IN VITRO TECHNOLOGY OF HAPLOID RAPESEED PLANTS *(BRASSICA NAPUS* L.)

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Abstract. Cultivation conditions of isolated anthers, microspores and ovaries have been optimized in order to obtain haploid rapeseed plants under *in vitro* conditions. It has been established that B_5 hormones kinetin attended in the nutrient medium (1 mg per litre) stimulated the process of embryogenesis and also stimulated the formation of both secondary and tertiary embryos on hypocotil and leaf segments of sterile sprouts. The further cytologic analysis of plant regenerants proved their haploid origin.

Key words: rapeseed, haploid plants, microspore culture, isolated anthers, embryogenesis, in vitro.

Introduction

The most important direction for contemporary plant breeding is to develop improved and essentially new agricultural crop genotypes possessing individual, group or complex resistance for biotic and abiotic stress environmental factors and preserving the same or increased productivity and quality. The rational combination of classical breeding methods and biotechnological ones allows to solve above mentioned tasks in shorter periods of time.

Using cell and tissue biotechnology methods in plant breeding makes the traditional breeding process of new crop samples and varieties development more easy and rapid. One of such methods is the method directed to develop haploid plant *in vitro* that allows obtaining quickly genetically stable homozygous lines. To induct haploidy usually are used traditional (classical) method, such as remote hybridization or crossing, phytohormones, creation of stressful conditions, etc. However these techniques are labour-consuming, require a lot of time and they are inefficient due to low coefficient of haploid plants output. To increase efficiency of haploid induction is possible to use the following methods of cultivation *in vitro*: 1) androgenesis in anther and pollen culture; 2) ginogenesis in isolated ovules culture and 3) parthenogenesis in culture of a hybrid germ that lost fatherly parental chromosomes [1,2].

The method of anther and pollen culture is one of the perspective ways to obtain haploid plants. The microspore and pollen culture is a convenient experimental object for basic fundamental investigations related to regeneration in monocelled systems. Besides, correct identification of cultivation conditions, providing differentiation of embryoids

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from microspores and comparison of this process with the somatic and zygote germ formation are subjects of biochemical and morphological investigations and experiments on study of molecular mechanisms specific for embryogenesis as well. Potential opportunities for theoretical and applied application of anther and pollen culture method are beyond haploid breeding. So, double haploid plants (dihaploids) can be used successfully for mutant identification in cellular structures. Efficiency of mutagenesis on haploid level is obvious as dihaploid cells on the same locus need double mutation and it is enough for haploid expression to concentrate mutation in one locus with subsequent doubling chromosomes.

The most widespread method of development homozygous lines of many species of *Brassica* is to obtain a plant from microspores in anther culture. As a rule authors carried out research to obtain haploid plants at the certain stage of androgenesis, for example, under conditions of donor plants cultivation, their preliminary processing in low temperature or biologically active substances, and investigated dependence of morphogenesis from plant-donor genotype features, microspores development stage in an anther, a nutrient medium structure and anther cultivation conditions, as well as other conditions stimulating androgenesis [3-5]. Obtained results have shown that till now embryogenesis in anther culture *in vitro* for different *Brassica* species happend spontaneously and offered technologies are difficultly reproduced and poorly investigated within every stage of androgenesis. Therefore, development and improvement of existing methodological approaches must be carried out for each certain genotype.

Material and methods of investigation (research methods)

In this work rapeseed *Brassica napus* variety Hans and hybrids F, (WF x Titan, Galicia x TGI) donor plants were grown in greenhouse during a year in Vegetable experimental station named after V.I. Edelshtein, Russian State Agrarian University — Moscow Agricultural Academy named after K.A.Timiryazev. The work was carried out on buds isolated either the main shoot or lateral ones. Inflorescences were placed in a glass of water and were subjected with cool preprocessing under 4-6°C in home refrigerator within 16 hours. After that the buds were sterilized in 0,1% solution of corrosive sublimate during 4 minutes and were washed many times with the sterile distilled water. The anthers were aseptically taken from the buds under a binocular magnifier and were replaced onto inducting medium B_5 containing 3% of saccharose and hormones with cytokinin and auxin activities as well. In case of cytokinins were studied 6-benzylaminopurine, kinetin, 2ip in concentration from 0.5 up to 1.5 mg/1, in case of auxins 2.4-D, a-naphtaleneacetic acid, indoleacetic acid in 0.5-2.0 mg/1 concentration have been investigated. The influence of nutrient medium consistence (solid, semi-liquid and liquid) as well as combination of liquid and solid media (two-layer media) on embryogenesis process also has been investigated. Cultivation of obtained embryoids and plants-regenerants has been carried out in MC nutrient medium.

Petri's dishes (double dishes) with a vegetative material have been placed in thermostat and incubated under 350°C temperature 24 hours, then they have been carried in usual cultivation conditions (25°C temperature, photoperiod of 16 hours and illumination by white fluorescent lamps with light intensiveness in 5 thousand lk). Experiments have been conducted according to rules of work under sterile conditions, developed at the Agricultural Biotechnology department. Cytologic researches on chromosomes and chloroplasts estimation in stomata closed cells have been carried out in accordance with requirements of practicum on cytology and cytogenetic of plants.

Results and discussion

Investigations carried out have shown anthers and microspores cultivation under the medium containing 1 mg/1 kinetin and 1 mg/1 a-naphtaleneacetic acid has resulted to strong formation of anthers and microspores vacuole cells. Moreover, free of anther somatic tissues microspores under the solid medium start their division and form embryoids, further developing plants (fig. 1). Despite of low frequency of somatic embryogenesis that gave only 1%, average number of inducted embryoids per an anther was 16-25 items. Moreover, this process happened asynchronously (fig. 2).

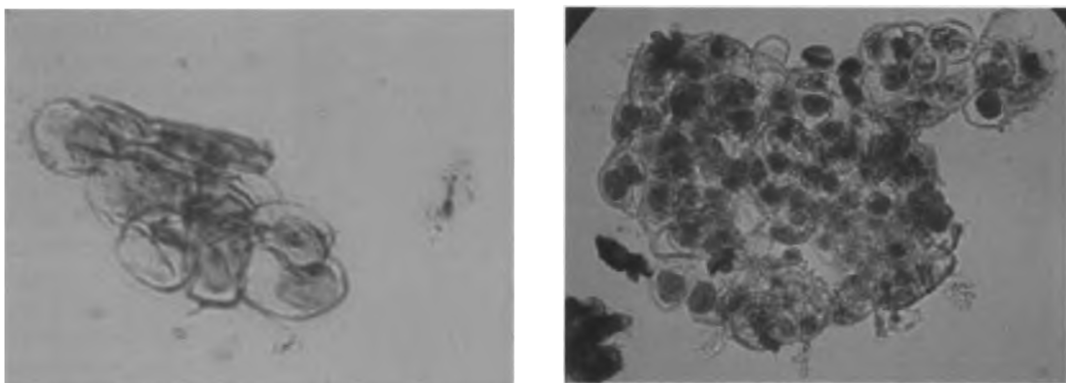

Fig. 1. Process of embryoids formation from isolated microspores:
a) formation of vacuole cells; b) formation of embryoid structures

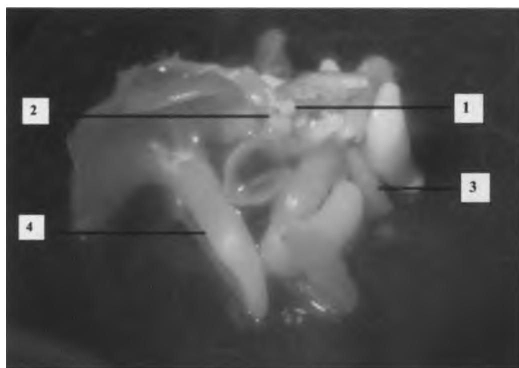

Fig. 2. Asynchronous formation of embryoids:
1 — globule stage; 2 — heard-shaped stage;
3 — torpedo stage; 4 — a sprout

In the future generations formed embryoids have been separated from the primary explant and from each others and placed in tubes under media containing mineral salts according to MC, as well as various hormones and agar concentration: 1) solid hormone-free MC medium with 2% saccharose, 8g/l agar; 2) liquid hormone-free MC medium with application of filtering paper (on bridges) with 2% saccharose; 3) solid MC medium with 2% saccharose, 8g/l agar, 0.5 mg/1 indoleacetic acid, 0.5 mg/1 6- benzylaminopurine.

The investigations have shown rapid development of embryoid into sprouts took place under the agar nutrient medium containing hormones while under liquid cultivation conditions (on bridges) it was the root system that firstly developed and then sprout hypocotyl part began its development (fig. 3).

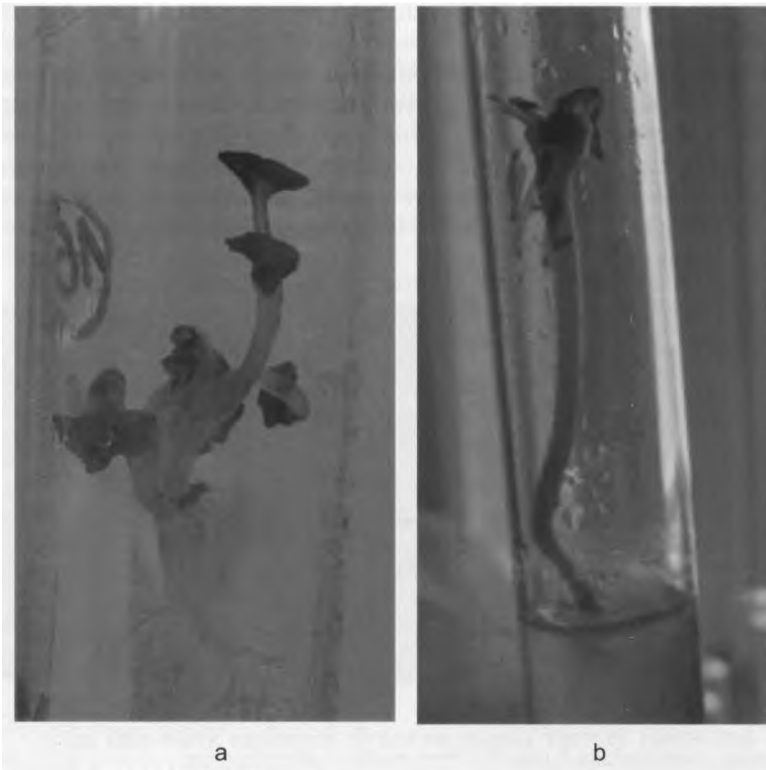

Fig. 3. Formation of plants under various media conditions: a) under liquid medium on bridges; b) under solid agar medium

Under long cultivation of plants in liquid medium it was observed the secondary formation of embryoids that developed from epidermal cellular layers of hypocotyls and petioles and as well as from bottom side of leaf plates of spouts (fig. 4) was observed.

Generated secondary embryoids have been aseptically taken with binocular magnifier and placed under inducting MC medium containing 2% saccharose, as well as 0.5 mg/16-benzylaminopurine, 0.5 mg/1 indoleacetic acid. Under these conditions embryoids gave sprouts than later have been replaced into soils for growth to obtain plant regenerants (fig. 5).

To have indirect demonstration of haploid chromosome set for obtained plant regenerants from isolated anthers of rapeseed chloroplasts calculation method for stomata closed cells has been used. It was experimentally identified that chloroplasts quantity in stomata

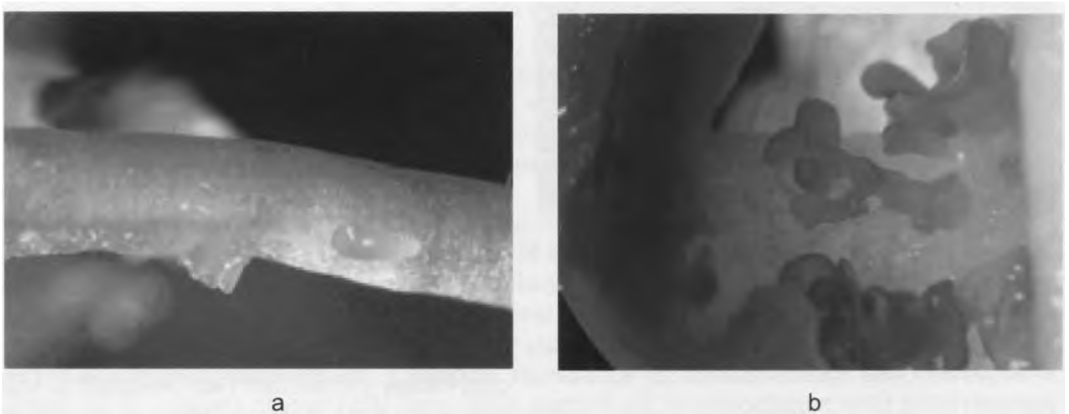

Fig. 4. Formation of secondary embryoids: a) on hypocotyls segment; b) on base of leaf plates

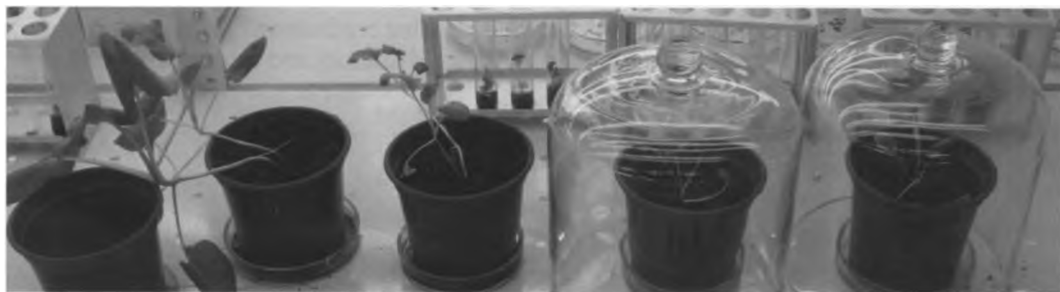

Fig. 5. Adapted plant regenerants

cells of a haploid plant amounted from 10 to 15 items, while initial donor plants had from 35 up to 45 items (fig. 6).

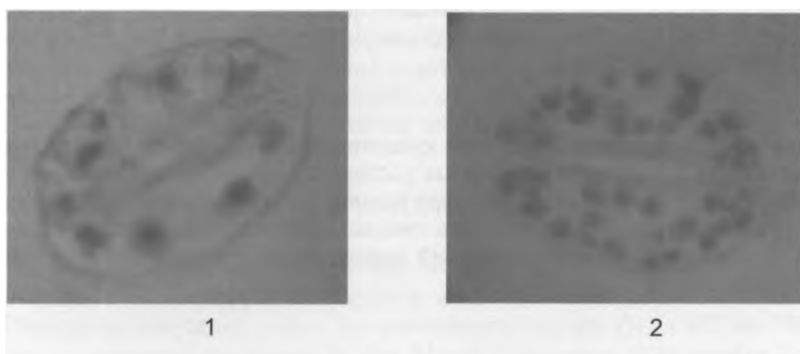

Fig. 6. Chroloplasts in stomata closed cells: **1** — haploid plants; 2 — dihaploid plants

Reduced quantity of chloroplasts was also observed in plants obtained from secondary and tertiary embroids.

Direct proof of haploid nature of rapeseed plants is cytological method of chromosomes calculation in root meristem. We have identified that plant regenerants obtained in isolated microspores had single set of chromosomes (n=19) (fig. 7).

Fig. 7. Rapeseed root meristem cells: a — haploid plants; b — diploid plants

Thus, according to our obtained data we have selected cultivation conditions providing development of rapeseed plant regenerants from isolated anthers and microspores and their haploid origin have been demonstrated.

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Аннотация. Оптимизированы условия культивирования изолированных пыльников, микроспор и завязей для получения гаплоидных растений рапса *in vitro.* Показано, что кинетин B_5 в питательной среде (1 мг/л) стимулировал процесс эмбриогенеза, а также образование как вторичных, так и третичных эмбрионов на гипокотилях и листовых сегментах стерильных проростков. Последующий цитологический анализ растений-регенерантов подтвердил их гаплоидное происхождение.