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Some features of anther wall formation and microsporogenesis in tetraploid maize

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ABSTRACT

Maize (*Zea mays* L.) is the world's third plant among the bread cereals by its economic value. Compared to diploid maize, tetraploid one has heavier vegetative mass, larger caryopsis, better seeds' quality and resistance to unfavorable factors but the abnormalities in reproductive structures' development may cause the decrease in grain productivity. As material for studies, we used autotetraploid lines ATL1280 and ATL1180, and population Krasnodarskaya TetraI of maize. Development of male reproductive sphere in three objects under investigation generally passes in a similar way and was resemble to diploid maize. The stamens in maize are tetrasporangiate; the locules are associated pairwise in two thecae. The formed anther wall consists of four layers: epidermal layer, fibrous one (endothecium), middle layer and tapetum. The type of anther wall development corresponds to the III type Umbellifera or Monocotyledonous type. Microspore formation proceeds according to successive type. As a result of meiosis, we observed the formation of not only isobilateral tetrads of microspores, which is typical for maize in general, but also tetrahedral ones. Abnormalities in shape of microsporocytes, microspores, pollen grains may be caused by their relative position in the anther and by deviations in the course of meiosis. The first reason – "positional" one – is connected with the features of growth and relative positions of microsporocytes in anther. It leads some of them to take atypical shape (trapezoidal, angulated, with elongated tip). The second reason – "mechanical" – is that some microsporocytes stay attached to callose column in the center of locule and hangs as a drop on it. Such a position of microsporocytes leads the cell to take a shape with strongly elongated tip. The third one – "meiotic" – is aligned with disturbances in meiotic divisions. These deviations lead to changes in cytoplasm's allocation within the microsporocyte and to formation of irregular tetrads, pentads, etc. and break the isobilateral symmetry of cells. The abnormalities observed probably are the consequence of the tetraploid nature of the lines studied and cause violation of male fertility and a decrease in grain productivity, which is characteristic to some tetraploid maize lines.

Keywords : *Zea mays* L., tetraploids, anther wall formation, microsporocytes, abnormalities

Maize (*Zea mays* L.) is the world's third plant among the bread cereals by its economic value. It regularly undergoes genetic and selection improvement. Tetraploid maize originally was obtained by Randolf L.F. in 1932 (Randolph 1932) by the means of contrast temperature influences on the zygote in the moment of its first division. Later, the methods for obtaining tetraploid plants changed, and research was carried out to find the optimal one (Kagermazov and Khachidogov 2018).

Compared to diploid maize, tetraploid one has heavier vegetative mass, larger caryopsis, better seeds' quality and resistance to unfavorable factors (Shcherbak 1970, Khadzhinov 1974, Khatefov *et al*. 2010, 2012, Palii and Batiru 2013, Kagermazov and Khachidogov 2018). The increase of heterosis' amplitude also was noticed when passing from diploid level to tetraploid one (Riddle and Birchler 2008). That is why tetraploids are involved in maize breeding. For example, some attempts take place to use diploid-tetraploid crosses in order to breed apomictic maize lines (Tsvetova *et al*. 2016) For the other hand, the abnormalities in reproductive structures' development of tetraploid maize are found out that may cause the decrease of crop (Shcherbak 1970, Khadzhinov 1974, Kagermazov and Khachidogov 2018).

Different abnormalities in meiosis during microsporogenesis leading to formation of faulty pollen were remarked in tetraploid maize lines. Abnormally developing pollen grains were observed in the tetraploid maize line KrP-1 and in hybrids KrP-1 with the AT-1 parthenogenetic line (Lobanova *et al*. 2010). Later, a complex of meiotic disorders was found in maize of the KrP-1 line, including incorrect separation of chromosomes, incomplete cytokinesis or its failure, a change in the orientation of spindles, etc., as well as

the formation of abnormal sporades of microspores and microspores having an atypical form (Lobanova *et al*. 2018, 2019).

An unusual abnormality was found out in the process of meiosis in maize autotetraploid ATL1280. In some microsporocytes (1-2%), peculiar outgrowths were observed, in which callose septa were formed, and conglomerates of membrane vesicles were also observed. After the meiosis, one of the cells of tetrad inherited this outgrowth (Shatskaya and Shamina, unpubl. data).

The above mentioned investigations are very important for understanding of the processes that take place during microsporogenesis in tetraploid maize. However, these research works are incomplete by virtue of the methods intending the study of single microsporocytes, sporads of microspores and pollen grains. They do not represent interactions of the generative cells with each other and with the surrounding tissues anther, although these ideas are of great practical and theoretical interest. The goal of our research is to get an idea of anther development in a whole and to add new information on microsporogenesis in tetraploid maize already available in terms of the lines in study.

MATERIALS AND METHODS

The material – autotetraploid lines ATL1280 and ATL1180, and population Krasnodarskaya TetraI of *Zea mays* L. – was grown in 2016 on the fields of Federal State Budget Scientific Organization "National Center of Grain named after P.P. Lukyanenko" in Krasnodar (FSBSO «NCG P.P. Lukyanenko»). Plant lines and populations under consideration were bred in this organization for different years.

Taking into account the early publications of V. S. Shcherbak (1970), we can assume that the Krasnodar tetra (KrTetraI) population was created on the basis of tetraploid analogues of five simple hybrids of diploid corn. Colchicine was used to chromosom doubling of diploid hybrids.

Autotetraploid lines ATL1280 and ATL1180 were bred from single (unique) diploid hybrid plants treated with colchicin with simultaneous self-pollination. These hybrid plants were obtained by crossing two simple hybrids of inbred lines with haploinduser ZMK1U (Shatskaya, 2010) during the maize haploid production program. Colchicination was performed according to the conventional for FSBSO «NCG P.P. Lukyanenko» technique for breeding of maize doubling haploids (Shatskaya *et al.* 1994). Seed reproduction of these samples is performed by self-pollination or by pollination with a mixture of pollen from two-four plants. An incomplete seeds number in the ear is characteristic for lines ATL1280 and ATL1180 (from 30 to 100 seeds number in the ear), while the plants of the population KrTetraI show the complete seed set (from 200 to 480).

The flower buds from the male inflorescence were fixed with a modified Navashin retainer (Wada and Kusunoki 1964) in the development phase of 7-8 leaves. Further, the material was treated according to the conventional methods of permanent preparations making for light microscopy. The buds were dehydrated by carrying through the series of alcohols and alcohol-chloroform mixtures up to the pure chloroform and enclosed in Histomix® (Biovitrum Company). This method is described in more details in: Voronova and Babro, 2018.

The blocks of Histomix with embedded flower buds were cut by microtome with section thickness of 5 ìm. The slides were stained by "triple staining" (Kamelina *et al.* 1992), Felgin's Shiff reagent with alcyan blue (it is simplified triple staining without Erlich hematoxyline), or Heidenhain's hematoxyline with alcyan blue (Zhinkina and Voronova 2000).

Slides and photographing were analyzed by microscope Zeiss Axioplan 2 Imaging with digital camera and AxioVision computer application.

RESULTS AND DISCUSSIONS

Development of male reproductive sphere in three maize lines under investigation generally passes in a similar way.

The male inflorescence of tetraploid maize, as well as in diploid one (Weatherwax 1955, Vollbrecht and Schmidt 2009), is a spreading panicle including the main axis and a number of branches. Every branch has four rows of spikelet's pair. One spikelet in a pair is sessile, another is pediculate. Each tassel spikelet produces two glumes, an outer and inner glume, which enclose two florets, an upper and lower floret; one of them is better developed. Each floret includes a floral shoot that originates from the axil of an additional bract called lemma. The floret consists of a single lemma and palea, two lodicules, three stamens and a rudiment of a pistil (Fig. *1a,b*).

The male flower of maize includes three anthers, two lodicules and rudiment of a pistil.

According to the literature data, the development of male reproductive structures in every flower begins with the formation of the three primordia of the prospective stamens on the male inflorescence's apical point. A number of cells of the primary archesporium of subepidermal origin can be distinguished under the two-layered tunica of these primordia (Chebotaru 1972, Vollbrecht and Schmidt 2009).

Our investigation gave the option to retrace the processes of anther wall formation and microsporogenesis from the archesporial cells' initiation up to the stage of microspores' segregating.

The stamens in maize are tetrasporangiate, the locules are associated pairwise in two thecae (Fig. *1b*).

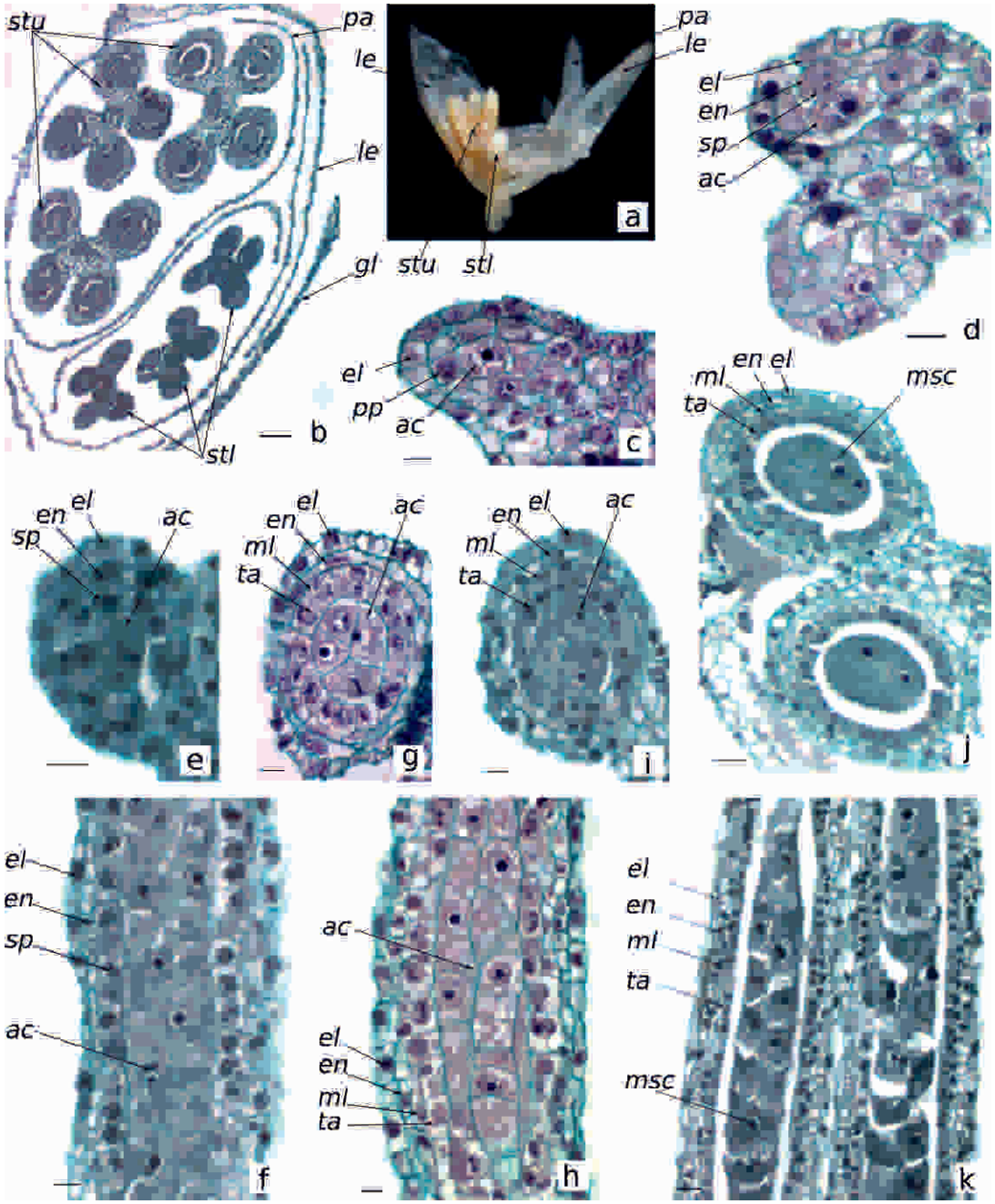

Fig. 1. Male flower and early stages of anther development in *Zea mays*.
a – general view of a male spikelet, glumes, lemma, palea and stamens are visible;
b – male spikelet, two flowers are located inside it, each of them is bounded by lemma and palea, three stamens are visible in each flower, one flower is at a more advanced developmental stage, the other flower has the stamens at an earlier stage of development (cross section); c – part of the stamen, in the center of the anther locule there is an archesporial cell surrounded by two layers of anther wall cells (cross section); d – part of the stamen, there is the archesporial cell in the upper locule of the anther. Pairs of cells that have recently divided periclinally are above the archesporial cell. These periclinal divisions leads to the formation of the third layer of the anther wall (cross section); e, f – the locule, anther wall consists of 3 layers of cells (e - cross section, f - longitudinal section); g, h – the locule, formation of the 4th layer, archesporial tissue consists of several rows of cells (from 4 to 6), the metaphase of mitotic division can be seen in one of the cells, archesporial cells stretch along the polar axis of the anther and take an angular, triangular or trapezoidal shape, often with pointed tip (g - cross section, h - longitudinal section);
i – the locule, the anther wall is 4-layered (cross section); j, k – the locule, archesporial cells differentiate into sporogenous cells (microsporocytes), stretch along the polar axis of the anther and take an angular, triangular or trapezoidal shape, often with a pointed tip, the anther wall is 4-layered, differentiation of cell layers is in progress (j - cross section, k - longitudinal section).
ac – archesporial cells, *el* – epidermal layer, *en* – endothecium, *gl* – glume, *le* – lemma, *ml* – middle layer, *msc* – microsporocyte, *pa* – palea, *pp* – premier parietal cell (layer), *sp* – secondary parietal cell (layer), *stl* – stamen of lower floret, *stu* – stamen of upper floret, *ta* – tapetum. a,b,e,i –KrTetraI, c,d,f,g,h,k –ATL1280, i– ATL1180 Bars: b – 50mkm, c,d,e,f,g,h,i – 10 mkm, j,k – 20 mkm

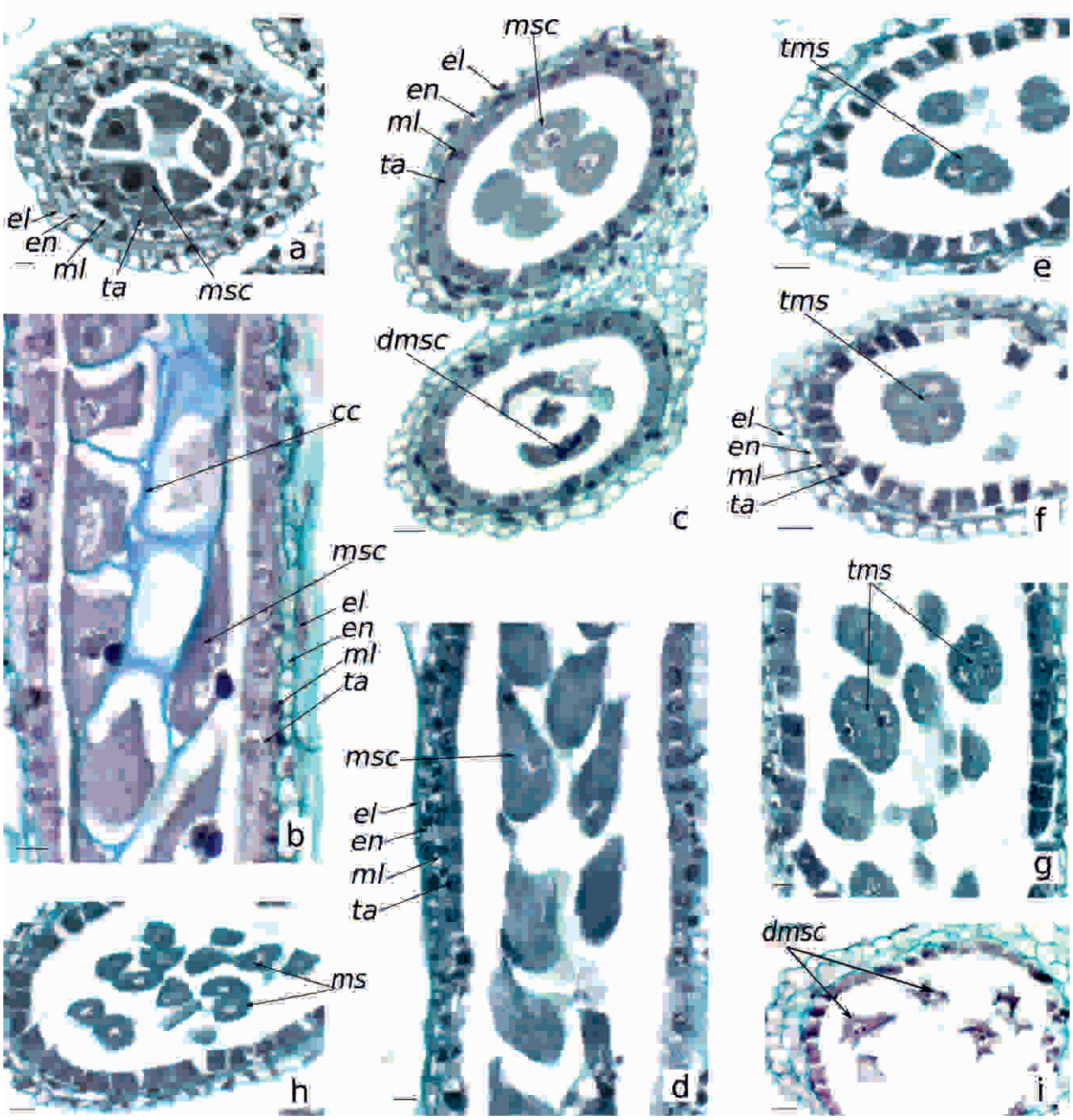

Fig. 2. Microsporogenesis and further stages of anther development in *Zea mays* (ATL1280).
a, b – the locule, the anther wall is 4-layered, there are five microsporocytes with strongly thickened walls in the center of the locule ; especially high amount of callose is deposited in the center of the locule, forming a callose column *(a*-cross section, *b* - longitudinal section); c – part of the stamen, the anther wall is 4-layered, in the upper locule MSC are in the prophase of meiosis I, in the lower one there are degenerating MSCs with developmental abnormalities (deformed nuclei, irregular cell shape), the MSC covers are slightly thickened; remnants of callose are observed in the center of the locule (cross section); d – part of the locule, the anther wall is 4-layered, the MSC covers are slightly thickened, the remains of the callose column are observed in the center of the locule (longitudinal section); e, f – part of the locule, the anther wall is 4-layered, there are tetrahedral (e) and isobilateral (f) tetrads of microspores in the center of the locule (cross section); g – part of the locule, the anther wall is 4-layered, there are tetrads of microspores (tetrahedral and isobilateral ones) in the center of the locule (longitudinal section); h – part of the locule, the anther wall is 4-layered, the former tetrads dissociating into separate microspores can be seen in the center of the locule; some of the microspores have a normal rounded-oval shape, while others have angular protrudences and elongated tips (cross section);
i – part of the locule, the anther wall is 4-layered, in the center of the anther locule there are degenerating microspores of an unusual stellate shape (cross section). *cc* – callose column, *dmsc* – degenerated microsporocyte, *el* - epidermal layer, *en* - endothelium, *ml* - middle layer, *ms* - microspore, *msc* - microsporocyte, *ta* - tapetum, *tms* - tetrad of microspore.
Bars: a,b,d, – 10mkm, c,e,f,g,h,i – 20 mkm

At the earlier developmental stage we can see the primary archesporial cells in the locule's center and parietal cells situated in subepidermal layer (Fig. *1c*). Anther wall consists of the two layers of cells – epidermis and primary parietal layer.

The third layer forms later by periclinal divisions of parietal cells. The layers of the anther wall are not highly differentiated at this stage and have no differences (Fig. *1d*).

The division of epidermal and subepidermal cells, as well as their growth, significantly accelerates by the beginning of microsporangium's differentiation. The intensive divisions of cells of the primary archesporium and their growth in radial direction can be observed at the same time. The cells of the primary archesporium divide mitotically, and the number of their longitudinal strands in the middle of each locula increases to four or more (Fig. *1e,f,g,h*).

It is characteristic for both the diploid (Korobova 1962) and tetraploid maize that only anticlinal divisions are noted in the formed anther wall.

The formed anther wall consists of four layers: epidermal layer, fibrous one (endothecium), middle layer and tapetum (Fig. 1i).

Epidermis is the outer cell layer, developing directly from epidermis of initial stamen primordium. The fibrous, middle and tapetal layers arise due to the periclinal divisions of subepidermal layer. Tapetal and middle layers are the youngest, while epidermal and fibrous ones are the oldest.

Therefore, the type of anther wall development in maize corresponds to the III type Umbellifera (according to Batygina et al., 1963), or Monocotyledonous type (on classification by Davis 1966, Kamelina, 2011).

An anther elongates intensively. Growth is carried out in the cells of the tapetum due to division, in the archesporial cells - due to division and stretching of cells, and in the cells of the middle layer and epidermis, mainly only by stretching. About 14-15 tapetal cells, approximately the same number of cells of middle layer and 7-8 epidermal cells accounts for 10 archesporial cells on the longitudinal se ction of an anther within the single locality of anther wall (Fig. *1f,h*). It should be noted that the cords of archesporial cells at the center of the locule are not strictly ordered cell rows. Archesporial cells stretch irregularly, shift relative to each other and take a trapezoidal, angular form under conditions of limited volume. The cells of the anther wall layers have strict order in their arrangement and their shape on the sections is closer to a regular rectangle.

Furthermore, the divisions in archesporium decrease and entirely stop. Since that time, archesporial cells become microspore mother cells – microsporocytes (MSC) that come into the deep mitotic dormancy. At this developmental stage of the anther, MSC are closely appressed to each other and have triangular form on transverse section. Dense cytoplasm fills the entire cell's volume; rather large nucleus is situated in the center of the cell. The cell walls of MSC thicken, and callose deposits on them. The most of it accumulate in anther's center, forming a sort of column with pockets where MSC locate (Fig. 2*a,b*).

The growth of MSC and an anther can be noted during the prophase of the first division of meiosis. An anther enlarges more than 2 times in length and 1,5-2 times in width. MSC intensively grows in size and considerably stretches, its cytoplasm becomes vacuolated.

An anther grows in width and also in length by means of stretching of all its cells. As a result, MSC stretch lengthwise. They are seen as elongated angular, sometimes as drop-shaped cells on the longitudinal section (Fig. 2b).

The anther continues to grow. Callose column with pockets where MSC locate begins to dissolve gradually and ceases to be a consistent system. It leads the majority of MSC to be loose-lying in the locule (Fig. 2*c,d*). S.N. Korobova described the similar pattern for the diploid maize (Korobova 1962).

Differentiation in the cells of anther wall is observed during the first division of meiosis. The cells of the outermost layer stretch strongly without any anticlinal divisions. Two adjacent layers also tend to stop divisions. There are divisions of nuclei without cytokinesis in the tapetal layer. Its cells become binucleate (Fig.2*c,d*).

By the time of prophase of meiosis MSC have rounded-ovate shape on transverse sections and ovate or ovate-oblong, sometimes droplike shape with elongated "beaks" on longitudinal ones. A few of callose on the periphery of the locule and the residuals of callose column in its center can be seen at this stage (Fig. 2*d*).

In addition to normally formed MSCs, we have seen MSC with different abnormalities in their shape: strangulated, angular-shaped, compressed ellipsoid-shaped, semicircular, falcate or formless cells, cells with distorted nuclei (Fig.2*c*). In addition, most of MSC remained in connection with each other and with the residuals of callose column in the center of locule and they hung down and take on a form of a drop (Fig. 2*d*).

Microspore formation in maize proceeds according to successive type (Reeves 1928, Korobova 1962).

As a result of meiosis, we observed the formation of not only isobilateral tetrads of microspores (Fig. 2*f,g*), which is typical for maize in general (Korobova 1962), but also tetrahedral ones (Fig. 2*e,g)*.

There were very few specimens at the stage of meiosis proper among the material in this study and in the present work we can talk about the possible disturbances in meiosis only by its result – the stage of microspore separation, when different abnormalities were observed. In addition, we were basing on the earlier studies on one of the tetraploid maize lines (Lobanova *et al.* 2010, 2018, 2019).

Authors of those papers have seen atypical shaped MSC with a frequency of 0,8 - 9,8% in different plants of KrP-1 line and also various disturbances of caryo- and cytokinesis during meiotic division such as: asynchronous nuclei divisions, scatter of chromosomes in the spindle or their ejection outside it, disorder of spindle's orientation and others (Lobanova *et al.* 2019). These disturbances resulted in formation of abnormal microspore tetrads – with atypical spore number (other than four) and abnormal shape (Lobanova *et al*. 2018). According to O.A. Shatskaya, the samples of KrP-1 line (Saratov) and population KrTetraI (Krasnodar) come from the same source.

Soon after the completion of meiosis, the lysis of the membranes and the release of microspores from the tetrads begin. This was observed not only for the tetraploid lines, we studied, it is also typical for maize in general (Korobova 1962). Callose covers dissolve, and microspores released from them begin to obtain spherical shape. The majority of microspores have round or rounded-ovate shape on the sections at this time but there are some microspores of atypical shape – half-round, angular or with elongated tip (Fig. 2*h*).

It is worth pointing out that the star-shaped cells at the sections of locules of some anthers were noted (Fig. 2*i*). Such cells were seen in the anthers of all three mostly developed stamens within the flower and in some flowers of the same fixation (i.e. the same plant). These anthers came under our notice not only by unusual cells' shape but by the difficulties to identify whether stage they should be attributed to. Anthers's size, cells' state in different wall's layers and lack of callose column are characteristic to the stage of microspores. But on the other hand, these cells may be MSC that did not undergo meiosis and become degenerating because the appearance of nuclei and the amount of cytoplasm around them (taking into account cytoplasm's obvious compression) is more characteristic for MSC at the premeiotic stage.

The microspores of atypical shape cause different abnormalities in pollen grains in the plant lines under investigation (Shatskaya unpublished data). The pollen grains of atypical shape were noted in above mentioned line KrP-1, too (Lobanova *et al*. 2010). It is important to note that such a rather rare phenomenon as an abnormal shape of microspores and pollen grains is sometimes found in diploid maize too. In the collection of local breeding lines, O. A. Shatskaya discovered the presence of anomalous pollen grains with different frequencies in plants of two lines of diploid maize (unpublished data).

Meiosis abnormalities and formation of atypical in shape microspores were observed also in the inbred line of diploid maize CMS-43 (Ricci *et al.* 2007). The authors described different abnormalities in karyo- and cytokinesis, including formation of pentads. They also presented the photographs of the atypical in shape MSC with elongated tips, like ones seen in above mentioned tetraploid KRP-1 line and in lines under our investigation. The authors associate abnormalities' formation with inbreeding and state that the largest number of abnormal cells was seen in the first self-pollinated generation.

In the other research carried out by squash technique on the unique endogamous plant from the inbred line various abnormalities of meiosis leading to deviant MSC, sporads and microspores formation including cell shape aberrations (with frequency 1,06% from the total observed cells number) were also discussed (Utsunomiya *et al.* 2002). The half-round, angular cells with elongated tips, looking like those we have observed in our study can be seen on the photographs in their paper.

Poliads formation with microspores of atypical shape as the result of disturbances in tetraploids' meiosis was mentioned not only in maize but also in some other plant species, for example in *Brachiaria ruziziensis* (Gamineae) (Risso-Pascotto *et al.* 2005).

The question on the spatial arrangement of MSC, microspores and pollen grains within the anther and on the relationship between cells' location and shape is more interesting and more difficult than at first glance. The cells studies carried out by squash technique do not give an answer on it. The organs and tissues investigations based on the light microscopy partially lift the curtain of secrecy on this question but can't provide the conclusive decision. As it is known, some shrinkage of cytoplasm occurs during fixation for light microscopy that may cause cells' distortion on the sections. Moreover, the longitudinal and transverse sections do not give a clear vision of the anther's space structure because they need intellectual or computer-based 3D-reconstruction of the unit of analysis. The use of confocal microscopy can partially help to resolve this problem whereas the objects with smaller anthers fit this method better than maize (Kelliher, Walbot, 2011).

With reference to above mentioned the data obtained by cryo-SEM study is of the great interest. The investigation of this kind was carried out by the group of researchers in order to study anther's development and its dehydration process in *Zea mays* and *Oryza longistaminata* (Tsou *et al*. 2015). The authors showed that the locule is filled by liquid and gas that have physical impact on developing reproductive cells. After dissolution of tetrads' callose covers, microspores appear to be not exactly "loose lying" inside the locule but pressed against the tapetum in one layer in the result of pressure on the microspores. This causes microspores to become wedge-shaped. Only a small number of microspores positioned in the locule's center appear to be sphere-shaped.

Data analysis lets us suggest that formation of atypical-shaped microspores is a result of a complex of reasons. The first of them – "positional" – is associated with the features of growth and relative positions of MSC in anther. It leads some of them to take atypical shape (trapezoidal, angulated, with elongated tip). The second reason – "mechanical" – is that MSC stay attached to callose column in the center of locule

and hangs as a drop on it. Maybe, such a position of MSC leads the cell to take a shape with strongly elongated tip. The third one – "meiotic" – is aligned with disturbances in meiotic divisions. Isobilateral tetrad consisting of four similar to sphere-shaped microspores forms in the course of normal meiosis. If not four microspores (five or more), form as a result of any aberrations during meiosis (e.g. incorrect chromosome disjunction or micronuclei formation) especially if microspores vary in size, they will obtain an abnormal shape. This shape keeps even after microspore's exit from the sporad's callose cover, and takes hold in the course of callose deposition during pollen grain formation.

CONCLUSION

There are no conceptual differences in anther wall development in tetraploid maize in comparison with diploid one. Abnormalities in shape of microsporocytes, microspores, pollen grains, which can be seen in some lines, may be caused by their relative position in the anther and by deviations in the course of meiosis. These deviations lead to changes in cytoplasm's allocation within the microsporocyte and to formation of irregular tetrads, pentads, etc. and break the isobilateral symmetry of cells. The abnormalities observed probably are the consequence of the tetraploid nature of the lines studied and cause violation of male fertility and can decrease in grain productivity, which is characteristic to some tetraploid maize lines.

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