THE ULTRASTRUCTURE OF THE MATURE EMBRYO SAC IN THE NATURAL TETRAPLOID OF RED CLOVER (*TRIFOLIUM PRATENSE* L.). THAT HAS A VERY LOW RATE OF SEED FORMATION

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ABSTRACT

In this study, ultrastructural organization of cells in the mature embryo sac of natural tetraploid *Trifolium pratense* L. was investigated. The mature embryo sac of this plant contains an egg cell with two synergids at the micropylar end, and a central cell with two polar nuclei. The ultrastructure of these cells agrees with what is known for most angiosperms studied with the electron microscope. The egg cell is a large and highly vacuolate cell, partially surrounded by a wall. Much of the cytoplasm is located around the nucleus at the chalazal end and there are few numbers of channel-shaped endoplasmic reticulum, mitochondria, plastids and numerous ribosomes distributed throughout the cytoplasm. Unlike the egg cell, much of the cytoplasm in synergid cells is located at micropylar part of the cell and the synergid cytoplasm contains especially, large numbers of rough endoplasmic reticulum, free ribosomes, mitochondria and plastids. The central cell of *T. pratense* L. contains two large polar nuclei which lie close to the egg apparatus. Each polar nucleus has a single, large, dense nucleolus that contains several nucleolar vacuoles. Much of the central cell cytoplasm consisting of granular and agranular endoplasmic reticulum, mitochondria, plastids, ribosomes, dictyosomes and lipid bodies are placed around polar nuclei.

KEY WORDS: embryo sac, *Trifolium pratense* L., ultrastructure.

INTRODUCTION

Red clover (*Trifolium pratense* L.) is one of the most important forage legumes grown in many areas of the world. Tetraploid *T. pratense* L. which is grown naturally is a feed plant having a great economic value because of having properties of a tetraploid plant and also, a high protein capacity. A large amount of seeds are required for natural tetraploid *T. pratense* L. to be grown in a large areas. However, the amount of seeds can not be raised to a large proportion because of seed setting problem of this plant.

Although the factors responsible for the loss of potential seed production in red clover were investigated in a substantial number of morphological and anatomical (Martin and Watt 1934; Fedortschuk 1944; Povilaitis and Boyes 1959, 1960; Hindmarsh 1964; Rembert 1969; Shimada 1978a) and genetic (Povilaitis and Boyes 1956; Shimada 1977, 1978b; Pozdnyakov 1985; Ogorodnikova 1989; Leduc et al. 1990) studies, little success in understanding the problem or increasing actual yields has been obtained.

Ultrastructural and cytochemical studies have been made on synergids before and after fertilization of *Gossypium hirsutum*

(Jensen 1965a), *Capsella bursa-pastoris* (Schulz and Jensen 1968a), *Petunia hybrida* (van Went 1970a), *Aquilegia formosa* (Vijayaraghavan et al. 1972) and *Crepis capillaris* (Kuroiwa 1989). The egg and central cell of *G. hirsutum* (Jensen 1965b), *C. bursa-pastoris* (Schulz and Jensen 1968b, 1973) *P. hybrida* (van Went 1970b), *Nicotiana tabacum* (Mogensen and Suthar 1979), *Penstemon gentianoides* (Olgun and Jensen 1987), *Brassica campestris* (Sumner and van Caeseele 1989) and several other species have also been studied at ultrastructural level. Subsequent investigations by Folsom and Peterson (1984), Tilton et al. (1984), Kennel and Horner (1985) and Dute et al. (1989) have been made in relation to development of embryo sac and ovule of *Glycine max*. But up to now no study has been done on the ultrastructure of the natural tetraploid *T. pratense* L.

In the previous paper, we used light microscope to examine the development of embryo sac and formation of egg in the natural tetraploid red clover (Algan and Bakar 1990). We observed that mature embryo sac was formed in 18.1% of ovules examined, but seed formation was obtained to be only 5.8%. As the rate of seed set was lower than formation of the mature embryo sac, this investigation was done to determine whether there is a degeneration in the cells of embryo sac.

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MATERIALS AND METHODS

Plants of the natural tetraploid *T. pratense* L. were grown under natural conditions. For light microscopic observations, some of the floral buds, flowers and fruits at various stages of development were fixed in ethanol: acetic acid (3:1) for 12 h and subsequently transferred to 70% ethanol. Dehydration was carried out in an ethanol-xylol series and the material was embedded in paraffin. Sections were cut at 8-12 μ m thickness and stained with Heidenheim's iron alum haematoxylin. For electron microscope observations, ovules were dissected under moist condition and samples were placed immediately into 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 (Millionig 1961) for 3h at room temperature. Then post-fixed with 1% O_3O_4 in 0.1 M phosphate buffer at room temperature for 3h. Samples were prestained with 2% aqueous uranyl acetate for 2h. Materials were dehydrated with a gradual ethanol series followed by propylene-oxide and embedded in Epon 812 (Luft 1961). Ultrathin sections were double stained in a saturated solution of uranyl-acetate in 50% ethanol for 30 min (Stempak and Ward 1964). Followed by a treatment Sato's (1967) lead citrate for 10 min and examined using a JEOL 100 CX II transmission electron microscope (TEM) at 80 kV.

RESULTS

The structure of mature embryo sac.

According to literature the mature embryo sac of red clover is monosporic, 8-nucleated and is of the Polygonum type (Martin 1914). Thus the mature embryo sac should contain an

egg apparatus composed of an egg cell and two synergids at the micropylar end, a central cell with two polar nuclei, and three antipodals at the chalazal end.

The large bases of synergid cells were seen to cover the egg cell partially and it was observed that polar nuclei were located very close to the egg apparatus (Fig. 1). At serial sections of ovules in which mature embryo sacs were formed all the cells existing in a embryo sac were not observed at the same section as they could be observed at the serial sections (Fig. 2A-B). As the embryo sac matures it always gets enlarged and somewhat curved. The mature embryo sac of the natural tetraploid *T. pratense* L. has a length of about 645 μ m and a width of about 79.9 μ m at the micropylar end with a width of about 129.2 μ m at the chalazal end.

In studied tetraploid plants no antipodal cells were found in all examined ovules containing mature embryo sacs.

Egg Cell.

At longitudinal section, the egg cell is large and ovate. Its width is broader at the chalazal end and gets narrower towards the micropylar end. It is attached to the embryo sac slightly above the base of the two synergids. In some sections, a big vacuole and in some others a few big vacuoles with a plenty of small vacuoles in various sizes are present at the micropylar part of the cell. The nucleus and most of the cytoplasm are located at the chalazal end of the cell and the egg nucleus contains a single large nucleolus (Fig. 3). The nucleus of the egg cell has a length of about 10.8 μ m to 13.1 μ m and a width of about 10.4 μ m to 12.6 μ m. The egg cytoplasm contains only a few organelles. Ribosomes are more concentrated in the micropylar region of the egg cell cytoplasm (Fig. 4). They are either attached to the endoplasmic reticulum (ER) or

Fig. 1. Schematic drawing of a medium longitudinal section of the micropylar end of the mature embryo sac showing the egg cell (E); two synergids (Sy) and two polar nuclei (PN); Mi Micropyle Bar = 50 μ m

Fig. 2. Light microscopic photographs of the successive paraffin sections of the micropylar end of the mature embryo sac. **A.** an egg cell (E) occupies the micropylar region, one of the two polar nuclei (PN) is in almost central position. **B.** two synergids (Sy) and other polar nucleus (PN) are seen in serial section. Bar = 50 μ m

Figs 3-6. Electron micrographs showing a longitudinal section of the egg cell.

Fig. 3. Electron micrograph of the chalazal cytoplasm adjacent to the nucleus (N); Nu – nucleolus; V – vacuoles. Note the different size and distribution of vacuoles in the egg cell. Bar = 2 μ m

Fig. 4. Micropylar part of the egg cell cytoplasm which contains mitochondria (M); r – ribosomes. A portion of the synergid cell wall (CW) is seen near the egg cell. Bar = 1 μ m

Fig. 5. Chalazal part of the egg cell cytoplasm, adjacent to the nucleus, which contains channel-shaped endoplasmic reticulum (ER); V – vacuoles. Bar = 1 μ m

Fig. 6. Chalazal part of the egg cell cytoplasm showing plastid (P); V – vacuole. The cytoplasm appears quiescent; ribosome density and plastids, endoplasmic reticulum and dictyosome abundance is low. Bar = 1 μ m

are free in the ground cytoplasm throughout the egg cell. Mitochondria are numerous and they are more concentrated in the micropylar region of the egg cytoplasm. They contain relatively few short cristae. An extensive system of channel-shaped ER is present in the chalazal region of the cytoplasm (Fig. 5). Plastids and dictyosomes are extremely rare in the egg cell cytoplasm (Fig. 6). The egg cell is surrounded by a partial cell wall. The wall is relatively thick in the micropylar part. The egg cell shares a common wall with each synergid and with the central cell. The wall has a regular appearance in the micropylar half of the cell, but assumes an irregular and unusual morphology at the chalazal end, where the egg borders the central cell cytoplasm. In this region the plasma membranes of the egg and the central cell are separated by wide gaps.

Synergids.

The two synergids partially surround the egg. Each synergid has a common wall either with the egg or the other sister synergid. The wall gradually thins toward the base of the synergids where only a thin wall separates them from the egg cell. The micropylar end of both cells is occupied by the complex filiform apparatus (FA). The FA consists of an extensive labyrinth of wall projections extending deep into the synergid cytoplasm. Most of the synergid cytoplasm is situated at the micropylar part of the cell. There is a large vacuole and there are several small vacuoles located throughout the cytoplasm at the chalazal end of the cell. The nucleus is located near the center of the cell, just above the big vacuole and contains a single, large nucleolus. The synergid cytoplasm shows a complex organization and is rich in organelles. Mitochondria are abundant, with large accumulations adjacent to the FA (Fig. 7). They are spherical to slightly oval with well-defined short tubular cristae. The dictyosomes consisting of 3-4 flat cisternae are present at the micropylar side of the cytoplasm (Fig. 8). Profiles of rough ER often appear in masses of parallel stacked cisternae. Ribosomes are attached to the ER or are free in the cytoplasm (Fig. 9). Few plastids are present which contain small starch grains and they are more concentrated in the chalazal region of the cytoplasm (Fig. 10). Lipid bodies are also, present in the synergid cytoplasm. In some of the unfertilized megagametophytes examined, both of the synergids are degenerated (Fig. 11). The big vacuole located at the chalazal part of these synergid cells disappears. The cytoplasm is highly electron dense and lipid bodies, some remains of ER and numerous polysaccharide vesicles can be identified. More lipid droplets are accumulated at the cell border and, also the lipids around the degenerated organelles.

Central Cell.

The central cell occupies the major portion of the embryo sac. The two polar nuclei are located near the egg apparatus. Each polar nucleus contains one nucleolus approximately having the same size (Fig. 12). The cytoplasm of the central cell is rich in organelles. Much of the central cell cytoplasm consisting of numerous granular and agranular ER, mitochondria, ribosomes, plastids, dictyosomes and lipid bodies is placed around polar nuclei. Various-shaped plastids are found in central cell cytoplasm which is at the micropylar side and some of them contain starch grains (Fig. 13). Dictyosomes are composed of 3-5 flat cisternae. There are different sized mitochondria and numerous ribosomes which are free or bound to ER and distributed all around the cytoplasm (Fig. 14). Plenty of lipid bodies in different sizes are located in the central cell cytoplasm.

Polar nuclei were observed to be fused in the most of the ovules in which mature embryo sacs were formed (Fig. 15). The fusion of nuclei starts after polar nuclei come together and it occurs by invagination of the nuclear membrane. Two polar nuclei which are located close to each other form extensions towards cytoplasm at several places as if they were pulling each other. Cytoplasmic masses are found at the places where these invaginations exist between two nuclei membranes. At this side of the cytoplasm abundant mitochondria were observed (Fig. 16). The shape of the fused nucleus is oval and it is very big. It has a granular nucleoplasm and the nucleolus contains a big central vacuole (Fig. 17). Secondary nucleus is bigger than egg cell nucleus and nuclear membrane has a partially undulated structure.

DISCUSSION

In the mature egg of tetraploid *T. pratense* L., the nucleus is present in the chalazal region of the cell and a large vacuole is present in the micropylar side. The majority of plastids, ER, dictyosomes and ribosomes are perinuclear, although some of these organelles are present throughout the egg cell cytoplasm. The cytology of the mature egg cell of *T. pratense* L. is similar to *Gossypium* (Jensen 1965b), *Capsella* (Schulz and Jensen 1968b), *Petunia* (Van Went 1970b) and *Nicotiana* (Mogensen and Suthar 1979).

In most of angiosperms, the egg cell has been shown to be a polarized cell. The micropylar region of the cell contains a large vacuole with the nucleus and the most of organelles located in the cytologically inactive chalazal cytoplasm (Willemse and van Went 1984).

The egg cell wall is thicker at the micropylar end than that of the chalazal end. A similar progressive thinning of walls separating the mature egg and synergid cells from one another was also, noted in another cultivar of *Gossypium* (Jensen 1965a, b), *Capsella* (Schulz and Jensen 1968a, b) and *Glycine* (Folsom and Peterson 1984, Dute et al., 1989). The cytoplasm of the egg cell in *T. pratense* L. appears quiescent compared with other embryo sac cells. This state is very similar to that described for *Gossypium* (Jensen 1965b), *Capsella* (Schulz and Jensen 1968b) and *Brassica* (Sumner and van Caesele 1989).

This study notes that among the cytoplasmic features of the egg cell there is a small number of undifferentiated plastids situated around the nucleus at the chalazal end. Elsewhere, plastid development and abundance remains low, and the mitochondrial concentration and dictyosomes activity are much lower than observed in the synergids.

The cytoplasm of the synergids in *T. pratense* L. shows a complex organization rich in organelles including mitochondria, ER, plastids and dictyosomes. There is a tendency for a high concentration of mitochondria to accumulate near the FA, which has also been shown in *Gossypium* (Jensen 1965a) and *Capsella* (Schulz and Jensen 1968a). In *Petunia* (Van Went 1970a), *Nicotiana* (Mogensen and Suthar 1979) and *Brassica* (Sumner and van Caesele 1989) mitochondria are uniformly distributed. The morphology of the FA is highly variable among angiosperm species (Kapil and Bhatnagar 1981). FA represents the system of absorption and transportation of nutrients from the surrounding tissue to the egg cell and the central cell (Jensen 1965a).

In *T. pratense* L. synergid cytoplasm contains especially large numbers of mitochondria, plastids, dictyosomes, ribosomes and ER. In synergid cytoplasm granular and agranular ER which is in vast amount is found in close parallel associ-

Figs 7-10. Electron micrographs showing a longitudinal section of the synergid cells.

Fig. 7. Micropylar region of a synergid showing the filiform apparatus (FA). The FA consisting of electron translucent material lies at the top of the cell. Numerous mitochondria (M) are located near the FA Bar = 1 μ m

Fig. 8. Micropylar part of the synergid cytoplasm showing mitochondria (M); d – dictyosomes. Bar = 1 μ m

Fig. 9. Chalazal part of the synergid cytoplasm showing the rough ER (RER). The rough ER is present in masses of parallel stacked cisternae. r – ribosomes; l – lipid body. Bar = 1 μ m

Fig. 10. Chalazal part of the synergid cytoplasm which contains plastid (P) with small starch grain (S). V – vacuole. Bar = 1 μ m

Fig. 11. Electron micrograph of the degenerated synergid cells (Dsy) located at the micropylar end of the embryo sac. The cytoplasm of the degenerated synergid cells is electron dense and contains many lipid bodies (arrows). Bar = 1 μ m

Figs. 12-14. Electron micrographs showing a longitudinal section of the central cell.

Fig. 12. Electron micrograph of the central cell (CC) cytoplasm which contains polar nucleus (PN). Nu – Nucleous. Bar = 2 μ m

Fig. 13. Micropylar region of the central cell cytoplasm showing plastids (P), mitochondria (M) and endoplasmic reticulum (ER). Bar = 1 μ m

Fig. 14. Chalazal region of the central cell cytoplasm showing ribosomes (r); vacuole (V). Bar = 0.5 μ m

Fig. 15. Light micrograph of a paraffin section showing a longitudinal section through the central cell. The two polar nucleoli (PN) of the cell (arrows) can be seen here just prior to fusion. Bar = 20 μ m

Fig. 16. Electron micrograph of the central cell (CC) cytoplasm showing the two polar nuclei (PN) during fusion. Note the nuclear bridge between two nuclei. Fusing polar nuclei show cytoplasm pockets between nuclear bridges that contain numerous mitochondria (M). Bar = 2 μ m

Fig. 17. Electron micrograph showing the fused nucleus (N) which has a granular nucleoplasm and the nucleolus (Nu) containing a big central vacuole (V); CC – central cell. Bar = 3 μ m

ation with the wall and plasma membrane of the synergid. As conjectured by Schulz and Jensen (1968a) this arrangement could facilitate the transfer of substances into the egg.

In some of the ovules with mature embryo sacs, two synergids have been degenerated. One of the synergids degenerates earlier than the other. The cytoplasm of the degenerated synergids is mostly electron dense and organelles are degenerated in cytoplasm.

The polar nuclei in the mature embryo sac is very close to egg apparatus. Central cell cytoplasm is rich in organelles as in *Gossypium* (Jensen 1965b), *Capsella* (Schulz and Jensen 1968b), *Petunia* (van Went 1970b), *Nicotiana* (Mogensen and Suthar 1979), *Penstemon* (Olgun and Jensen 1987) and *Brassica* (Sumner and van Caeseele 1989). Plastids of central cell cytoplasm are of various shapes and some of them contain small starch grains. Besides, lipids are found as storage material in central cell cytoplasm. Jensen (1965b) and Schulz and Jensen (1968b) stated that central cell kept the metabolites as starch and lipid and used them in the first phase of formation of endosperm after fertilization.

In most of the ovules, in which the mature embryo sac is formed polar nuclei are fused. Fusion of polar nuclei was observed in one of the samples which was examined at ultrastructural level. Jensen (1965b) determined that polar nuclei fused before fertilization and it lasted for a few days. Schulz and Jensen (1973) showed the fusion of polar nuclei in *Capsella*; polar nuclei were completely fused just before the fertilization. The fused nucleus of *T. pratense* L. has approximately a diameter of 14.8 μ m and is formed of the granular nucleoplasm. The nucleolus as seen in Fig. 17 contains a big

central vacuole. Schulz and Jensen (1973) had the same results in their ultrastructural study as we had in our observations; nucleolus in fused nucleus of the mature embryo sac contains a big central vacuole and a few small vacuoles.

Antipodal cells existed in none of the ovules containing mature embryo sacs. The ultrastructure of the mature embryo sacs of tetraploid *T. pratense* L. show no basic differences in comparison with the embryo sacs described for other species of angiosperms. We concluded that cells forming the mature embryo sac are normal in the natural tetraploid *T. pratense* L. and they do not handicap the seed formation. There are probably different reasons for low seed set in this plant. More detailed studies are necessary in order to determine the underlying causes of the low seed set in the natural tetraploid *T. pratense* L.

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ULTRASTRUKTURA DOJRZAŁEGO WORECZKA ZAŁĄŻKOWEGO NATURALNEGO,
TETRAPLOIDA CZERWONEJ KONICZYNY *TRIFOLIUM PRATENSE* L.
(SŁABO ZAWIAZUJĄCEJ NASIONA)

STRESZCZENIE

Zbadano ultrastrukturę komórek dojrzałego woreczka załączkowego tetraploidu *Trifolium pratense* L. Dojrzały woreczek zawiera komórkę jajową i dwie synergidy na mikropylarnej stronie oraz dwa polarne jądra komórkowe w środku. Ich struktura jest typowa dla większości okrytozałączkowych. Duża, zwakuolizowana komórka jajowa jest okryta niekompletną ścianą, a większość cytoplazmy ubogiej w kanały ER, mitochondria i plastydy, a bogatej w rybosomy, leży wokół jądra.

Większość cytoplazmy synergidu, inaczej niż w komórce jajowej, leży w po mikropylarnej stronie i zawiera dużą liczbę kanałów szorstkiego ER, wolnych rybosomów, mitochondriów i plastydów.

Dwa jądra polarne komórki centralnej *T. pratense* leżą przy aparacie jajowym: zawierają duże jąderka z kilkoma wakuolami jąderkowymi. Wokół jąder polarnych leży główna część cytoplazmy komórki centralnej zawierająca agranularne retikulum, mitochondria, plastydy, rybosomy, diktiosomy i ciała tłuszczowe.

SŁOWA KLUCZOWE: woreczek załączkowy, *Trifolium pratense* L., ultrastruktura.