

UNIVERSITY OF DEBRECEN
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Faculty of Agriculture
Institute for Land Utilisation, Technology and Regional Development

**DOCTORAL SCHOOL OF INTERDISCIPLINARY AGRICULTURAL AND NATURAL
SCIENCES**

Head of Doctoral School:
Prof. dr. János Nagy
Doctor of the Hungarian Academy of Sciences

Tutors:
Prof. dr. Beáta Barnabás
Corresponding Member of the Hungarian Academy of Sciences
Prof. dr. János Nagy
Doctor of the Hungarian Academy of Sciences

THESES OF DOCTORAL DISSERTATION

**EXAMINATION OF THE EFFECT OF *IN VIVO* AND *IN VITRO*
ENVIRONMENTAL FACTORS ON THE DEVELOPMENT OF
THE REPRODUCTIVE GENERATION OF HUNGARY'S
MAIN CULTIVATED CROPS**

Prepared by:
Ilona Timár
candidate

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1. OBJECTIVES OF WORK

Yield reliability of agricultural plants is significantly affected by various biotic and abiotic environmental factors in Hungary. Yield reliability is a key element of economic plant production, being an important aim in seed production as well. Seed production is directly connected to the basic science of flowering biology. The sexual reproduction and ontogenesis of angiospermal plants are based on the fusion of gametes. The life cycle of the new sporophyte generation begins with the dual fertilisation. Therefore, issues in relation to reproduction biology directly influence the basis of seed production and thus considered as high priority fields of research. Improving the genetic diversity of our important agricultural crops is one of the main aims of plant breeding research activity, which is implemented by the simultaneous application of traditional genetic techniques, embryological methods and *in vitro* manipulation procedures.

Plants exposed to changing environmental effects such as extreme variation of weather conditions respond mainly with yield reduction to different abiotic stress factors in the generative stage of their ontogenesis. Consequently, it is reasonable to examine and reveal the most vulnerable critical phases of male and female gametophytic development.

Environmental factors can be reproduced under laboratory conditions by employing e.g. artificial plant cultivation techniques. The so-called *in vitro* environment provides the opportunity to trace the processes of ontogenesis and differentiation, and to directly study the effects of different environmental factors.

Interventions to plant organisations or derivative *in vitro* cultured cells can be efficient if they are carried out expediently in the appropriate phases of ontogenesis or cell cycle.

The knowledge available for us at present about the sexual reproduction of plants serves as basic information for *in vitro* fertilisation researches being an important field of reproduction biology. By fusing isolated sperm cells and egg cells, viable progenies can be produced and – in theory – hybrids of wheat and its related species can be created by gametoplast-fusion being similar to protoplast-fusion.

For implementing interspecific *in vitro* gametes fusion, detailed information is required on the developmental process of pollen and embryo-sac of certain *Triticum* species under natural conditions *in situ* and *in vitro*.

Today, plant breeding activity greatly demands viable dihaploid lines originating from dihaploid, anther or microspore cultures in order to expand the genetic basis of plant breeding and shorten the time required for producing lines. In the gene-bank established in Martonvásár, exotic maize genotypes with high androgenetic capacity can be used as genetic basis. In the anther cultures of genotypes with high inductive ability, numerous microspore-derived structures are formed, however, only a part of these structures develops into fertile plants. Our aim is to identify the characteristics of

processes occurring *in vitro* by comparative morphologic examinations of zygotic and androgenetic embryogenesis and callogenesis, making haploid induction more efficient and available for practical application.

The importance of examinations on grape is justified by the high position of Hungary's quality wine production at the world market. The results of our research provide basis for elaborating new biotechnical methods, making grape breeding – primarily resistance breeding – shorter and more efficient. Studies on the two wine-grape genotypes aimed at examining the male and female gametophytes of grape were performed.

The summary of the objectives of our examinations is as follows:

- Tracing the differentiation of the male and female gametophytes of *Triticum* species with different genome compositions and ploidy levels under *in vivo* and *in vitro* conditions.
- Revealing the synchronistic relation of the male and female gametophytes of wheat. Defining the developmental phases of the female wheat gametophyte using an indirect examination method.
- Studying the development of the egg cell of wheat *in planta* during the maturation of embryo-sac.
- Characterising the isolated wheat egg cell morphologically and functionally from juvenile stage to aging using optical and electron microscopes.
- Specifying the characteristics of young embryos of *Triticum* species developed *in planta* on the basis of the changes in their size and their morphological features. The results assist the studying of interspecific and intergeneric embryos produced by *in vitro* fertilisation.
- Investigating the embryogenesis of maize microspores undergone cold treatment by optical and electron microscopes.
- Defining the dynamics of the development of grape flower, characterising the developmental stages of the male and female gametophytes.

2. ANTECEDENTS OF RESEARCH

Research on flowering and flowering biology has been carried out for decades in our Institute. The yield of numerous plant species has been increased significantly by using hybrid varieties. The thorough investigation of the flowering biology of wheat was inspired by the demand to develop productive hybrid wheat varieties.

The correlation between the age of pistil and fertilization as well as the development of seeds were examined just as the difference between the average weight of wheat seeds (Rajki-Cicer 1961b). Macromorphologic inspections carried out *in vivo* were later completed with histological data

collected with light microscopic examinations. The effect of the pistil's age on the structure of developing wheat seeds were also studied (Molnár-Láng, M., Rajki-Cicer, E. 1983).

Exact, reproducible and controlled research on the male generative generation, the pollen has been carried out *in vivo* and *in vitro* in the phytotron of the Institute since 1972. Studies dealing with the viability of the pollen were accelerated by the demands of plant breeding and modern genetic research activities. Using deep-freezing pollen conservation (Barnabás, B., Rajki, E. 1976.), dehydrated maize, rye and triticale pollens can be preserved for long in liquid nitrogen at the temperature of -196°C. Conserved functional pollens enable the hybridization of plants flowering at different times or in great distance as well as the international exchange of genetic materials via the pollen bank established in Martonvásár.

The biotechnologic examinations of pollens were carried out on maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.). Pollen callus were induced and examined and plants were regenerated from maize and wheat haploid calli and embryoid structures.

The cytological and ultrastructural examinations of gametes *in planta* and *in vitro* were promoted by the application of high resolution microscopes and electron microscopes. The genetic transformation of wheat is enabled by the fusion of isolated gametes, the application of *in vitro* fertilisation techniques and the injection into the egg cell using micromanipulation methods.

3. MATERIALS AND METHODS

Examinations were carried out at the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár between. Studies accomplished in co-operation:

Agricultural Biotechnology Centre, Gödöllő, electron microscopic examination of wheat;

ELTE Department of Plant Anatomy, Budapest, LSM examination of wheat embryo-sac;

Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental de Zaidín, CSIC. Granada, Spain, optical and electron microscopic examinations of maize microspore embryogenesis; FVM Research Institute for Viticulture, Eger, sampling of grape flower, optical microscopic examination of pollen.

Plants used for the examinations

- The following *Triticum* species were used for the comparative examination of the *in planta* and *in vitro* development of male and female gametophytes of wheat with different genome compositions and ploid levels:

Triticum aestivum L. cv. Mv 15 (AABBDD) (2n=6x=42)

Triticum araraticum Jakubz. (AAGG) (2n=4x=28)

Triticum monococcum L. (AA) (2n=2x=14).

- The fine structure and the dimensional changes of wheat egg cell as well as its morphological features were studied on the spring wheat varieties called Siete Cerros and Chinese Spring.
- For analysing the size of mature pollen grains, the spring wheat variety Chinese Spring was used.
- *Triticum aestivum* L. cv. Mv 15 (AABBDD) ($2n=6x=42$), *Triticum araraticum* Jakubz. (AAGG) ($2n=4x=28$), *Triticum monococcum* L. (AA) ($2n=2x=14$) were used to compare the embryonic development of wheat species with different ploidy levels.
- The process of *in vitro* androgenesis of maize was studied on the anthers of the hybrid 'DH 109 x ZK191/a' having high frequency of haploid induction.
- The following genotypes were used for characterising the dynamics of the development of grape flower and for the structural and morphologic examinations:
Muscadinia rotundifolia Planch. x *Vitis vinifera* L. BC₃,
Leányka.

Cultivating conditions of the plants examined

- At open ground:

out of the plants examined, wheat and grape were grown at open ground.

For the comparative examination of the *in vivo* and *in vitro* development of male and female gametophytes of wheat, plants were grown in small plots. When the male gametophyte was in its Mu (Mid uninucleate) stage, the main spike of some plants were grown further as *in vitro* spikelet culture up to flowering.

The flowers of the two grape genotypes were collected from the plants growing in the open ground variety collection at the FVM Research Institute for Viticulture in Eger – Kőlyuktető.

- In phytotron:

For excising wheat egg cells and studying the size of mature pollens and the examining of young embryos and maize androgenesis, plants were grown under controlled environmental conditions in phytotron.

Examinations

- Examination of the *in vivo* and *in vitro* development of wheat pollens and embryo-sacs

The development of three wheat species with different ploidy levels and genome compositions grown in different environment was compared. For tracing the *in vivo* and *in vitro* development of the male and female reproductive generation of wheat, 20 main spikes in the same developmental stage of each species were used. Main spikes were isolated with cellophane bags. The developmental state of the microspore was determined from the anther obtained from the middle segment of the spike.

Starting from the mid uninucleate (Mu) state of microspore development, 5 anthers and 10 pistils were removed each day up to the state of anthesis meaning the formation of tricellular pollen. To detect the developmental state of microspores, anthers were fixed in fixing solution (3:1 mixture of absolute alcohol and glacial acetic acid) while pistils were fixed in 2.5% glutaraldehyde solution prepared with 0.1M phosphate-buffer for microtechnical examinations.

The sampling of anthers and the determination of the developmental state of the microspore were carried out simultaneously with that of the female gametophyte. After fixation, the anthers and microspores were stained, the developmental state of the pollen grains was specified and their diameter was measured. The data characterising the changes in the sizes of the pollens of the three wheat species were analysed and evaluated by analysis of variance.

The development of the embryo-sac was studied on half-thin (1.5 µm) longitudinal sections acquired with ultramicrotechnical procedures from pistils (10 pistils per species and per samplings) embedded in Spurr resin. The sections were stained with 0.5% toluidine blue (O'Brien et al. 1981) and the examinations were carried out with Zeiss Ultraphot-III optical microscope. On the series of the longitudinal sections of pistils with different levels of development, the largest size of the embryo-sac in the ovule was searched for, and the length and width of embryo-sacs were determined with ocular micrometer. On the basis of the data gathered, schematic figures indicating the changes in the embryo-sac's form and size were prepared using CorelDraw computer programme.

- Examination of embryo-sacs with laser scanning microscope

Fixed and dehydrated wheat pistils (Mv 15) were embedded in polyethylene-glycol (PEG 1500). After solidification, 10-40 µm thick sections were prepared using Reichert section cutter with steel blade. The sections were stained with 1 µl/ml acridine-orange (SIGMA, c.i. 46005).

- Comparison of the development of male and female gametophytes

Our tests included the comparison of the development of the male and female gametophytes of wheat species with different ploid levels. Altogether eight samplings were carried out, and six and five developmental states were assessed.

The developmental stages of the male gametophyte observed:

Mu (mid-uninucleate)

Lu (late uninucleate)

Pm (premitotic)

eBn (early binucleate)

Bc (bicellular)

Tc (tricellular)

The developmental stages of the female gametophyte observed:

tetrad
macrospore
bicellular
tetracellular
7-cellular (8-nucleate)

These observations provide important information on the timing of plant growing and the synchronisation of gamete isolation so that the *in vitro* fusion of gametes can be successful.

The structural, plant anatomical, morphological and histological examinations of the dynamics of the development of grape flower were accomplished on 5 samples for each tests.

Developmental states of grape pollens

After disclosing the anthers, dissections were prepared and the microspores were stained (Alexander 1969). Samples were taken in the following developmental states for each genotypes:

1. PMC (pollen mother cell),
2. Tetrad,
3. Early uninucleate: asymmetric form, thin pollen wall that is stained pale green, no nucleus can be observed,
4. Mid-uninucleate: Rugbyball-shaped microspore, thick pollen wall that is stained dark green, the nucleus can be dimly seen in the centre of the microspore,
5. Late uninucleate: round shape, the cytoplasm is stained darker and darker, the nucleus – together with the strongly stained nucleolus – moves toward one pole of the microspore, while a vacuole is formed on the other pole,
6. Early binucleate: the cytoplasm is so dark that the nuclei can be hardly observed, one of the nuclei can be found in a lentoid formation,
7. Mature pollen: the cytoplasm is evenly stained dark purple, the nuclei cannot be seen at all, the pollen wall is dark green.

Examination of grape pistils

After determining the developmental state of the pollen, 5-5 flowers with microspores in the states of tetrad, mid-uninucleate and transitional late uninucleate – binucleate were selected. The pistils (15 samples from each genotypes) of these flowers were embedded in artificial resin to prepare half-thin sections.

● Examination of wheat embryos

7-cellular (8-nucleate) embryo-sacs were compared to 2-week-old embryos and mature embryos.

Three isolated spikes were castrated of each plants of *Triticum* species (*T. aestivum*, *T. araraticum*, *T. monococcum*) grown in Conviron PGV phytotron chamber applying t2 spring climate programme (Tischner et al. 1997). The spikes were pollinated artificially using rotational method.

Fourteen days after the pollination and fertilization, the 2-week-old seed buds were fixed in 2.5% glutaraldehyde solution. The embryos were removed under stereomicroscope (Zeiss Stemi 2000-C), their length and width were measured using the ocular micrometer of the microscope. For the morphologic comparison of the embryos, pictures were taken by the electron microscope Zeiss EM910 in SEM mode.

The mature grains of the three *Triticum* species were swelled for 48 hours in distilled water at room temperature. After ripping the husk of the swollen grains, the mature embryos were removed under stereomicroscope and they were measured. The data obtained were put into a table.

- Examinations of egg cells

In planta egg cells

The changes in the size of the egg cells were examined for the three *Triticum* species. The sections prepared from the pistils that enabled us to define the largest diameter of the egg cell located in the embryo-sac next to the micropyle were selected. The data relating to the *in planta* egg cells were put into a table, each developmental state was photographed, providing information for us about the process of the ripening of the egg cell to reach functional maturity.

Excised egg cells

Egg cells were excised from the hexaploid spring wheat varieties of Siete Cerros and Chinese Spring under sterile and controlled conditions.

From the castrated flowers the ovaries and egg cells were excised using microsurgical method (Kovács et al. 1994) every third day (3, 6, 9, 12, 15, 18, 21 days after castration) under ZEISS STEMI 2000C binocular microscope. The isolated egg cells were taken over to microdrops of 5 microlitres using WPI A203XVZ nanolitre-injector (Pónya et al. 1999). Gametes were embedded in agarose gel with low melting point (SIGMA, mp = 30°C, bp= 65°C, No. A-9414). The solidificated agarose drops containing the egg cells were fixed in 0.2 M Sodium-cacodylate ((CH₃)₂As(O)ONa x 4H₂O) buffer with 2.5% glutaraldehyde. The diameters of the morphologically intact cells and nuclei preserving their shapes were measured using ocular micrometer. Egg cells were embedded in artificial resin in order to carry out optical microscopic and TEM examinations.

- Examination of the mature pollens of wheat

For examining the 3-cellular mature pollens of Chinese Spring, the anthers containing mature pollens were opened. Five anthers were selected, and from each anthers ten pollens were chosen in the microscope's visual field. When preparing the dissection, the stained (1% carmine acetic acid) and covered pollen grains placed on the glass slide were cleared by heating above flame because of the

accumulated starch content. The generative and vegetative cells in the mature pollen were measured with the ocular micrometer of the optical microscope ZEISS Ultraphot-III. Data relating to their sizes were collected, tabulated and analysed.

- Anther cultures

At least 15 maize anthers were collected randomly from the Petri dish 0, 1, 2, 3, 4, 5, 6, 13, 17 days after starting the cell culture. The anthers were subjected to the preparation procedure of light and TEM examinations. The anthers were fixed at 4°C for one night in a solution containing paraformaldehyde of 4%, glutaraldehyde of 0.1% and 0.1 mol/l Sodium-cacodylate buffer (pH 7.2). After embedding the fixed samples in Unicryl resin, sections were prepared.

- Ubiquitin immunolocalisation

Half-thin sections of maize anthers were placed on glass slides coated with 3-aminopropyl-triethoxy-silane. The glass slides were placed into TBS (10 mM/l Tris, 115 mM/l Na Cl, pH 7.4) for 10 minutes at room temperature, then they were put into the blocking solution (2% BSA TBS) for one hour at room temperature. Afterwards, rabbit polyclonal anti-ubiquitin antiserum (Sigma, St. Luis, MI, USA) was added to get 1:10 rate with the blocking solution (4°C for one night) and they were placed in TBS (3x20 minutes at room temperature), they were incubated with rabbit anti- IgG (Fe) – alkaline phosphatase conjugate (Promega Coop., Madison, WI, USA). It was diluted in the rate of 1:200 at 4°C for 4 hours, followed by a TBS treatment (3x20 minutes at room temperature), the detection buffer contained: Tris-HCL 100mmol/l pH 9.5, Sodium chloride 100mmol/l, Mg Cl₂ 5mmol/l, levasimole 1mmol/l (20 minutes at room temperature), and finally NBT/BCIP substrate was added (Armesco Inc., Solon, OH. USA) (5 hours at room temperature in dark). Control slides were prepared without adding antiubiquitin antibodies.

Histological examinations

- Embeddings

Two types of embeddings were applied for wheat pistils: Spurr resin (AGAR SIENTIFIC LTD, U.K. R1032) Spurr (1969) and Polyethylene glycol (PEG 1500).

Embedding in unicryl resin

After being washed in buffer solution, maize anther samples were dehydrated in ethanol series, then the temperature was gradually decreased to –25°C according to the instructions of the embedding procedure of unicryl resin (BB International, Cardiff, UK). Polymerisation occurred at –25°C for 3 days with exposure to UV light.

Embedding in agarose

The embryos removed from 2-week-old wheat seed buds were put in LMP (SIGMA, mp=30 °C, bp=65 °C, No. A-9414) drops of agarose-glucose (1:1) placed on glass slides. The partially solidificated material was covered with an ending drop. A block with the embryo in the centre of it was cut out of the totally solidificated agarose. The blocks were stored in 2.5% glutaraldehyde solution until being examined (SEM).

• Preparing of sections

Artificial resin blocks containing wheat pistils and isolated egg cells were cut using Reichert-Jung ULTRACUT E 701704 microtome with glass knife and diamond knife. Half-thin (1-2 µm) sections were stained with 0.5% toluidine blue. Examinations were carried out and pictures were taken with OPTON ULTRAPHOT III microscope. Polymerised egg cells were identified under phase contrast microscope, and ultra thin (70 nm) sections were prepared with microtome using diamond knife for electron microscopic examinations (TEM).

Sections with the thickness of 10-40 µm were prepared from the wheat pistils embedded in PEG using Reichert (steel bladed) microtome. The sections were stained with 1 µl/ml acridine-orange (SIGMA, c.i. 46005) for the LSM examination.

Half-thin sections of 1 µm were prepared from the maize anthers embedded in unicryl using Reichert-Jung ultramicrotome. The sections were then stained with 0.5% methylene blue and 0.5% toluidine blue. The stained sections were kept in 1% boric acid for 10 minutes and examined with Zeiss Axioplan photomicroscope. The 70 nm thick ultra thin sections were also prepared with this microtome. They were then placed on 300-mesh nickel grids and stained with uranyl acetate and lead citrate. The observations were implemented using Zeiss EM 10C/CR transmission electron microscope at 60 kV.

• Examinations with optical microscope

The stained microspores and the 1-2 µm thick half-thin sections were examined with OPTON ULTRAPHOT III and Zeiss Axioplan optical microscopes.

• TEM examination

The ultra thin sections were placed on metal grids and the contrast was intensified with uranyl acetate and lead citrate (Venable et al. 1965), the sections were then examined with Zeiss EM10C/CR and Zeiss EM910 electron microscopes.

• SEM examination

The length and width of young wheat embryos were measured with the microscope's ocular micrometer. The embryos placed on single-hole grids coated with formvar were covered with carbon

coat and stained with uranyl acetate. They were examined with Zeiss EM910 electron microscope at 60 kV.

For the morphologic comparison of the 2-week-old embryos, pictures were taken by electron microscope in SEM mode.

Mathematical methods applied

Levels of significance were calculated with one factorial randomized block analysis of variance and multifactorial ANOVA method.

On the basis of the data gathered on wheat embryo-sacs, schematic figures indicating the changes in the embryo-sac's shape and size were prepared using CorelDraw computer programme. The volume of the embryo-sac and the embryo were calculated with the formula: $4/3\pi*r^2*R$.

4. MAJOR ESTABLISHMENTS OF THE THESIS

1. The *in vivo* and *in vitro* development of the male and female gametophytes of three wheat species (*T. aestivum* cv Mv 15, *T. araraticum* and *T. monococcum*) with different ploidy levels and genome compositions was examined from the state of meiosis to the formation of mature pollen and embryo-sac.

Significant difference in the dynamics of the differentiation of male gametophytes due to the difference in ploidy levels was not found up to the tricellular developmental state. The difference in the development of haploid male gametophytes could be observed at the rapid grower hexaploid variety during the spreading of mature pollens. Microspores developed at a slower pace *in vitro*, the uninucleate state was found to be slightly longer at the first pollen mitosis comparing to that of the control. Genotype did not have considerable influence on the *in vitro* pollen development. The development from uninucleate microspores to functional trinucleate pollens completed within one week both *in vitro* and *in vivo*. However, the length of the different developmental states varied under *in vitro* and natural conditions.

The *in vivo* and *in vitro* development of the male gametophyte was studied on the basis of the diameter of pollen grains in three developmental states, namely Mu (mid uninucleate), Bn (binucleate), Tc (tricellular). In accordance with the result of the analysis of variance, the differences are reliable at $p=0.1\%$ level for the *Triticum* species. Difference was found on one occasion at $p=0.5\%$ level, and there were two cases where significant difference could not be found comparing any of the combinations.

2. The development of the embryo-sac in the *in vitro* spikelet culture was similar to that of the *in vivo* control, however, there was a difference in size for all three species. The embryo-sac of the hexaploid species is significantly larger than that of the other two species. The female

gametophytes reached their maximal dimension two or three days before the ripening of the male gametophytes. The dynamics of the differentiation was influenced by the ploid level, the differentiation of the 7-cellular female gametophytes took one day longer for the tetraploid and diploid species than for the hexaploid species.

3. The development of the 7-cellular female gametophyte is faster than that of the male gametophyte. For the species examined, the embryo-sac formed in three or four days from the macrospore as a result of three consecutive rapid mitosis, while the trinucleate pollen developed as the outcome of two microspore mitosis could only be observed on the seventh day. The development of the male and female gametophytes is asynchronous, confirming the protogyny of *Triticum* species.

The egg cells developing *in planta* from the initiation to the receptive state undergo a maturing process accompanied by growth and the changing of their shapes.

4. The volume of the 2-week-old embryos of the *Triticum* species demonstrated a difference in the intensity of development comparing to the volume of either the embryo-sacs at anthesis, or the mature embryo. On the basis of the averages, the volume of the 2-week-old embryos of the diploid wheat exceeded even that of the hexaploid wheat. It was also verified when the two-week-old seed buds were compared to the mature grains. The size of the two-week-old diploid seed bud was 60% of the mature grain, while it was 50% for the hexaploid and 23% for the tetraploid wheat.

5. The studying of the egg cells developed *in planta* was followed by the examination of excised or isolated egg cells, which took spherical shape after being removed. The changes in the size of egg cells and the nuclei are connected with their age. The diameter of the egg cells was examined on the two hexaploid wheat varieties Chinese Spring and Siete Cerros on 3, 6, 9, 12, 15, 18 days old egg cells calculated from castration. The average growth of the egg cells and the nuclei was similarly increasing from day 3 to day 18 for both varieties. However, the diameters of the cells and the nuclei of the two varieties reached their maximum values at different times. The diameters of 6 and 9 days old cells were 63,49 μm and 73,32 μm .

The morphologic examinations were carried out on half-thin sections prepared from 3, 6, 9, 12, 15, 18 days old egg cells. The morphologic features were also connected to their age. The largest difference could be observed in the case of young (3 days old), receptive (9 days old) and old (18 days old) egg cells.

6. By examining the isolated egg cells with optical microscope, more detailed ultrastructural observations could be taken using TEM.

In the cytoplasm of the 3 days old egg cell prior to anthesis, lipid bodies, mitochondria, amiloplasts, starch granules can be found among the peripheral vesicles.

The 9-day-old receptive egg cell was the closest to anthesis. It can be characterised by dense cytoplasm, large number of mitochondria, the endoplasmatic reticle in the nucleus and polarisation.

The old egg cell has several features. The chromatin in the nucleus is degraded. Chromatin residues are adhered to the membrane of the nucleus forming the so called blebbings. Lipid, starch and

protein are accumulated in the cytoplasm. Lysis could be observed in the vesicles, and autophag vacuoles appeared. The old egg cell can be characterised by the features of programmed cell death.

7. The generative and vegetative features of the mature, 3-cellular pollens of the wheat variety Chinese Spring were compared. The dimorphism of the two male gametes is indicated by the consistent difference in their sizes. The diameter of the pollen is similar to that of the hexaploid egg cell, while the size of the male gamete is only the quarter of the size of the mature egg cell.

8. The maize anthers of the hybrid line 'DH109 x ZK 191/a' were isolated at late uninucleate state. The embryoid structures were formed on YP medium by cold treatment.

On the basis of the optical microscopic examinations, 2-cellular microspores were found on the sections prepared from the anthers 2 days after the starving treatment. Several variations of the cultured microspores such as multicellular microspores (MCM) and multinuclear microspores (MNM) were observed in the population from day 5, as well as degenerate and non-induced microspores. The occurrence of several microspore sub-populations drew our attention to the fact that microspore types differing in cell division and development can be observed in the culture.

The ultramicroscopic examinations showed that in these cellular forms and cell clusters special characteristics can be detected such as the nucleus pores in the early stage of induction and the formation of thick cell wall under the intine. A certain degree of polarity could also be observed in these structures. Proembryonic structures could be found 10-13 days after the initiation of the culture, while embryonic structures appeared on approximately day 16.

Commercially available polyclonal antibodies were used for the ubiquitin examination to complete immunocytochemical detection on half-thin sections of the anthers in co-operation with Spanish researchers. Marking could be found for all of the cell types mentioned above except for degenerate and non-induced microspores. The intensity of the marking was higher in the case of MCM's and androgenic microspores. A model was set to demonstrate the role of ubiquitin in the pollen cell cycle and the induction of androgenesis, and in the ensuring of the degrading route.

9. For examining the dynamics of the grape flower's development, the methods applied for wheat were adapted and the developmental state of the male and female gametophytes were detected at both varieties.

In the development of grape pollen, seven phases were defined. The typical structural features were specified. Correlation was searched between the individual developmental states of the seed bud and pollen grains by systematic samplings.

The cell development in the embryo-sac and the optimal stage of development for tissue cultures were determined using an indirect method.

5. NOVEL RESULTS OF THE THESIS

The results of complex comparative examinations were obtained on the development of male and female gametophytes of plants grown under *in vivo* and *in vitro* circumstances. Cytological, histological and morphological data were gathered on the gametophytes and young embryos of *Triticum* species with different genomes and ploid levels.

During our *in planta* examinations, a series of sections were prepared from the initial development of the egg cell to the receptive egg cell. The phases were recorded by microscopic images.

Sections of 1-2 μm were prepared from the egg cells excised periodically following a microtechnical procedure, thus the morphological and fine structural studies were made possible. The data gathered contribute to the selection of egg cells with optimal conditions for different micromanipulation methods.

The microspore-derived structures in maize anther cultures are heterogeneous following the androgenetic induction. The existence of different forms were verified by microscopic examinations and immunocytochemical markings. Necrotic, non-stained microspores, multicellular and multinuclear microspore structures were investigated.

The developmental stages of grape pollen and pistil were defined and documented, assisting the successful application of androgenic and gynogenic tissue culture methods.

6. PRACTICAL APPLICABILITY OF RESULTS

The examination of the flowering and reproduction biology of cultivated wheat and its related species provides important background information for modern plant breeding researches using biotechnological methods as well. The detailed cytological and histological examination of the formation and development of the male and female gametophytes (pollen and embryo-sac) and the studying of the synchronisation of reproductive processes supply information on the gametophytic procedures of different cereals, and provide an opportunity to analyse the direct and indirect effects of environmental changes as well as for the *in vitro* micromanipulation of gametes for biotechnological purposes.

Our results showed that the time required for the formation of the 3-cellular pollens of *Triticum* species with different genome compositions and ploid levels was 1 week after the meiosis both *in planta* and *in vitro*. The ripening and spreading of 3-cellular pollens are greatly influenced by abiotic environmental factors (primarily temperature). The development of embryo-sac antedated the development of 3-cellular pollen both under natural conditions and *in vitro*, thus the protogyny of the *Triticum* species examined was proved. However, no reliable data are available whether the very young egg cell can be fertilized, therefore, further examinations are required on this question.

Our ultrastructural examinations demonstrated that the morphologic, structural and thus functional features of very young, mature (at anthesis) and old wheat egg cells differ. Further studies can answer basic questions such as how parthenogenetic egg cell activation can be generated and which developmental state is the most suitable for this, or which developmental state of the egg cell is the most appropriate for injecting agronomically valuable genes in order to ensure stable integration.

The sizes of the embryo-sac and the egg cell were the largest for the hexaploid species. Sizes decreased in connection with the reduction of ploid level. However, this tendency was not observed in the case of the embryos. The sizes of the embryo-sacs and egg cells did not correlate with the size of the embryos in the case of the *Triticum* species examined. Young embryos can be used for research on gene transformation.

The improvement of our knowledge on the female gametophyte contributes to the successful implementation of the *in vitro* fertilisation activity and plant “test-tube baby” programme carried out at our Plant Cell Biology and Physiology Department as well as that of the gene transformation to the egg cell, induced parthenogenesis, egg cell cryopreservation and the creation of copyDNS library from the egg cell.

The cytological and ultrastructural examinations of the *in vitro* androgenesis of maize are pioneer works even from international aspect as the induction frequency relating to microspores is so minor that the process cannot be followed. At our Institute, maize genotypes with great haploid induction capacity were available and the effective anther culture method was on hand, enabling us to examine the detailed ultrastructure of microspore-derived structures and to biochemically analyse the process in co-operation with Spanish researchers. The practical applicability of the maize haploid induction method elaborated by us is examined and evaluated together with the maize breeders at our Institute.

For the induction of haploid/diploid grape plants from macrogametophyte cells, it is essential – beside several other factors – to determine the developmental state of the embryo-sac and choose the optimal developmental state for executing tissue culture methods. Gynogenetic haploid and homozygous diploid plants can be produced from *in vitro* ovary and ovule cultures. These plants can be used for breeding and genetic examinations.

7. Publications in the subject of the dissertation

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