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Polar nuclei behaviour in the mature megagametophyte of the natural tetraploid *Trifolium pratense* (Fabaceae)

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Abstract: The mature female gametophyte in *Trifolium pratense* L., a natural tetraploid plant, consists of 7 cells: the egg cell, 2 synergids, the central cell, and 3 antipodal cells. This type of mature female gametophyte is known as polygonum. The central cell occupies the largest portion of the mature female gametophyte and the polar nuclei are situated close to the egg apparatus. The cytoplasm of the central cell is rich in organelles. A large number of rough and smooth endoplasmic reticulum, mitochondria, plastids, dictyosomes, ribosomes, starch, and lipid bodies were observed. Differences were identified in this plant between the sizes of polar nuclei and secondary nuclei generated by the fusion of the former. It was observed that the polar nuclei had fused long before the pollen tube entered the female gametophyte, and that the secondary nuclei in some female gametophytes were degenerated before fertilisation.

Key words: Central cell, female gametophyte, polar nucleus, *Trifolium*

1. Introduction

Tetraploid *Trifolium pratense* L. is an economically important forage legume naturally grown in Turkey for its tetraploid characteristics and high protein capacity. This naturally occurring tetraploid (2n = 4x = 28) is generally superior to the diploid forms in yield, disease resistance, and persistence (Taylor & Smith, 1979). A large number of seeds are required for natural tetraploid *T. pratense* to be grown in large areas, but there are problems with seed setting in this plant.

Female gametophyte development of some genera of Fabaceae has been studied (Folsom & Cass, 1992; Faigon Soverna et al., 2003; Moço & Mariath, 2004; Zulkarnain, 2005; Rodriguez-Riano et al., 2006; Chehregani & Tanaomi, 2010; Chehregani et al., 2011). Recently, ontogenic studies on female gametophyte development have been carried out in Fabaceae, such as *Caesalpinia* (De Pádua Teixeira et al., 2004), *Pterodon emarginatus* Vogel. (Oliveira et al., 2005), *Astragalus cemerinus* Beck. and *A. Ruscifolius* Boiss. (Riahi & Zarre, 2009), and *Onobrychis schahuensis* Bornm. (Chehregani & Tanaomi, 2010).

We observed that the megagametophyte was formed in 18% of the ovules examined, and about 13.9% of the total number of ovules observed were fertilised, but seed was formed by only 5.8% of the ovules (Algan & Bakar, 1997a). Algan and Bakar (1990) reported that seed abortion might be due to several factors:

1. There may be some problems in microspore and megaspore formation due to difficulties in the male and female gametophyte, or 2. there may be problems in the fertilisation and postfertilisation stages. Female gametophyte development in the natural tetraploid *Trifolium pratense* has been examined by light microscopy. Embryo endosperm (Algan & Bakar, 1996) and the ultrastructure of the megagametophyte have been investigated (Algan & Bakar, 1997). This study was based on the assumption that failure in seed setting might be caused by fertilisation failure in the poorly developed female gametophyte of this natural tetraploid (Bakar Büyükkartal, 2008).

2. Materials and methods

We used plants of the natural tetraploid *Trifolium pratense* variety E2, which have a diploid chromosome number of 2n = 4x = 28. Chromosome counting was done at the root tips. Plants were collected from the Tortum region of Erzurum (Turkey) by Elçi (1982). Plants were grown under natural conditions (Figure 1) (outside in the ground), and, when they started to bud, samples were taken from the flower buds at various stages of development.

For light and electron-microscopy observations, developing ovules were dissected from ovaries under a stereoscope. Three developmental stages were analysed, and the stages were established according to ovary length (Table). Two hundred and twenty-five ovules were examined.

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Table. Number of ovules analysed for the natural tetraploid *Trifolium pratense* in each developmental stage. Stages were assigned according to the length of the ovary.

Developmental stages of examples	Ovary lengths of examples (µm)	The number of examined ovules
Developmental stage 1	between 750 µm and 950 µm	58
Developmental stage 2	between 950 and 1150 µm	109
Developmental stage 3	between 1150 and 1350 µm	88
	Total number of examples	255

Figure 1. Habit of the natural tetraploid *Trifolium pratense* variety E2.

Ovules were fixed in a 96% ethanol:acetic acid (3:1) solution for 12 h. Dehydration was carried out in an ethanol–xylol series, and the ovules were embedded in paraffin (Algan, 1981).

For electron microscopic examinations, ovules were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate (pH 7.2) for 3 h at room temperature. Materials were postfixed in 1% osmium tetroxide for 3 h at room temperature. Samples were dehydrated in an ethanol series, transferred to 100% propylene oxide, and embedded in Epon 812 (Luft, 1961). Ultrathin sections were stained with uranyl acetate and lead citrate. Ultrastructural observations were made with a JEOL CXII transmission electron microscope (TEM).

3. Results

The polar nuclei are situated in the centre of the female gametophyte close to the egg apparatus and surrounded by thin cytoplasm (Figure 2). The central cell contains a large central vacuole and many small vacuoles are dispersed throughout the cytoplasm. In 12% of the ovules at developmental stage 1, the polar nuclei exhibited variation in size (width 8.8 µm, length 9.7 µm) and had a larger volume (Figure 2). These nuclei have electron-dense materials.

The central cell is rich in organelles (Algan & Bakar, 1997). A large number of RER, SER, mitochondria, plastids, dictyosomes, ribosomes, and lipid bodies were observed (Figure 2). In 22% of the ovules at developmental stage 2, fusion of the polar nuclei was examined at the ultrastructural level (Figure 2).

Fusion occurs after the polar nuclei make contact with each other through a burst in the nuclear membrane. The nuclear membranes of 2 opposite polar nuclei form finger-like extensions. A great number of mitochondria were observed in the cytoplasm bodies around those extensions. Once formed, the secondary nucleus is situated just beneath the egg cell (Figure 3). The sizes of the secondary nucleus range between 14.8 µm and 16.5 µm (Figure 3). The secondary nucleus is composed of granular nucleoplasma, and the nucleolus contains a large central vacuole in some cases. The nucleus membrane has a slightly undulating structure (Figure 3).

In the cytoplasm of the central cell, dictyosomes are composed of 4 to 5 systems. The endoplasmic reticulum (ER) is poorly developed and is formed of short structures and canals. Both types of ER, with and without granules, were present. The mitochondria vary in size (Figure 3). The cristae are long and numerous. Many free and ER bound ribosomes were dispersed within the cytoplasm. In the cytoplasm of the central cell, near the membrane at the micropylar side, plastids of different sizes occur. Some of these plastids contain more than one starch grain and a small number of lamellae (Figure 3).

In terms of storage structures (other than starch), many lipid bodies occur in the central cell cytoplasm. Mitochondria that vary in structure appear in some parts of the surrounding cytoplasm. These mitochondria were not regular in shape and their partitions were parallel to their long axes. The wall towards the nucellus (surrounding the female gametophyte) was thick and irregular. There appears to be a strong bond between the wall and nucellus cells. During the maturation of the female gametophyte, the diameter and volume of the central cell increase and the surrounding nucellus cells degenerate.

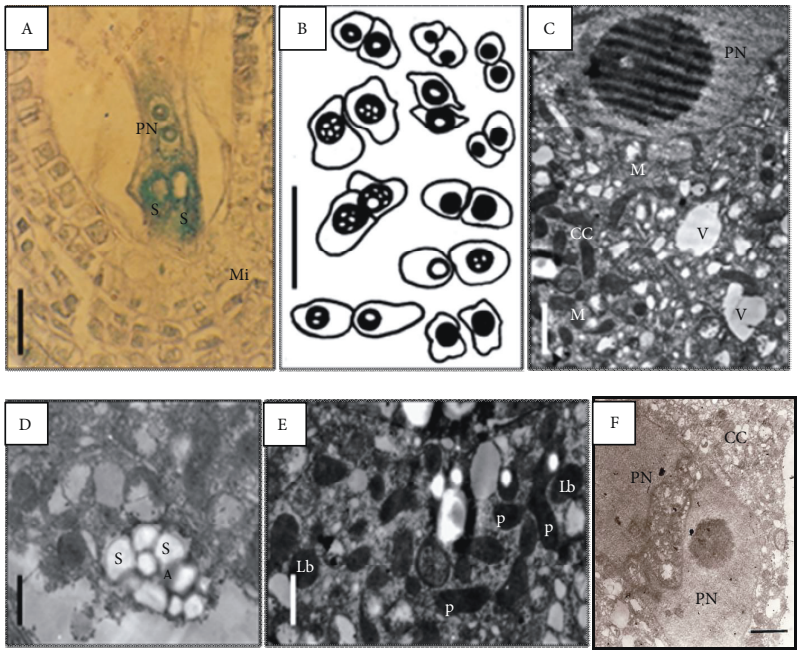

Figure 2. A- Light microscopic photograph of the paraffin section of the micropylar end of the mature female gametophyte showing 2 polar nuclei (PN) and synergid cells (S); B- Schematic drawing of a medium longitudinal paraffin section showing size variation in several almost-fused polar nuclei; C- Electron micrograph of the central cell (CC) cytoplasm containing a polar nucleus (PN), mitochondria (M), and vacuoles (V); D- Amyloplast (A) with starch grains; E- Electron micrograph of the central cell cytoplasm containing plastids (P) and lipid bodies (Lb); F- Electron micrograph of the central cell cytoplasm with fusing polar nuclei. Scale bars: A = 25 µm, B = 20 µm, C, D, and E = 2 µm, F = 3 µm.

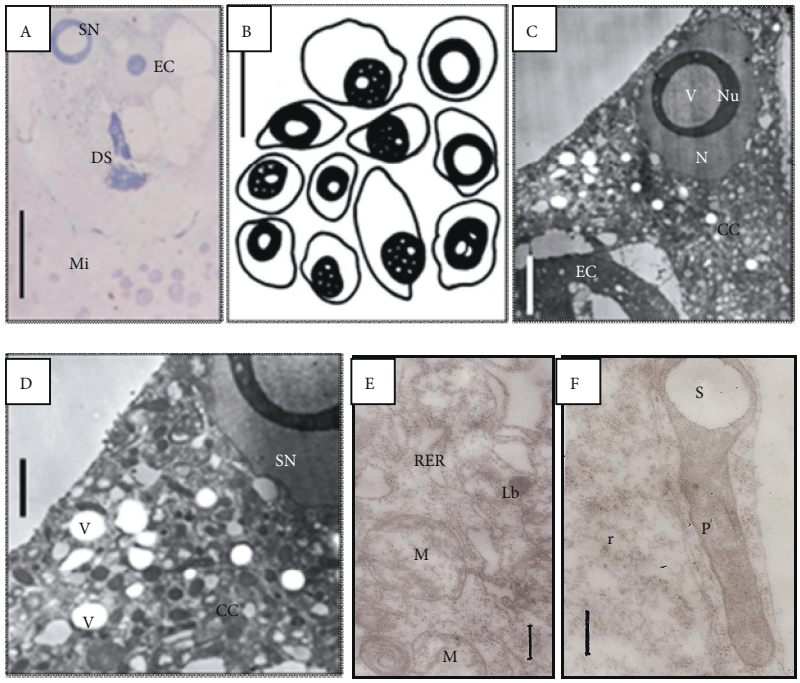

Figure 3. A- Semithin section of the micropylar end of the mature megagametophyte showing degenerated synergids (DS), egg cell (E), and secondary nucleus (SN); B- Schematic drawing of a medium longitudinal paraffin section showing the size variation in some of the fused nuclei (secondary nucleus); C- Electron micrograph showing a fused nucleus (N) that has a granular nucleoplasm and the nucleolus (Nu) contains a large central vacuole (V), central cell (CC), and egg cell (EC); D- Electron micrograph of the central cell (CC) cytoplasm showing vacuoles (V); E- Granular ER (RER), lipid bodies (Lb); F- Plastids (P) with starch (S) grain, and ribosomes (r). Scale bars: A = 25 µm, B = 20 µm, C = 1 µm, D = 5 µm, E and F = 1 µm.

From the total number of ovules analysed for developmental stage 3, only 7.6% of the ovules contained female gametophytes. These ovules contained secondary nuclei that were degenerated, vacuoles in the nucleoli increased, and the nuclear membranes became emaciated (Figure 4). The nucleolus has a greater electron density than does the nucleus. In the secondary nuclei, which were severely degenerated, vacuolisation increased; a large central vacuole and a great number of small vacuoles appear in the nucleolus (Figure 4).

4. Discussion

It has been observed that the polar nuclei in the mature female gametophyte were situated close to the egg apparatus, and the cytoplasm of the central cell was rich in terms of organelles (Algan & Bakar, 1997a). In a group of ovules (22%) containing mature female gametophytes, the polar nuclei fuse to form secondary nuclei (Bakar Büyükkartal, 2008) as is the case in the majority of angiosperm taxa (Faigon Soverna et al., 2003; Rodriguez-Riano et al., 2006; Gotelli et al., 2006; Vardar et al., 2012). Two polar nuclei fuse before fertilisation, and in this experiment the secondary nucleus was found to be of different sizes in a number of samples. During female gametophyte maturation, the diameter and volume of the central cell increases.

The central cell is the largest cell in the mature female gametophyte of *T. pratense*. It contains a large central vacuole and shares common cell walls with the egg apparatus. One of the structural characteristics of the central cell is the wall ingrowth adjacent to the nucellus. This ingrowth, which increases the cell surface area, suggests its important role in the absorption of metabolites from the adjacent tissue (Pate & Gunning, 1972; Folsom & Cass, 1986; Chamberlin et al., 1993; Bakar & Algan, 1997b).

The cytology of the central cell of *Trifolium pratense* is shown by this study to be essentially similar to that of other legume species (Folsom & Cass, 1989; Johansson & Walles, 1993; Chamberlin et al., 1994), but the behaviour of the polar nuclei in a certain number of the ovules examined seems to be different from that found in other species.

In 18% of the examined ovules of natural tetraploid *Trifolium pratense* there were mature female gametophytes (Algan & Bakar, 1997a). Although they appeared normal, in some of them (7.6%) the secondary nucleus was degenerated before fertilisation, so that the triple fusion with one of the nuclei from the sperm could not take place because of failure in the fusion of the polar nuclei. The failure in fertilisation in both cases is concluded to be one of the factors (Bakar Büyükkartal, 2008) affecting the seed setting rate of this plant, and the results reported here support this conclusion.

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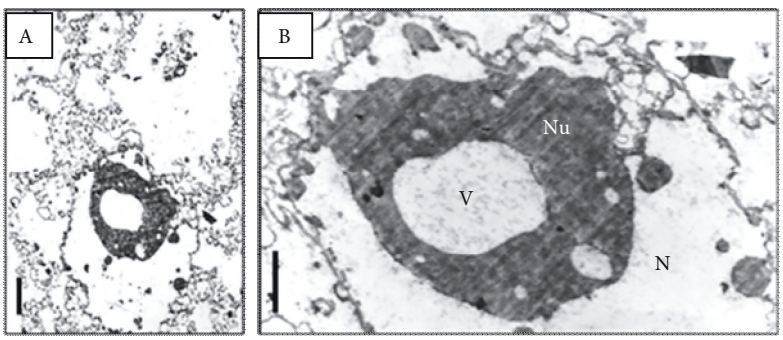

Figure 4. A- Light micrograph of the semithin section showing the micropylar end of the mature megagametophyte containing degenerated secondary nucleus; B- Enlarged portion of Figure 4A of the central cell showing degenerated secondary nucleus. Scale bars: A = 10 µm, B = 3 µm. Abbreviations: N = Nucleus, Nu = Nucleolus, V = Vacuole.

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