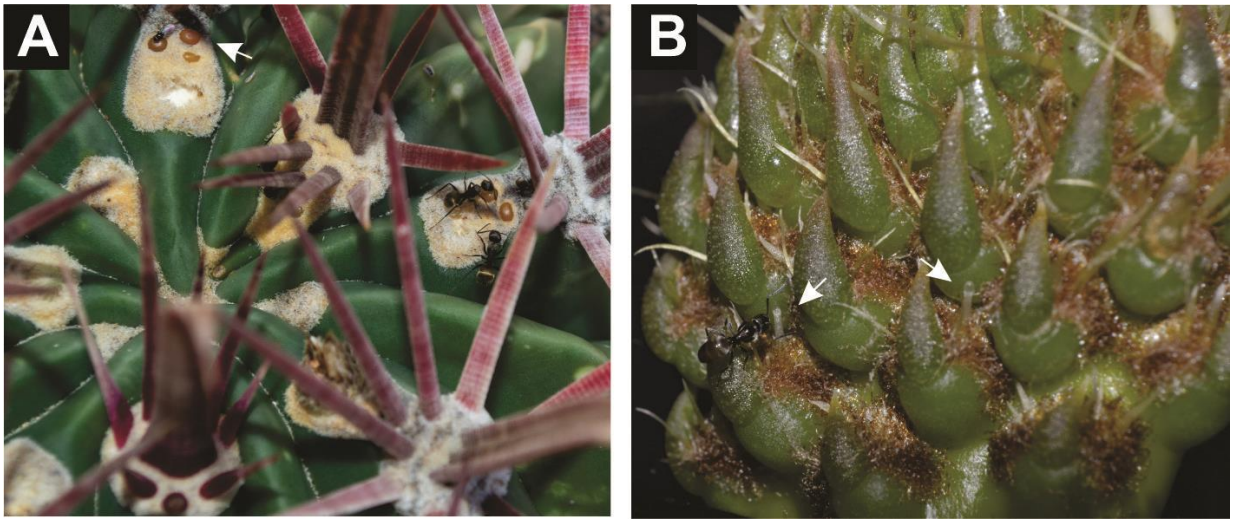

Figure. 2. Scanning electron micrograph of extrafloral nectaries of *Ferocactus recurvus* and *Opuntia streptacantha.* Extrafloral nectaries of *F. recurvus:* A) Full view of EFN; B) Secretory gland with a lump at the tip; C) Transition region between the body of EFN and the apical lump, showing a broken cell and the intercellular space (arrow). Extrafloral nectary morphology of *O. streptacantha:* D) Full view of areole, showing the two secretory glands (arrows), surrounded by trichomes and glochids; E) Whole modified spine acting as EFN, with an apical secretory cone; F) Detailed view of the apical secretory cone. ASC – apical secreting cone; EFN – extrafloral nectary; GL – glochids; TR – trichomes.

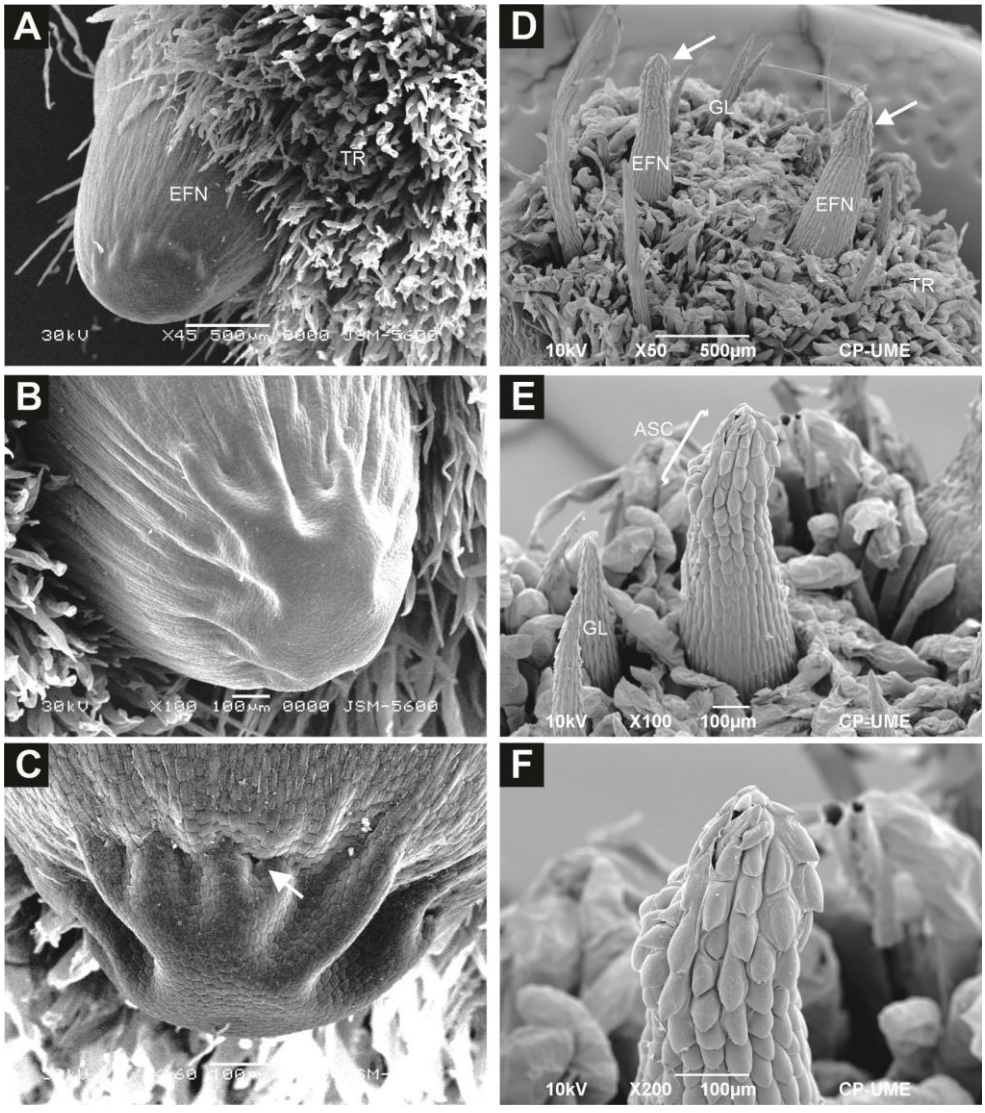

Figure. 3. Anatomy of the extrafloral nectaries of *Opuntia streptacantha.* A) Whole nectary showing the apical secretory cone in the apical region; B) Middle elongation region of the nectary; C) Basal section of the nectary; D) Transversal section of the apical secretory cone; E) Transversal section of the middle region of the apical secretory cone. ASC—apical secretory cone; BS — basal section; EP — epidermis; IN — internal tissue; MS — middle section; NU — nuclei; VA — Vacuole.