Culture of potato microspores (Solanum tuberosum L.)
Nevsky variety

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Abstract. Within 24 days of cultivation, the ratio of the number of potato microspores at different stages of development was studied depending on the content of 6-BA in nutrient media. Four media options were used: No. 1 MS + 6-BA 1 µM, No. 2 MS + 6-BA 2 µM, No. 3 MS + 6-BA 3 µM, No. 4 MS + 6-BA 4 µM. It was found that the use of MS-based culture media with 1-4 µM 6-BA is effective for the induction of embryoidogenesis in potatoes in microspore culture. 88.0-95.0% of the microspores were in a living state for 24 days. The frequency of embryoid formation in the experiment was 0.5-1.0% of the total number of microspores and 4.7-6.6% of the number of multicellular structures. Monad could be the initial cells of the embryoids of the tetraploid variety of potato in the period up to 11-12 days. In addition to single germ-like structures, in the suspension of potato microspores, embryoid conglomerates - polyembrioids - were noted on days 11-12. In the light, microspores and germ-like structures of Nevsky tetraploid potato variety acquired a green color. On the 24th day of cultivation, developing potato embryoids were detected in the phase of organogenesis.

1. Introduction
More than 300 million tons of potatoes are produced annually in the world [1]. A third of potato production is concentrated in developing countries, and over 1 billion people consume potatoes as their staple food. Potatoes are the most important food source of starch, proteins, vitamins, antioxidants in the diet of the planet’s population [2]. Potato is second only to soy in protein content. Patatin is the main reserve protein of potato tubers, one of the most balanced among the known plant proteins in human nutrition [3]. The great importance of potato as a culture at the international level, especially in developing countries, was presented by the UN in 2008, which was the year of potatoes [4]. Potatoes grow in a wide range of habitats, rising to 4000 m above sea level [5].

The technology for producing doubled haploids (DH (double haploid) technologies) through the anther/in vitro anther/microspore culture is one of the methods for the genetic improvement of agricultural plants. Despite the fact that great success has been achieved in the production of haploids in some plant species, nevertheless, there are many factors that can contribute to the improvement and optimization of DH-technologies. A deeper fundamental understanding of the processes of induction of embryogenesis of microspores and the development of haploid plants, which will ensure high efficiency in the production of DH-lines, is necessary [6].

To date, numerous data have been accumulated that make it possible to identify relatively optimal cultivation conditions for anthers of potatoes for the induction of callus and embryoidogenesis, as well as to achieve the regeneration of fully formed plants. An important role in the formation and
development of tumors in vitro and subsequent regeneration of plants is played by the component composition of the nutrient medium. A number of authors have shown the requirements of different potato genotypes for the content and ratio in the environment of nitrogen-containing salts, various macro- and microelements, amino acids, sugars, vitamin supplements and hormonal factors, binding and adsorbing agents [7-13].

The aim of this work was to determine the effectiveness of the induction of embryoidogenesis in a microspore culture in a potato cultivar (Solanum tuberosum L.) Nevsky, depending on the content of 6-BA (6-benzylaminopurine) in culture media.

2. Experimental part

The object of the study was the domestic potato of Nevsky variety. The flowers were collected in the field during the budding phase and, for preliminary treatment with low temperatures, they were placed in a refrigerator for 4 days at 4-5°C. The plant material was sterilized with a 1% sulfochlorantin solution for 10 min, then washed three times with sterile distilled water. To isolate the anthers, buds 5.0-7.0 mm long were used. Microspores from anthers were released with a household blender in a liquid nutrient medium for 1.5 minutes. 100 anthers were isolated and broken up in 100 ml of liquid nutrient medium. The microspore suspension was filtered and in a volume of 20.0 ml was layered on solid agar media in vials and plastic containers.

A liquid nutrient medium was prepared on the basis of the Murashige-Skoog (MS) prescription, 500 mg/L of L-glutamine, 1 μM kinetin, 1 μM 2,4-D (2,4-dichlorophenoxyacetic acid) were added. Solids were also prepared on the basis of MS with 1-4 μM 6-BA (6-benzylaminopurine). Four media options were used: No. 1 MS + 6-BA 1 μM, No. 2 MS + 6-BA 2 μM, No. 3 MS + 6-BA 3 μM, No. 4 MS + 6-BA 4 μM.

Osmotic pressure in the medium is a critical factor for the development of an in vitro embryoid. Sucrose is used in an in vitro plant cell culture as a source of carbohydrates and an osmotic pressure regulator. Typically, high osmotic pressure is required for the embryoid in the early stages of development (48 h), and in the later stages it should be lower. Sucrose in high concentrations can act as an osmotic stressor, on the other hand, it is necessary for the formation of embryoids. In our experiment, liquid media contained 9% sucrose. It was added in the agar media in an amount of 3%.

The cultivation was carried out in a thermostat at a temperature of 26-27°C, in the dark.

3. Research methods and discussion of the results

We used a method of culture of isolated microspores, based on the use of the biological phenomenon of androkline, or androgenesis in vitro, in which haploid cells of microspores develop along the sporophytic pathway to embryoid-like structures - embryoids and then to regenerant plants containing half the set of chromosomes.

Cytological studies were performed every 3-4 days of cultivation. For this, 100 μl of a suspension of microspores was taken, the preparations were stained with acetocarmine, 200 microspores were studied from one glass.

As a result of a series of successive mitotic divisions, microsporocytes, or maternal cells of microspores, are formed from primary sporogenous cells. From each microsporocyte, a tetrad (four) of haploid microspores is formed as a result of meiotic division. At the beginning of the prophase of meiosis, a thick layer of callose, a water-insoluble polysaccharide, begins to be deposited around microsporocytes. In the period between the end of meiosis and the release of sister spores from the callosal membrane, the processes of sporderm formation (microspore membrane) proceed. The formed microspores are released from the tetrad, as a result of the rapid dissolution of the callosal membrane. Released microspores are rapidly increasing in size. But, as was shown by a number of researchers, the main structural features of ectexia are determined in an earlier tetrad period, and subsequent changes concern only the development of existing structural features. From this it is concluded that in isolated microspores, the main morphogenetic processes die out very soon after the tetrad decay. But although morphogenetic activity ends, there is a differential growth that changes the proportions of the
various elements of ectxins. Finally, shortly after the release of microspores from the tetrad, the development of intina also begins. The morphological activity of the cytoplasm of the microspore ends at the beginning of the division of its nucleus, when all triggers switch to completely different processes. The microspore in which the division began, thereby already ceases to be a microspore in the exact sense of the word. From the microspore itself, only its shell remains, and the contents are already the two-cell or three-cell stage of the development of male gametophyte [14].

At the time of isolation of potato microspores, the Nevsky cultivar noted the following stages of development: tetrads, decaying tetrads into monads, early, middle, and late (highly vacuolated) microspores. Two and three cell pollen were not observed. In the field of view of the microscope, 2-4% of the microspores had an atypical structure. Among the middle and late microspores, binuclear ones with symmetrical nuclei were found. The appearance of atypical microspores may have induced cold pretreatment of flowers for 4 days, or this is due to disturbances in meiosis during microsporogenesis.

At this stage of the work, we studied the effect of the concentration of 6-BA in an induction nutrient medium on the frequency of embryoid formation from in vitro cultured isolated microspores in order to develop a method for the targeted production of embryoids. Within 24 days of cultivation, normally developing microspores, atypical and dead, were counted.

Already on the 3rd day of cultivation, changes in the ratio of different stages of the development of microspores in the experiment were noted. On Wednesday, No. 1, the number of monads is reduced due to an increase in the proportion of early microspores. The ratio of tetrads, middle and late microspores remains almost unchanged. On Wednesday, No. 2, it is likely that the processes of passage through the microspores of the development stages are faster. The proportion of tetrads is significantly reduced, while the number of late microspores is increasing. With an increase in the 6-BA content to 3 μM on medium No. 3, these processes are further accelerated. Along with a sharp decrease in the proportion of tetrads, monads and early microspores, the number of late microspores and bicellular pollen significantly increases. On Wednesday, day 4, we did not find tetrad on the 3rd day of cultivation, monads and early microspores became much smaller, and middle and late microspores were observed in equal proportions. At the same time, 1% of the dead microspores were revealed.

On the 7th day of cultivation on media with 6-BA 1 μM, the proportions of monads, early, middle and late microspores are almost equal. With an increase in the content of 6-BA in nutrient media, the percentage of medium and late microspores significantly increases. Those, with an increase in the concentration of cytokinin, the normal development of microspores accelerates. But, nevertheless, by 7 days, death is observed up to 5% of microspores and the proportion of atypical increases to 3.8%. Along with the asymmetric division of the nucleus into large vegetative and small generative, atypical binuclear microspores with equal nuclei. Such microspores are found among the middle and late. Also, by 7–11 days, as a result of a series of divisions, multicellular structures were formed that were inside the shell of the microspore (figure 1).

Figure 1. Multicellular structures inside the shell of microspores: a) 7 days, b, c) 11 days. (enlarged × 1000).
According to J. M. Dunwell and U. Sunderland, the realization of the androgenetic potential of potato plants is no less important than the nutrient medium factors when choosing the stage of microspore development during explantation [15]. For potatoes, the optimal explantation phase is between the exit of microspores from the tetrads and the first pollen mitosis (the stage of late strongly vacuolated microspores) [7, 15]. In our experiments, we observed the atypical development of microspores at the stage of tetrads and monads, where nuclear fission took place. By 11 days, in a suspension of microspores, we observed nucleus-like structures (figure 2 a).

As it was shown by a number of researchers, in isolated microspores, the main morphogenetic processes die out very soon after the tetrad decay [14]. In our opinion, under artificial conditions, under the influence of external factors, the probability of switching the microspore development program at the monad stage to the direct formation of a new plant from it is very high. We are inclined to the version that the initial cells of the embryoids of the tetraploid variety of potato in the period up to 11-12 days were monads.

After 11 days of cultivation, part of the microspore culture vials was transferred from thermostats to shelves with 2000 lux illumination. Microspores and germ-like structures of the tetraploid Nevsky potato cultivar acquired a green color (figure 2 b, c).

Figure 2. a) potato embryoid, b), c) potato embryo grown in the light (enlarged × 400).

The nutrient medium contains 6-BA cytokinin. It is known that cytokinin is a request for photoassimilates. If the tissue is able to form chloroplasts, then under the action of cytokinin, chlorophyll is synthesized. At the same time, cytokinins activating the synthesis of chlorophylls and apoprotein CCKII contribute to the formation of the chloroplast structure [16]. The potato microspore culture was kept in the thermostat in the dark for 11 days, then 4 days in the light. Under the influence of culture media and cultivation conditions in microspores, certain shaping processes have passed. It can be assumed that even if, due to their specialization, microspores are not able to form chloroplasts, during the cultivation in artificial conditions this ability appeared in them.

On days 16-24 on different types of media, the ratio of multicellular structures had its own characteristics. In our experiment, multicellular structures were formed inside the shell of microsporocytes, monads, and microspores (figure 3a). Multicellular formations such as callus and embryoids were found in the field of view of the microscope. The largest number of multicellular structures in the shells of microsporocytes and monads was observed on medium No. 1., as well as undifferentiated tissue of the callus type. Bicoreal middle and late microspores with symmetrical nuclei were detected on media No. 2 and No. 3. According to the data of Ludmila Rthova and Jaroslav Tupy [17], stress-induced embryogenesis in potatoes is initiated by symmetric nuclear fission in microspores, with the formation of two nuclei of the same size.

According to O.A. Seldimirova and N.N. The circular main problem of biotechnology for obtaining wheat haploids through embryogenesis is the low yield of regenerated plants. The solution to the problem can be the production of polyembryoids, a special type of embryoids characterized by the
formation of multiple growth points in their apical part [18]. In our experience, we observed the formation of polyembryoids in potatoes in a microspore culture (figure 3 b).

On the 24th day of cultivation, the frequency of formation of embryos (single germ-like structures) and the frequency of formation of polyembryoids in the experiment amounted to 0.5-1.0% of the total number of microspores and 4.7-6.6% of the number of multicellular structures.

It should be noted that in our experiments, 88.0-95.0% of the microspores were in a living state for 24 days. Czech scientists Ludmila Rihova and Jaroslav Tupy [18] noted that only 33% of potato microspores were viable within 14 days of development.

On the 24th day of cultivation, developing potato embryoids were detected in the field of view of the microscope in the phase of organogenesis (figure 3 c).

![Figure 3](image)

**Figure 3.** a) fissile microsporocytes on the 11th day of cultivation (enlarged × 1000), b) potato polyembryoid grown in the light (enlarged × 1000), c) potato embryoids in the phase of organogenesis (enlarged × 100).

4. Conclusion
The use of MS-based culture media with 1-4 μM 6-BA is effective for inducing embryoidogenesis in potato in microspore culture. The frequency of formation of embryos (single germ-like structures) and the frequency of polyembryoid formation in the experiment amounted to 0.5-1.0% of the total number of microspores and 4.7-6.6% of the number of multicellular structures. 88.0-95.0% of the microspores were in a living state for 24 days.

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