

Development of methodological procedures for culturing sunflower anthers *in vitro*

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Sunflower is one of the most important crops worldwide. Therefore, of much importance are the techniques which would allow accelerating the production of new lines and varieties with desired agronomic properties. One of such techniques is experimental haploidy involving the introduction of haploid plant cells (e. g. microspores) in culture and subsequent regeneration of plants and chromosome doubling. The current work reports the search for the optimum conditions for *in vitro* culturing of sunflower anthers. Among other parameters, the growing conditions for donor plants in the controlled environment of the growth chamber have been optimized. The optimal developmental stage of explants at which they may be introduced in culture has been determined as well as the explant pretreatment conditions. It has been demonstrated that both the sunflower plant genotype and the composition of the culturing medium, in particular the presence of auxins and cytokinins, and their combinations, have significant effect on the callus genesis in the anther and microspore culture *in vitro*. In the course of the study, the optimal composition of the culturing media was suggested, including the phytohormones concentrations, the latter being chosen individually for each sunflower genotype.

Keywords: sunflower; *Helianthus annuus* L.; anther culture; callus genesis; regeneration; culturing medium composition; α -NAA; 6-BAP; AgNO₃; genotype.

Abbreviations: FAO: Food and Agriculture Organization; EU: European Union; FASO: Federal Agency of Scientific Organizations; MS: Murashige & Skoog medium; α -NAA: α -Naphthaleneacetic acid; 6-BAP: 6-Benzylaminopurine.

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Introduction

Sunflower is the main oil crop in Russia and one of the four most important oil crops in the world. The oil extracted from sunflower is used in the food industry and for technical purposes. In last decades, there has aroused considerable interest in using sunflower oil for biofuel production.

According to experts, the demand for sunflower seeds (especially high-oleic) and oil is rising steadily in EU countries, while Russia and the countries of Eastern Europe are considered as the key suppliers of these products due to the shortage of land for growing sunflower in Western Europe [1].

One of the key components of competitiveness of the final breeding product is the speed and energy intensity of its production. The key end

product of the breeding program for the development of the so-called "heterotic" crop is the genetically pure line. The commonly used approach for creating such lines is forced self-pollination and selection of the most uniform offsprings during 6-8 generations. Experimental haploidy (*in vitro* cultivation of haploid plant cells on artificial nutrient media with subsequent doubling the chromosome number in the haploid plants regenerated from these cells) has significant potential for accelerating the breeding programs for hybrid industry. As a result, it becomes possible to obtain genetically pure lines in one year, or a year and a half, such lines by virtue of their complete homozygosity being perfectly fit to serve as parental lines for F₁ hybrids.

Haploid technologies are currently in the focus abroad. Research work concerned with the development of efficient methods for obtaining haploids is supported by such organizations as FAO (Food and Agriculture Organization of the United Nations) and the European Union. In particular, EU has established the program COST 851 "Gametic cells and molecular breeding for crop improvement". In the largest foreign breeding companies (Syngenta, Bayer, etc.) the production of doubled haploids is pipelined along with other biotechnology techniques.

In the Russian Federation, the research work aimed at producing doubled haploids of the main crops is currently performed by the laboratories, concentrated mostly in the FASO (Federal Agency of Scientific Organizations) research institutes. For example, positive results were obtained in the development of androgenic double haploid lines in rapeseed. However, *in vitro* haploid cells techniques are not used, and have never been used earlier, to obtain genetically stable sunflower lines with the purpose of developing the hybrids on their basis. First of all, this is because it is quite a challenging task to apply the gametic cell technologies to this plant species. Therefore, no works are available which would report the use of such techniques for this crop breeding in our country. On the

other hand, doubled haploids of this plant are in extremely high demand in various breeding institutions.

In foreign literature, there can be found few, and for the most part old, works reporting single successful attempts to regenerate sunflower plants *in vitro*, using anthers as explants. In particular, in 1994 the researchers from Pakistan noted the instability of such regeneration [2].

Sometime later, the group of Indian scientists stated *in vitro* regeneration in the anther culture of sunflower with the frequency of 8.3%, without, however, achieving the doubling of the chromosomes in the obtained regenerated plants. In 1998, they published the experimental protocol for callus induction with subsequent regeneration of *Helianthus annuus* L. plants. Murashige & Skoog (MS) medium was used as a basal medium. The authors studied the effects of phytohormones, α -Naphthaleneacetic acid (α -NAA) and 6-Benzylaminopurine (6-BAP), on callus genesis and embryogenesis from anthers. They have also demonstrated a statistically significant effect of the sucrose and agar-agar concentrations on callus genesis in the plants of the studied genotypes. The differences due to the incubation of explants in the dark vs. in the light, cold pretreatment of antheridia for 1 - 6 days prior to inoculation of anthers onto the surface of the nutrient medium, as well as the genotype of donor plants were shown to be insignificant in the discussed work. However, all these factors exerted statistical effects on the embryogenesis, when microcalli were transferred from different nutrient media to the medium containing 0.1 mg/L α -NAA and 0.5 mg/L 6-BAP with the frequency of callus genesis reaching 100% and the frequency of embryogenesis being as high as 44%. But even in the cases of embryo formation, the frequency of regeneration from these plants was rather low (14.3%). The researchers attempted to replace the embryogenic developmental pathway with morphogenesis to obtain multiple shoots. This was achieved by transferring the embryogenic microcalli with developing embryo-like structures on the MS

nutrient medium containing 0.5 mg/l 6-BAP. Elongated shoots were rooted on half-strength MS containing 0.5 mg/l α -NAA. Cytological analysis of the embryogenic callus and somatic embryos revealed the presence of haploids with the 30% frequency, while the rooted shoots produced haploid regenerated plants with the 8.3% frequency [3].

In 2011, the US Department of Agriculture launched the project "Doubled haploid production in sunflower", aimed at the development of a technique for the mass production of dihaploid sunflower lines using the anther (microspores) culture *in vitro* [4].

The results of the project have not been reported to the global scientific community, nor were the experimental protocols, culturing media formulations, and etc.

On the other hand, the interest in this issue is still acute due to huge technological advantages, which, if successful, the doubled haploid production scheme may bring to hybrid sunflower industry. For example, at the 19th International Sunflower Conference held in last May-June, few attempts to achieve plant regeneration in the anther culture of sunflower *in vitro* were reported.

In particular, the Turkish scientists from Trakya University (Edirne, Turkey) investigated the effect of different cytokinins and auxins on sunflower androgenesis. Parental lines of hybrids were used as material for research. Anthodia were sterilized, and the anthers taken from the anthodia of different size were surface sterilized and placed on the Murashige & Skoog nutrient medium with agar. The effects of four concentrations (0, 0.5, 1.0, and 2.0 mg/l) of α -NAA and 6-BAP were studied. Anthers were cultured in darkness or with photoperiod. The studies have shown that mononuclear microspores are contained in the flowers with the length of 3 – 4 mm.

When culturing the anthers under photoperiod conditions, the maximum percentage of callus genesis (87%) was observed on the medium containing 2.0 mg/l α -NAA and 1.0 mg/l 6-BAP, whereas when cultured on the same induction medium, but in the darkness, the percent of callus genesis was higher (90%). The phytohormones had no effect on the androgenesis when used separately, but when introduced together to the nutrient medium in the increasing concentrations, their stimulating effect on androgenesis gradually increased [5].

Another group of Turkish scientists subjected sunflower anthers containing microspores at the late mononuclear stage to osmotic stress (0.5 M mannitol) with subsequent optimization of the qualitative and quantitative composition of phytohormones to stimulate embryogenic (morphogenic) processes in callus tissues [6].

The success of the Serbian scientists turned to be very promising as they managed to achieve the formation of callus masses, organogenesis (formation of roots, shoots), and even embryogenesis, and regeneration of plants (with low frequency) in the anther culture of sunflower *in vitro* [7].

The Ukrainian scientists from the Yur'ev Crop Research Institute, Kharkov, studied the capacity for androgenesis among the cultivated sunflower *Helianthus annuus* L. lines (X114B, X526B, X711B, X720B, X762) and wild species *H. divaricatus* L., *H. giganteus* L., *H. microcephalus* L., *H. nuttallii* L., *H. decapetalus* L. According to the authors, sunflower genotype proved to be a critical factor of regeneration. The highest regenerated plant producing capacity was detected in *H. giganteus* L. Anthers at the appropriate stage of development were isolated from the flowers under a binocular microscope in aseptic conditions of the laminar box and placed in the tubes onto the induction media. The induction media was based on the MS medium. The number of chromosomes was calculated on temporary squash preparations of cauline regenerants by the standard method.

After 30 days of culturing, regenerated plants were transferred to the new medium containing 3.0 mg/l α -NAA. The largest number of cauline regenerants was obtained from the anthers of *H. giganteus* L. The other samples cultivated on the regeneration medium showed the presence of calli of differing density. Further cultivation allowed to obtain cauline shoots only in the case of *H. giganteus* L. and *H. decapetalus* L. Chromosomes counting confirmed that regenerated plants were haploid. Rhizogenesis was observed only in *H. giganteus* L. and *H. decapetalus* L. [8].

The aim of the research work carried out by the scientists from the Saratov State Agrarian University was to study the influence of marker genes and the composition of the nutrient medium on the effectiveness of androgenesis in the culture of sunflower anthers *in vitro*. The donor plants of the studied genotypes were grown in the field. In order to check the correspondence between the morphological criteria of the size of the calathide and the size of the flowers at the optimal stage of vacuolated mononuclear microspores, a cytological analysis was performed under a microscope on squash preparations stained with acetocarmine according to the method of Pausheva [9]. Analysis of the neoplasms obtained with the anthers was carried out on the 30th day of cultivation. The authors convincingly demonstrated that the efficiency of callus genesis depended on the carbohydrate composition of the nutrient medium and the effect of the genotype.

The use of sucrose in a concentration of 30 g/l compared with maltose for 3 years on average significantly increased callus genesis in the studied lines: for the standard line by 61%, for the remaining lines by 31 to 62%, or by 42% on average. Screening of the lines on a medium supplemented with 30 g/l of sucrose showed that all four lines significantly exceeded the callus output of the standard line by 7 to 21%, or by 14% on average. On a nutrient medium with the addition of maltose, one line significantly

exceeded the standard by 9.33%, the remaining lines were at the level of the standard [10].

It should be noted that there have never been developed any techniques for the mass production of doubled haploids of sunflower from the anther culture *in vitro*, which are essential for the production of F1 hybrids in breeding programs. In view of this, any such technique would be obviously innovative. It will at least 10 times increase the intensity of breeding process and more than 2 times reduce the duration of the breeding process (and hence the entry to the seed market) when creating F1 sunflower hybrids with new agronomical properties.

In the view of this, the main objective of the current research was to develop methodological procedures for the cultivation of sunflower anthers *in vitro*. Based on the purposes of this research, the following tasks have been formulated:

1. selection of the optimal growth conditions for sunflower donor plants in the controlled environment of growth chambers;
2. identification of the optimal stage for explants (anthers) collection for further introduction in culture;
3. optimization of the explant sterilization methods;
4. adjustment of the cultivation conditions and formulation of the culturing media for the *in vitro* cultivation of sunflower anthers.

Material and methods

The material for research was two commercial hybrids (Orpheus and Oracle), and the plants from seven F2 segregating populations obtained by cross-breeding sunflower lines from the working collection of the Agroplasma Breeding and Seed Company nos. 6 p, 189, 67, 85, 12, 00NI, NK.

Donor plants of the studied genotypes were grown in the controlled environment of the

growth chambers at the temperature of 20-22°C with 40% humidity, and 12-hour photoperiod. From three to five days prior to anther collection, the temperature was lowered to 17°C.

Cold pretreatment of explants

Before planting anthers on the nutrient media, the cut antheridia were incubated for 1 – 6 days at 10°C.

Sterilization of explants

The antheridia were washed with warm water, surface sterilized for 15 min in 30% Belizna commercial bleach preparation, and then washed again with sterile distilled water for three times.

Nutrient medium for the cultivation of anthers

To inoculate sunflower anthers, basal Murashige & Skoog medium with agar was used [11], with varying qualitative and quantitative content of phytohormones α -Naphthaleneacetic acid (α -NAA) and 6-Benzylaminopurine (6-BAP) as well as AgNO_3 .

Statistical processing of data

The data was processed statistically using the MSTAT-C program [12], Statistica [13], and Microsoft Excel [14]. In the tables, in most cases, the data is presented as averages with a standard error. The essential difference was assessed by the method of one-factor or two-factor analysis of variance, and also using the LSD criterion [15].

Cytological analysis

The cytological analysis was performed under a microscope on squash preparations stained with acetocarmine according to the method of Pausheva [9].

Staining with acetocarmine without fixation in acetic alcohol was performed as follows. A fresh anther was placed on a slide in a drop of acetocarmine, and one of its ends was cut off with a razorblade. Holding the anther with tweezers, a needle was taken from the tweezers

to the cut off end of the anther, squeezing out its contents. After removing unnecessary tissues, the preparation was covered with a cover slip and gently heated using a spirit lamp.

Staining with acetocarmine with fixation in acetic alcohol (squash method) was performed as described below. Prior to staining, anthers were fixed in acetic alcohol (3:1), and pollen grains then were stained with acetocarmine. The fixative (acetic alcohol) contained CH_3COOH and $\text{C}_2\text{H}_5\text{OH}$ in the ratio of acetic acid to alcohol as 3:1. To obtain the staining solution, 1 g of carmine was dissolved in 45 ml of glacial acetic acid and 55 ml of distilled water and the mixture was incubated in a water bath for 1 hour.

Preparation and staining of anthers included the following steps:

1. Material (anthers) fixing in the acetic alcohol for a day at low temperature.
2. Washing in 70% alcohol for 15 minutes (the material can be further stored in 70% alcohol).
3. Washing in distilled water for 15 minutes.
4. Staining in acetocarmine for 15-20 minutes;
5. Short washing.
6. Maceration in 45% acetic acid from 1.5 to 2 hours. Under the cover glass, the stained and macerated anther splits into one layer of cells.

The state of nuclei in the pollen grains was also examined *in vivo*, by placing fresh pollen in a 5-10% sucrose solution. In the field of vision, in a light or dark field, under a microscope, there can be seen the tetrads of microspores, mononuclear pollen grains, first mitoses, generative cells, and the vegetative core.

Results

Identification of the stage at which explants are selected for the introduction into the *in vitro* culture

The stage of development of the anther and pollen used to initiate callus-, embryo-, and

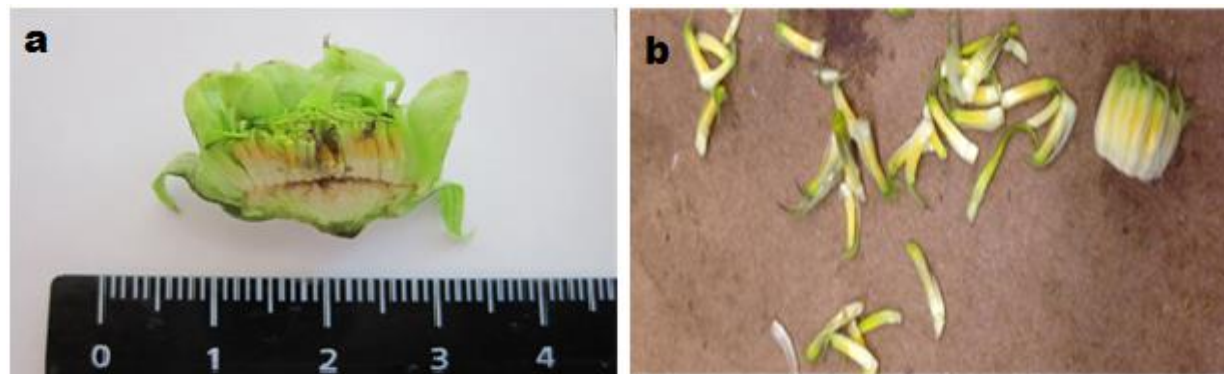


Figure 1. Identification of the optimal stage of development for explants collection for further introduction in culture. (a) Optimal size of sunflower anthodia for the *in vitro* cultivation of anthers; (b) Sunflower disk flowers before the anther introduction in the *in vitro* culture.

morphogenesis is very important. It is believed that the success in obtaining haploid structures depends much on the appropriate selection of the developmental stage. This stage is critical [16].

In this period, the microspores in the sunflower are at the mid-to-late mononuclear stage. In this view, for anther cultivation, the anthodia were collected immediately after the opening of the marginal sexually sterile ray flowers, but prior to the opening of the outer disk flowers.

For each target sunflower genotype, the size of anthodium should be chosen experimentally by visual parameters. The morphological criteria, which have to be considered, are the size and color of disk flowers. In our experiments, it was found that for the plants cultivated in growth chambers, the optimal anthodium size for *in vitro* cultivation of anthers ranged from 10 to 50 mm in the studied sunflower genotypes, in individual plants of segregating F_2 populations it was up to 50 mm (in hybrid plants) (Figure 1a). The disk flower was of the light-yellow color (Figure 1b).

Effects of the quantitative and qualitative composition of phytohormones on callus genesis and regeneration processes during the *in vitro* cultivation of the anthers of sunflower hybrids

In the current work, we studied the effects of the quantitative and qualitative composition of phytohormones, 6-BAP, α -NAA, and AgNO_3 (silver nitrate) on callus genesis and regeneration processes during the *in vitro* cultivation of the anthers of Oracle and Orpheus sunflower hybrids. The following concentrations of the indicated substances were used: 6-BAP: 0.5, 1.0, and 2.0 mg/l; α -NAA: 1.0 and 2.0 mg/l; AgNO_3 : 0.0, 2.0, 4.0, and 6.0 mg/l.

Our experiments demonstrated that callus genesis in the Orpheus hybrid was at the same level when the nutrient medium variants with either 0.5 mg/l or 1.0 mg/l 6-BAP were used. Optimum concentrations of 6-BAP for this hybrid were found to lay within the range from 0.5 mg/l to 1.0 mg/l. For the Oracle hybrid, the concentration of this phytohormone optimal for callus genesis stimulation is 0.5 mg/l.

The increase in the 6-BAP concentration in the callus inducing nutrient medium up to 2.0 mg/l had a negative effect on callus genesis in both studied hybrids (table 1).

Analysis of the data on the use of α -NAA showed that the increase in this substance concentration led to the increase in callus genesis in the Oracle hybrid. The Orpheus hybrid showed a slight reduction of this parameter, which indicates that it is necessary to choose the appropriate

Table 1. Callus genesis in the *in vitro* anther culture of sunflower hybrids in the presence of 6-BAP.

Hybrid	6-BAP (mg/l)	Mean value (%)	Error of mean	Min ÷ max
Orpheus	0.5	34.95	6.17	22.52÷47.38
	1.0	30.10	6.17	20.67÷45.53
	2.0	18.58	4.36	9.79÷27.36
Oracle	0.5	32.20	6.17	19.77÷44.63
	1.0	22.57	5.04	12.42÷42.71
	2.0	12.75	4.36	13.96÷41.54
LSD				
P= 0,05 level		2,90		

concentrations of this auxin with respect to a particular genotype (table 2).

Table 2. Callus genesis in the *in vitro* anther culture of sunflower hybrids in the presence of α -NAA.

Hybrid	α -NAA (mg/l)	Callus genesis (%)
Orpheus	1.0	28.6
	2.0	25.5
Oracle	1.0	24.4
	2.0	31.1
LSD		
P= 0,05 level		1,18

Effect of silver nitrate concentration on the callus genesis in sunflower hybrids

The effect of silver nitrate on callus genesis and regeneration in the sunflower hybrids was studied for three different concentrations: 2.0, 4.0, and 6.0 mg/l (table 3).

Analysis of variance made it possible to assess the effects of AgNO_3 concentration and genotype on callus genesis. The maximum callus genesis was observed with the silver nitrate concentrations of 2.0 mg/l in the case of the Oracle hybrid and 4.0 mg/l in the case of the Orpheus hybrid.

For the cultivation of anthers of the sunflower plants of the segregating F_2 populations, eight variants of nutrient media based on MS with

different phytohormone ratio, as well as with and without casein hydrolysate, were tested. The anthers were not responsive to the composition of the first four variants of the culturing medium (nos. 1-4).

Table 3. Callus genesis in the *in vitro* anther culture of sunflower hybrids in the presence of silver nitrate (AgNO_3) in the nutrient medium.

Hybrid	AgNO_3 (mg/l)	Callus genesis (%)
Orpheus	0.0	6.3
	2.0	7.3
	4.0	20.0
	6.0	6.9
Oracle	0.0	7.1
	2.0	13.3
	4.0	9.7
	6.0	7.3
LSD		
P= 0,05 level		2,52

Due to the weak inducing effect of these media, we didn't observe microspore divisions, and hence, there was no callus formation.

The following variants of the nutrient media composition were tested:

Variant 1: MS + 0.02 mg/l α -NAA + 0.01 mg/l BAP + 30.0 g/l sucrose + 7.0 g/l agar;

Variant 2: MS + 0.1 mg/l α -NAA + 0.2 mg/l 2,4-D + 0.5 mg/l 6-BAP + 30.0 g/l sucrose + 7.0 g/l agar;

Table 4. Callus genesis in the *in vitro* anther culture of the sunflower plants from the segregating F₂ populations on different nutrient media.

Genotype	Nutrient medium No. 5		Nutrient medium No. 6		Nutrient medium No. 7		Nutrient medium No. 8	
	Callus genesis (%)	Morphogenic callus (%)	Callus genesis (%)	Morphogenic callus (%)	Callus genesis (%)	Morphogenic callus (%)	Callus genesis (%)	Morphogenic callus (%)
6 p	66.7	-	66.7	-	80.0	20.0	46.7	20.0
189	10.0	-	40.0	-	23.3	6.7	53.3	3.3
67	33.3	-	23.3	-	13.3	-	16.7	-
85	8.3	-	21.7	3.3	11.7	-	13.3	5.0
12	32.8	-	42.8	0.56	43.3	0.56	66.7	1.7
00 NI	23.3	6.67	30.0	-	55.0	-	58.6	25.0
NK	38.4	1.1	36.0	3.8	30.7	19.6	35.1	3.3

Variant 3: MS + 0.1 mg/l α -NAA + 0.1 mg/l 6-BAP + 0.1 mg/l gibberellic acid + 30.0 g/l sucrose + 7.0 g/l agar;

Variant 4: MS + 0.5 mg/l 2,4-D + 30.0 g/l sucrose + 7.0 g/l agar.

The most significant results were obtained with the following four (nos. 5-8) variants of the media with MS salts and added auxin and cytokinin growth regulators and casein hydrolysate (table 4).

The nutrient medium composition in these four variants were as follows:

Variant 5: MS + 2.0 mg/l α -NAA + 2.0 mg/l 2,4-D + 0.5 mg/l 6-BAP + 250 mg/l Casein hydrolyzate + 30.0 g/l sucrose + 7.0 g/l agar;

Variant 6: MS + 0.1 mg/l α -NAA + 0.2 mg/l 6-BAP + 30.0 g/l sucrose + 7.0 g/l agar;

Variant 7: MS + 500 mg/l Casein hydrolyzate + 2.0 mg/l α -NAA + 1.0 mg/l 6-BAP + 30.0 g/l sucrose + 7.0 g/l agar;

Variant 8: MS + 0.1 mg/l α -NAA + 0.5 mg/l 6-BAP + 30.0 g/l sucrose + 7.0 g/l agar.

There were planted 7,605 anthers in total. The average percentage of callus genesis was 31.6% with the percentage of regeneration of 2.4%.

It was found that callus genesis in the studied F₂ populations of sunflower differed significantly, which indicated that callus formation depends on the studied genotype.

The sample No. 6 p was characterized by the maximum responsiveness, while the sample No. 85, by the minimum responsiveness. No. 6 p was also superior to all other samples by the mean value. The wide confidence interval in the No. 00NI population indicated that the careful selection of the inducing environment and cultivation conditions is required (Figure 2).

Evaluation of the morphogenic potential of the callus obtained in the anther culture, suggested that the studied populations are characterized by about the same regeneration capacity. Only the sample No. 67 may be distinguished as having the maximum number of morphogenic calli and the NK sample, as having the minimum number of morphogenic calli (Figure 3).

The narrow confidence interval for the populations Nos. 67 and NK reliably indicates the effect of genotype on callus genesis and regeneration.

The analysis of the effect of nutrient medium composition on callus genesis in the anther

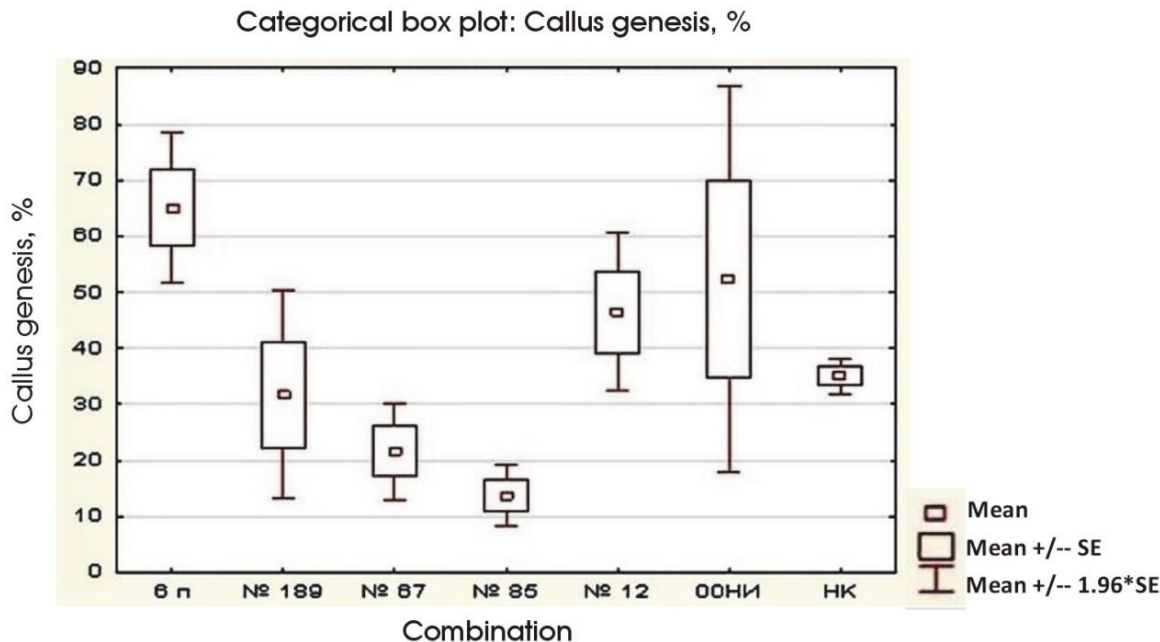


Figure 2. Effects of sunflower genotype on callus genesis.

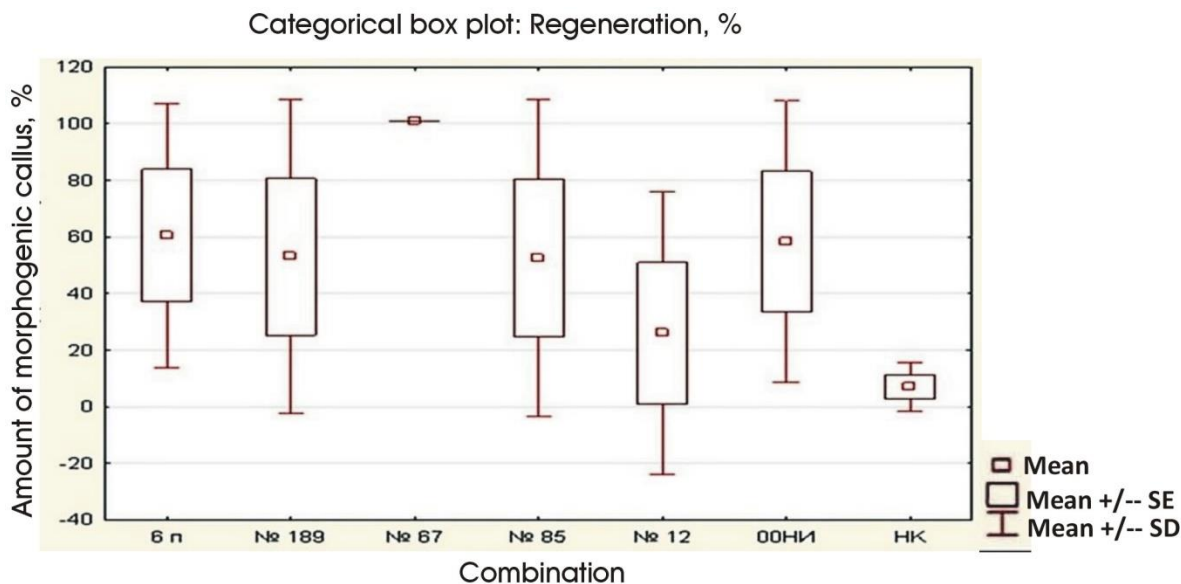


Figure 3. Effects of sunflower genotype on regeneration.

culture of sunflower hybrids, revealed that the differences in the stimulation of callus genesis were insignificant. The inducing medium variant no. 8 (MS + 0.1 mg/l NAA + 0.5 mg/l 6-BAP) proved to be the best for callus proliferation, with the average callus genesis rate exceeding

the corresponding average values for other variants of the nutrient medium (Figure 4).

When analyzing morphogenic processes depending on the variant of the nutrient medium used it was found that the composition

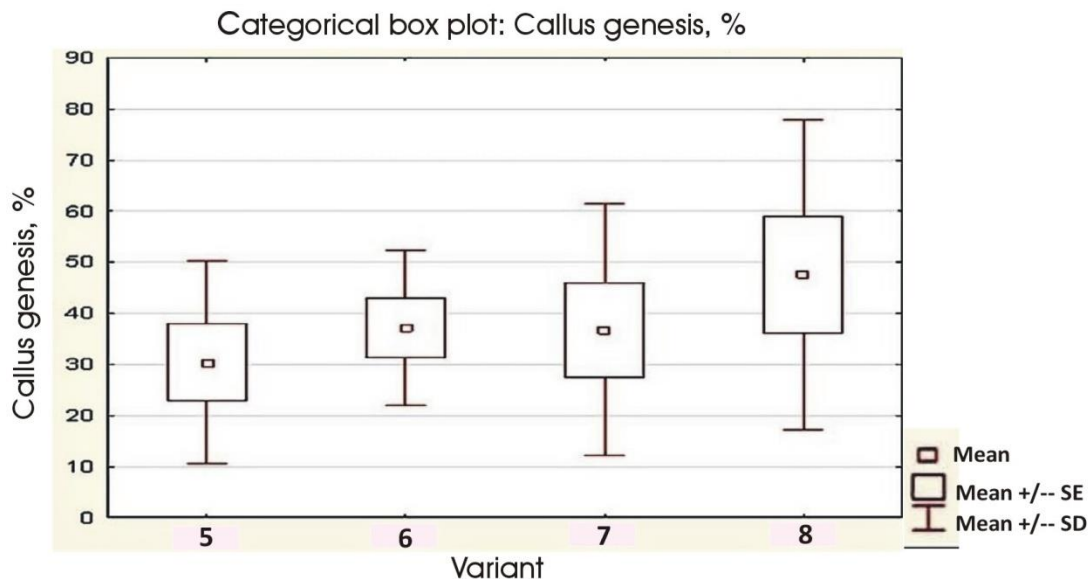


Figure 4. Dependence of callus genesis on the nutrient medium composition.

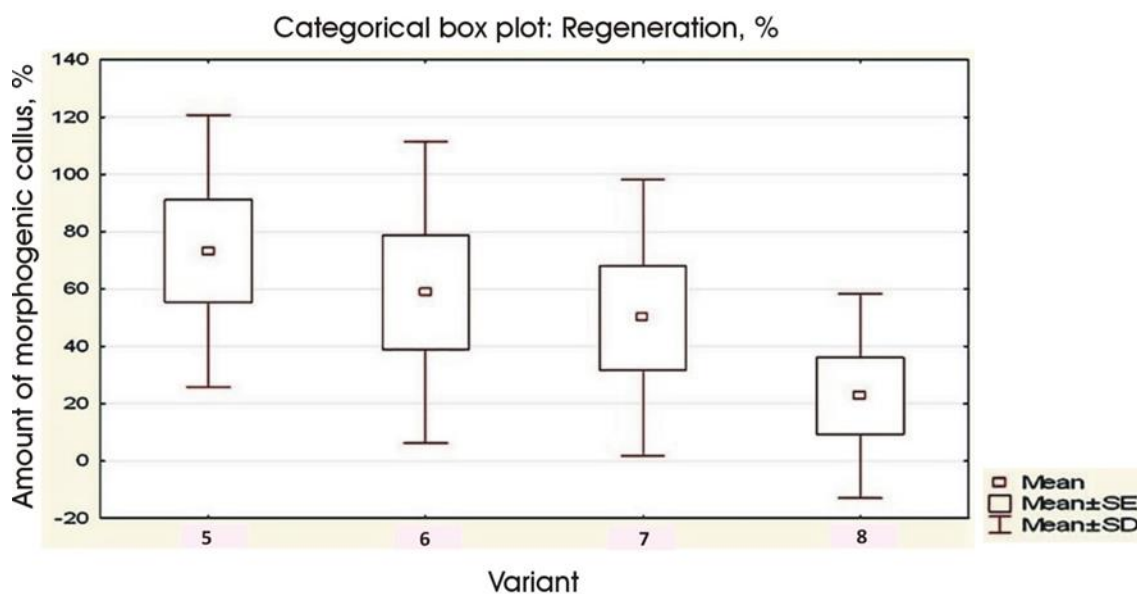


Figure 5. Dependence of regeneration on the nutrient medium composition.

of the variant no. 5 is more preferable than that of the variant no. 8. No significant differences between the variants nos. 5, 6, and 7 were identified, although the average morphogenesis rate in the case of the 5th variant was higher, which implies that this variant is universal for all genotypes. The variation is higher in the variant

no. 6, which indicates its greater specificity (Figure 5).

The callus morphotypes (6/1 - 6/7) obtained during the cultivation of the anthers of the studied sunflower genotypes are presented in Figure 6 (Figures 6a- 6g).

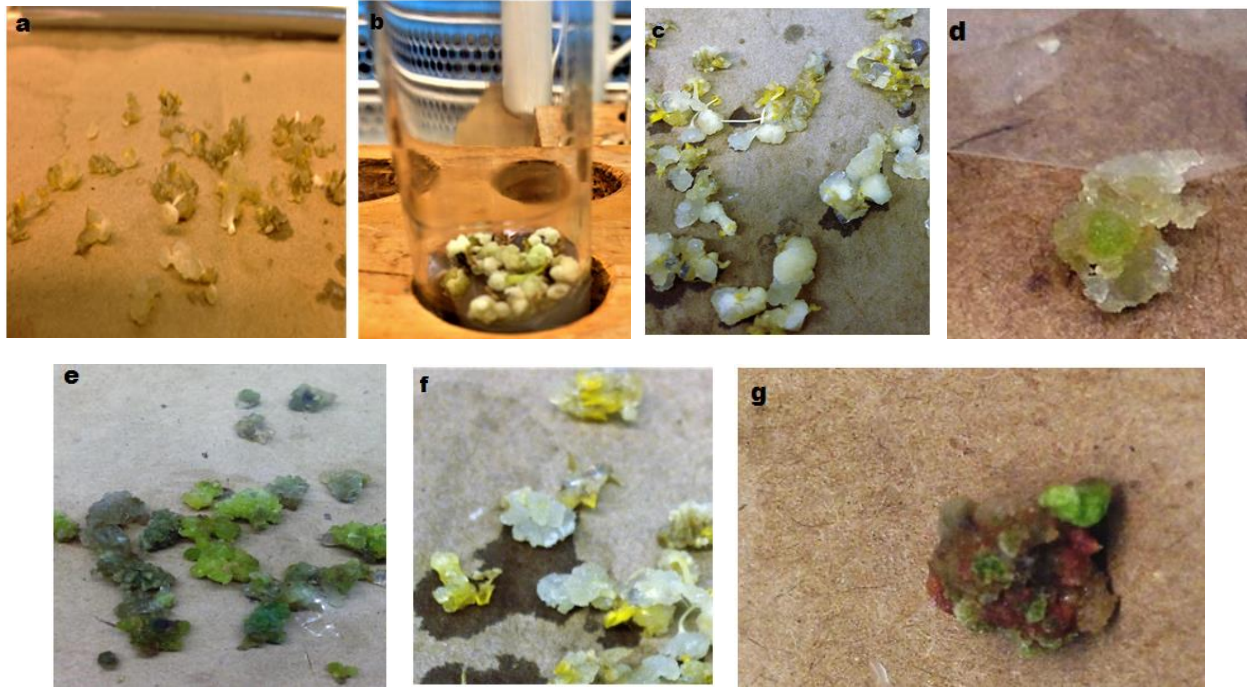


Figure 6. Morphotypes of sunflower callus obtained in the course of sunflower anthers cultivation in different variants of the agar nutrient media. (a) 6/1; (b) 6/2; (c) 6/3; (d) 6/4; (e) 6/5; (f) 6/6; (g) 6/7.

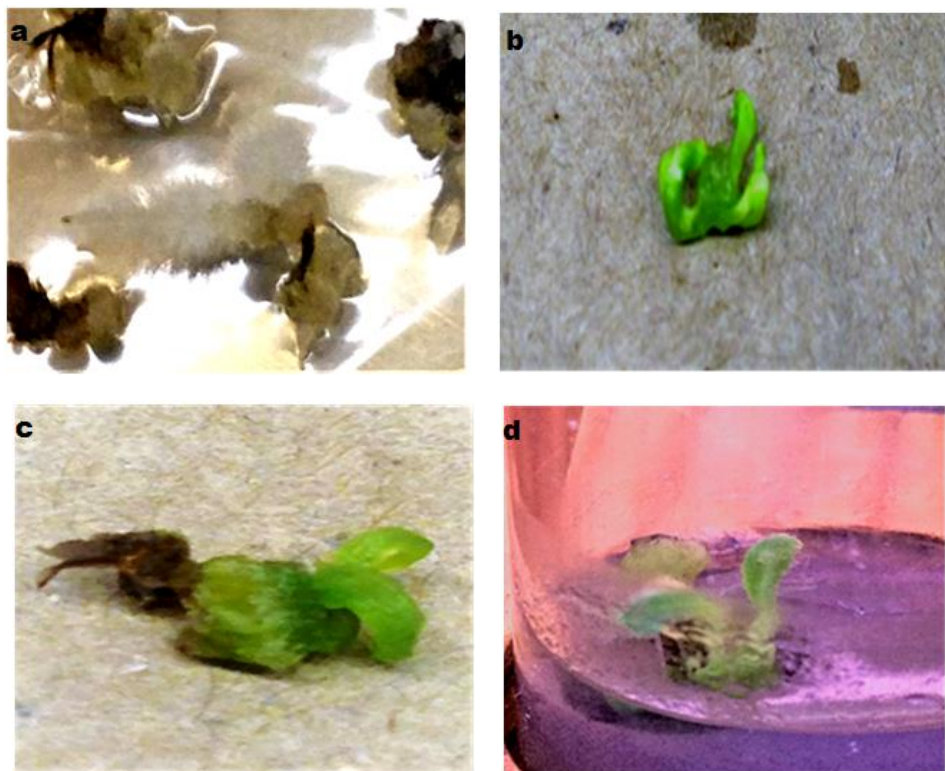


Figure 7. Regeneration processes in the obtained callus masses. (a) Rhizogenesis; (b) Regenerated plant without a root system; (c) Regenerated sunflower plant with a root; (d) Androgenic regenerated sunflower plants.

According to their morphogenic or non-morphogenic properties, the calli of different types were described as follows:

- 6/1. Watery transparent, yellowish-brown, non-morphogenic;
- 6/2. Dense, globular, white, morphogenic;
- 6/3. Combined, watery, transparent with dense white impregnations, partially morphogenic;
- 6/4. Watery, transparent, yellowish with zones of meristem cells, morphogenic;
- 6/5. Dense, matte, green, morphogenic;
- 6/6. White, transparent, non-morphogenic;
- 6/7. Red (pigmented with anthocyanins), morphogenic.

Regeneration processes which were observed in the obtained callus masses are presented in the Figure 7.

Discussion

The positive effect of the following concentrations of auxins, cytokinins and substances with a hormone-like action on the rate of callus genesis from the anthers of the studied sunflower genotypes has been revealed experimentally: 2.0 mg/l of α -NAA, 0.5-1.0 mg/l 6-BAP, and 2.0-4.0 mg/l AgNO_3 .

According to the results, nutrient media of the following composition are optimal for the cultivation of anthers of the two studied sunflower hybrids:

The first variant: MS + 2.0 mg/l α -NAA + 1.0 mg/l 6-BAP + 30.0 g/l sucrose + 7.0 g/l agar;

The second variant: MS + 2.0 mg/l α -NAA + 0.5 mg/l 6-BAP + 2.0 mg/l AgNO_3 + 30.0 g/l sucrose + 7.0 g/l agar.

When studying callus genesis in the F_2 sunflower populations its strong dependence on the genotype was observed.

Thus, the obtained experimental data revealed a significant influence of both the genotype and

the composition of the nutrient medium on the morphogenic processes in the anther culture of sunflower genotypes studied in this work.

In our studies, the morphogenetic potential of the obtained callus lines was realized in the following types of organogenesis (rhizogenesis, gemmogenesis, gemmorhizogenesis) (Figure 7):

- organogenesis by the type of rhizogenesis - root formation (Figure 7a);
- organogenesis by the type of gemmogenesis - the formation of the bud and leaf-like organs without a root (Figure 7b);
- organogenesis by the type of gemmorhizogenesis - formation of the bud and root.

The process of gemorhizogenesis in this case consisted of two stages: first, in the morphogenic callus, a bud was formed on the surface of the morphogenetic focus, then within the callus, the root was endogenously formed (Figure 7c).

Formation of the roots occurred in the basal (at the base) or in the middle part of the callus at the varying distance from the surface of the morphogenetic focus and in different locations relative to the bud. The degree of development of the buds was rather high. Exogenously on the surface of the morphogenetic foci, primordia of the first leaves occurred, which gradually developed into the first leaves. After the formation of the root meristem, the leaves gradually formed in the bud. With the development of the buds and roots between them, a connection is gradually established by the formation of the vascular system elements in the callus.

Well-developed buds, combined with a root into a single system, were then transferred to MS nutrient medium without hormones, in order to obtain regenerating plants. As a result, single regenerants were obtained, formed by the type of gemmorhizogenesis (Figure 7d).

As a general conclusion, it should be noted that in most cases, the cells of the sunflower

morphogenic callus showed weak regenerative capacity. Further careful selection of nutrient media components is necessary to induce regeneration processes.

Conclusions

To summarize, we have optimized the following parameters of *in vitro* cultivation of the anthers of the two locally adapted Russian sunflower hybrids Orpheus and Oracle (the originator – Agroplasma Company) and seven segregating F2 populations obtained by cross-breeding the sunflower lines from the working collection of the Agroplasma Breeding and Seed Company, LLC (Krasnodar):

- growing conditions for donor plants;
- optimal development stage of explants and their pretreatment;
- nutrient medium composition (to obtain actively proliferating callus mass, it is recommended to include the combination of phytohormones 6-BAP and α -NAA in the nutrient medium).

We have obtained morphogenic callus and single regenerant plants. It was demonstrated that morphogenesis in the anther and microspore cultures is affected by different factors. First and foremost, the success of cultivation of anthers and microspores depends on the nutrient medium composition. Among the hormonal factors, the primary role in the differentiating of morphogenesis is played by auxins, cytokinins, and their combinations. The important role is played by the plant genotype; therefore, it is necessary to apply an individual approach for each specific hybrid, varying the concentrations of phytohormones used.

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