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# Induction of Embryogenesis in the Culture of Isolated Microspores of Wheat (*Triticum aestivum* L.)

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**ABSTRACT**

**Wheat (*Triticum aestivum* L.) haploids and doubled haploids are widely used in breeding, the investigations of a combinative variability and its stabilization in homozygotes. In four domestic varieties of winter wheats (Moskovskaya 56, Moskovskaya 39, Galina, Nemchinovskaya 24) and three domestic varieties of spring wheats (Ester, MIS, Amir). With spring wheat variety Falat as a control, the efficacy of embryogenesis in isolated microspores was tested using standard protocol for induction of direct embryo formation in the isolated microspore culture. In all winter varieties there was shown a low frequency of cytoplasmic strands, which are typical for the embryogenic microspores, whereas in the spring varieties it was high. After 4 days cultivation in the medium used for induction, the microspore viability decreased in winter varieties. and another 10 days later the Viable cells were not observed. The spring varieties developed the multicellular structures, which could produce embryos. The reference variety Falat produced 28 % of proembryoids, able mostly to further embryonic formation. Basing on these results, the protocol for inducing direct embryogenesis in wheat microspores was modified, including maltose concentration in medium, the conditions of spikelet heat treatment, the number of ovaries and time when they were added to the culture, the combination and concentration of hormones in the media for induction and cultivation.**

***Keywords***- microspores, in vitro, wheat, embryogenesis, doubled haploids, plant growth regulators.

## I. INTRODUCTION

Haploids and doubled haploids of wheat are valuable material for breeding and solving a number of scientific and practical problems, including those related to the study of combinative variability and the search for approaches for its effective use and fixation in homozygous lines. Initially, methods of chromosome elimination and anther culture were used to obtain wheat haploids (1, 2).

The method of culture of isolated microspores has been developed relatively recently (3). Its essence is as follows. Wheat plants are grown under certain conditions (vernalization stage, temperature 15-20 ° C, illumination 5-15 thousand lux and 16-hour photoperiod). For culture, microspores are used that are at a late single-core or early dual-core stage of development (4-6). Spikelets or isolated microspores are first cultivated on nutrient media that block gametophyte spores and induce the formation of embryogenic microspores (usually poor media with mannitol and / or sorbitol) at low temperatures (4-7 ° C). Next, embryogenic microspores are transferred to a nutrient medium with carbohydrates and hormones, incubated at 22-25 ° C in the dark until the formation of embryoids, which, in turn, are transferred to dense nutrient media for plant regeneration containing phytohormone, low sugar concentrations, and maintain a certain light mode (3-5). The whole process usually takes about 2 months.

Despite the constant improvement of methods, obtaining homozygous dihaploid wheat lines is possible only by adapting existing protocols for specific plant genotypes.

The purpose of this work was to determine the efficiency of the induction of mbryogenesis in the culture of isolated microspores in spring and winter wheat varieties of domestic selection using previously developed protocols.

## II. METHODOLOGY

We used wheat varieties (*Triticun aestivum* L.) - winter (Moskovskaya 56, Moskovskaya 39, Galina, Nemchinovskaya 24) and spring (Ester, MIS, Amir). The spring wheat cultivar Falat served as a control. It was previously shown that it has the highest susceptibility to the induction of embryogenesis and the maximum frequency of regeneration (7). Donor plants were cultivated according to standard methods (4,7). The material was removed when 80% of the microspores in the anthers of the spike were at the one-cell stage.

Our work was based on the protocol for the induction of direct embryogenesis in the culture of isolated wheat microspores (7). According to this technique, stress treatment of microspores was not expected.

The optimal stage of development was determined using an inverted light microscope LaboMed IZ (Labor-microscopes, Russia). Anther preparations were stained with acetocarmine or [) APl (4 ', 6diamidino-2-phenylindole) (8, 9). Microspores were isolated from freshly cut spikelets.

Spikelets were sterilized in 70% ethanol by soaking for 1 min. The anthers of lateral flowers were isolated and washed in liquid AB medium (7). Anther

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microspores were released using centrifugation for 2-3 minutes (600 rpm). The resulting suspension was placed in tubes for centrifugation, passing through a filter (6 () μm). Then, the microspores were washed in the AB medium by 2-fold centrifugation (5 min at 600 rpm). The precipitated microspores with a density of approximately 2x104 were carefully suspended in 1.5 ml of A2 medium (7).

Cultivation of microspores was carried out in sealed Petri dishes (35x10 mm) in the dark at a temperature of 25 ° C. After 7 days from the beginning of cultivation, plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mg / L), kinetin (0.2 mg / L), as well as ovaries (6-7 pcs. ) and continued cultivation under the same conditions.

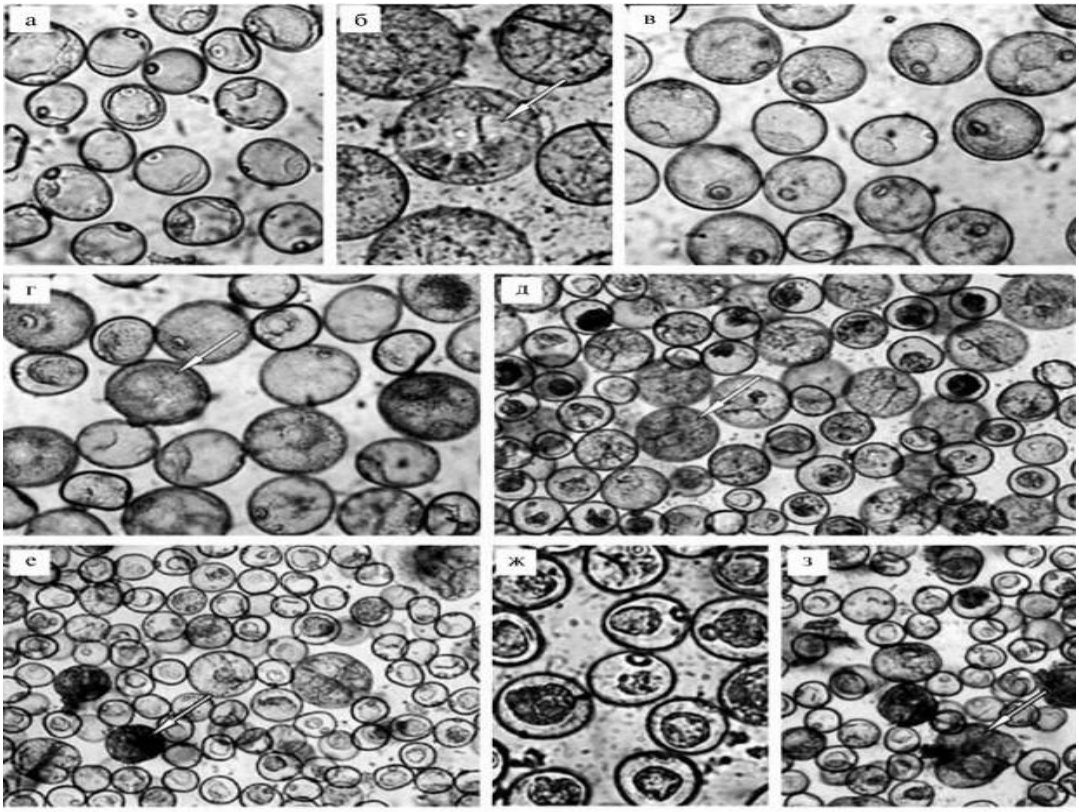
The ovary was removed under aseptic conditions from the ear and cultured on solid A2 medium at a temperature of 25 ° C (7). If necessary, the ovary was placed in a liquid induction medium.

The presence of starch in the cultured cells was determined under an inverted light microscope LaboMed IZ (Labor-microscopes, Russia) after staining with Lugol's solution.

## III. RESULTS

The viability of isolated wheat microspores immediately after isolation was 58.2% for cultivar Falat and 31% for other cultivars. Microspores at the late mononuclear stage had a large vacuole, a thin layer of cytoplasm, and a round nucleus opposite the embryonic pore (Fig. A).

After 4 days of cultivation, their viability decreased to 42.3% in Falat variety, to 32.2-41.0% in spring varieties and to 8.0-11.5% in winter varieties. In most cases, the nuclei moved to the center of the cell, the vacuole disappeared, and the density of the cytoplasm increased. Some microspores increased in size up to 60-80 microns. Some of them had star-like structures, which are altered vacuoles with cytoplasmic strands (see Fig., B). Visually, they were similar to the previously described structures of tobacco microspores, characteristic of an embryogenic microspore (4, 7).




Isolated and vitro cultured microspores of the Amir variety: a - microspores immediately after isolation from freshly cut spikelet's, b - embryonic microspores after 4 days of cultivation at 25°C (the arrow indicates a star-like structure), c - immature two-celled pollen grains, d - microspores in the two-cell stage after 4 days of cultivation on a nutrient medium A2, e - multicellular structures formed as a result of mitotic division after 1-2 weeks of cultivation, f - embryo-like structures (marked with an arrow). g - plasmolysis of microspores after 10 days of cultivation on a nutrient medium A2 3 - formation of multicellular structures without their further development (light microscopy, magnification x600).

If early two-celled immature pollen grains were used for cultivation (see Fig., C), then starch accumulated in the cells during the first 4 days of incubation and they

died. The addition of growth regulators and ovaries led to rupture of membranes and a significant decrease in cell viability, although some still formed multicellular structures (see Fig., H), a small fraction of which could develop into embryoids.

One week after the addition of ovaries and growth regulators, the proportion of living cells was 33.1% for cultivar Falat and 0-9.2% for other cultivars (table). At the same time, living cells were absent in all winter varieties. Within 1-2 weeks, microspores passed through a series of mitotic divisions and formed embryoid precursors (see Fig. E). Some of the multicellular proembryoids ruptured the cell wall and went outside, while others ejected cytoplasm and nuclear material and died. Some developed into globular embryo-like structures, then into embryoids through direct embryogenesis and could grow as normal zygotic embryoids with various shapes and sizes, while the rest formed callus (see Fig., H).

**Viability of microspores in wheat of spring and winter sodas at different stages of development in vitro**

| Sort | Percentage of live mic by% | | |
|---|---|---|---|
| | After isolation | After 4 days | One week after the addition of elyato to the ovary and ovary |
| Falat (control) | 58,2 | 42,3 | 33,1 |
| Moscow 56 (winter) | 35,2 | 9,3 | 0 |
| Moscow 39 (winter) | 39,6 | 8,3 | 0 |
| Galina (winter) | 33,4 | 11.5 | 0 |
| Nemchinovskaya 24 (winter) | 31,5 | 10,2 | 0 |
| Esther (spring) | 48,3 | 41,0 | 9.2 |
| MIS (spring) | 42,2 | 35,8 | 7,3 |
| Amir (spring) | 38,5 | 32.2 | 7,4 |

2-3 weeks after the addition of ovaries and growth regulators, multicellular structures in spring cultivars were less than 5. Later, they did not develop into embryoids. In the control cultivar Falat, the proportion of proembryodic structures was 28%, while most of them later developed into embryoids.

Thus, all studied winter wheat varieties of domestic selection showed a low frequency of formation of star-like structures characteristic of embryogenic microspores. After 4 days of cultivation in the induction medium, a decrease in the viability of microspores was noted, and after 10 days, no living cells were found in the culture. Spring cultivars demonstrated the ability to form multicellular structures that stopped developing after 3 weeks of cultivation. Based on the data obtained, it became necessary to modify the existing protocol, namely, to change such parameters as the concentration of maltose in the induction medium, temperature treatment of wheat spikelets, the number of ovaries and the time of their addition to the medium, the type and concentration of hormones in the induction medium and in the medium. for cultivation.

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