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EFFICIENCY OF *IN VITRO* ANDROGENESIS IN ANTHER CULTURE OF BREAD WINTER WHEAT (2021-2023)

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Summary. Androgenesis *in vitro* in the anther culture of 105 genotypes of soft winter wheat of different genetic origin was studied. Differences regarding the frequency of callusogenesis induction (from 0.17 to 21.25 percent of planted anthers) and the ability to regenerate plants (from 0 to 4.94 percent of planted anthers) were found. The dihaploid lines in anther culturefor almost 50% of the studied genotypes were obtained. Spontaneous diploidization averaged 33.73 % of the obtained regenerants. 168 dihaploid lines of bread winter wheat were obtained.

Keywords: dihaploids, wheat, androgenesis in vitro, callus, regeneration.

Introduction

Conventional crossbreeding programs were the dominant tool in wheat breeding until recently. Modern biotechnological methods make it possible to increase the efficiency of selection and speed up the selection process. These methods include *in vitro* androgenesis (anther culture) and remote crosses. Anther culture has many advantages for cultivated plant researchers. One of the most important advantages is obtaining a homozygous line within one generation. The rapid spread of doubled haploid (DH) lines provides an opportunity for rapid selection of recessive alleles. Also, DH makes it possible to obtain new forms of wheat, resistant to various adverse factors, in the shortest possible time and without the use of large sown areas [5, 10, 13, 18, 19]. In addition, *in vitro* anther culture can be further combined with other valuable biotechnological methods such as marker-assisted selection (MAS), QTL analyses, genetic transformation or induced mutation to achieve desired selection and research goals [4, 12, 14, 15, 17].

The efficiency of obtaining dihaploid lines in the culture of bread wheat anthers strongly depends on the genotype, which does not make it possible to ensure predictability of results when working with any genotype. All this prompts researchers to search for possible activation of morphogenetic competence in in vitro conditions [5, 7-10].

The aim of the research was to evaluate the effectiveness of single stages of androgenesis for the creation of homozygous dihaploid lines of bread winter wheat in the anther culture *in vitro*.

Materials and methods

Research material was provided by the Department of Wheat Breeding and Seed Production, the Department of Wheat Breeding and the Department of Phytopathology of the National Institute of Entomology of the PBGI-NCNS. 105 selection samples (varieties, parental selection lines, F_1 hybrids and F_4, F_8 populations) were used to obtain dihaploid lines of bread winter wheat. This material were obtained according to two breeding directions: 1) – it is characterized by complex resistance to brown and stem rust [2, 3], and 2) – it is the result of a breeding program for obtaining high-protein wheat genotypes:

2021 year: 43 breeding samples of various origins, distinguished by resistance to brown and stem rust; 2022 year: 25 breeding samples (11 parental lines and 14 F_1 hybrids) distinguished by resistance to brown and stem rust; 13 breeding hybrids of various origins, which are the result of a

selection program for obtaining high-protein genotypes from crosses of wheat with stable forms, carriers of the GPC-B1 gene: from *Aegilops tauschii* (line 3238;) and from *Triticum dicoccoidles* (lines 9225; 9300), with subsequent selection and saturating crosses). 2023 year: 13 breeding lines with increased protein content; a line carrying the GPC-B1 gene; 5 F_1 hybrids: Kuyalnik x GPC-B1, Optima x GPC-B1, Kantata x GPC-B1, Er.261 x 1944-1, Er.261 x 1944-2; and three F8 populations under № 800, 900, 1001, obtained from the crossing of Odeska 267 bread winter wheat with original amphiploids (2n=42, AABBStSt), previously isolated from the combination – *T. durum* "Chornomor" x *Elytricum fertile* (material provided leader researcher of General and Molecular Genetics, Ph.D. Ivan Motsnyi.

As a method for obtaining doubled haploids (DH) of wheat, *in vitro* culture of isolated anthers was used. Donor plants of winter bread wheat were grown in paddy fields at the experimental plots of the PBGI-NCSCI in Odesa, Ukraine. The ears were cut when vacuolated microspores of most of the anthers were in the early and middle-late uninucleate stage. The cytological control of the development stage of microspores in anthers was conducted by preparing temporary micropreparations of anthers stained with acetocarmine (4%) under a light microscope (Axiophot, Opton, West Germany). The cut ears in the covering leaf were placed in water solution of abscisic acid (ABA) at a concentration 0.5 mg L^{-1}, wrapped with film, and placed in a climate chamber at the temperature of +2–4°C for 3–5 days.

Sterilization was carried out as follows: spikes were freed from the covering leaves, cut off the awns and placed in Petri dishes with a diameter of 150 mm; dishes were poured over with a solution of the commercial product Bilyzna (including sodium hypochlorite (NaOCl), active chlorine and alkalis) for 40 minutes and then drained. Further, the Petri dishes with spikes were filled with 0.05 N HCl (hydrogen chloride) solution for 10 minutes, after which they were washed five times with sterile distilled water.

Anthers were isolated aseptically in laminar box conditions and were cultured on the hard 190-2 nutrient medium, which 10 ml mowed in test tube (ø = 15 mm, h = 200 mm) and exposed to the dark at +30°C first three days and further at +24°C for callus induction. For each combination, 600-700 anthers were explanted in three replicates. The 190-2 nutrient medium was supplemented with 90 g L^{-1} of sucrose, 400 mg L^{-1} L-glutamine, 400 mg L^{-1} L-proline, 100 mg L^{-1} myo-inositol, 3 g L^{-1} gelrite and different phytohormones: 2,4-D (2,4-dichlorophenoxyacetic acid) 1.5 mg L^{-1} and kinetin 0.5 mg L^{-1} [19]. Subsequently, after 3–6 weeks of cultivation, formed ELS on surface of the anthers (1–2 mm in size) were transferred to the proliferation and regeneration Murashige and Skoog (MS1) basal medium with addition of 0.2 mg L−1 2,4-D, 0.5 mg L−1 kinetin, 200 mg L−1 L-glutamine, 200 mg L−1 L-proline, 100 mg L−1 myo-inositol, 6 g L−1 agar (Agar Plant tissue culture grade, AppliChem) and 60 mg L−1 sucrose [6, 10] and cultivated in the dark for 21 days at 24 ± 2°C before the emergence of regeneration centers. Subsequently, the calli or embryo-like structures (ELS) were transplanted to enhance the regeneration of green plants on MS2 medium with the 0.5 mg L−1 gibberellic acid and 25 mg L−1 malic acid, 1 g L−1 potato starch (Sigma), 30 mg L−1 sucrose, 6 g L−1 agar and were cultivated 2-3 weeks at 10 - 12°C, of 16-hour photoperiod, 2500 - 3000 lux.

Regenerants of green plantlets formed on ELS were transplanted into glass jars (200 ml) to the ½ MS hormone-free basal medium for rooting. The developed plantlets were acclimatized and transplanted into a greenhouse for further vernalization (45 days), growth to seed and evaluation. The ploidy level of the regenerated DH plants was also evaluated by fertilisation analysis.

For each genotype, the following parameters of in vitro androgenesis were determined: the number of the induction of the androgenic callus, i.e. embryo-like structures (ELS), green plantlets (GP) per 100 anthers. Also, we were determinated the acclimatized plantlets per 100 regenerated green plantlets (ADP/100GP), and the doubled haploid plants per 100 acclimatized plantlets (DH/100ADP) [20],

The evaluation of the obtained data of the androgenic parameters was carried out by the methods of statistical research [1] using software *Excel*.

Results and discussions

This paper presents the results of the work of the tissue culture laboratory from 2021 to 2023 [11, 20] regarding the study of 105 genotypes of winter bread wheat in the in vitro culture of anthers, most of which had an unknown sensitivity to androgenesis *in vitro* (Table 1). Stabilization of genetically unstable breeding material with specific marker traits, which is the result of crossing wheat with introgressive lines (obtained on the basis of crossings with wild relatives) is an urgent task of biotechnology today.

Table 1

Efficiency of the haploproduction process in the culture of anthers *in vitro* of different genotypes of bread winter wheat (2021-2023)

Parameters of androgenesis *in vitro*		2021 year	2022 year	2023 year	Total
Genotypes have been studied	ps.	43	39	23	105
Genotypes for which DH lines were obtained	ps. / %	21 / 48.84	18 / 46.15	13 / 56.52	52 / 49.52
Planted anthers	ps.	76645	92142	44821	213608
Callus induction (ELS)	ps.	1605	1123	1650	4378
	%	2.09 ± 0.05	1.22 ± 0.04*	1.92 ± 0.06	2.05 ± 0.03
	min - max	0.17 – 18.48	0.36 – 6.38	0.61 – 21.25	
Green plantlets (GP)	ps.	207	208	199	614
	%	0.27 ± 0.02	0.23 ± 0.02	0.31 ± 0.03	0.29 ± 0.01
	min - max	0.04 – 3.26	0.04 – 1.01	0.05 -4.94	
Acclimatized plantlets (ADP)	ps.	180	189	129	498
	% from GP	87,0 ± 2.3	90.9 ± 2.0	64,9 ± 3.4*	81.1 ± 1.6
Fertile plants (DH lines)	ps.	70	52	46	168
	% from ADP	38.89 ± 3.63	27.51 ± 3.25*	35,66 ± 4.22	33,73 ± 2.12

Note: *differences compared to the control are significant at P<0.05

It was shown that under the given conditions of the experiment, all the examined samples were sensitive to the first stage of androgenesis *in vitro* - callus induction (Table 1). The percentage of calluses formed from planted anthers ranged from 0.17 ± 0.08 (F_1 5/21 / 237/21) to 21.25 ± 1.29 (genotype No. 1968), while the spread of this indicator in 2021 and 2023 was quite large. In the indicator "callus induction" in the anther culture of the studied samples mostly average values were found. Thus, 31 genotypes had a value of the percentage of calli formed from planted anthers of less than 1%; 48 genotypes – from 1% to 3%, 11 genotypes – from 3% to 5%; ten - from 5% to 10%, and five - above 10%.

Plant regeneration is a complex process that depends on many factors, the main one of which is the genotype [16]. The ability of individual genotypes to regenerate green seedlings is different. Thus, in our studies, the percentage of green regenerants from planted anthers ranged from 0 to 4.94%, on average - 0.29%, and the range of indicators was smaller than that at the first

stage of in vitro androgenesis and was the same in all studies years. The percentage of regeneration of the vast majority of genotypes was less than one (25 genotypes), only in eight of them this indicator was within 1-3%. A total of 614 green plants were obtained. If we evaluate both stages of in vitro androgenesis of different wheat genotypes with complex resistance to rust, this process was most effective in the anther culture of two lines: CR 132/20 and CR 352/20.

One of the most critical stages of any in vitro biotechnology is the adaptation of regenerative plantlets to ex vitro conditions. It was shown that at the stage of adaptation "nutrient medium - soil" on average up to 40% of regenerants obtained in the culture died. In our study, the percentage of survival after the stages of adaptation to ex vitro conditions and 45-day vernalization of the obtained plantlets was quite high - on average 81.1 ± 1.6% of all obtained green regenerants. In 2023, regenerated plantets the worst suffered of soil adaptation and vernalization. Only about 65% of green regenerants were further grown in artificial climate conditions - this is the worst indicator in 3 years, which we can associate with systematic power outages in Ukraine.

As a result of the study, 168 dihaploid lines from 52 genotypes were obtained, which is almost 50 percent of the genotypes that regenerated green plants (Table 1). The average frequency of spontaneous diploidization was 38.89 ± 3.63 (2021), 27.51 ± 3.25 (2022) and 35.66 ± 4.22 (2023), which corresponds to similar results in the culture of wheat pollen from other authors: 32.72 % [8] and 28.40% [9].

Conclusions

The success of *in vitro* androgenesis metod for obtaining linear material (DH) of bread winter wheat depends on the donor material were shown. The investigated hybrids carrying in the genome foreign genetic material with the GPC-B1 gene from *Ae. tauschii* and *T. dicoccoidles*, have been low and medium haploproduction potential. However, different genotypes of wheat with complex resistance to various types of rust and other diseases have been medium and high indicators of androgenesis in vitro. 168 homozygous dihaploid lines of different genotypes of soft winter wheat were obtained. The frequency of spontaneous diploidization amounted to an average of 33.73% of the acclimatizated regenerants.

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