

Research Article

Optimization of the Androgenesis Method in the In Vitro Culture of Anteneson the Basis of *T. Aestivum* Hybrids

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Abstract. This study aimed to examine the regularities of the regeneration processes of haploid plants, the dependence of in vitro microspore morphogenesis in anther culture on optimization factors, and their efficiency in F1 hybrids of *T. aestivum* of different ecological and geographic origin. It was found that heterosis contributed to an increased yield of haploid chlorophyll-bearing regenerants from hybrids obtained from the crossing of parental forms with different responsiveness to androclina. Results were obtained for the complex optimization of the androgenesis method for the in vitro anther culture of *T. aestivum*, in order to create diploidized haploid lines (DHL) regardless of the influence of the genotype. The agroecological properties for a complex of economically useful traits were also assessed. DHLs were created that combined high yield (5.1-6.8 t / ha) with lodging resistance (straw height – 60-80 cm) and consistently high grain quality; these were characterized by increased resistance to major leaf diseases in comparison with the standard variety in the conditions of the Central Economic Region of the Non-Black Earth Zone of the Russian Federation.

Keywords: spring soft wheat, androgenesis, embryoidogenesis, callusogenesis, diploidized haploids, in vitro, yield and quality

1. Introduction

Modern farming systems require varieties of spring bread wheat (*Triticum aestivum* L.) with consistently high productivity and high grain quality, ecological stability, including resistance to biotic and abiotic stressors [1, 2]. Prospects for the intensification of the breeding process are opened by methods of biotechnology in combination with hybridization and selection. Gamete selection in combination with haploidy is of practical interest. Diploidization of haploids created on the basis of hybrid genotypes, along with a high morphogenetic process, ensures the fixation of homozygotes and increases the selection efficiency. The realization of the potential of haploidy in hybrids is hindered

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Dates

Published 13 January 2022

Publishing services provided by
Knowledge E

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Selection and Peer-review under the responsibility of the 8th Scientific and Practical Conference Conference Committee.

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by the lack of simple and reliable methods of mass production of haploids, regardless of the influence of the genotype, which limits the practice of haplo-biotechnology in breeding, and puts it on the empirical path of work [3-5]. The competence of *T. aestivum* L. microspores to androclinic development in vitro is under the control of the multigenic system, the induction of multicellular structures (MCS) is determined by a complex of exogenous physical and biochemical factors, which ultimately determines the effectiveness of the androgenesis method [6-9].

The aim of the research was to study and carry out a comparative assessment using the example of intervarietal hybrids F1 *T. aestivum* L. of factors for optimizing androgenesis methods in anther culture in vitro and the creation of diploidized haploid lines on their basis, regardless of the influence of the genotype.

2. Materials and methods

The research was carried out in the laboratories of biotechnology, selection and primary seed growing of spring wheat at the Federal State Budgetary Scientific Institution FRC 'Nemchinovka'. The object of the study is the varieties of spring bread wheat: Amir, Ester, Lada of the selection of the Moscow Research Institute of Agriculture 'Nemchinovka', Visa (Belarusian Research Institute of Agriculture), Tasos (SAATEN, Germany), Saratovskaya 68 (Research Institute of Agriculture of the South-East), Tulaykovskaya 10 (Samara Research Institute of Agriculture) and reciprocal hybrids obtained on their basis. In vitro studies were carried out in accordance with generally accepted methods of plant biotechnology [10]. The ears were treated superficially with a saturated solution of sodium hypochlorite, followed by repeated washing in sterile water. Cold pretreatment of the spike material of the hybrids was carried out at a temperature of 5-7 ° C in the dark for 1-14 days. Anthers were inoculated on induction media in the phase of mononuclear microspores to establish status on temporary acetocarmine preparations on agar and liquid culture media in Petri dishes. They were cultivated at 24 ° C until neoplasms appeared. Multicellular structures: calli, embryoids, and embryonic-cell complexes were explanted on regeneration media and exposed at an illumination of 5 Klux / m² and a 16-hour photoperiod until stable regeneration. Basics of induction and regeneration media - MS (Murashige, Scoog, 1962), N6 (Chu, 1978), Blaydes (1966), 190-2 (Wang, Hu, 1984), phytohormones – 2,4-D, HA, ABA, amino acids - glutamine, histidine. Normally developed androclinic chlorophyll-bearing regenerants were transferred to a hormone-free MS medium, then, according to the establishment of the haploid status (counting the number of chromosomes on temporary compressed acetocarmine root preparations),

TABLE 1: Induction of MCS (%) in vitro depending on the genotype of *T. aestivum*, the type of sugars and the consistency of the MS medium

Variety, hybrid F1	Agarized			Liquid		
	c^{Et}	g	m	c	g	m
Lada	0,00	0,00	0,45	0,21	0,67	1,34
Amir	0,50	0,56	1,57	1,00	1,14	2,84
Visa	1,25	1,78	3,42	2,35	2,98	3,51
Averagebygrade	0,58	0,78	1,81	1,19	1,60	2,56
Visa x Amir	0,00	0,00	0,35	0,00	0,20	0,75
Thassos x Lada	1,23	2,37	0,89	1,78	5,08	3,52
Saratov 68 x Visa	0,00	1,21	4,43	0,40	3,14	7,12
Tulaykovskaya 10 x Thassos	0,78	0,31	2,64	3,12	1,56	3,15
[(Saratov 68 x Visa) F1 x (Tulaykovskaya 10 x Thassos) F1]	3,54	5,12	7,23	4,21	6,84	11,24
[(Visa x Amir) F1 x (Thassos x Lada) F1]	0,00	0,48	1,62	0,98	1,75	3,07
Averageforhybrids	0,93	1,58	2,86	1,75	3,10	4,81
HCP _{0,5}		1,42		2,55		

Note: Et – reference standard; c – sucrose (50 g / l); g – glucose (50 g / l); m – maltose (50 g / l).

they were diploidized with colchicine, followed by adaptation to soil conditions in growing vessels in an artificial chamber. Diploidized haploid regenerants (CO) were grown in pots, DG lines (C1 and C2) were propagated in soil.

3. Results and discussion

It was found that the level of MCS induction from microspores in anther culture in vitro in *T. aestivum* on MS agar medium containing sucrose (50 g / L), which we adopted as a reference standard, varied significantly depending on the genotype of the donor material. In the studied genotypes, the ISC indicator ranged from 0.00% to 1.25% in varieties and from 0.00% to 3.54% in hybrids. The highest induction rate of ISS was noted in the Visa variety (1.25%) and in the hybrid [(Saratovskaya 68 x Visa) F1 x (Tulaykovskaya 10 x Thassos) F1] F1 (3.54%) (Table 1).

Significant differences were observed in the ability of genotypes to develop microspores androclinically depending on the type of sugars used and the consistency of the medium. Substitution of maltose for sucrose in agar medium increased the frequency of ISS induction in varieties and hybrid genotypes by an average of 3.1 times (from 0.58% to 1.81% and from 0.93% to 2.86%, respectively). The subsequent transfer of the in vitro culture to the liquid type (soluble starch 1.0 mg / l) contributed to the manifestation of a positive synergistic effect, which relative to the reference standard

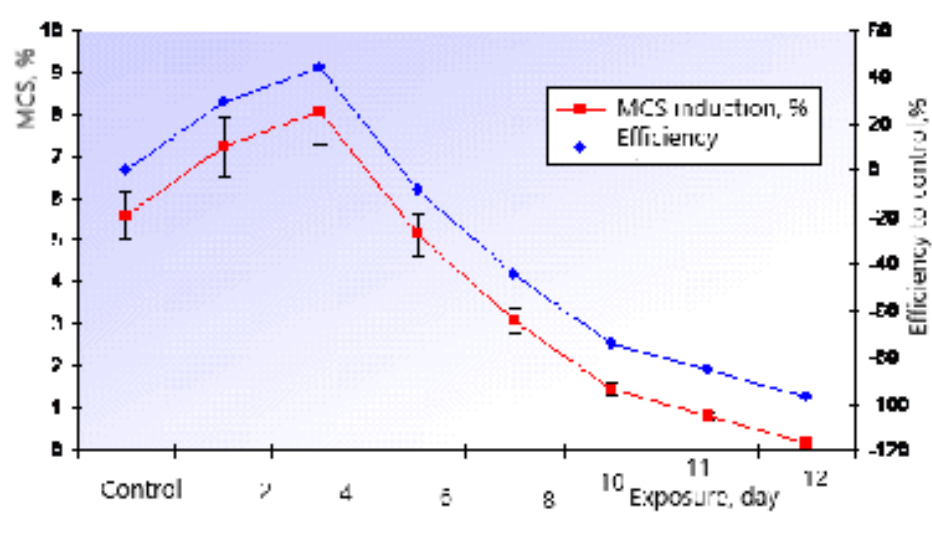


Figure 1: MCS frequencies from the complex of procedures on average by genotypes: hypothermal exposure (60C) and conditioning by co-cultivation of ovules (1: 2) on a liquid MS medium - efficiency (%) is shown relative to control (0-factor).

was 56.9% for varieties and 73.1% for hybrid genotypes. The highest percentage of MCS induction was observed in the Viza variety (3.51%), among simple hybrids – in (Saratovskaya 68 x Viza) F1 (7.12%) and complex – in [(Saratovskaya 68 x Viza) F1 x (Tulaykovskaya 10 x Thassos) F1] F1 (11.24%). The competence of microspores to develop in vitro in genotypes with a high degree of heterozygosity was significantly higher than the induction responsiveness of microspores produced by simple hybrids and varieties; the frequencies of negative results in the sum on Wednesdays were respectively: 0.08, 0.17 and 0.21, ie, there was a manifestation of a heterotic effect on this trait [11].

Cold pretreatment of spike material for 4-6 days at a temperature of 6 ° C before the explantation procedure, followed by the application of the method of conditioning the medium by co-cultivation of ovules in the ratio of 1 anther to 2 ovaries, contributed to an increase in the frequency of the haploproduction process on average in F1 *T. aestivum* by 44.3% , in comparison with the absence of those. Thus, in a simple hybrid (Viza x Amir) F1, the ISS frequency increased from 2.64% to 7.81%, and in a complex hybrid [(Saratov 68 x Viza) F1 x (Tulaykovskaya 10 x Thassos) F1] F1 – from 9, 23% to 15.38% (Figure 1).

In order to optimize the method of androgenesis, the salt composition of the medium and the concentration of 2,4-D were established as the most effective in increasing the frequency of induction of embryoids and morphogenic callus. The intensity of embryoidogenesis per 100 inoculated anthers on MS and 190-2 media was the same: 5.2 pcs. and 5.6 pcs., respectively (Table 2).

TABLE 2: Influence of the salt composition of nutrient media on the induction of embryoids in vitro (%)

Hybrid F ₁	Medium			
	MS	Blaydes	N ₆	190-2
Visa x Amir	0,8±0,4*	0,0	1,5±1,1*	2,6±0,4
Thassos x Lada	3,5±0,9	0,2±0,4	0,00	4,5±1,1
Saratov 68 x Visa	7,2±1,2	2,1±0,6	3,5±1,5	6,5±1,3
Tulaykovskaya 10 x Thassos	3,3±0,8*	0,0	2,4±0,9*	5,1±0,9
[(Saratov 68 x Visa) F1 x (Tulaykovskaya 10 x Thassos) F1]	11,2±2,1	4,7±1,7	6,8±1,3	9,2±1,5
average	5,2±1,2	1,4±1,1	2,8±1,2	5,6±1,1
CV, %	79,5	147,9	92,1	43,8

Note: * - differences are significant at P <0.05

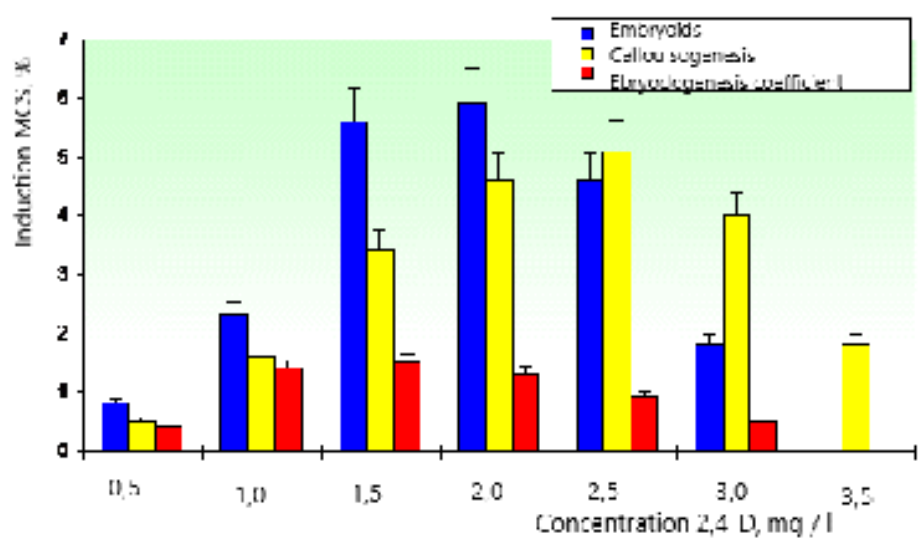


Figure 2: Morphogenesis of neoplasms in a liquid medium 190-2, depending on the concentration 2,4-D (average for F1 T. aestivum).

The smallest degree of genotypic variation (43.8%) was observed on medium 190-2; on media Blaydes and N₆, the coefficients of embryoidogenesis (the ratio of frequencies of induction of embryoids to frequencies of callusogenesis in summa) were insignificant. The highest percentage of direct embryoidogenesis on average for F1 T. aestivum was observed on medium of composition 190-2 at a concentration of 2,4-D of 1.5 mg / l (5.6%); the coefficient of embryoidogenesis was 1.5. A further increase in 2,4-D concentration led to an increase in the proportion of total callusogenesis (Figure 2). The introduction of the amino acids glutamine and histidine at 10 mg / l in medium 190-2 contributed to an increase in the proportion of manifestations of positive effects, the frequency of induction of direct embryoidogenesis on average increased from 5.4% to 11.8% (Figure 3).

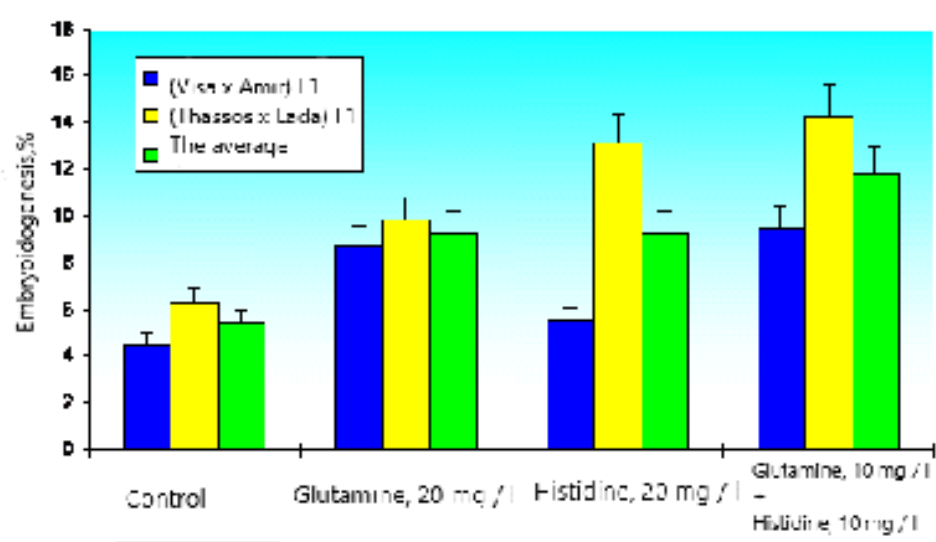


Figure 3: Frequency of embryoidogenesis in liquid medium 190-2 (2,4-D, 1.5 mg / l) depending on the introduction of amino acids.

The highest percentage of regeneration of chlorophyll-bearing haploids was observed when phytohormones HA and ABA were introduced into the liquid medium MS at concentrations of 0.50 mg / l (geometric mean was 12.5% and 5.3%, respectively), with their complex application (0.50 mg / l and 0.25 mg / l, respectively), the efficiency of haploproduction increased to 19.9%. The use of a set of optimization additions contributed to an increase in the efficiency of haploproduction processes in vitro and normal regeneration of chlorophyll-bearing haploids F1 T. aestivum relative to the initial procedure (reference standard) by the index (Es,%) of a qualitative assessment of the efficiency of Marshall-Edgeworth-Bowley changes [12, 13] by 156 , 8% (Table 3).

The study of DG lines C1 of spring wheat in an artificial climate made it possible to establish that the indicators of the main elements of productivity are at the level of the parental forms and in some cases exceed them. Thus, the height of plants of DG lines was on average 8.6 cm lower, the degree of grain content on an ear was higher by 7.9% of parental varieties, i.e., there is a manifestation of both positive and negative transgressions in the inheritance of economically useful traits. Comparison of the coefficients of variation of the values of morphological traits and elements of the productivity structure shows that their variation in the offspring of doubled haploid regenerants (C1) is 1.5-2 times lower than the degree of variability of the same traits in parental forms. Variability of traits: plant height, spike length, number of spikelets and grains per spike, degree of graininess of the spike, grain weight per spike and 1000 grains, between families of doubled haploids is much higher than the intrafamily variability of the same traits in sister DG lines forming these families ... The largest

TABLE 3: Efficiency of haploproduction processes of the initial and optimized method of androgenesis F1 T. aestivum in vitro.

HybridF ₁	Straight embryoidogenesis, %		Regeneration, %						E _s , %
	Ind-MS*	opt-190-2	MS			opt-MS			
			Ch	Ab	Kr	Ch	Ab	Kr	
Visa x Amir	0,0	9,4	0,7	3,9	0,2	21,6	5,2	4,2	88,3
Thassos x Lada	1,2	14,2	0,0	3,8	0,0	18,2	5,1	3,2	44,4
Saratov 68 x Visa	0,0	10,5	0,0	5,8	0,0	25,3	5,9	4,3	74,4
Tulaykovskaya 10 x Thassos	0,8	6,5	0,0	3,7	0,0	15,7	2,8	5,6	35,7
P	0,9	9,8	0,0	4,2	0,0	19,9	4,6	4,2	56,8

Note: * Ind - induction medium (reference standard); opt - optimization add-ons in the complex for this type of environment; Ch - chlorophyll-bearing haploid regenerants and Ab - abnormal regeneration in total: nonmorphogenic callusogenesis, albinos, rhizo- and hemogenesis; Kr is the coefficient of normal regeneration; P - geometric mean; E_s,% - index of qualitative assessment of the effectiveness of changes.

TABLE 4: Characteristics of families of DG lines (C1) T. aestivum.

DG linefamily	Height plants, cm	Earlength, cm	Number per ear, pcs.		Weight, g	
			spikelets	grains	grain from an ear	1000 grains
Esther x Thassos	69,1±0,6	10,2±0,1	20,0±0,2	30,0±0,3	0,80±0,02	28,67±0,50
R (max - min) *	69,1-56,0	10,3-8,7	20,0-15,7	33,2-27,0	0,93-0,57	28,67-24,33
Cv _(l) ** / Cv _(f)	4,5 / 12,1	3,4 / 6,7	5,0 / 19,6	5,0 / 10,7	12,5 / 29,1	8,7 / 16,4
Esther x Visa	45,0±0,5	10,0±0,1	17,5±0,2	46,0±0,2	0,66±0,02	17,45±0,42
R (max - min)	51,6-37,4	10,8-9,7	20,6-17,5	53,7-34,0	1,08-0,41	29,11-17,45
Cv _(l) / Cv _(f)	5,6 / 11,8	3,0 / 6,1	5,7 / 16,4	2,2 / 17,3	15,2 / 37,6	12,2 / 17,8
Visa x Amir	50,3±0,6	9,5±0,1	18,7±0,1	31,7±0,2	0,92±0,02	31,65±0,49
R (max - min)	56,5-41,0	10,8-9,0	21,0-16,5	31,7-19,5	0,92-0,52	36,65-25,17
Cv _(l) / Cv _(f)	6,0 / 10,8	4,7 / 7,9	2,7 / 18,5	3,2 / 24,4	10,6 / 31,9	7,9 / 15,1
Visa x Lada	62,2±0,7	11,5±0,1	16,5±0,3	46,0±0,3	1,10±0,01	26,91±0,60
R (max - min)	74,2-58,3	11,5-11,0	19,5-16,5	55,7-40,0	1,25-0,68	26,91-19,23
Cv _(l) / Cv _(f)	5,7 / 13,6	2,2 / 5,1	9,0 / 17,3	3,3 / 12,0	9,1 / 33,7	11,6 / 14,8
Average	56,7	10,3	18,2	38,4	0,88	25,67

Note: * R (max - min) - the range of the limits of the values of the attribute; ** Cv_(l) - coefficient of variation of the trait in DG lines (%), Cv_(f) - intrafamily variation of the trait in DG lines (%).

steppe variation was noted for the grain weight per ear – from 29.1% in the family of DG lines obtained on the basis of a hybrid (Ester x Thassos), to 37.6% in the family of DG lines (Ester x Visa) (Table 4).

According to the results of a small ecological study of families of DG lines (C3 - C4), it was found that as a method of increasing the accuracy and reliability of their

TABLE 5: Index of integral estimation of productivity of initial forms of *T. aestivum* using lines of diploidized haploids, 2011.

Cross combination	breeding	DG-lines ¹ highlyproductive, %	Average productivity of lines relative to (%)		IIAP ² , %
			bestparent	standardgrade	
Visa x Lada		0,0	111,3	111,3	111,3
Tulaykovskaya 10 x Esther		4,7	107,0	117,7	112,4
Esther x Thassos		4,7	110,6	121,8	116,2
Visa x Amir		9,3	108,1	124,2	116,2
Thassos x Lada		2,3	118,2	118,2	118,2

Note: ¹ - Highly productive line - DG-line of spring wheat, exceeding the standard variety by more than 20% in yield (the percentage of lines was calculated from the total number of tested DG-lines - 41 pcs.); ² - IIAP - index of the integral assessment of productivity [15].

assessment by a set of economically useful characteristics, testing and selection of the best of them sister lines in early regenerations should be carried out. Evaluation of DG lines using the method of orthogonal regression of the Puss indicator (%), which allows taking into account both the level and stability of the yield of the variety [14], provides a more accurate differentiation of families and the selection of the sister lines most adaptive to specific soil and climatic conditions. In this case, the most valuable is the I group of orthogonal distribution, which includes lines with an average level of productivity higher than that of the standard Lada cultivar by the NSR value, and the Puss indicator (%) at and above the standard. The distribution of DG lines in classes does not violate the normality in the assessment according to the Shapiro-Wilk (W) goodness-of-fit criterion, which, when analyzed, makes it possible to reveal the individuality of crossing combinations. As a result of the assessment of families of DH lines according to the index of the integrated assessment of productivity (IIAP), it was found that their breeding value is determined by the recombination ability of the original forms. In most cases, the average productivity of families of DH lines is higher than the best parent and the standard variety. The largest number of highly productive DG lines was created on the basis of a hybrid (Visa x Amir) F1 – 9.3% (Table 5).

According to the complex of limiting signs in the conditions of the Central Economic Region of the Non-Black Earth Zone of the Russian Federation and high baking qualities, a number of DG lines were identified, the yield level of which was 51.0-67.5 c / ha, grain weight per ear – 1.36-1, 64 g, protein content in grain – 15.10-16.51%, gluten in flour – 35.4-37.4%; having the quality of gluten IDK – 73-80 units. sc., sedimentation level – 10.0-11.5 ml, the total score for standard baking was 4.5-5.0. These lines are recommended

for involvement in the breeding process as sources of valuable traits and for further evaluation in competitive variety testing.

4. Conclusions

Based on the studies carried out, it was found that the optimized method of androgenesis in the culture of F1 *T. aestivum* anthers in vitro, including cold pretreatment of spike material for 4 days at 60C, liquid induction medium 190-2 conditioned by co-cultivation of gynoecium (1: 2), and containing maltose (50 g / l), 2,4-D (1.5 mg / l), glutamine (10 mg / l) and histidine (10 mg / l), MS regeneration medium containing HA (0.50 mg / l) and ABA (0.25 mg / l), which in combination contributes to an increase in the yield of normally developed chlorophyll-bearing haploid regenerants on average by genotypes by 1.5 times relative to the initial procedure. Dihaploid haploid lines of *T. aestivum* (C1 - C3) have a high degree of genotypic and phenotypic homogeneity in morphological and biological characteristics ($r = 0.75 \pm 0.11$). The degree of variation of quantitative traits is on average 2-3.5 times lower than that of the original forms. On this basis, as a method of intensifying the breeding process, it is recommended to select the best families and sister DH lines in early regenerations. The possibility of creating DG-lines based on the use of an optimized androgenesis method, combining high yield (5.1-6.8 t / ha) with lodging resistance (straw height – 60-80 cm) and consistently high grain quality and characterized by increased stability to major leaf diseases in comparison with the standard variety Lada.

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