

Ultrastructure of mature pollen grains in diploid and the natural tetraploid *Trifolium pratense*

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Abstract

Within the scope of this study, the ultrastructural changes in diploid and natural tetraploid *Trifolium pratense* L. (Elçi red clover) were examined from tetrad stage to mature pollen stage. In both diploid and natural tetraploid types, it was identified that the mature pollens were two-celled, the generative cell was oval-shaped and situated adjacent to pollen wall and the vegetative cell was elipsoidal-shaped. In both types, the vegetative cell included abundant vesicles, ribosomes and mitochondria.. Agranular ER, plastids and dictyosomes were low in number. In the tetraploid type, abundant ER foldings parallel to the cell wall were remarkable around the generative cell. The generative cell, containing a low quantity of mitochondria, ribosomes and vesicles, had a large nucleus at the center. It is noteworthy that in both types, there were great quantities of electron-dense vesicles.

Key words: mature pollen grains, *Trifolium pratense*., ultrastructure

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Diploid ve doğal tetraploid *Trifolium pratense*'de olgun polenin ince yapısı

Özet

Bu çalışmada, diploid ve doğal tetraploid *Trifolium pratense* L. (Elçi çayırucüğü) de tetrad evresinden olgun polen evresine kadar polenlerdeki ultrastrüktürel değişimler incelenmiştir. Hem diploid hem de tetraploid örneklerde olgun polenlerin iki hücreli olduğu, generatif hücrenin oval şekilli ve polen çeperine yakın yer aldığı, vejetatif hücrenin ise elipsoidal şekilli olduğu gözlemlendi. Her iki tipde de vejetatif hücrede çok sayıda küçük vesiküller, ribozom ve mitokondri mevcuttur. Granülsüz endoplazmik retikulum (ER), plastidler ve diktiyozomlar az sayıdadır. Tetraploid polenlerde generatif hücre çevresinde bol miktarda hücre membranına paralel ER katlanmaları dikkati çekmiştir. Az sayıda mitokondri, ribozom ve vesikül içeren generatif hücrenin merkezi olarak yer alan büyük bir çekirdeğinin bulunduğu gözlemlenmiştir. Her iki tipde de elektronca yoğun çok miktarda vesiküllerin bulunması dikkati çekmiştir.

Anahtar kelimeler: olgun polen, *Trifolium pratense*., ince yapı

1. Introduction

Tetraploid Elçi Red clover (*Trifolium pratense* L.) is an economically important forage legume naturally grown in our country for its tetraploid characteristics and high protein capacity. Naturally occurring tetraploid *T. pratense* L. ($2n = 4x = 28$) is generally superior to the diploid forms in yield, disease resistance and persistence (Taylor and Smith, 1979).

There are many studies on the pollen morphology and anther development of the family Fabaceae (Güneş and Çırpıcı, 2010; Vardar and Ünal, 2011; Kahraman *et al.*, 2012; Çeter *et al.*, 2013). *Astragalus hamosus* and *Astragalus glycyphyllos* have been investigated for crystals in anther and filament by Yılmaz *et al.*, (2013).

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Morphology (Pinar et al. 2001), in-vitro germination and fertility of pollens, characteristics of pollen tubes as well as callose formations (Büyükkartal, 2003) in diploid and natural tetraploid *Trifolium pratense* L. were examined. In addition to fertile pollens, a great quantity of sterile pollens (%60-70) were also determined in natural tetraploid *Trifolium pratense* L. (Büyükkartal, 2003). This study was conducted in order to display the ultrastructural changes in both types of *Trifolium pratense* L. from tetrad stage to mature pollen stage based on the observed variation among pollens and observed failures in meiosis of microspore mother cells (Büyükkartal and Çölgeçen, 2007) that may lead to low seed setting.

2. Materials and methods

Plants of natural tetraploids of *Trifolium pratense* L. (Elçi red clover) type E2 ($2n = 4x = 28$) (Figure 1A and B) were collected in 1982 by Elçi from the Tortum region of Erzurum (Turkey). Diploids of *T. pratense* L. ($2n = 14$) were collected in Ankara (Turkey).



Figure 1A and B. Habitat and flower of the natural tetraploid *T. pratense* L. (Elçi red clover) type E2
Şekil 1. A ve B Doğal tetraploid *T. pratense* L. E2 tipi (Elçi çayırüçgülü) bitkisinin habitatı ve çiçeği.

Floral buds at various stage of development were taken everyday in the morning between 9. 00 and 10.30. Approximately, 243 anthers were examined for light microscope studies and some 132 anthers for electron microscopy studies. Samples were firstly fixed in % 3 glutaraldehyde buffered with 0.1 M phosphate (pH 7.2) for 3h at room temperature. Then post-fixed with % 1 osmium tetroxide in 0.1 M phosphate buffer at room temperature for 3h. Samples were dehydrated with a gradual ethanol series followed by propyleneoxide and embedded in Epon 812 (Luft, 1961). Semithin sections were stained with 1 % methylene blue or toluidine blue. Ultrathin sections were stained with uranyl acetate (Stempak and Ward, 1964) and lead citrate (Sato, 1967), and examined with Jeol CXII transmission electron microscope (TEM) at 80 kV.

3. Results and dicas

Observations in the cytoplasm of microspores and pollens in diploid and natural tetraploid *Trifolium pratense* L. were similar. In the tetraploid type, microspore tetrads were tetrahedral-shaped (Figure 2A-B). There was a large nucleus at the center. A quite thick, electron-transparent callose wall was observed around the tetrads (Figure 2C).

These observation agree with main description of *Amygdalus nana* (Chudíková *et al.*, 2011), *Onobrychis* (Chehregani *et al.* 2011), *Leucojum aestivum* (Ekici and Dane, 2012) and *Nicotiana tabacum* (Mursalimov and Deineko, 2012).

At small vacuolated stage, callose was dissolved and microspores were dispersed throughout the anther loculus (Figure 2D). The nucleus was first situated at the center (Figure 3A) and then it migrated to one of the poles in the cell (Figure3B).

At early microspore stage, the pollen was developed. The nucleus was at the center and contained an electron-dense nucleolus (Figure 3C). The nucleolus was stained quite dark. The nuclear membrane displayed an irregular outline in some parts. The cytoplasm contained a couple of dispersed large vacuoles and a few number of small vacuoles (Figure 3D). In addition, ribosomes, ER, dictyosomes, mitochondria and a great quantity of electron-dense granules were observed as well.

Like in many species (McCormick, 1993; El-Ghazaly *et al.* 2001; Teixeira *et al.* 2002; Vinckier and Smets, 2005; Rosenfeldt and Galati, 2005; Unal, 2006; Vardar and Ünal, 2011) the pollens in this plant were also two-celled.

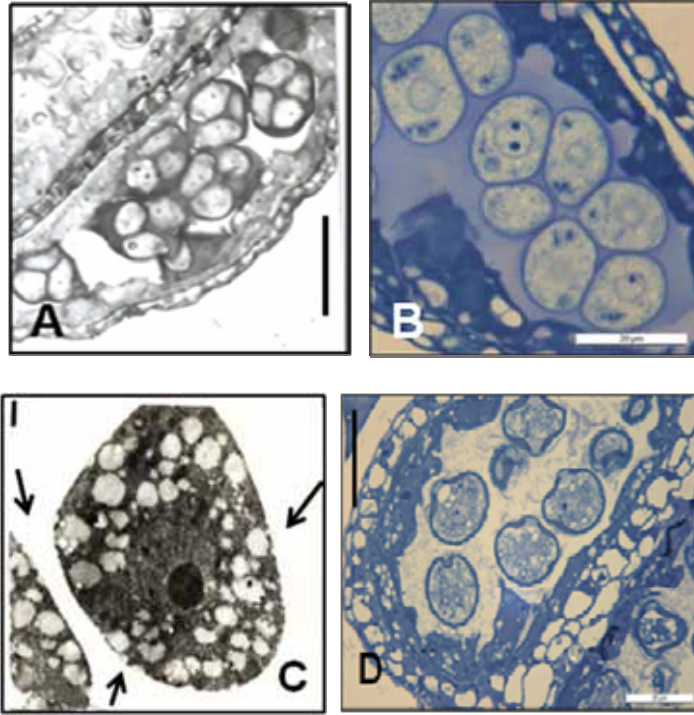


Figure 2. An anther of the natural tetraploid *T. pratense* L. A-B. Cross sectioned anther of the natural tetraploid *T. pratense* L. at tetrad formation stage. C. Tetrad callosic wall (arrows). D. Microspores and tapetum of the natural tetraploid *T. pratense* L. at the early free microspore stage. Scale bars = 20 μ m.

Şekil 2. Doğal tetraploid *T. pratense* L. anterinin enine kesitinde tetrad evresi. C. Tetrad’ da kaloz çeper (ok ile gösterilen). D. Doğal tetraploid *T. pratense* L. ‘ de erken mikrospor evresinde tapetum ve mikrosporlar

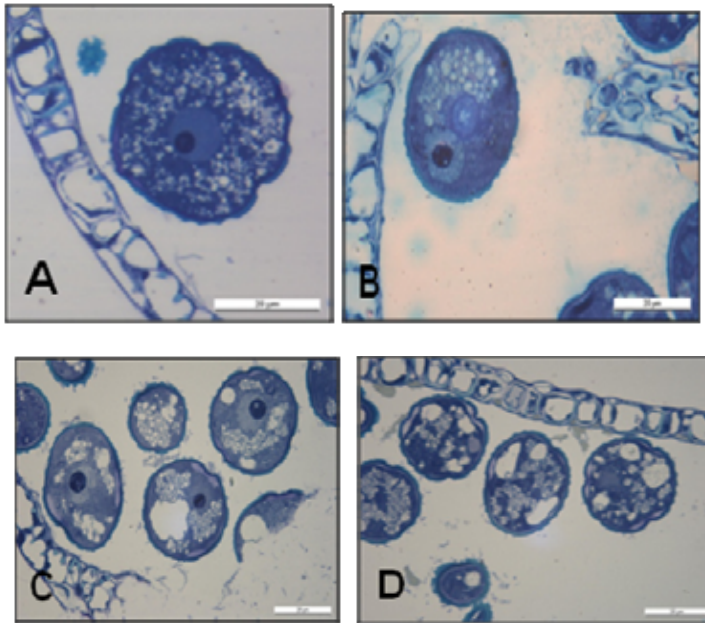


Figure 3. Semi-thin sections of anthers of the natural tetraploid *T. pratense* L. A. Microspore of the natural tetraploid *T. pratense* L. at the early free microspore stage. Note the nucleus situated at the center of the cell. B. Nucleus migrated to one of the poles in the cell. Bar = 20 μ m. C. Microspores at the early free microspore stage. Bar = 20 μ m. D. Cytoplasm of the microspores at the early free microspore stage. Bar = 20 μ m.

Şekil 3. Doğal tetraploid *T. pratense* L. anterlerinin yarı ince kesitleri. A. Doğal tetraploid *T. pratense* L. de erken mikrospor evresinde mikrosporlar. Çekirdek hücrenin merkezinde yer alır. B. Çekirdek hücrenin bir kutbuna doğru yer değiştirir. C. Erken mikrospor evresinde mikrosporlar. D. Erken mikrospor evresinde mikrosporların sitoplazması

The generative cell was oval-shaped; about 5 μm in length and 3 μm in width in diploid *T. pratense* and about 12.85 μm in length and 6.3 μm in width in natural tetraploid *T. pratense*. The generative cell in diploid pollens was half the size of the generative cell in tetraploid pollens. It was situated adjacent to the pollen wall. The vegetative cell was ellipsoidal-shaped and occupied a larger space (Figure 4A). An irregular and thin wall separated the generative cell from the vegetative cell (Figure 4B).

Polowick and Sawhney (1993a,b) have also stated that an irregular and thin wall separated these two cells in *Lycopersicum esculentum* pollens. Abundant ER foldings parallel to the cell wall were remarkable around the generative cell (Figure 4B). Van Went (1981) have emphasized the occurrence of excessive membrane foldings in the cytoplasm of *Impatiens wallariana* pollens that have cytoplasmic sterility. A great number of vesicles as well as small, irregular vacuoles were determined as a result of the examinations in the pollen cytoplasm. (Figure 4C). Some of the vacuoles contained granular materials.

In *Arabidopsis thaliana*, Yamamoto et al.(2003) have stated that vacuoles were dispersed throughout the cytoplasm and the electron-density of the vacuoles was the same with that of the cytoplasm.

Mitochondria were arranged in a crystal-like pattern and they some contain electron-transparent sections. Dictyosomes were more scarcely dispersed throughout the cytoplasm compared to agranular ER ribosomes. Many round and oval-shaped electron-dense vesicles of varying sizes were dispersed throughout the cytoplasm (Figure 4D-E). While 16.3 vesicles per 50 μm^2 were determined in diploid *T. pratense*, 30 vesicles per 50 μm^2 were determined in natural tetraploid *T. pratense*. The amount of electron-dense vesicles in diploid pollens was the half of that in natural tetraploid pollens. Hargrove and Simpson (2003) have stated that in *Cryptantha intermedia* pollens, there was a central region of intine wall material, and a concentration of cytoplasmic vesicles.

At vacuolated pollen stage, a change in the shape of the generative cell was observed. The cell, formerly oval-shaped, became spindle-shaped or ellipsoid and approached to the pollen wall (Figure 4F-G). In the center of the pollen, a large vacuole was observed containing granular materials.

The cytoplasm occupied a lesser space close to the wall and was electron-dense. The spindle-shaped generative cell had a large, dark stained nucleolus. It was observed also in diploid types that callose was dissolved and microspores were dispersed throughout the anther loculus (Figure 5A) at the small vacuolate stage. The nucleus was first at the center (Figure 5B-C) and then migrated to one pole of the cell.

At vacuolated pollen stage, no significant change was observed in the shape of the generative cell as such occurred in the tetraploid type. The cell has almost preserved its oval shape and approached to the pollen wall (Figure 5D). The quantity of round and oval-shaped electron-dense vesicles of varying sizes was lower and these were dispersed throughout the whole cytoplasm (Figure 5E-G).

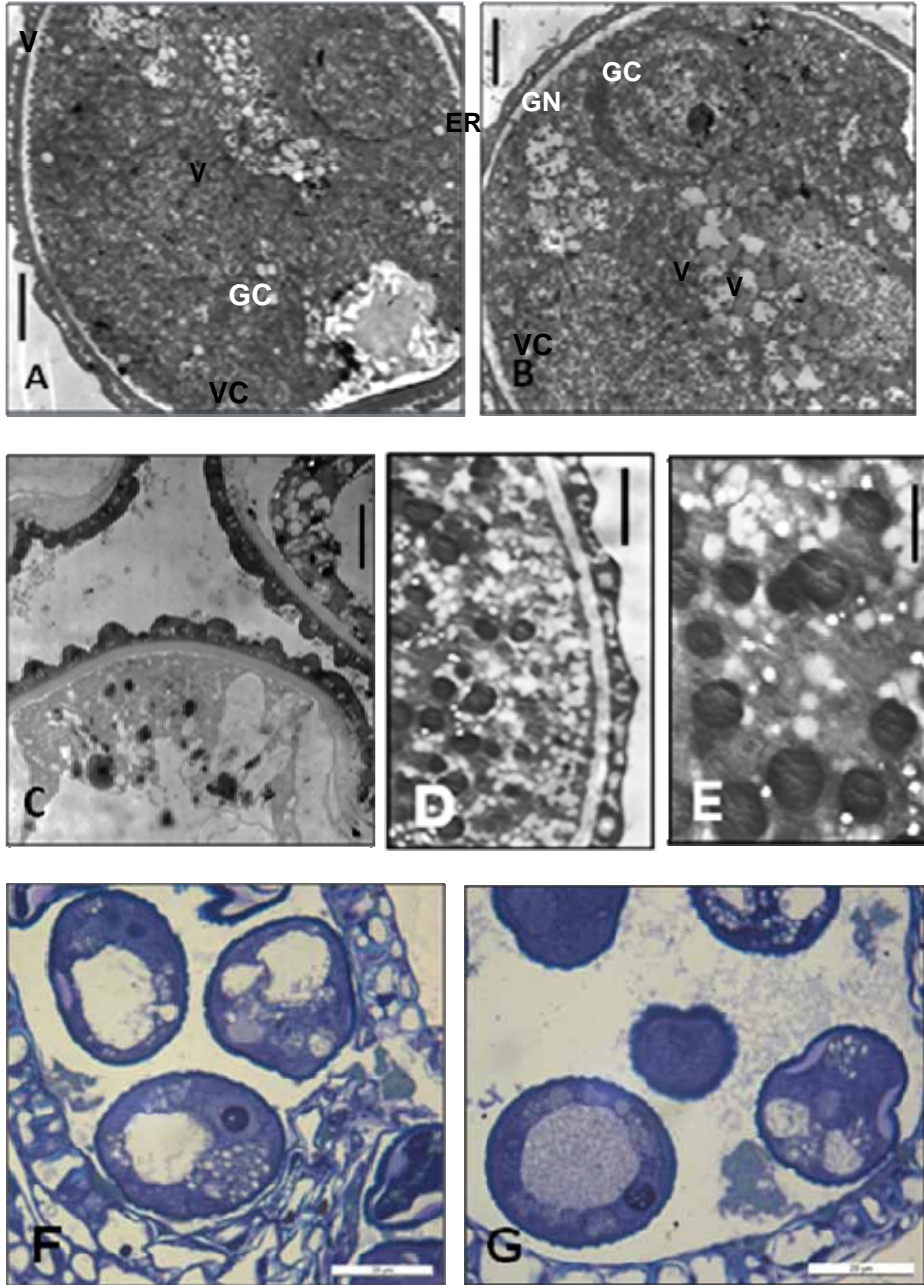


Figure 4. Electron micrographs of the natural tetraploid *T. pratense* L. A-B. Electron micrographs of the generative and vegetative cell showing the cytoplasm in the natural tetraploid *T. pratense* L. Bar = 20 µm. C. Young pollen grains showing vesicles and vacuoles are distributed throughout the cytoplasm. Bar = 20 µm. D. Numerous electron-dense vesicles are scattered in the cytoplasm. Bar = 20 µm. E. Detail of the cytoplasm in pollen cells showing numerous electron-dense vesicles Bar = 20 µm. F-G. At vacuolated pollen stage showing the generative and vegetative cell Bars = 20 µm

Şekil 4. Doğal tetraploid *T. pratense* L. elektron mikrografları. A-B. Doğal tetraploid *T. pratense* L.'de vejetatif ve generatif hücre sitoplazmasının elektron mikrografları. C. Genç polen tanesinin sitoplazmasında dağılmış halde bulunan vakuol ve vesiküller. D. Sitoplazmada bulunan elektronca yoğun vesiküller. F-G. Vakuollü polen evresinde generatif ve vejetatif hücreler

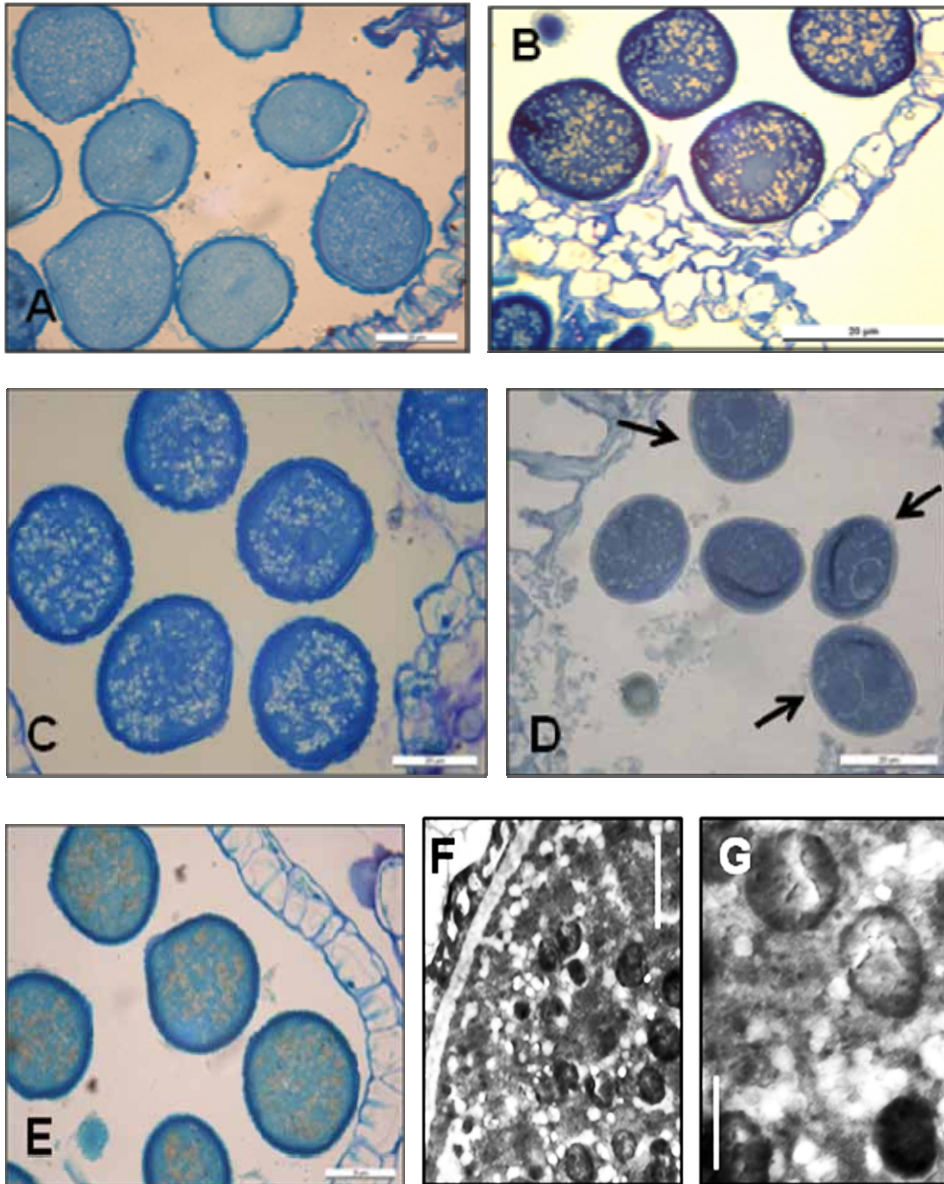


Figure 5. Anthers of the diploid *T. pratense* L. A. Microspores of the diploid *T. pratense* L. at the early free microspore stage. Bar = 20 μ m. B-C. Note the nucleus situated at the center of the cell. Bar = 20 μ m. D. Vegetative and generative cells Bar = 20 μ m E-F. Numerous electron-dense vesicles are scattered in the cytoplasm. Bar = 20 μ m G. Detail of the cytoplasm in pollen cells showing numerous electron-dense vesicles Bar = 20 μ m

Şekil 5. Diploid *T. pratense* L. anterleri. A. Diploid *T. pratense* L.'de erken mikrospor evresinde mikrosporlar. B-C. Çekirdek hücrenin merkezinde yer alır. D. Vejetatif ve generatif hücreler. E-F. Sitoplazmada dağılmış çok sayıda elektronca yoğun vesiküller. G. Sitoplazmada çok sayıda elektronca yoğun vesiküller

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