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## Ultrastructural studies on the sporogenous tissue and anther wall of *Leucojum aestivum* (amaryllidaceae) in different developmental stages

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### ABSTRACT

In this study, ultrastructures of anther wall and sporogenous tissue of *Leucojum aestivum* were investigated during different developmental stages. Cytomictic channels were seen between pollen mother cells during prophase I. Polar distribution was described in the organelle content of pollen mother cells and microspores in early phases of microsporogenesis and also in pollen mitosis. Active secretion was observed in tapetal cells. Previous reports about developmental stages of male gametophyte were compared with the results of this study.

**Key words:** *Leucojum aestivum*, Amaryllidaceae, ultrastructure, sporogenous tissue, anther wall.

### INTRODUCTION

Study of anther development in angiosperms has spanned more than a century and has provided a stable foundation for later work in the areas of physiology and more recently, in molecular biology. Much of the early work is compiled by Maheshwari (1950); the later studies have been surveyed by Bhandari (1984) and the others (Raghavan 1997).

Although most of the published papers dealing with male gametophyte, only a few species have been observed with the electron microscope. Ultrastructural studies about male gametophyte were done in *Helleborus foetidus* L. (Echlin and Godwin 1968), *Sorghum bicolor* (L.) Moench. (Gramineae) (Christensen et al. 1972), *Ginkgo biloba* L. (Wolniak 1976), *Ribes rubrum* (Geneves 1976), *Helianthus annuus* (Horner 1977), *Solanum nigrum* Linn.

(Bhandari and Sharma 1987, 1988), *Stangeria* (Rodkiewicz et al. 1988), *Malvaceae* (Kudlicka and Rodkiewicz 1990), *Triticum aestivum*, *Allium cepa* (Wang et al. 2004) and recently in *Lygeum spartum* L. (Abdeddaim-Boughanmi and Kaid-Harche 2009), *Nicotiana tabacum* L. (Wang et al. 2004, Mursalimov et al. 2010).

In this study, *Leucojum aestivum* L. is used. *L. aestivum* is a member of Amaryllidaceae. According to recent records, this family has 60 genus and 800 species (Watson and Dallwitz 2005). Genus *Leucojum* has only two species: *L. vernum* and *L. aestivum* which has two sub-species: subsp. *aestivum* and *pulchellum* (Crellin 2005). *L. aestivum* is naturally spread in the North Africa, Europe, Southeast Asia and in the Mediterranean (Darlington and Ammal 1945, Crellin 2005). This genus is represented with “*L. aestivum* subsp. *pulchellum*” in Turkey which is conserved due to destroyed habitats.

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The aim of this study is to explain the ultrastructure of anther wall and sporogenous tissue in *L. aestivum* during different developmental stages. Such investigations will furnish useful information about the structural changes at the subcellular level for Amaryllidaceae family and the others. And also this study will contribute to opinions about polarity of organelle distribution during microsporogenesis and microgametogenesis (Ekici and Dane 2004).

#### MATERIALS AND METHODS

In this study, *L. aestivum* plants were collected from the natural population at Tavuk Forest of Edirne A1(E) in European Turkey, between April and May in 2004 and 2005. They were brought to the Botanical Garden of Trakya University. Voucher specimens were placed in the herbarium of Trakya University (EDTU). Anthers were removed from flowers and fixed in 3% glutaraldehyde in 0.1M Millonig's phosphate buffer pH 6.8, for 2 hours (Millonig 1962). The anthers were then washed several times in buffer, fixed overnight with 1% buffered OsO<sub>4</sub> and dehydration was made with gradually increasing acetone-propyleneoxide series. They were stained in 70% acetone containing 1% uranyl nitrate overnight. The material was embedded in Epon (Freeman and Spurlock 1962). Semithin sections (1000 nm thick) of anthers were stained with toluidin blue. Thin sections [15-30 nm thick] were cut on a RMC (MTX) ultramicrotome with a glass or diamond knife. Sections were stained on grids with lead citrate (Reynolds 1963) for one minute and observed by a JEOL Jem 1010 electron microscope.

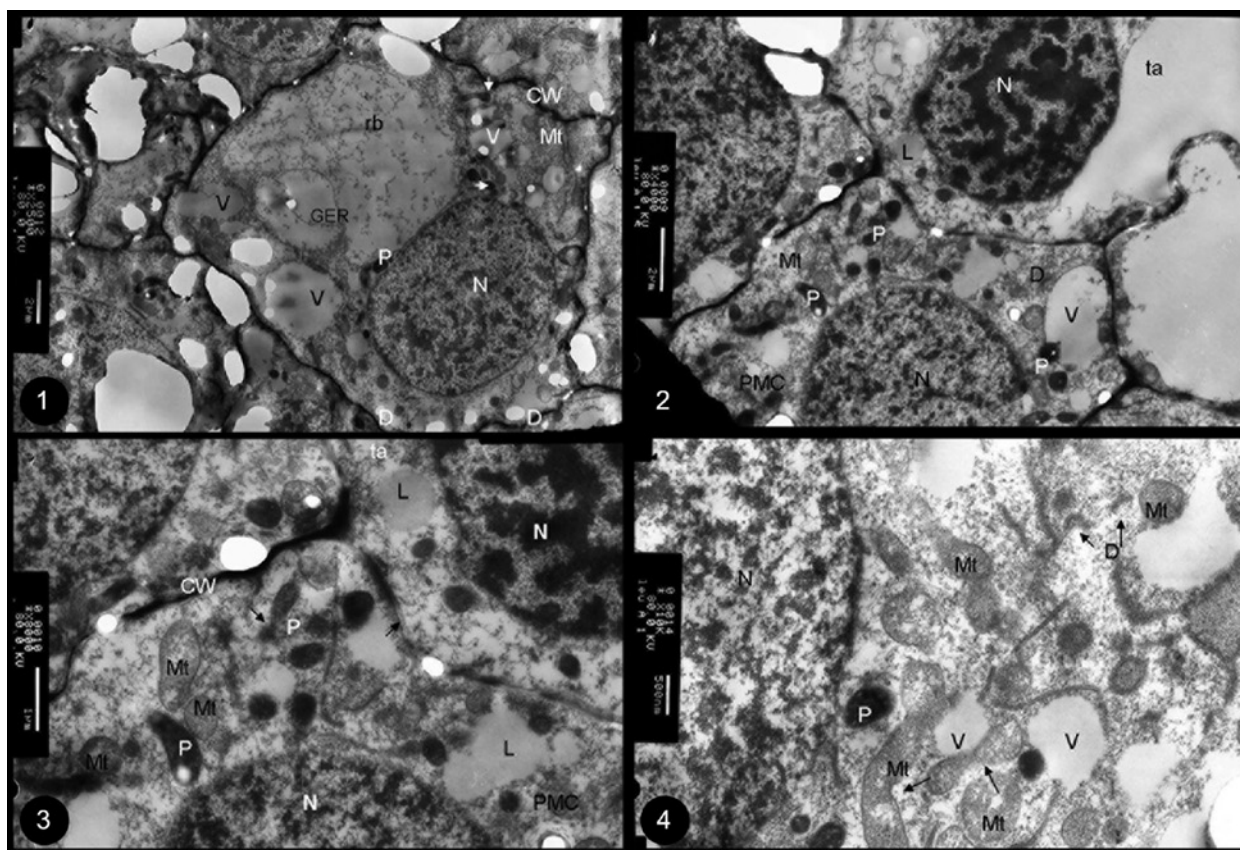
#### RESULTS

Ultrastructures of the anther wall and sporogenous tissue cells in *L. aestivum* were studied at the early microsporogenesis, tetrad, one-nucleated microspore and mature pollen phases. It was observed that nucleus had been in one side of the cell in sporogenous tissue cells before microsporogenesis and polarity was seen in distribution of the organelles. Organelles were mostly gathered around the nucleus and polisomes

were dense on the other side of the cell. There were few plastids, mitochondria, rough endoplasmic reticulum (RER), some dictyosomes and vacuoles in various sizes around the nucleus. It was observed that RER and dictyosomes were less-developed, vacuoles were usually nearby the cell wall and the plastids were divided. The crista of mitochondria was not well developed. The cell wall thickenings were homogenous (Figure 1).

When the parts neighbouring to the tapetum layer of pollen mother cell (PMC) were examined, the nuclei of PMCs were observed that they were rich in terms of euchromatin whereas nuclei in tapetum cells were rich in terms of heterochromatin (Figure 2). Thinner layers were seen in cell walls between PMCs and also between PMCs and tapetum cells. Organelle content of PMCs is denser than tapetum cells. Moreover, in various sizes, dividing plastids (Figure 3 -arrow) and mitochondria (Figure 4-arrow) were observed in this phase. And also RER was less developed, polisomes were dense and there were few vacuoles.

In the early prophase I, it was observed that the nucleus of PMC had been in one part of the cell due to a large vacuole; on the other hand, cell organelles had been between nucleus and cell wall. It was determined that there had been many mitochondria, a few plastids and less-developed RER (Figure 5). Nucleus membrane had many pores and polisomes were dense around it (Figure 6). Furthermore, the plasma membrane and thin areas in the cell wall between PMCs were observed. (Figure 7). In the next phase, an increase in the number of mitochondria and plastids, and a decrease in the size and the number of vacuoles were observed. Euchromatin was dense in the nuclei with nucleolus (Figure 8). Thin regions in some areas of the cell wall between PMCs were also observed in this phase (Figure 9). And also there was a single and large nucleus with dense heterochromatin in the middle of the tapetum cells which were not rich in terms of organelle content. Organelles were accumulated around the nucleus



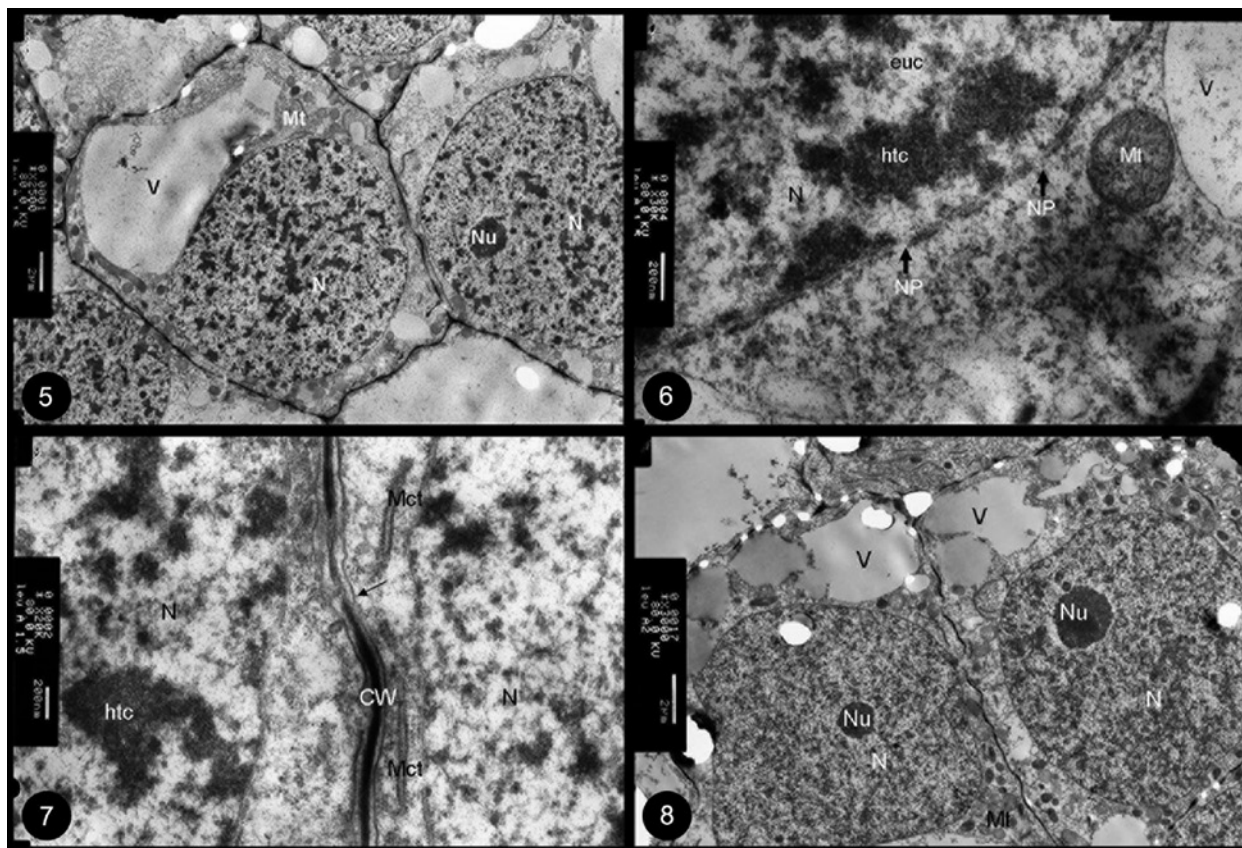
**Fig. 1** - Electron micrograph of sporogenous tissue cell in *L. aestivum* (CW, cell wall; D, dictyosome; GER, granular endoplasmic reticulum; Mt, mitochondria; N, nucleus; P, plastid; rb, ribosome; V, vacuole). **Fig. 2** - Electron micrograph of the pollen mother cell and tapetum cell in *L. aestivum* (D, dictyosome; L, lipid; Mt, mitochondria; N, nucleus; P, plastid; PMC, pollen mother cell; ta, tapetum; V, vacuole). **Fig. 3** - Electron micrograph of the cell membrane between pollen mother cell and tapetum cell in *L. aestivum* (CW, cell wall; L, lipid; Mt, mitochondria; N, nucleus; P, plastid; PMC, pollen mother cell; ta, tapetum). **Fig. 4** - Electron micrograph demonstrating organelle content of a part in pollen mother cell in *L. aestivum* (D, dictyosome; Mt, mitochondria; N, nucleus; P, plastid; V, vacuole).

(Figure 10). Anther wall was consisted of 4 or 6 cell layers. Epidermis, endothecium and middle layer cells were poor in terms of organelles. No organelles were seen except nucleus (Figure 11).

At the end of meiosis, formation of the exine was observed in the cell walls of microspores in tetrad and the cell wall of them was wrinkled. Nucleus covered the most part of the cell and organelles were seen in a small area between nucleus and cell membrane in this phase (Figure 12). Microspores of tetrad were swollen by taking fluid from the anther locus. On the other hand, some of them remained wrinkled. Nucleus, cytoplasm and organelles of swollen microspores were normal, but

chromatin distribution in nucleus was not typical in wrinkled ones and they had very small amount of both cytoplasm and organelles (Figure 13). Some changes were seen especially in epidermis cells of anther wall in this phase. There were a few large vacuoles and nucleus had dense heterochromatin in these cells; and cytoplasm was nearby cell wall, surrounding large vacuoles. An increase in the number of mitochondria and lipid granules was observed according to the content of anther wall cells in the early phases of meiosis. On the other hand, a large vacuole and cytoplasm including lipid granules and a few mitochondria were seen in the endothecium cells (Figure 14).





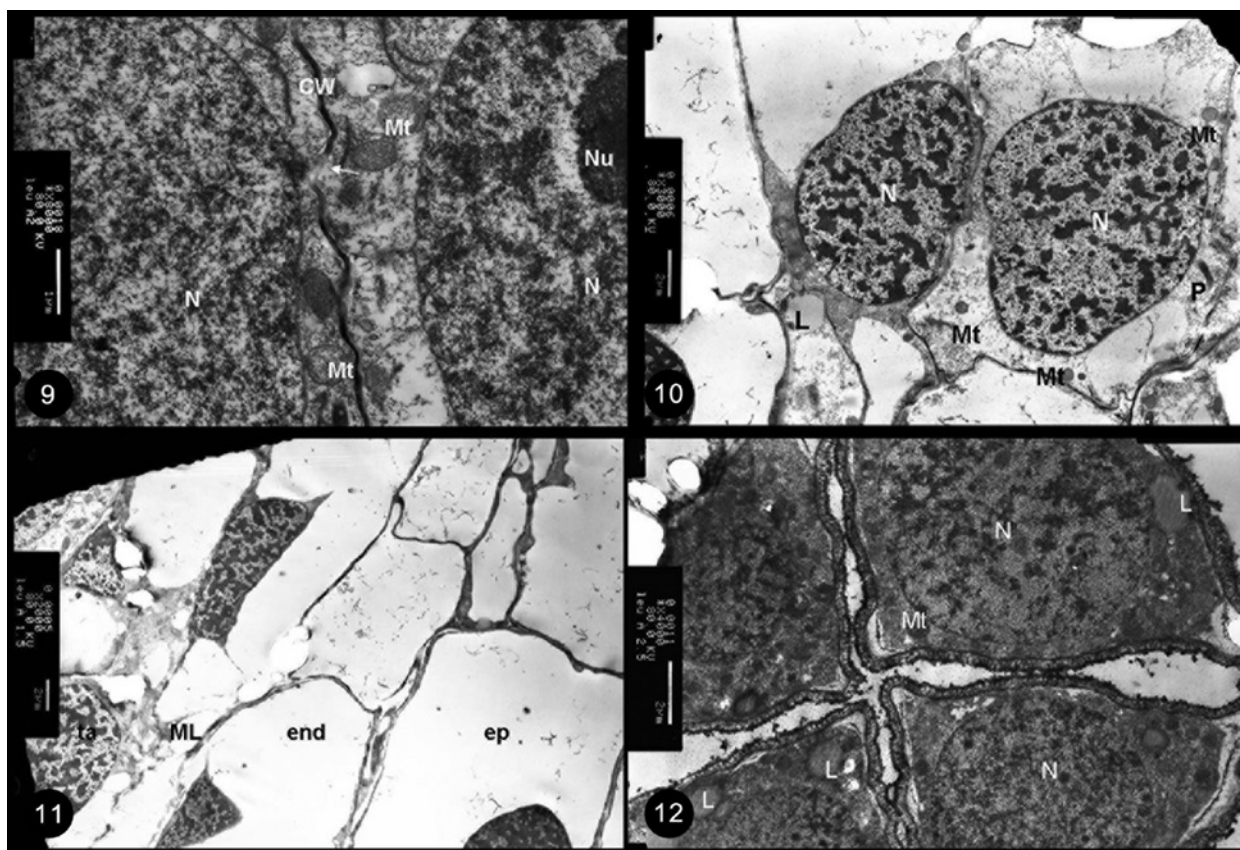
**Fig. 5** - Electron micrograph of pollen mother cell at the early phase of microsporogenesis in *L. aestivum* (Mt, mitochondria; N, nucleus; Nu, nucleolus; V, vacuole). **Fig. 6** - Electron micrograph of nucleus membrane of pollen mother cell at the early phase of microsporogenesis in *L. aestivum* (euc, euchromatin; htc, heterochromatin; Mt, mitochondria; N, nucleus; NP, nuclear pore; V, vacuole). **Fig. 7** - Electron micrograph of the cell membrane between two pollen mother cells at the early phase of microsporogenesis in *L. aestivum* (CW, cell wall; htc, heterochromatin; Mct, microtubule; N, nucleus). **Fig. 8** - Electron micrograph of pollen mother cells at microsporogenesis in *L. aestivum* (Mt, mitochondria; N, nucleus; Nu, nucleolus; V, vacuole).

Swollen microspores were released from the tetrad and turned towards the tapetum. In this phase, some changes were determined in the ultrastructure of anther wall. An increase was observed in the number and volume of lipid granules and organelle content especially in peripheral cytoplasm of epidermis cells (Figure 15). Nuclei of endothecium cells were become smaller and a large central vacuole and a few organelles in peripheral cytoplasm were detected. Cytoplasm and organelle contents were less in the cells of middle layer with large vacuoles and the tapetum cells started to degenerate. Exine formation was continued in the cell walls of microspores in this phase. Nucleolus, euchromatin and heterochromatin regions of nucleus were seen normal and it is placed in the centre of the

cell. Vesicle formation was occurred around nucleus. A lot of mitochondria with different sizes and many polysomes were seen in cytoplasm (Figure 16). Secretion granules were released from tapetum cells to the region between microspore and tapetum cells in this phase. Mitochondria were accumulated in the side of microspore facing tapetum (Figure 17).

Microspore polarity was seen before pollen mitosis. Nucleus was migrated to the one pole of the cell whereas on the other pole there were dense polysomes in the cytoplasm. Vesicle formation was observed around the nucleus. Many plastids and mitochondria were distributed periphery of the cell wall (Figure 18).

Lens shaped generative cell nucleus and lobular shaped vegetative cell nucleus were seen in



**Fig. 9** - Electron micrograph of cell wall between two pollen mother cells at the early phase of microsporogenesis in *L. aestivum* (CW, cell wall; Mt, mitochondria; N, nucleus; Nu, nucleolus). **Fig. 10** - Electron micrograph of the tapetum cells with one nucleus at the early phase of microsporogenesis in *L. aestivum* (L, lipid; Mt, mitochondria; N, nucleus; P, plastid). **Fig. 11** - Electron micrograph of the anther wall at the early phase of microsporogenesis in *L. aestivum* (ML, middle layer; end, endothecium; ep, epidermis; ta, tapetum). **Fig. 12** - Electron micrograph of tetrad phase of microsporogenesis in *L. aestivum* (L, lipid; Mt, mitochondria; N, nucleus).

the two celled mature pollen grains. The generative cell nucleus was rich in heterochromatin. There were numerous starch granules in the cytoplasm of the vegetative cell and it had euchromatin rich nucleus. Many lipid granules were detected around the pollen (Figure 19). Ubisch granules were seen between the pollen grains and tapetum cells. Degeneration of the tapetum was increased and the material exchange was continued (Figure 20). While the tapetum cells were degenerating, other anther wall cells were very active in this phase and an increase was detected in number of organelles than the previous phases (Figure 21). Many mitochondria and amyloplasts were seen in the epidermis cells (Figure 22). Endothecium cells

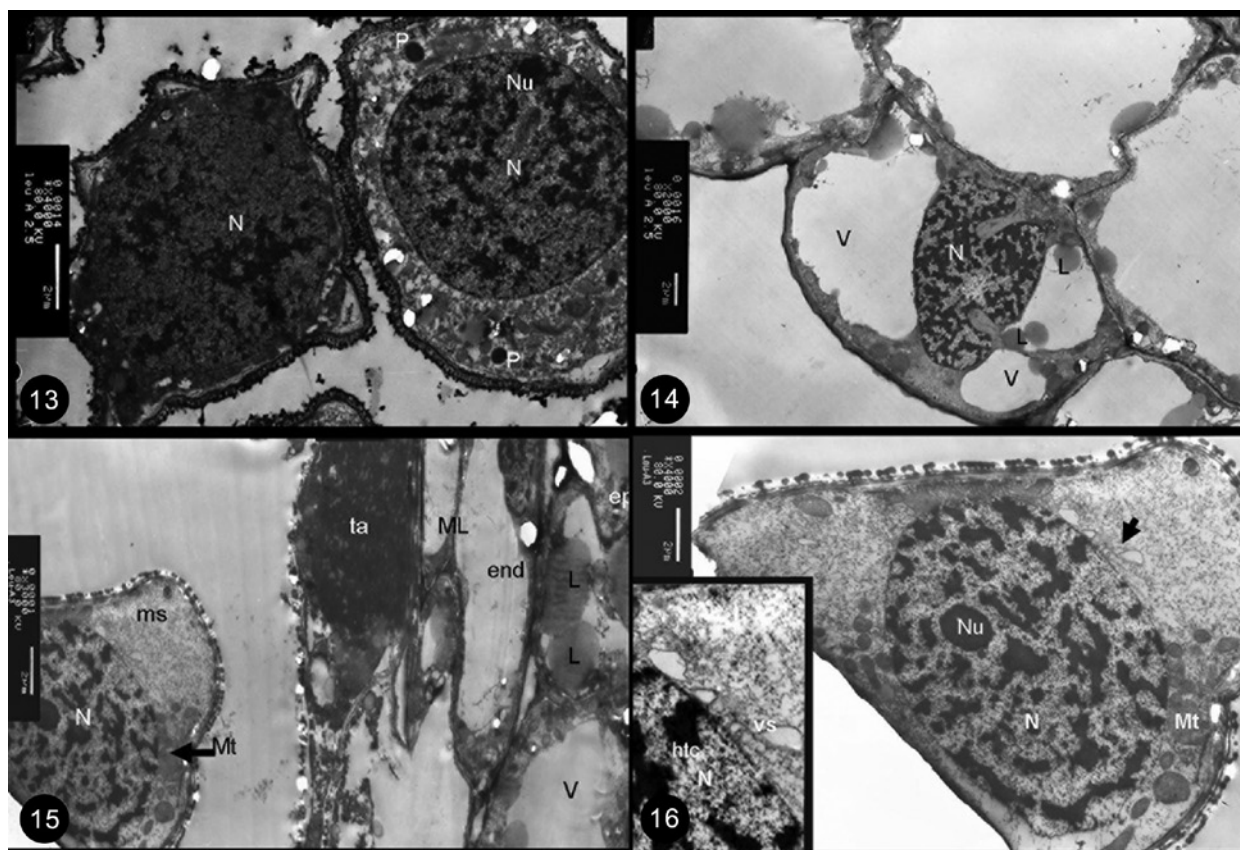
showed the same characteristics with the epidermis cells. Cell wall thickenings were not detected in the ultrastructure of endothecium (Figure 21).

#### DISCUSSION

The ultrastructure of the sporogeneous tissue cells and the anther wall were discussed in *L. aestivum* during different developmental stages with the other species from other families due to the lack of information about ultrastructural studies in Amaryllidaceae.

Nuclei of the sporogenous tissue cells were seen in one side of the cell at the early microsporogenesis in *L. aestivum* and there was a polarity in distribution of the organelles. It was observed that



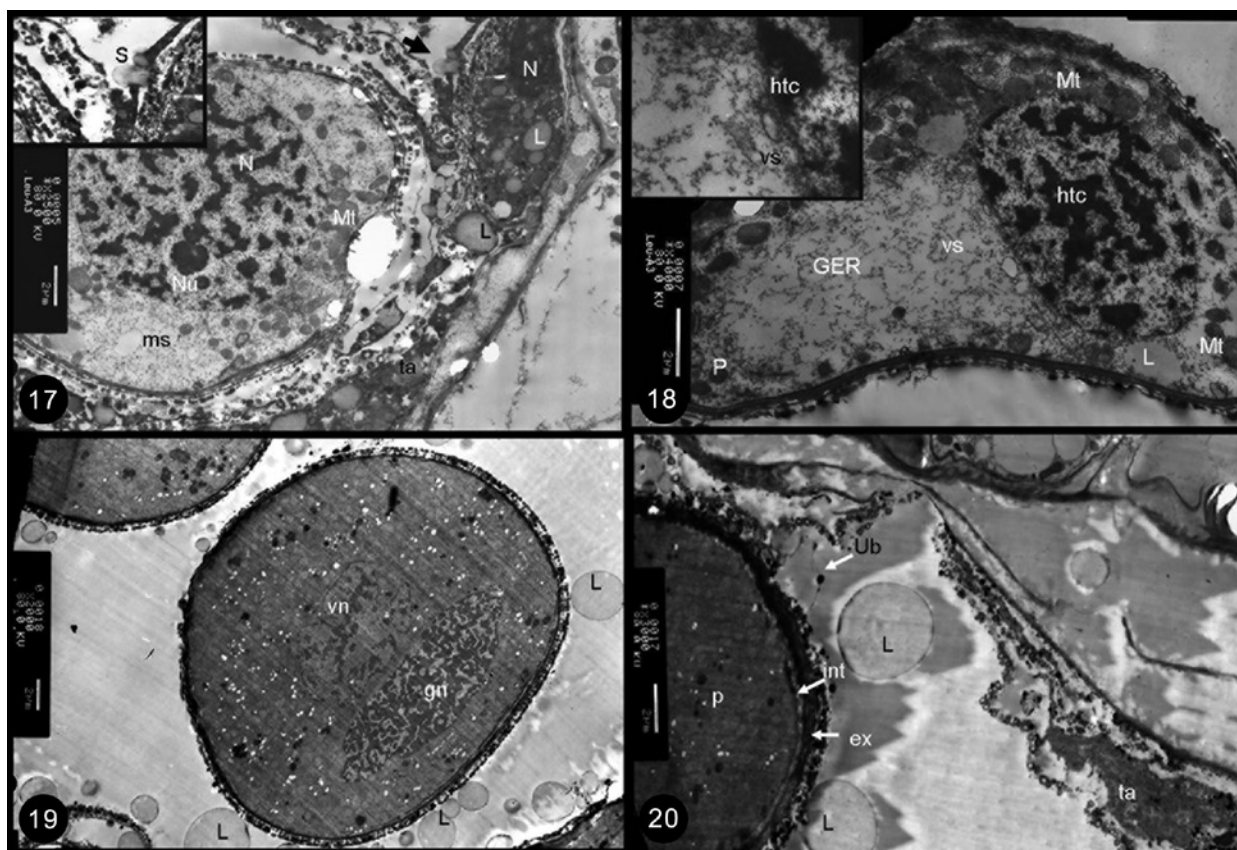


**Fig. 13** - Electron micrograph of the dispersing microspores at the end of tetrad phase in *L. aestivum* (N, nucleus; Nu, nucleolus; P, plastid). **Fig. 14** - Electron micrograph of the epidermis layer of anther wall at the tetrad phase in *L. aestivum* (L, lipid; N, nucleus; V, vacuole). **Fig. 15** - Electron micrograph of the microspore and anther wall at the end of microsporogenesis in *L. aestivum* (ML, middle layer; end, endothecium; ep, epidermis; L, lipid; ms, microspore; Mt, mitochondria; N, nucleus; ta, tapetum; V, vacuole). **Fig. 16** - Electron micrograph of the microspore in *L. aestivum* (Vesicle formation around nucleus; arrow- figure at the left bottom) (htc, heterochromatin; Mt, mitochondria; N, nucleus; Nu, nucleolus; vs, vesicle).

cell organelles were mostly gathered around the nucleus and polysomes were mostly dense on the other side. A polar distribution of organelles in the sporogenous tissue cells was not seen in *Solanum nigrum* (Bhandari and Sharma 1987). There were few plastids and mitochondria, RER, dictyosomes and vacuoles in various sizes around the nucleus. RER and dictyosomes were poorly developed. Vacuoles were usually placed near the cell membrane. Plastids were divided and mitochondria had poorly differentiated cristae. The primary sporogenous cell contained poorly differentiated plastids, mitochondria and short strands of RER in *Solanum nigrum* (Bhandari

and Sharma 1987). In *Helleborus foeditus*, no plastids were reported by Echlin and Godwin (1968) in the same stage.

In *L. aestivum*, during early prophase I, polar distribution of the organelles was seen in PMCs because of a large vacuole as in *Stangeria eriopus* (Rodkiewicz et al. 1988). Cell organelles were situated between the nucleus and cell wall. Many mitochondria, a few plastids and less-developed RER were seen. Plastids and mitochondria were clustered on one side of the nucleus in *L. aestivum*. On the contrary, in the same phase a polar distribution of the organelles was not observed in *Lavatera L.* and *Malva L.* (Kudlicka



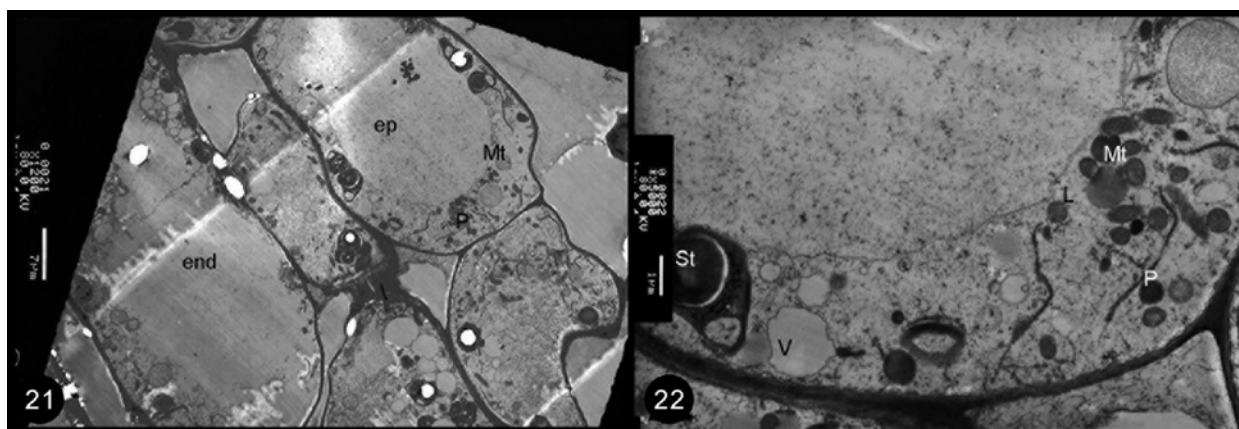
**Fig. 17** - Electron micrograph of the microspore attached to the tapetum before the pollen mitosis in *L. aestivum* (Exocytosis in the tapetum cell, arrow-small figure at the left top) (L, lipid; ms, microspore; Mt, mitochondria; N, nucleus; Nu, nucleolus; ta, tapetum; S, secretion). **Fig. 18** - Electron micrograph of the microspore before the pollen mitosis in *L. aestivum* (Vesicle formation around nucleus, arrow-small figure at the left top) (GER, granular endoplasmic reticulum; htc, heterochromatin; L, lipid; Mt, mitochondrion; P, plastid; vs, vesicle). **Fig. 19** - Electron micrograph of the mature pollen grain in *L. aestivum* (gn, generative nucleus; L, lipid; vn, vegetative nucleus). **Fig. 20** - Electron micrograph of the pollen and tapetum cell at the end of the pollen mitosis in *L. aestivum* (ex, exine; int, intine; L, lipid; p, pollen; ta, tapetum; Ub, ubish granule).

and Rodkiewicz 1990). Mitochondria, plastids and many vesicles were gathered in the perinuclear region at the late prophase. Organelles formed a dense layer which tightly coated the nucleus. Furthermore, aggregation of organelles was seen in the equatorial space between two nuclei of a meiotic cell during the telophase I in *Lavatera* and *Malva* (Kudlicka and Rodkiewicz 1990), *Ginkgo biloba* L. (Wolniak 1976) and *Solanum nigrum* L. (Bhandari and Sharma 1988). The aggregation of organelles in an equatorial region of a meiotic cell may facilitate their equal apportionment during the second meiotic division (Geneves 1967, Wolniak 1976, Kudlicka and Rodkiewicz 1990). Nucleus

membrane was consisted of many pores and nucleus was surrounded densely by polysomes. The nuclei with nucleolus were rich in terms of euchromatin in *L. aestivum* during the prophase I. This situation showed that PMCs were very active and also RNA and protein synthesis occurred in these cells. In addition, cytoplasmic channels were seen between PMCs at the early prophase I.

Cytoplasmic channel represents a huge inter-cellular connection other than plasmodesma. They also called cytoplasmic connections or cytomictic channels (Wang et al. 2004). An important role in the regulation of the growth and development of plants belongs to the intercellular cytoplasmatic





**Fig. 21** - Electron micrograph of the anther wall at the mature-pollen phase in *L. aestivum* (end, endotecticium; ep, epidermis; Mt, mitochondria; P, plastid). **Fig. 22** - Electron micrograph of the epidermal cell in the anther wall at the mature- pollen phase in *L. aestivum* (L, lipid; Mt, mitochondria; St, starch; P, plastid; V, vacuole).

contacts, realized via plasmodesmata and cytomictic channels. (Mursalimov et al. 2010). Cytomictic channels were widespread in meiocytes of all species with a secretory tapetum (Heslop-Harrison 1966, Pacini and Cresti 1978, Pacini et al. 1986, Mursalimov et al. 2010), but they were never reported in those species with ameboid tapetum. Cytomictic channels in the angiosperms had not been always seen in the same phase. This process occurs for instance between the first and the second meiotic division in *Lycopersicum peruvianum* (Pacini and Cresti 1978), *Olea europaea* L. (Pacini et al. 1985) and *Prunus avium* (Pacini et al. 1986); at the onset of first meiotic division in *Helleborus* (Echlin and Godwin 1968). On the contrary, in gymnosperms cytomictic channels were detected only between tapetal cells, but never in the meiocytes (Dickinson and Bell 1976). In this study, cytomictic channels were seen in the early prophase I of meiosis in *L. aestivum* as in the *Helleborus* (Echlin and Godwin 1968).

There were no cytomictic channels in the PMC walls of *Ceratozamia mexicana* (Audran 1979) from the gymnosperms at meiosis even though cytomictic channels were frequent in ferns and angiosperms during meiosis. According to Heslop-Harrison (1966) and Audran (1979), cytomictic channels were

holes in the meiocyte callose wall through which cytoplasmic connections were formed in adjacent meiocytes. These structures are important because they facilitate the movements and intermixing of cytoplasmic components of the cells within the microsporangium. They appear to be responsible for the synchronization in the anther locus.

Exine formation begins in the cell wall of the microspores in tetrad at the end of the meiosis in *L. aestivum* and the cell wall was appeared to be wrinkled. Microspores have a large nucleus in this phase. Microspores moved towards the tapetum by taking the fluid in the anther locus after leaving from the tetrad. Similar situations have also seen in *Sorghum bicolor* (Christensen et al. 1972) from Poaceae, in *Oryza sativa* (Dane and Meriç 2005) and in *Triticale* cv Tri-1 (Bhandari and Khosla 1982) from Gramineae.

In *Citrus limon* while the microspores were still in tetrads, active secretion (proorbicules) and tapetal wall dissolution began in the tapetum. And also there had been an increase in ER and dictyosomes in that phase (Horner and Lersten 1971). The tapetal ribosomes increased in number similar to that reported in *Zea mays* (Moss and Heslop-Harrison 1967). These events were also identified in *L. aestivum*. Ubisch granules or orbicules are still an

argument for the participation of the tapetum in the development of the exine (Abdeddaim-Boughanmi and Kaid-Harche 2009). The ubisch granules were seen between plasmalemma and the tapetal cell wall in *Ceratozamia mexicana* (Audran 1979).

In this study, vesicle formation was observed around nucleus in *L. aestivum* during one nucleated microspore phase. Vacuolization had played a role in cellular elongation (Willemse 1981). In this phase, secretory granules of tapetal cells were seen to move towards the area between the microspore and the tapetal cell. Mitochondria in the microspore were observed to accumulate mostly in one side of the microspore facing tapetum. This showed that the substances secreted by the tapetum had been taken by the microspore via active transport. Similarly, ubisch granules were seen to form in the tapetal cells in *Crinum* (Davis 1966). And also it was shown that microsporogenesis was affected by the tapetum in *Lilium longiflorum* (Miki-Hirosige and Nakamura 1982).

At the end of pollen formation, the generative cell nucleus was lens-shaped whereas the vegetative cell nucleus was lobed in *L. aestivum*. The generative cell nucleus was rich in heterochromatin; on the other hand, the vegetative cell nucleus was rich in euchromatin and there were numerous starch granules in the cytoplasm. A large number of lipid droplets and ubisch granules were seen around the pollen. And substance exchange between the degenerating tapetal cells and pollen grains had also continued throughout this phase. These observations were similar to the results of the previous studies carried out (Horner 1977, Bhandari and Khosla 1982, Tanaka 1997). While the tapetal cells were degenerating, the other cells of anther wall were in their most active periods. The anther wall cells were richer in terms of the organelles. They contain nucleus, mitochondria, lipid granules and starch grains. These features showed that the anther wall is also active in the late phases. There was no study related to the ultrastructure of anther wall cells in this phase in literature.

In conclusion, ultrastructures of anther wall and sporogenous tissue during various developmental stages in *L. aestivum* were explained for the first time. There was polarity in organelle distribution almost every developmental phase of microsporogenesis. Cytomictic channels were seen between PMCs at the early prophase I of meiosis. Anther wall cells had function in the formation of microspores. Tapetum contributed exin formation by secreting ubisch granules. These data will contribute to ultrastructural studies about Amaryllidaceae.

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#### RESUMO

Neste estudo, ultraestruturas da parede da antera e tecido esporogênico de *Leucojum aestivum* foram investigados durante diferentes estágios do desenvolvimento. Canais citomíticos foram vistos entre células – mãe de pólen durante a prófase I. Distribuição polar foi descrita no conteúdo da organela de células – mãe de pólen e em micrósporos nas fases iniciais da microesporogênese e também na mitose do pólen. Secreção ativa foi observada nas células tapetais. Registros prévios referentes aos estágios do desenvolvimento do gametófito masculino foram comparados com os resultados deste estudo.

**Palavras-chave:** *Leucojum aestivum*, Amaryllidaceae, ultraestrutura, tecido esporogênico, parede da antera.

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