INDUCTION OF SPRING RAPESEED UNFERTILIZED OVULES (*BRASSICA NAPUS* L.) IN *IN VITRO* CULTURE

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Abstract

Analysis of the results of the studies of nutrient media showed that the addition of phytohormone 2,4-D increases the number of responsive ovules. The best responsiveness of ovules was gained in the variant where 6-BAP – 0.3 mg/l, NAA – 0.1 mg/l, kinetin – 0.4 mg/l, 2.4-D – 1.0 mg/l have been added, the callus induction frequency was 40.9%.

Keywords: *rapeseed, dedifferentiation, nutriculture medium, growth regulators, seed-bud ovule, ovule, callus tissue, genotype.*

Introduction

Haploid biotechnology in our time is becoming an indispensable component of various breeding programs. In this case, the main ad`vantage of haploidy is used - the possibility to obtain stable homozygous lines for 1-2 generations, which can be immediately assessed for the prospects for breeding practice. In addition, haploids significantly expand the genetic spectrum of the initial selection material: firstly, due to recombination during meiotic division during gametogenesis; secondly, due to mutations arising in the process of culture cells *in vitro*. Using haploids, recessive mutations can be easily detected, cell lines resistant to pathogens, salinization, high and low temperatures, and other stress factors can be selected.

Obtaining haploid plants in an *in vitro* culture from non-pollinated ovules is the result of the transition of germinal cell cells from the hematophytic pathway to the sporophytic pathway with the formation of embryoids or morphogenic callus from them [1].

In plants with male sterility, the cultivation of unfertilized ovules is the only way to get haploids that can be used for heterotic selection based on CMS [2].

Obtaining haploid plants from unfertilized (non-pollinated) ovules is one of the poorly studied areas of biotechnology of spring rape.

Improving the efficiency of obtaining plants from the ovules of spring rape in artificial nutrient media is an actual area of research.

Research methodology

In the researches used donor plants were grown in the field conditions. For introduction into the culture, buds 2-6 mm sized, located on central inflorescences, were used.

When introduced into the culture, a 7% *Domestos* solution was used as a sterilizing agent. The exposure time of the buds in an aqueous solution is 10-15 minutes. Then the buds were washed with sterile water 3-4 times and the ovules were isolated with sterile needles. Ovule cultivation was carried out in a nutrient medium Murashige and Skoog (MS) [3].

As growth regulators, 2,4-D (2,4-dichlorophenoxyacetic acid) was added to the composition of the nutrient medium at concentrations of 0.2, 1.0, 2.5, 5.0 mg/l, kinetin 0.4, 5, 0 mg/l, 6-BAP (6-benzylaminopurin) - 0.2, 0.3 mg/l and NAA (naphthylacetic acid) - 0.1 mg/l. As a control medium, MS nutrient medium with the addition of 6-BAP — 0.3 mg/l, IAA — 0.1 mg/l and Gibberellic Acid (HA) —0.1 mg/l was used [4].

Observations of the morphological state of the ovules were performed after 2 weeks during the entire cultivation period. Callus, dedifferentiated from the ovules, was transplanted into fresh medium until growth points formed, which were then transplanted onto a hormone-free MS medium for regeneration. Regenerants in the phase of 2-3 leaves with a well-developed root system were planted in a mixture of soil and sand (in a 3:1 ratio) and covered with plastic glasses. After the appearance of a new true leaf, the glasses were carefully opened, after the acclimatization of the seedlings, the glasses were removed.

In the flowering phase, fertile plants were isolated for selfing. Sterile (haploid) plants were cut and placed in an aqueous solution of colchicine at a concentration of 0.5%, in which they were kept for one day at room temperature.

After seed ripening, the plants were cut, threshed. Harvested seeds were propagated in a field or greenhouse.

When processing the experimental data, statistical methods were used according to Dospekhov B.A. [5], as well as the application package *Microsoft Excel* and *Statistica 6.0* on a personal computer.

Research results

The cultivation of unfertilized ovules (ovules) was carried out in test tubes with a nutrient medium, on the surface of which isolated ovules were placed from one pistil (Fig. 1).

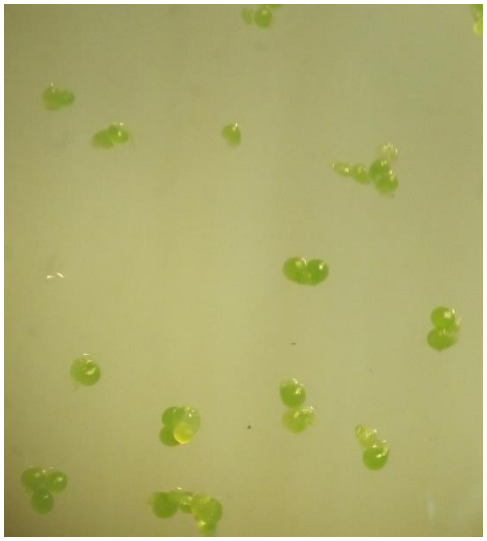


Figure 1 - Non-pollinated ovules in a nutrient medium

As a result of the cultivation of ovules, most of them underwent necrosis. Ovules responsive to dedifferentiation formed the following types of callus tissue: globular light green (Fig. 2 a), globular opaque (Fig. 2 b), rough green dense and rough transparent.

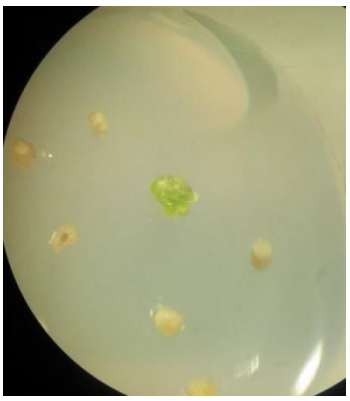
а  б 

Figure 2 - Globular callus: a) transparent green,
b) matte.

The globular tissue was slowly developing; at the end of the passage, the formation of green structures was observed, which, being transplanted onto a hormone-free medium, formed embryoids (Fig. 3).

Figure 3 - Morphogenic callus with embryoids.

The rough callus had a loose, rapidly growing granular mass. Transplantation on fresh medium did not contribute to the formation of morphogenic structures. Similar results were obtained from other authors [6, 7].

Table 1 presents the results of the cultivation of ovules on variants of the medium in which the frequency of induction of callus is better than the control variant.

Table 1 - Effect of growth regulators in the composition of the initial nutrient medium of MS on the induction of unfertilized ovules

Medium variant	The concentration of growth regulators, mg/l	The number of ovules		Frequency of responsive seed,%
		cultivated, pcs	responsive, pcs	
5	6- BAP – 0,2, NAA – 0,1	267	14	5,2
8	6- BAP – 0,2, NAA – 0,1, kinetin – 0,4	915	58	6,3
11	6- BAP –0,3, NAA – 0,1, kinetin – 0,4, 2,4-Д – 0,2,	2150	731	34
14	6- BAP – 0,3, NAA – 0,1, kinetin – 0,4, 2,4-Д – 1,0,	1306	534	40,9

15	6- BAP – 0,3, NAA – 0,1, kinetin – 0,4, 2,4-Д – 2,5,	753	244	32,4
18	kinetin -5,0, 2,4-Д – 5,0	808	264	32,7
the control	6- BAP – 0,3 mg/l, IAA – 0,1 mg/l, HA – 0,1 mg/l	340	15	4,2

The addition of 2,4-D to the medium increased the dedifferentiation of ovules and ovules. On the 6-7th day, a rapidly growing greenish callus of green color was formed from the seedstalks. When transplanting responsive ovules to a fresh medium, this callus was necessarily removed, since in the future it was difficult to distinguish it from callus obtained from ovules.

The best responsiveness of ovules was gained on the 14 variant; the frequency of callus formation was 40.9%. The differences between the 11 and 14 variants in the frequency of callus induction are insignificant ($НСР_{05}$ - 3.46). The frequency of formation of callus tissue from ovules in variants 11, 14, 15, 18 significantly exceeds the dedifferentiation of ovules cultivated on the control variant medium.

Thus, according to the results of the cultivation of ovules on the studied variants of nutrient medium, it was concluded that the addition of the phytohormone 2,4-D increases the number of responsive ovules.

Secondary structures (callus obtained from ovules and ovules synthesizing chlorophyll) were transplanted into MS medium with the addition of 6-BAP - 0.5 mg/l, kinetin 3.0 mg/l.

Some of the ovules continued to form callus tissue of rough or embryoidogenic types (Fig. 4a), while some formed solid green structures (Fig. 4b).

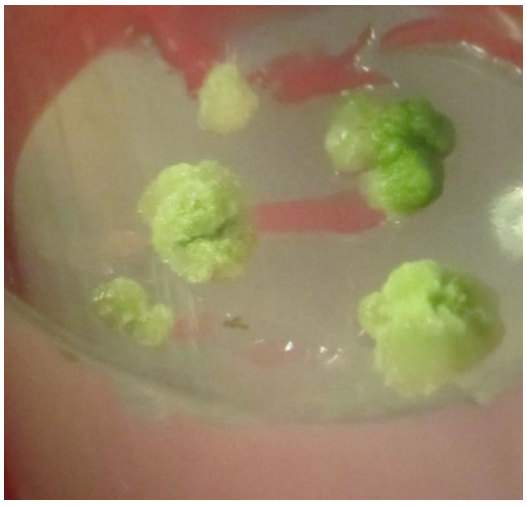
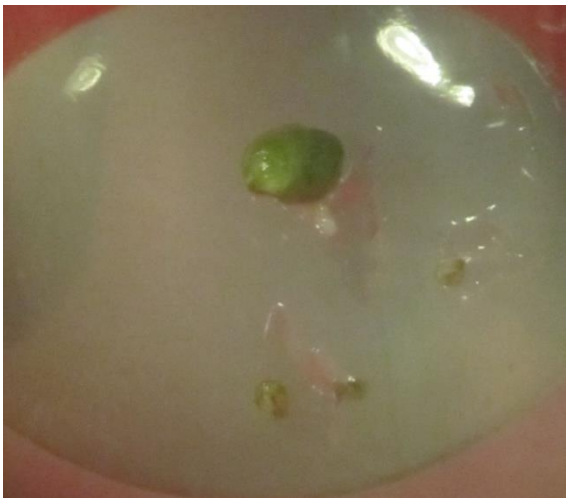
а  б 

Figure 4 - Cultivation of non-pollinated ovules in nutrient media:
a - callus tissue, dedifferentiated from the ovules; b - green ovule.

After two weeks of cultivation, the shell of green structures (ovules) was carefully damaged with a needle and transplanted into a hormone-free medium. At the end of the passage, leaflets appeared from the ovules, and then seedlings, from which the plants were obtained (Fig. 5)

a 

Figure 5 - Development of seedling from the ovule

Morphogenic callus cultivated in this medium formed embryoids and green dense structures, but most of the morphogenic callus perished.

Thus, from the analysis of dedifferentiation of unfertilized seeds, it was concluded that responsive ovules form different types of callus tissue, the addition of 2,4-D phytohormone to the nutrient medium increases the number of responsive ovules. The best responsiveness was obtained with the option with the addition of 6-BAP - 0.3 mg/l, NAA - 0.1 mg/l, kinetin - 0.4 mg/l, 2.4-D - 1.0 mg/l, frequency callus induction was 40.9%.

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