Anomalous pollen grain development after nondifferentiating microspore division in *Eremurus* *

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Abstract

Anomalous pollen grain development in *Eremurus* is caused by an anomalous position of the mitotic spindle and microspore protoplast division into two cells different than in differentiating division. The nuclei of the abnormal gametophytes are always spherical and that of the smaller of the two cells, notwithstanding the shape and position of the latter, has a more compact structure resembling rather that of the generative cell nucleus. Binucleate abnormal gametophytes always have equal nuclei.

The wall separating the cells in abnormal pollen grains at first contains callose and, after disappearance of the latter, probably pectins and cellulose. Abnormal pollen grains contain less cytoplasmic RNA than normal ones and most of them degenerate. If their viability is preserved they do not form normal pollen tubes *in vitro*.

The frequency of anomalous microspore division is higher in *E. robustus* (max ca. 50%) than in *E. himalaicus* (max. ca. 30%) and shows considerable seasonal variations. The results obtained suggest that disturbances in microspore development in *Eremurus* have a genetic background, but are stimulated by temperature variations in the period preceding mitosis in the microspore.

INTRODUCTION

Abnormal pollen development in Angiosperms is usually the consequence of disturbed meiosis or postmeiotic mitosis in the microspore. The background of these changes may be genetic or they may be caused by external factors among which the most important role is ascribed to considerable temperature variations. The position of the nucleus in the

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microspore during mitosis, the orientation of the mitotic spindle and later of fragmoplast decide of the normal or disturbed development of the gametophyte. An asymmetric microspore division resulting in the development of typically differentiated cells — the generative and the vegetative one — is a differentiating division. A microspore division giving an abnormal gametophyte may be considered as nondifferentiating and it is in this sense that this term is further used in the paper. In mature pollen of two species of *Eremurus* which was available to us a certain per cent of pollen grains was found differing morphologically from normal ones. The low basic number (7) of large chromosomes and the relatively large pollen grains with thin exine encouraged us to perform cytological observations on microspore and pollen grain development in these plants to establish type of developmental abnormalities and their eventual causes.

MATERIAL AND METHODS

The investigations were performed on *Eremurus robustus* Reg. and *E. himalaicus* Baker. from the Botanical Garden of the Warsaw University, on the same 5 specimens in two successive vegetation seasons. The material was collected at the end of May and beginning of June, 1972 and in the second half of May in 1973. The racemose inflorescences of *Eremurus* with several hundred flowers mature basipetally, this making possible simultaneous fixation of anthers at various phases of development. The development of microspores and pollen grains within the anther is not strictly synchronized, however, on the basis of the size of flower buds and anthers the developmental stage can be approximately recognized (data below).

The anthers with the apical part cut off were fixed in Navashin's modified fixative, CrAF 0.5-1-20 and UF (uranyl nitrate and formalin) 0.2-10 for 24 h for preparation of microtome paraffin sections.

The sections were stained by Feulgen and PAS methods (after Pearse, 1968), toluidin blue pH 3.4 and alkaline fuchsin (after Teleżyński, 1973). Smears made after fixation of the anthers for 2 h in AA 1:4 or immediately and stained with acetoorcein with HCl (0.5% orcein solution in 45% acetic acid with 1 N HCl vol./vol. (9:1).

Callose was identified by the method of aniline blue fluorescence (Eschrich and Currier, 1964).

The test of pollen grain germination on liquid medium was run by the standing drop method according to Kwack and Kim (1967).

For determining the frequency of nondifferentiating microspore division the percentage of abnormal pollen grains was calculated in the same 5 specimens of *E. robustus* and *E. himalaicus* in both vegetation

Dependence between flower bud length and stage of anther development

Species	Flower bud length mm	Anther length mm	Development stage of pollen grains
<i>Eremurus himalaicus</i>	6	3	mitosis
	8	4	bicellular just after cytokinesis
	11	4	bicellular older
	13	4	bicellular with generative nucleus elongated
	18—19 (flower open)	5	mature pollen grains
<i>Eremurus robustus</i>	7	3	mitosis
	8	4	bicellular just after cytokinesis
	10—12	4	bicellular older
	15	5	bicellular with generative nucleus elongated
	20 (flower open)	5	mature pollen grains

seasons. The calculations were done on squash preparations of the anthers in which normal pollen grains had slightly elongated generative cells. Preliminary trials demonstrated that there are no significant differences in the percentage of abnormal pollen grains in the anthers of the same flower and between flowers lying at various distances from the inflorescence apex. Therefore the per cent of abnormal pollen grains was calculated in 10 randomly collected flowers from the central part of each inflorescence. Samples were taken from only one anther in each flower. (In 20 randomly chosen samples from both pollen sacs the number of abnormal grains per 100 grains was calculated).

RESULTS

Normal microspore and male gametophyte development in *Eremurus*

In both the studied *Eremurus* species the microspores and pollen grains develop in the same way. After meiosis and simultaneous cytokinesis the pattern of the microspores in the tetrad is tetrahedric. An aperture forms on the distal microspore pole, in the shape of a slit parallel to the cell long axis. The exine is thin with a delicate relief (Plate I, photo 1).

The elongated shape of microspores and pollen grains later and also the presence of the aperture allows to distinguish distal and proximal poles after the release of the microspores from the callose wall. The maturation of microspores of *Eremurus* is accompanied by cytomorpho-

gical transformations which are characteristic for the majority of Angiosperms: 1. early microspore with centrally located nucleus and weakly vacuolized cytoplasm, 2. central vacuole formation and the transformation of the microspore nucleus to the cytoplasm layer adjacent to the pollen wall near the cell long axis end, 3. change of vacuole configuration and the nucleus translocation to the proximal pole of microspore in the central cytoplasm wisp between two vacuoles. The mitosis proceeds in this position.

The mitotic spindle is perpendicular to the long axis of the cell during the differentiating microspore division. The hemispherical cell plate separates the generative cell from the vegetative cytoplasm on the proximal pole (Plate I, photo. 2 and Plate III, photo. 16) After translocation towards the centre of the pollen grain, the generative cell, at first spherical, gradually elongates during pollen grain maturation and in the ripe grain it is greatly elongated and fusiform (Plate I, Photos 3—7). The change of the cell shape is accompanied by changes in the shape and structure of the generative nucleus which in mature pollen grain is also fusiform. The generative nucleus which at first is reticular (Plate I, photo. 3) shows in maturing pollen grains a structure similar to that in early prophase (Plate I, photo. 6). The chromosomes with folded arms bent in the centromere are linearly arranged within the nucleus superposed on one another on some length. Such a state is characteristic for the elongated generative nucleus before opening of the flower and it changes within a dozen or so hours before pollen shedding. In mature pollen grains (shed from the anther) the generative nuclei have again a reticular structure and it is not possible to distinguish the particular chromosomes within the nucleus (Plate I, photo. 7). The nucleolus in the generative nucleus is visible immediately after the end of microspore division and in the nucleus of ellipsoid shape.

The vegetative nucleus of *Eremurus* has very loose chromatin and a large nucleolus which disappears in the end phase of pollen maturation. It has a very low affinity to the dyes with which the generative nucleus chromatin gives a strong colour reaction. In the Feulgen reaction it also stains very weakly. The intensity of staining in this reaction increases only in mature pollen grains. With toluidin blue the vegetative nucleus stains orthochromatically weaker than the generative one. Alkaline fuchsin gives a stronger staining of the vegetative nucleus chromatin than does Feulgen staining and it reveals its reticular structure. The vegetative cell cytoplasm in a normal gametophyte stains metachromatically with toluidin blue and in the pollen grains with elongated generative cell it contains numerous minute structures staining strongly metachromatically.

Normal pollen grains development in *Eremurus*

Fig. 1. Pollen grain of *E. robustus*, aperture and exine relief are visible

Fig. 2—7. Two-celled pollen grains of *E. himalaicus*

Fig. 2. Lenticular generative cell several hours after the microspore division

Figs 3, 4 and 5. Young pollen grains

Fig. 6. Pollen grain 24 h before pollen shedding; fusiform generative cell with nucleus showing characteristic chromosome pattern

Fig. 7. Generative cell in mature shed pollen grain

Fig. 3. Fixed with UF 15, not stained, the remaining figures after staining with

Abnormal pollen grain of *Eremurus* developing after nondifferentiating microspore division

Fig. 8. Young binucleate *E. robustus* pollen grain

Figs 9—14. Two-celled pollen grains (Figs 9, 10, 12. *E. himalaicus*, Figs 11, 13, 14 *E. robustus*). In the grains in which the gametophyte cells differ in size, the nucleus of the smaller grain has a more compact structure

Fig. 15. Degenerating *E. robustus* pollen grains

Fig. 8. Fixed with CrAF, Feulgen reaction, Figs 9-15 — staining with acetorcein. ca. $\times 1200$

Fig. 16. *Eremurus* pollen grains after differentiating microspore division, fixed with CrAF, stained with alkaline fuchsin

Fig. 17. Callose wall of normal (Figs 18 and 19) and abnormal grains (Fig. 20) of *Eremurus*, fluorescence with aniline blue

Figs 16 and 17 ca. $\times 1200$, figs 19 and 20 ca. $\times 500$

Anomalies in the development of the male gametophyte in *Eremurus*

In neither of the examined *Eremurus* species were disturbances noted in meiosis. In the premitotic period the microspores did not differ morphologically. The nuclei of all microspores gave an equal intensive Feulgen reaction, toluidin blue showed no differences in staining of the nucleus and cytoplasm of the microspores. In all of the latter vacuolization occurs in the same way. Microspores from which an abnormal gametophyte will develop differ during mitosis by a different position of the mitotic spindle than in the case of differentiating division. The spindle lies parallelly or at an angle different than 90° to the long microspore axis. Mitosis occurs without disturbances in the function of the spindle. Usually mitosis is accompanied by cytokinesis which mostly leads to complete separation of the protoplast into two cells. The nuclei of the abnormal gametophytes are either similar or they differ in size and structure (Table 2, photos 8—14). Identical nuclei have a structure similar to that of the vegetative nucleus of normal pollen grains. When they differ, the smaller ones show a more compact structure. In abnormal gametophytes the nuclei, however, never have such a loose structure and large nucleoli as are seen in the nucleus of the vegetative cell.

There is a dependence between the differentiation of nuclei and the mode of division of the microspore: an unequal division results in wide differences between nuclei, the nucleus of the smaller of the two cells has a more compact structure similar to that of the generative cell nucleus (Plate II, photos 11 and 13).

An equal division of the microspore or mitosis without cytokinesis always give a gametophyte with two identical nuclei (e.g. Plate II, photo. 8).

In a dozen or so cases we found in *E. himalaicus* bicellular young pollen grains joined in tetrads. In several cases there were in the same tetrad two normal and two abnormal gametophytes formed from one meiocyte.

In binuclear abnormally differentiated pollen grains sporadically mitosis was observed in one or both nuclei resulting in tri- or tetranucleate pollen grains. This was more frequent in *E. robustus* than in *E. himalaicus*.

Most of the gametophytes formed as the result of nondifferentiating microspore division degenerate before the normal pollen grains mature (Plate II, photo. 15). A small percentage of these grains preserve their viability during pollen shedding. These grains germinate on nutrient medium, but, in contrast to normal ones, they form only small pollen tubes.

Chemism of the cell wall after nondifferentiating division

Observations in the fluorescence microscope with the use of aniline blue demonstrated that a callose wall separates both the lenticular generative cell from the vegetative one in normal pollen grains (Plate III, photos 18 and 19) and the two cells in abnormal pollen grains notwithstanding their shape (Plate III, photo. 20). In the course of further development callose disappears in normal and abnormal pollen grains of *Eremurus*. In normal ripening pollen grains the border between the generative and the vegetative cell is not of the character of a wall and it does not stain in the PAS reaction. In grains with an abnormal gametophyte a wall separating the two cells could be demonstrated at this time showing metachromatic staining with toluidin blue and a strong PAS reaction (Plate III, photo. 17). This may indicate that a cellulose pectin wall forms at the border between the two cells or that the callose in the cell plate has been substituted by pectin.

The intensity of nuclear staining (Feulgen, alkaline fuchsin, orthochromatic staining with toluidin blue) in the abnormally differentiated gametophytes depends on the degree of chromatin condensation. The cytoplasm of these grains stains metachromatically with toluidin blue much weaker than that of the vegetative cells of normal pollen grains.

Frequency of nondifferentiating divisions in *Eremurus robustus* and *E. himalaicus*

Analysing the cross and longitudinal sections of the anthers and taking samples for smears of their upper, middle and lower parts we found no relation between anomalous development of the microspores and their situation in the pollen sac.

The percent of abnormal pollen grains equal to the per cent of nondifferentiating microspore divisions is different in the two species examined and much higher in *E. robustus* specimens. Differences in the number of disturbances also occur between specimens of the same species. In the two successive vegetation seasons included in the investigations the difference in the number of abnormalities was considerable. In 1973 the number of nondifferentiating divisions was much higher (*E. robustus* in 1972 ca. 20% and in 1973 ca. 50%; *E. himalaicus* in 1972 ca. 6% and in 1973 ca. 30%). The results are illustrated by the diagram in Fig. 1.

DISCUSSION

Anomalies in pollen grain of *Eremurus* have so far not been described. In the study performed many years ago concerning the embryology

of *E. spectabilis* (Prosina, 1930) there is no mention of disturbances in microsporogenesis and pollen development in specimens of this species from the Botanical Garden in Moscow.

Fig. 1. The percentage of abnormal pollen grains of *Eremurus robustus* and *Eremurus himalaicus*. Numerals of five specimens (1—5) mean the same in two successive vegetation seasons. The lines parallel to horizontal axis show mean percent of abnormal pollen grains in each year

The position of the mitotic spindle different than in normal differentiating microspore division in *Eremurus* is the first morphological symptom of the developmental disturbances described by us and demonstrable in the light microscope. In both the species of *Eremurus* examined no cytological disturbances were found during meiosis, neither were morphological differences noted in interphase microspores from which the normal or abnormal gametophyte develops. There must, however, be differences even between the microspores arising from the same meiocyte, but they were not revealed by the method applied in the present study. These differences decide of the normal or abnormal division. It is probable that they concern the arrangement of the microtubules of the future mitotic spindle forming in the interphase microspore. Their modified localization was observed as late as mitosis in the microspore.

Abnormalities in the male gametophyte in various species of Angiosperms have been long reported (Maheshwari, 1949; Rodkiewicz, 1973), lately, however, no papers have appeared devoted to this problem. Differentiation of pollen grain nuclei different than in normal development has been described among others by Sax (1935, 1937) and La Cour (1949) in *Tradescantia*. Abnormalities in pollen grain develop-

ment in this plant were induced experimentally by treatment with low (6°C) and high (35°C) temperatures (Sax, 1935) or by alternation of low (8°C) and high (38°C) temperatures (Sax, 1937) in the case of glasshouse plants growing at 18°C . Sudden changes from low to high temperature were more effective than constant exposure to each of them. Drastic temperature changes produced disturbances of the meiotic division and of the polarity in part of the developing microspores manifested by changes in the position of the mitotic spindle in relation to that in normal division giving a generative and a vegetative cell. In contrast to our observations, Sax (1935) reports that microspores from which defective pollen developed differed in the degree of vacuolation and in the position of the nucleus during mitosis from the microspores giving normal pollen. According to Sax, at low temperatures, owing to delayed cytoplasm flow the microspore nucleus starts mitosis lying sideways before reaching the position characteristic for normal division. The action of high temperature accelerates cytoplasm streaming and the vacuoles normally present during mitosis partly or completely disappear before the nucleus enters the phase of mitosis. The mitotic spindle always "tends to occur in the long axis of the cytoplasmic mass" deviates in these microspores by various angles from the normal division plane. Normal differentiation of microspores is, according to Sax, dependent on the synchronization of cytoplasmic and nuclear activity, which may be disturbed by sudden temperature changes, and the position of the spindle is conditioned by the distribution of cytoplasm in the cell.

La Cour (1949) described in *T. bracteata* the formation of a high percentage of "dwarf" pollen grains developing from microspores which underwent mitosis without previous vacuolization characteristic for normal microspore development. Division, if it did occur, was always equal (in plane perpendicular to that of differentiating division) and the dwarf bicellular grains had two equal spherical nuclei. These developmental abnormalities occurred only in one clone of *Tradescantia* and their number increased under conditions of drought and elevated temperature. A temperature shock ($38^{\circ}-5^{\circ}\text{C}$) evoked in a large number of dwarf grains mitosis in both nuclei. In microspores usually developing normally it caused disturbances of the same character as those here described in *Eremurus*.

The wide seasonal differences found in the percentage of nondifferentiating divisions in both *Eremurus* species may be due to temperature changes. The diagram of maximal and minimal temperature in May in the years 1972 and 1973 plotted on the basis of the meteorological data of the Astronomical Observatory in the Botanical Garden of the Warsaw University shows the temperature distribution in the days preceding collection of material for investigation of the frequency of anomalies (Fig. 2).

Fig. 2. The temperature distribution in the days preceding the anthers fixation for the calculation of the abnormal pollen grains percentage. The areas between minimal and maximal temperature curves are indicated by horizontal and vertical dashes respectively for the two vegetative seasons

Fixed anthers in which normal pollen grains had a slightly elongated generative cell were used for the calculations. *Eremurus* pollen grains reach this stage of development about 3 days after the end of mitosis and cytokinesis. The microspore development after release from the tetrads to the end of microspore division lasts at least 5 days. In 1973 in this time interval wide diurnal temperature variations were noted in the early stage of microspore development (8th day before sampling) as well as a drastic maximal temperature fall in the course of several successive days (8th/7th day before sampling). Moreover in the period preceding immediately mitosis and during its duration a considerable minimal day temperature fall occurred. It is therefore probable that the particularly high per cent of nondifferentiating divisions in 1973 was the consequence of this influence of the environment.

The number of abnormal gametophytes in *Eremurus* does not depend on whether they lie in the middle or apical part of the anther or on their position in the neighbourhood of the tapetum. In some dozen cases in *E. himalaicus* we observed a bicellular gametophyte in tetrad arrangement. In several cases abnormal male gametophytes formed in the same tetrad next to two normal ones. This fact and the significant difference in the number of nondifferentiating divisions between *E. robustus* and *E. himalaicus* seems to suggest that the tendency to abnormalities is genetically conditioned.

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REFERENCES

- Eschrich W. and Currier H. B., 1964. Stain Technol., 39: 303-307.
 Kwack B. H., and Kim J. H., 1967. An important method for culturing *Tradescantia* pollen tubes for chromosomal analysis, Cytologia 32: 1-5.
 La Cour L. F., 1949. Nuclear differentiation in the pollen grain, Heredity 3: 319-337.
 Maheshwari P., 1950. An introduction to the embriology of Angiosperms, McGraw-Hill Book Comp., N-York, Toronto, London.
 Pearse A. G. E., 1957. Histochemia teoretyczna i stosowana. PZWL Warszawa.
 Prosina M. N., 1930. Embriologische Untersuchungen an *Eremurus spectabilis* var. *segeli*, Planta Archiv. fur Wissenschaftliche Botanik, Berlin: 532-544.
 Rodkiewicz B., 1973. Embriologia roślin kwiatowych. PWN, Warszawa.
 Sax K., 1935. The effect of temperature on nuclear differentiation in microspore development, J. Arnold Arbor. 16: 301-310.
 Sax K., 1937. Effect of variations in temperature on nuclear and cell division in *Tradescantia*, Amer. J. Bot., 23: 606-608.
 Teleżyńska J., and Teleżyński H., 1973. Double fertilization in *Helianthus*, Acta Soc. Bot. Pol. 42 (2): 323-343.

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Zakłócenia w rozwoju ziaren pyłku Eremurus spowodowane nie różnicującymi podziałami mikrospor

Streszczenie

U *Eremurus robustus* i *E. himalaicus* przebadano rozwój ziaren pyłku i wykazano, że rozwój nienormalnych gametofitów spowodowany jest odmiennym niż w podziale różnicującym położeniem wrzeciona w mitozie w mikrosporze. W postmeiotycznej interfazie nie wykazano cytomorfologicznych i cytochemicznych różnic między mikrosporami, z których rozwija się normalny i nienormalny gametofit.

Nie różnicujący podział mikrospor daje dwukomórkowy gametofit o kulistych jądrach, których wielkość i struktura zależy od wielkości komórek. Mitoza bez cytokinezy daje gametofity o dwu jednakowych jądrach. Ściana rozdzielająca komórki nienormalnego gametofitu podobnie jak w normalnych ziarnach pyłku zawiera kalożę. Po zaniku kaloży tylko w nienormalnych ziarnach przeprowadzone barwienia wskazują na wytworzenie ściany celulozowo pektynowej na granicy obu komórek lub zastąpienie kaloży w przegrodzie pierwotnej przez

substancje pektynowe. Sporadycznie, częściej u *E. robustus*, w nienormalnych ziarnach pyłku odbywa się druga mitoz. Nienormalne gametofity mają niższy poziom RNA w cytoplazmie niż komórka wegetatywna normalnych ziaren pyłku i wcześnie degenerują. Niewielki % zachowuje żywotność do momentu pylenia, ale w hodowli *in vitro* ziarna te nie wytwarzają normalnych łagiewek. Częstość zakłóceń w mitozie mikrospory jest wyższa u *E. robustus* i wykazuje znaczne różnice w dwu kolejnych sezonach wegetacyjnych. Nie wykazano zależności między rozwojem nienormalnych ziaren pyłku a ich położeniem w obrębie komory pyłkowej. Analiza rozkładu temperatury w okresie pobierania materiału nasuwa przypuszczenie o zakłócającym wpływie na różnicujący podział mikrospory dobowych wahań temperatury w okresie poprzedzającym mitozę. Istotne różnice w liczbie nienormalnych ziaren pyłku u obydwu badanych gatunków a także obserwowane przypadki rozwoju dwu nienormalnych i dwu normalnych gametofitów z jednego mejocytu sugerują, że podatność na zakłócenia ma u *Eremurus* podłoże genetyczne.