Anther development and cytochemistry of *Hibiscus syriacus* (*Malvaceae*)

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Received: February 23, 2023 ▷ Accepted: April 11, 2023

Abstract. The aim of this study is to examine anther development and cytochemistry of *Hibiscus syriacus*. The anther sections were stained with hematoxylin, Coomassie brilliant blue, periodic acid-Schiff and Sudan black B for cytochemical analyses. Characterization of cell death was checked by DAPI staining. The anther wall of *H. syriacus* consisted of epidermis, endothecium, middle layer, and glandular (secretory) tapetum. According to the authors' results, the epidermis was rich in insoluble polysaccharides and lipids, although the other anther wall layers were poor in organic materials. In the middle layer and tapetum, cellular degeneration started in the early pollen stage and continued in the vacuolated pollen stage. During the mature pollen stage, only the epidermis, endothecium and mature pollen grains remained in the anther lobes. The large-sized pollen grains were isopolar, spheroidal and pantoporate. Their cytoplasm was rich in protein and insoluble polysaccharides. Pollen ornamentation was echinate. The obtained results have provided new data on the male generative sphere of *H. syriacus* and the genus *Hibiscus*.

Key words: anther, *Hibiscus syriacus*, *Malvaceae*, pollen, tapetum

Citation: Doğan, M. & Vardar, F. 2023. Anther development and cytochemistry of *Hibiscus syriacus* (*Malvaceae*). -- Phytologia Balcanica, 29 (1): 53-62. -- ISSN 1310-7771 (print), 1314-0027 (online).

Introduction

Anther is the male reproductive organ related to microsporogenesis and microgametogenesis, both critical occurrences in plant reproduction with respect to pollen structure and function. Developmental events and critical stages in anther ontogenesis have attracted attention in relation to functional pollen production. Irregular pollen development induces reduction in successful pollination and fertilization (Bohdanowicz & al. 2005, Vardar & al. 2013). In plants, male sterility or unsuccessful reproduction generally originate from failure in microspore development or the surrounding anther wall layers, e.g., tapetum (Bohdanowicz & al. 2005). Tapetal cells, at first, undertake a secretory role to provide fundamental nutrients to the developing microspores and, subsequently, degenerate to facilitate pollen maturing and release (Pacini 2000). Furthermore, pollen exine components, especially sporopollenin, are secreted by the active tapetal cells, which undergo structural and biochemical alterations leading to cell death after their responsibility ends (Leśniews-

DOI 10.7546/PhB.29.1.2023.5

ka & Charzyńska 2000). Several studies have reported cell death in the middle layer, connective tissue and filament during anther development (Vardar & Ünal 2011, 2012). Mature pollen grains accumulate essential nutrients for pollen germination and tube growth (Rodriguez-Garcia & al. 2003).

Examination of the sexual process and reproduction also helps understand the phylogenetic relationships (Taylor & Osborne 2006). The present study reports observations of the development and cytochemistry of anthers and pollen in *Hibiscus syriacus* L. (*Malvaceae*), which is an ornamental horticultural shrub. *H. syriacus* is native to Korea and China, but also is widely spread across the world. Although *H. syriacus* is often used in horticulture, its dried leaves and flowers can be brewed as tea. Besides the flower, its root, stem and bark contain bioactive compounds widely used as medicines in Asia (Kim & al. 2022). Several studies have been dedicated to the bioactive components and effects of the *Hibiscus* species. There are also studies with numerous morphological analyzes aimed at clarifying the systematic distinctions between the taxa (Saifudin & Salamah 2019, Salamah 2021). Additionally, some tissue culture studies have been carried out (Sie & al. 2010, Ibrahim & al. 2014), as well as investigations of the reproductive cells and their relation to embryo-endosperm-fruit development (Sanyal 1958, Annahwi & al. 2017, Tapec & al. 2021), hormonal changes during flower development (Trivellini & al. 2011), and some transcriptomic analyzes (Trivellini & al. 2016). Von Balthazar & al. (2006) have investigated structure and evolution of the androecium in the Malvatheca clade (*Malvaceae* s.l.). The researchers had focused only on the androecial development and structure, and floral vasculature of six selected species (*Chiranthodendron pentadactylon*, *Fremontodendron californicum*, *Gyranthera caribensis*, *Huberodendron swietenioides*, *Ochroma pyramidale*, *Patinoa sphaerocarpa*). However, they have not provided any data on anther wall development of the indicated species. On the other hand, to the best of the authors' knowledge, there have been no published reports on the development and cytochemistry of anthers of the male reproductive organ in *H. syriacus*, or in other *Hibiscus* species. The present study aims to describe the development, cytochemistry, and cell death progression of the anther wall layers of *Hibiscus syriacus*.

Material and methods

Flower buds of *Hibiscus syriacus* L. (*Malvaceae*) growing in natural habitats in the vicinity of Çekmeköy (41°05'4"N - 29°19'3"E, Istanbul, Turkey) were collected in September-October 2020. They were fixed in FAA (formaldehyde: acetic acid: alcohol: distilled water, 10:5:50:35) for 24 h and stored in 70% alcohol at +4°C. Before embedding the buds in paraffin, one anther from each flower bud was gently dissected and squashed in 0.2% aceto-orcein, in order to determine the development (meiotic) stages, according to Vardar & Unal (2011, 2012). The paraffin sections (8-10 µm) were stained with Delafield's hematoxylin (Gill & al. 1974). For cytochemical observations, the sections were stained with Coomassie brilliant blue for protein (Fischer 1968), periodic acid & Schiff's (PAS) for insoluble polysaccharides (Feder & O'Brien 1968) and Sudan black B for lipids (Paerse 1961). To determine morphological changes in the nuclei of anther wall cells during development, the sections were stained with 1 µg/ml DAPI (4',6-diamidine-2' phenylindole dihydrochloride) for 30 min in the dark (Schweizer 1976). Slides were visualized with KAMERAM software, supported by a KAMERAM camera and Olympus BX-51 light microscope. DAPI stained samples were analyzed with an Olympus fluorescence microscope equipped with the appropriate filters (365 nm). A pollen morphology analysis was performed according to Woodehouse (1935). At least 500 pollen grains were counted and measured (pollen and aperture diameter, spine size) in each preparation.

Results

To determine flower morphology of *Hibiscus syriacus*, flowers at different development stages have been examined under a stereomicroscope (Fig. 1). *H. syriacus* is a perennial shrub, with a large number of flower buds produced in summer (June to September). How-

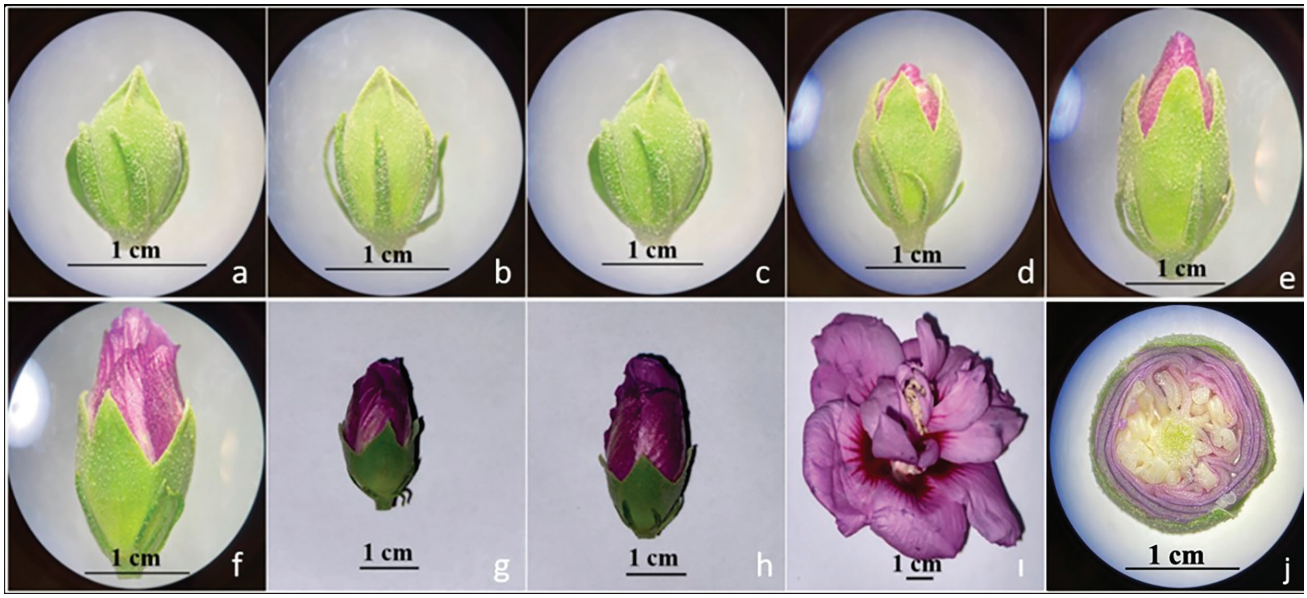

Fig. 1. Different development stages of *H. syriacus* L. flowers.

ever, they are open only for 2 or 3 days. Flower buds have been initially green and rounded and included five green hairy sepals (Fig. 1a-d); after blossoming, multilayered lilac-purple petals prevailed (Fig. 1e-i). The vase-shaped hermaphrodite flowers contained a superior (hypogine) ovary, a style with 5-partitioned stigma and a large number of stamens on the central column (Fig. 1j).

In order to examine anther development of *H. syriacus*, five stages correlating to the pollen development have been distinguished: prophase I (PI), tetrad (T), young pollen (YP), vacuolated pollen (VP), and mature pollen (MP).

According to the obtained results after hematoxylin staining, *H. syriacus* anthers were spherical-elliptical and consisted of meristematic cells at the very beginning of their development. The outer layer formed epidermal cells, the outer secondary parietal layer gave rise to the endothecium and middle layer, and the inner parietal layer developed directly into the tapetum (dicotyledonous type). *H. syriacus* anthers were tetrasporangiate and in each anther lobe hypodermal cells transformed into archesporial cells. Archesporial cells divided periclinally and gave rise to pollen mother cells (PMC). The anther wall of *H. syriacus* consisted of epidermis, endothecium, middle layer, and glandular (secretory) tapetum from outer to inner at the PI stage (Fig. 2a). Two- or 3-nucleated cells were also detected in the tapetum layer, which underwent mitosis (without cytokinesis) from PI to T stage (Fig. 2b). Morphological changes, such as nuclear and cellular degeneration, were observed in the cells of the middle layer and tapetum, beginning from YP to VP stage (Fig. 2c, d). The endothecium and epidermis layers were permanent and lasted during the mature pollen stage, although the middle layer and tapetum were ephemeral (Fig. 2e). The cytoplasm of the epidermis was determined as quite dense and stained dark from the beginning of development. At the MP stage, the endothecium layer increased longitudinally into a U shape, with no wall thickenings. Concurrent with anther enlargement, the pollen sacs were shaped by tissue fusion, subsequently to VP. At the final stage of development (MP), mature bicellular pollen grains with spiny outgrowths were released into the environment by dehiscence of stomium (Fig. 2f).

To examine the total presence of protein in the anther wall layers of *H. syriacus,* Coomassie brilliant blue (CBB) staining was applied. According to the obtained results, reaction of the epidermis, endothecium, and middle layer to protein content was feeble at all development stages (Fig. 3a-e). However, the nuclei of the anther wall cells reacted positively. Moreover, that tapetal degeneration was progressive at the VP

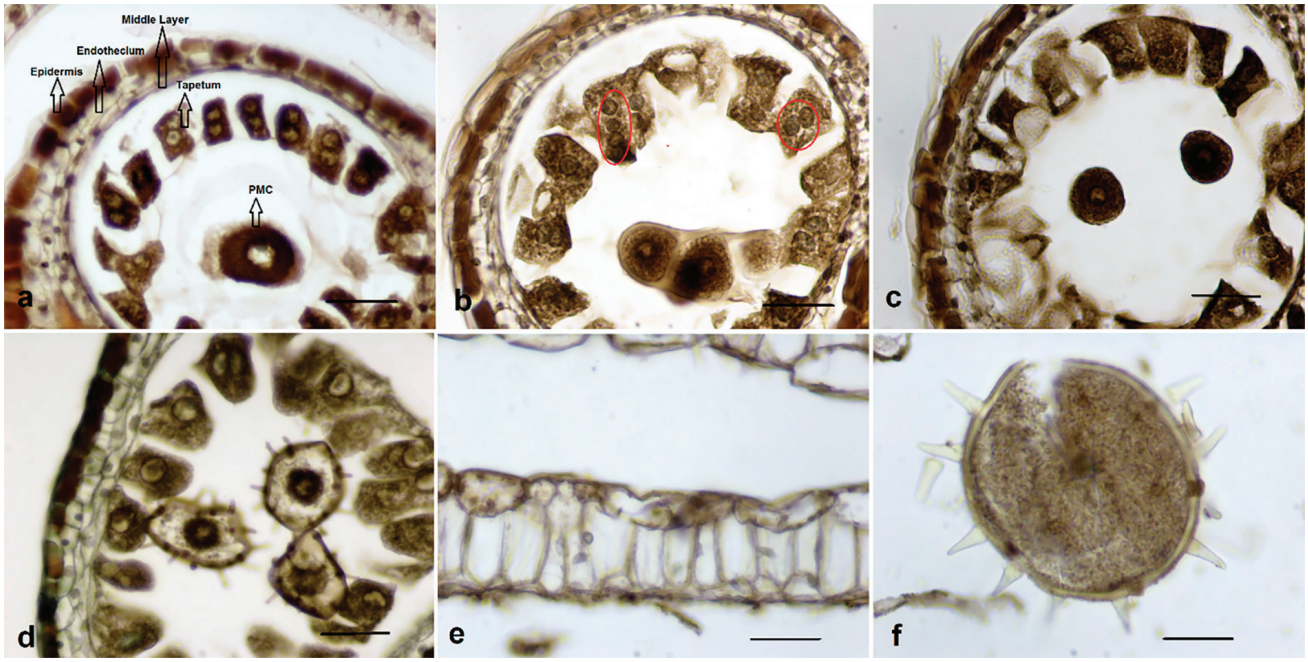

Fig. 2. General structure of *H. syriacus* anthers stained with hematoxylin: **a.** Prophase-I stage, **b.** Tetrad stage, 2 and 3 nucleated cells (in red circles), **c.** Young pollen stage, **d.** Vacuolated pollen stage. **e.** Mature pollen stage, **f.** Mature pollen. Bar a-d: 50 µm, e-f: 100 µm.

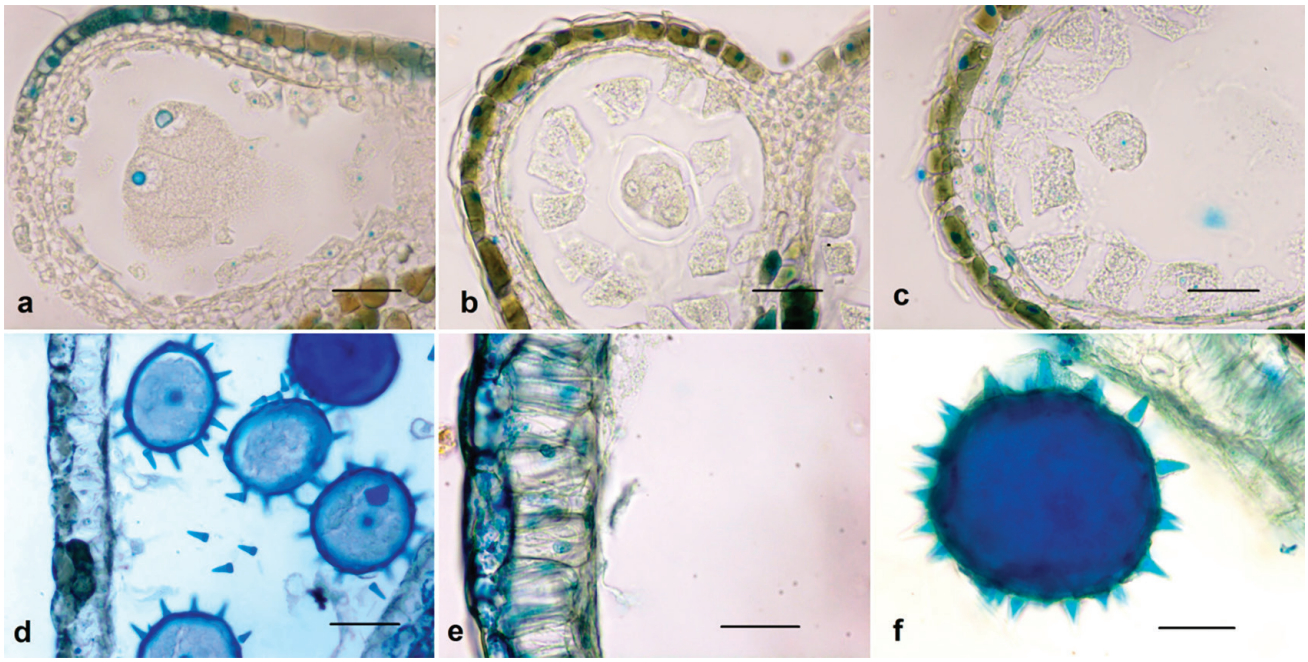

Fig. 3. Cytochemical features of *H. syriacus* anthers stained with CBB: **a.** Prophase-I stage, **b.** Tetrad stage, **c.** Young pollen stage, **d.** Vacuolated pollen stage, **e.** Mature pollen stage, **f.** Mature pollen. Bar a-d: 50 µm, e-f: 100 µm.

stage. The cytoplasm of mature pollen was very rich in protein; similarly, the pollen wall (exine and intine) also reacted positively (Fig. 3d, f). It was also found that the dense cytoplasm in the epidermis was not made of protein.

Periodic acid & Schiff (PAS) staining was applied to detect the presence of insoluble polysaccharides, such as starch and cellulose, in the anther wall layers of *H. syriacus*. The results showed a strong PAS positive reaction of the epidermis at all development

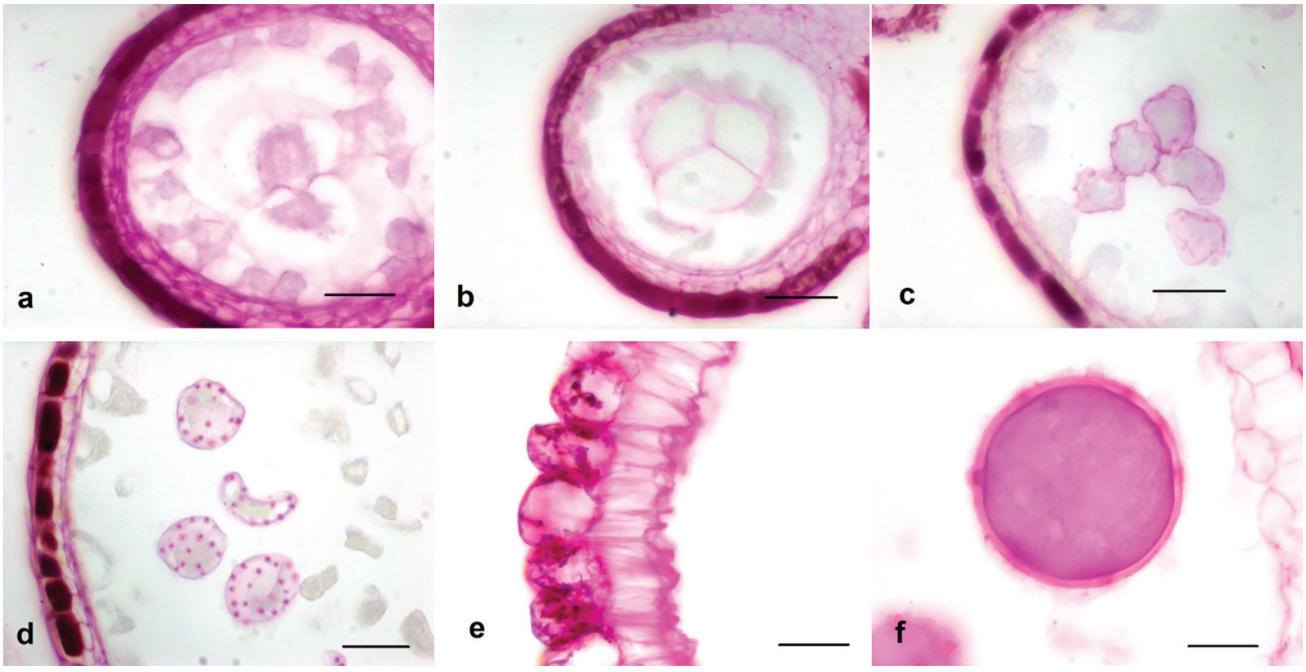

Fig. 4. Cytochemical features of *H. syriacus* anthers stained with PAS: **a.** Prophase-I stage, **b.** Tetrad stage, **c.** Young pollen stage, **d.** Vacuolated pollen stage, **e.** Mature pollen stage, **f.** Mature pollen. Bar a-d: 50 μm, e-f: 100 μm.

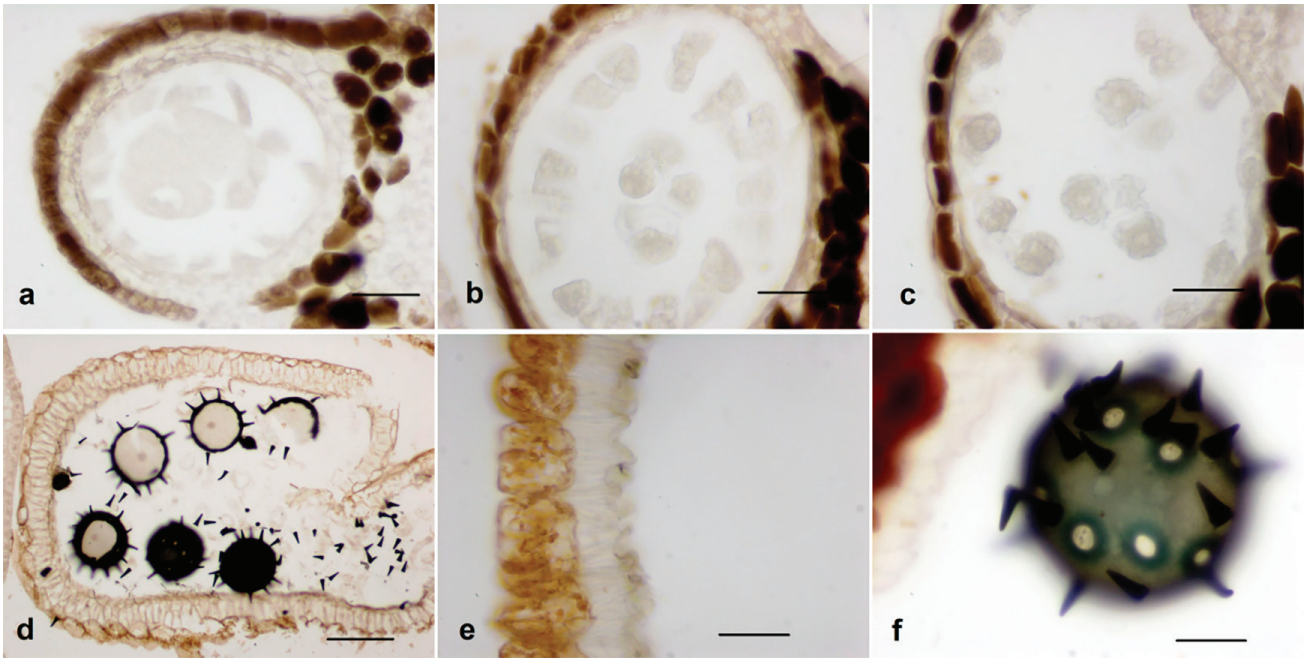

Fig. 5. Cytochemical features of *H. syriacus* anthers stained with SBB: **a.** Prophase-I stage, **b.** Tetrad stage, **c.** Young pollen stage, **d.** Vacuolated pollen stage, **e.** Mature pollen stage, **f.** Mature pollen. Bar a-d: 50 μm, e-f: 100 μm.

stages. However, the endothecium, middle layer and tapetum produced PAS negative results, except for their cell walls (Fig. 4a-e). Furthermore, the callose wall, progressively present at the T stage, reacted PAS positively. During degeneration of the tapetum cells, the cell walls also broke down and showed a PAS negative reaction (Fig. 4c-d). At the mature pollen stage, the cell walls of the endothecium also showed a strong PAS positive reaction (Fig. 4e). Furthermore, the cytoplasm and intine of the mature pollen were strongly

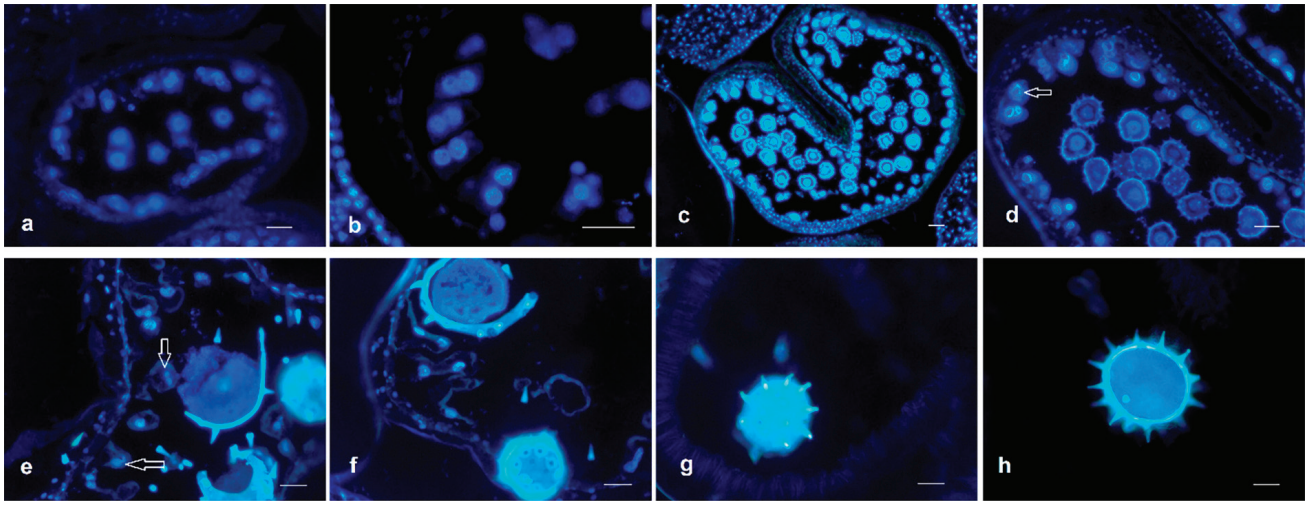

Fig. 6. DAPI staining of *H. syriacus* anthers: **a, b.** Tetrad stage, **c, d.** Young pollen stage, **e, f.** Vacuolated pollen stage, **g, h.** Mature pollen stage. Bar a, d, e, f, g, h: 50 µm, b: 100, c: 25 µm.

stained. Remnants of the spiny outgrowths tending to break off easily during sectioning were also stained as dark pink spots on the pollen grains (Fig. 4d, f).

Sudan black B (SBB) staining was used to examine the presence of lipids in the anther walls of *H. syriacus*. The epidermis and connective tissue cells proved quite rich in lipids in PI, T, YP, and VP stages (Fig. 5a-d). However, the endothecium, middle layer and tapetum cells were found to be lipid-poor. Although the epidermal cells lost lipid density at the MP stage (Fig. 5e), some lipids were still present in the cells, as compared to other anther wall cells. The exine walls made up of sporopollenin were stained strongly by the Sudan black B (Fig. 5d, f).

Changes in nuclear morphology have been revealed by DAPI staining. According to the obtained data, tapetal cells contained two or three nuclei as a result of mitosis (without cytokinesis). At the T stage, the nuclei of the tapetum cells preserved their spherical form and integrity (Fig. 6a, b). However, they seemed to have lost their normal shape and chromatin was distributed abnormally in the nucleus as from YP stage. Chromatin was found to condense and accumulate just below the nuclear membrane. In the VP phase, nuclear and cellular degeneration were obvious, both in the middle layer and tapetum (Fig. 6c-f). Degeneration terminated with cell death and no remnants were visible at the MP stage (Fig. 6g, h).

Morphological characteristics of *H. syriacus* pollen grains were examined with a light microscope. Polar axis (P) could not be distinguished from equatorial axis (E), due to its spherical shape and pollen aperture distribution. Therefore, only diameter measurements (R) were performed. According to the obtained results, the pollen grain diameter was 88.18 µm. A bicellular mature pollen grain was large, isopolar and spheroidal in shape. Pollen ornamentation was echinate and spin size was 14.46 µm. Pantoporate-type apertures with an average diameter of 9.46 µm were distributed irregularly across the pollen grain (Table 1).

Discussion

The anther wall of *H. syriacus* consisted of epidermis, endothecium, middle layer and glandular (secretory) tapetum. This structure is common for anther walls and varied in the number of layers and type of tapetum in the different species. All anther wall layers had special roles, but tapetum came to the fore for surrounding and supporting the sporogenous tissue. The anther layers underwent structural and biochemical changes during maturation (Lesniewska 2003, Vardar & Ünal 2012). Concurrently with anther development and maturation, the middle layer and tapetum degenerated to cell death, although the epidermis and en-

dothecium remained until anther dehiscence. Their degeneration stages varied from tetrad to vacuolated pollen stage, depending on their taxa. Cell death provided organic molecules for pollen development, especially in the tapetum. Tapetal cell death, accompanied with other anther wall cells, was needed for anther dehiscence and spread of mature and fertile pollen grains (Lesniewska 2003, Parish & al. 2010, Vardar & Ünal 2012). Several studies have confirmed programmed cell death during pollen maturation in the anther wall cells of *Lobivia rauschii* and *Tillandsia albida* (Papini & al. 1999), *Hordeum vulgare* (Wang & al. 1999), *Solanum melongena* (Xu & al. 1999), *Zea mays* (Schreiber & al. 2004), *Solanum lycopersicum* (Senatore & al. 2009), *Lathyrus undulatus* (Vardar & Ünal 2011, 2012), and *Brassica oleraceae* (Küçükali & al. 2020). Accordingly, early or delayed degeneration of the tapetum caused male sterility. It has been also revealed that male sterility may be caused by delayed degeneration of the middle layer. Limei & al. (2010) have examined sterile and mutant *Hibiscus cannabinus* anthers and have noticed that such abnormalities as delayed degeneration of the middle layer and premature separation of the tapetum from the middle layer cause male sterility. The reason for these mutations was functional loss of several receptor-like kinases causing defects in the middle layer and normal pollen formation (Mizuno & al. 2007, Cui & al. 2018). Tapetum and middle layer in *H. syriacus* were found to be ephemeral. No delay in their degeneration was observed.

Pollen diameters recorded for the species by Bae & al. (2015) were similar to the present results. According to the pollen morphology of Malvoideae described by Saensouk & Saensouk (2021), the pollen grains of *H. syriacus* could be defined as pantoporate, radially symmetrical, spheroidal and apolar. Their ornamentation was noted down as granulate, micro-network and subsilate.

After the cytochemical analyses, it was reported for first time that the pollen cytoplasm in *H. syriacus* was rich in protein and insoluble polysaccharides, and complemented anther development of the species. Although some studies have reported the development, morphology and spiny protrusions on *H. syriacus* pollen, information concerning cytochemistry of the mature pollen grains is insufficient (Takahashi & Kouchi 1988, Shaheen & al. 2009). Heslop-Harrison & al. (1973) reported that they encountered dense protein in the intine walls of different species of the *Malvaceae* family. In the same study, it was stated that the exine walls were rich in protein and carbohydrates. It was also explained that the exine material was produced by the tapetum released from the cisterna with granular-fibrillar content during the dissolution stage of the tapetum.

Tang & al. (2006) have investigated flowers, microsporogenesis and microgametogenesis of *Excentrodendron hsienmu* in the opening-functional flowers and non-opening flowers to reveal the evolutionary relationships of *Excentrodendron* (*Malvaceae*). The anther of *E. hsienmu* was tetrasporangiate, the tapetum was of the secretory type, and the pollen grains were two-celled, when shed. Development of the anther wall conformed with the basic type, it was five or six cells thick, and with a fibrous endothecium. Galati & al. (2007) have studied pollen and microsporangium development of *Modiolastrum malvifolium* (*Malvaceae*) in the light of orbicule development. The researchers reported plasmodial tapetum as invasive and non-syncytial, and formation of a peritapetal membrane with orbicules. Lattar & al. (2014)

Table 1. Some features of *H. syriacus pollen grains*

R (diameter, ±SD)	88.18 ±3.54 μm	**Size**	Large
Shape	Spheroidal	**Polarity**	Isopolar
Aperture	Pantoporate	**Aperture number**	>6
Aperture diameter (±SD)	9.46 ±1.29 μm	**Ornamentation**	Echinate
		Spine size (±SD)	14.46 ±1.29 μm

analyzed the anther development, microsporogenesis and microgametogenesis of six species of the genera *Corchorus, Heliocarpus, Luehea* and *Triumfetta* (Malvaceae). Those genera shared the following characters: a basic type anther wall ontogeny, simultaneous microsporogenesis, secretory tapetum, and bicellular mature pollen grains. The researchers claimed that that was the first embryological report of the *Grewioideae* subfamily contributing to characterization of the studied genera. As with *H. syriacus*, there has been no detailed study on the development and cytochemistry of the anther wall cell layers in other *Hibiscus* species. The present detailed study of anther development of *H. syriacus* has been the first of this kind and completes the characteristics of the male reproductive sphere of genus *Hibiscus* from the family of *Malvaceae*.

In conclusion, during the anther wall development in the light of structural development and accumulation of organic compounds (protein, insoluble polysaccharides and lipids), the epidermis cells of *H. syriacus* came to the fore, because the other anther wall cells were poor in organic compounds. The tapetal cells and middle layer degenerated entirely at the mature pollen stage. The present data have provided new information on the male generative sphere of *H. syriacus* and the genus *Hibiscus*. On the other hand, the timing of tapetal development, degeneration and pollen maturation may become the focus of future cell-death investigations. The developmental and cytochemical features of the anther wall cells and pollen may also provide useful characters for assessing the relationships within this genus and family.

Acknowledgement. This study is the MSc thesis of Bahar Doğan at Marmara University, Institute of Pure and Applied Sciences, Istanbul, Turkey.

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