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Creating Biotechnological Hybrids of Sugar Beet

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***Abstract*: Currently, implementation of the breeding programs, including the commonly recognized areas and classic breeding methods, cannot sufficiently ensure a quick and significant increase in the productivity of sugar beet hybrids, since its gene pool is almost exhausted. Based on the achievements in the field of genetics, new approaches to and opportunities in creating highly productive agrocoenoses of sugar beet have become popular.**

As a result of many years of work, results have been obtained about the nature of inheriting the resistance to glyphosate in individual heterozygous apo- and syncarpous forms in case of inbreeding and pair mating with the MC tester. The expression of target genes in the generations was monitored by the survival rate of sugar beet plants after the treatment with glyphosate. During the research, individuals with a high level of gene expression were selected.

Upon self-pollination of initial heterozygous original forms, deviations from Mendelian segregation were observed in most cases. The criterion for assessing the stability of expression of glyphosate resistance genes in case of seed breeding was the compliance with the laws of Mendel among the analyzed descendants.

In the initial stages of the research, the level of stability gene expression had been 10 – 15 % of the total number of analyzed plants. After four self-pollinations, the stability gene expression significantly increased, and genotypes with the resistance of 91 – 100 % were selected.

The first apo- and syncarpous self-pollinating lines of sugar beet with high tolerance in the role of resistance donors have been created. The positive results of preliminary tests of the first glyphosate-tolerant hybrids need confirmation. Seeds and roots of resistant forms have been obtained for further research.

***Index Terms*: Gene Expression, Glyphosate, Hybrid, Profitability, Self-Pollination, Sugar Beet, Sugar Content, Test, Tolerance, Yield Rate, Weeds.**

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I. INTRODUCTION

Sugar beet belongs to the crops of intensive agriculture with extensive consumption of material resources in cultivation. The cost of sugar beet cultivation has reached 70 thousand rubles per one hectare of the crop [1]-[3]. In the structure of the costs, the largest share is that of chemical products for plant protection (29 % or more) of the total cost [4]-[6]. The sugar beet industry still depends on imported seed material [7]-[9]. The share of the hybrids bred in Kuban in the total sugar beet crops is still very low. While in 2000 the seeds of Kuban-bred hybrids were sown on 80 %, and seeds of foreign-bred hybrids — on 20 % of the sowing areas, the ratio today is 2 and 98 %, respectively. Successful development of the sugar beet industry is possible if the ratio of own seed production and imported seeds is about 50:50 % [10], [11]. Higher quality of imported seeds and stable yield rate of the hybrids over the years are the main reasons for removing the seeds of domestic breeding from the market [12]-[14]. Due to the low availability of material and technical resources, delays in reconstruction and upgrading of equipment and machinery, destroyed seed breeding infrastructure, the creation and introduction of new profitable Kuban-bred productive hybrids is inhibited [7], [15]-[17]. Since sugar beet breeding and seed production are the most important components of the sugar beet industry, the situation requires immediate corrective actions [4], [18], [19]. In September 2013, the Government of the Russian Federation adopted Decree No. 839 "On the State Registration of Genetically Modified Organisms Intended to Be Released into the Environment, and the Products Obtained with the Use of, or Containing Such Organisms". The decree was accompanied by the rules of state registration for such products, including varieties and hybrids, and federal agencies responsible for the execution of certain registration forms appointed. More than two hundred years and several generations of breeders were required for continuous improvement and passing on their knowledge and experience to first create population varieties, and consequent abandoning them and switching to interline hybrids of sugar beet, which most fully met the requirements of beet growers.

These requirements primarily include high productivity, the level of which depends on the genotype of the components and a range of external factors; high quality of the roots tubers and seeds; the optimal cost of cultivation (services, equipment, transportation, etc.) combined with the minimum risk to the environment and human health; weeds, pests and disease control that determines the level of productivity and significantly affects the culture of farming; the scheme and the technology of seed production that ensure high profitability in the process of mass reproduction of hybrid seeds [20]-[23].

Only a third of a century ago the first genetically modified plants appeared, which were genetically modified using genetic engineering by the method of targeted induced mutation, and at that moment, the comprehensive study of new genotypes started, which was, above all, focused on safety to the environment and human health [24]-[27].

However, mass media started publishing negative information, same as about genetics in the middle of the twentieth century. There was an impression that geneticists and breeders who always created varieties and hybrids based on the quality indicators, their safety, have become less trusted by fellow breeders and some professionals that worked in other areas far from biology (economists, lawyers, etc.).

Large-scale checks (monitoring) of genetically modified forms of plants started. In the end, as noted in the report of the Directorate of European Commission for Science and Information, careful experiments performed at more than 130 scientific institutions, involving more than 500 independent research groups, have proven that biotechnology and its products, in particular, created using genetic engineering, varieties and hybrids recommended for cultivation and consumption were not more dangerous than the conventional varieties and hybrids obtained by traditional breeding methods [28]-[30].

This is evidenced by the reports and decisions of the WHO, FAO, National Academy of Sciences of the United States and the European Commission. While in the Russian Federation, since the nineties, such discussions were conducted and laws regulating and licensing genetic engineering activities were issued, the United States, Canada, China, Germany, Japan, India, and other countries had already been actively developing the methods for creating new source material. The spread of new transgenic varieties and hybrids has a very fast pace. For instance, in 1998, American breeders performed the first experiments with sugar beet; in 2005, they registered the hybrid, and in 2007, on the area of 1,000 acres in Wyoming, viable seeds for growing sugar beet hybrid Event H7-1 RR that was tolerant to treatment with Roundup (active substance — glyphosate) were sown for commercial purposes.

This beet hybrid allowed developing and using the innovative technology not only for plant breeding and seed production but also for production crops. In the countries that had adopted the use of these forms for commercial purposes, the cost of purchasing herbicides, as well as the number of treatments and the cost of introduction (fuel, wages, depreciation, etc.) significantly reduced. Biotechnology also allowed reducing environmental risks in relation to useful insects and, in general, to the animal world, making sugar beet production more profitable due to removing several technological operations [31]-[33].

Currently, in the area of more than 300 million hectares in various countries, biologically-inspired hybrids of the crops are cultivated: soy, sugar beet, maize, wheat, sunflower, cotton, rapeseed, and other plants, the creation of which involved biotechnological techniques and methods of classical breeding.

In recent years, various publications [34]-[36] have been increasingly focused on the need for creating biologically-inspired hybrids of sugar beet tolerant to herbicides, thus improving the environment, and minimizing the harm to the environment and to human health.

Until recently, in the Russian Federation, biologically-inspired hybrids of new generation resistant to drought, herbicides, low temperatures, and other adverse environmental conditions have virtually not been developed on a large scale.

The almost complete absence of instrumental and analytical base and reliable information on this issue were the impetus for developing a method of obtaining glyphosate-tolerant forms of sugar beet using the methods of classical breeding [32], [37], [38]. In the opinion of the authors, the most urgent and achievable at the first stage of the reserve study was testing for the presence of factors that determined tolerance to glyphosate of all heterozygous domestic and foreign-bred sugar beets source materials. The relevance of such studies was in the fact that the most significant losses to sugar beet productivity were caused by the weeds. In competing for soil nutrition, moisture, and sunlight, they often reduce the productivity of agrocenoses by 25 % or more. Weeds create problems not only in the process of growing but also in the process of harvesting and processing of raw materials [39]-[41].

The use of herbicides is associated with additional financial costs, the risk of environmental pollution, and harm to the health of humans and animals. For this reason, research with heterozygous forms of sugar beet of unknown origin were started in 2012 at the Pervomayskaya experimental station with the aim of creating lines of sugar beet with glyphosate-resistant recombinant DNA and RNA. The aim was to obtain glyphosate-resistant homozygous lines RRRR using the methods of breeding and genetic techniques, transferring the resistance gene RR to combination-capable lines, and creating biologically-inspired hybrids on their base.

II. METHODS

A. General description

The research was guided by the following provisions. Sugar beet is an ideal object for biotechnology, and the fears of glyphosate-tolerant hybrids spread by the mass media were considered incorrect. For the exploratory study, heterozygous sugar beet materials of unknown origin were used with the aim of identifying resistance to glyphosate; these materials differed by a number of economically important traits [13], [35], [42].

Glyphosate, $C_3H_8NO_5P$ is a nonselective system hybrid for fighting weeds. In terms of production, it is ranked the first among herbicides in the world. After plants spraying, unstable plants of sugar beet and weeds show the symptoms of nitrogen deficiency and die within 5 – 12 days. With that, glyphosate belongs to the herbicides that are safe for human health and for the environment. The affected enzyme is only found in plants, fungi, and bacteria. In order to successfully use glyphosate for fighting fields infestation with weeds, hybrids resistant to this herbicide were required [43], [44].

To accelerate the process of breeding, a greenhouse was used, where self-pollination of fertile forms of sugar beet and pair crossing according to the "recipient x donor" scheme occurred. The aim of the research was creating glyphosate-tolerant sugar beet lines as donors of resistance based on the domestic self-fertile apocarpous lines of O type, MC forms, and syncarpous pollinators. The practical result will be obtaining cost-efficient glyphosate-resistant TMC hybrids.

The choice of the material and research methods for determining the sugar beet genotype by its *tolerance to glyphosate* was based on the Mendel's idea about dominance and recessivity. It was tentatively assumed that tolerance was controlled by the dominant resistance gene and that RR was the dominance homozygote, rr was the recessivity homozygote, and Rr was the tolerance heterozygote. Plants with traits of tolerance to glyphosate were noted as T forms. For example, TMM pollinators, TO types, TMC forms, and TMC hybrids.

B. Algorithm

In the first stage, self-pollination of the suggested T forms had been used, descendants of which in the first and the second year of life either died or survived after spraying with glyphosate at certain concentrations. The assumption was that resistant (RR) plants could only be obtained by consistent (not less than three times) self-pollination and selection during the process of testing the forms in which this trait was constant.

In the experiments, with the aim of obtaining glyphosate-tolerant breeding materials, the *recipients* were the following common forms previously created at the Pervomayskaya breeding and experimental station:

1) Multiseeded fertile pollinator lines (MM) of various origin, which were father's forms for zoned and promising hybrids created by individual selection from populations in combination with subsequent inbreeding and evaluation of the combining ability;

2) Single-seeded fertile lines of O type (mm) tested for the fixing ability by the CMS trait (genotype Nxxzz) used for reproduction of MC lines of various types;

3) The MC testers and, maybe, the future mother components of the T-hybrid were the MC lines that were sterile in pollen — functionally female apocarpous analogs of the O-type lines with genotype mmSxxzz (MC). The MC testers were used for forced pair mating in isolators in order to refine the genotype of the father T form and on spatially isolated plots with free cross-pollination, for obtaining hybrid seeds of domestic trial TMC hybrids (tolerant to glyphosate).

In the process of self-pollination and reproduction by the type of sibs, individual and paired isolators, group and vegetation cabins were used, and for obtaining trial hybrids, components of the crossing were planted in spatially isolated areas (at the distance of 3 – 5 km from each other) for free repollination. The efficiency of crossing largely depended on the synchronism (or the lack of synchronism) in the flowering of the components of the crossing. The trial TMC hybrids and father components (TMM) obtained in the experiment were evaluated for resistance to glyphosate and compared to the reference hybrid by the yield, the quality of the product, resistance to diseases and premature seeding using conventional methods with some modifications and additions.

Plants of the experimental T forms, trial hybrids and numbers (samples) from analyzing and saturating crossings were treated with glyphosate in the phase of the first and/or the second pair of true leaves, and after that — in the greenhouse (or in the field, depending on the purpose of experiment) in the pericarp rosette phase in the second year of life. Dead specimens were considered to be of genotype rr. The surviving plants did not differ phenotypically, and by the genotype, most likely, were of type Rr or RR. It was impossible to determine which of these were predominant, and plants for further research were selected by the phenotype. The plants' preservation rate was accounted for after five, seven, and ten days after spraying with glyphosate. The plants in the first and the second year of vegetation in the field conditions were sprayed in doses using a backpack electric sprayer. The reference in all experiments was commercial hybrid Kubansky MC 95.

III. RESULTS

The observations, surveys, and analysis of the digital data were performed using standard methods. The results of the research include sample data of the surveys and observations of individual experiments.

Table 1 shows the results of testing the most valuable glyphosate-tolerant MM forms of sugar beet. The studied forms were in varying degrees tolerant to glyphosate (78 – 100 %) and needed additional confirmation of their stability in subsequent generations since it was not yet possible to veraciously determine genotype MMRr and MMRR among them.

Table I. Resistance of tolerant MM forms to glyphosate on the plots with various methods of isolation in 2017 and 2018. Planting: 09.04.2018. Date of treatment: 30.04.2018. Accounting: 10.05.2018

Catalog No. 2017	Resistance in the field in 2017	Methods of isolation in 2018		
		individual, %	group, %	spatial, %
742	78	100	100	-
747	78	90	96	98
750	97	100	100	100
772	100	100	100	89
781	98	100	100	99
782	100	100	100	-
844	91	95	100	-
795	100	100	100	-
819	100	100	83	-
825	83	100	100	-
829	84	89	100	81
840	83	100	100	-

In 2018, self-pollination and crossing of line MM with regular and partially tolerant MC lines in spatially isolated areas ("islands") continued, and individual lines were cultivated with free repollination within the plot ("in the clean"). MM lines with catalog numbers 747, 750, 772, 781, 782, 795, and 844 were scheduled for the research in 2019.

The characteristic of sample TMC mm lines tolerant to glyphosate is represented by the data of accounting in spatially isolated areas and group isolators (Table II).

Table II. Characteristics of sample glyphosate-tolerant sugar beet TMC mm lines

Catalog No.	Number of analyzed plants, pcs	Sterility, %	Apocarpy, mm, %	Resistance to glyphosate, %	
				rosette, 2017	seed plants, 2018
1. Spatially isolated plots, 2018					
860	189	100	100	90	100
864	74	100	100	96	100
865	68	100	50	93	100
866	64	100	100	94	100
867	50	100	96	88	100
2. Group isolators, 2018					
860	32	97	100	90	100
864	37	95	100	96	100
865	26	100	60	93	100
866	35	100	94	94	100
867	26	100	77	88	100

100 % sterility was shown by MC lines with catalog numbers 865, 866, and 867, 100% apocarpy — by mm lines with catalog numbers 860 and 864. The lines resistance in the phase of rosette at a breeding nursery in 2017 ranged from 88 to 96 %, and all seed plants in 2018 were stable. MC lines with catalog numbers 860, 864 and 866 were included in the programs for further studies.

Table III shows the tolerance of sugar beet lines capable of combining in the process of transferring stability after three and four self-pollinations. The average difference in tolerance is insignificant. The average weight of seeds in case of reproduction in group isolators amounted to 312 g, in case of individual isolation — to 19 g. The cultivation of steckling root tubers of each line was performed in the field conditions in eight-meter long single-row plots at the same seeding rate. The yield of root tubers per one meter in case of sibs reproduction and self-pollination was eight and five pcs, respectively.

Table III. Tolerance of interline hybrids of sugar beet to glyphosate in the process of transferring the stability to regular syncarpous lines capable of combination.

Catalog number	Presence of hybrid seeds, g.	Grown root tubers, pcs.	Stability, rosette phase in 2018, %
Seeds reproduced in group isolators in 2018 after four self-pollinations			
975	480	36	100
976	300	68	95
977	150	34	73
978	200	82	100
979	650	75	83
980	350	98	93
981	340	58	84
982	150	73	95
986	250	38	86
987	250	79	100
Average	312	64	91
Seeds obtained in individual isolators in 2018 after three self-pollinations			
1,089	22	70	100
1,090	12	30	96
1,093	19	25	93
1,096	19	9	95
1,097	24	47	84
1,098	31	64	87
1,100	15	27	100
1,102	12	37	87
1,106	18	27	100
1,108	15	22	80
1,111	26	28	86
1,112	20	10	87
1,114	21	10	75
1,115	23	25	87
1,122	23	54	83
1,126	13	58	91
1,127	13	37	91
1,137	20	51	81
1,138	17	66	100
1,148	27	44	86
1,149	17	26	94
1,150	21	64	88
1,152	15	23	100
1,154	28	13	74
1,161	16	84	80
1,162	19	66	85
Average	19	39	89

The initial self-fertile materials with the resistance of 93 – 100 % were included in the research program for 2019. Table 4 shows the results of preliminary testing of the first biologically-inspired TMC hybrids of sugar beet in 2018.

Table IV. Productivity of trial biologically-inspired hybrids of sugar beet according to tests in 2018

Catalog No.	Crossing combination	Resistivity., %	Yield, t/ha	Sugar, %	Sugar yield, t/ha	Quality of juice, %	Cercospora blight scores
875	TMC(8-93xOT 11301)x TOp 2-94	85	71.5	18.6	13.3	92.7	0.5
876	TMC(3-127xOT 4936)x TOp 2-94	91	68.3	18.7	12.8	92.7	0.5
882	TMC(8-93xOT 11301)x TOp 2-110	90	60.8	19.1	11.6	93.2	0.1
885	TMC(3-127xOT 4936)x TOp 2-110	81	56.9	19.5	11.1	93.3	0.1
891	TMC(8-93xOT 11301)x TOp 3-99	100	61.1	19.9	12.2	93.4	0.1
892	TMC(3-127xOT 4936)x TOp 3-99	100	54.7	20.1	11.2	93.8	0.5
898	TMC(8-93xOT 11301)x TOp Kp.22	98	52.0	19.8	10.3	93.4	1.0
899	TMC(3-127xOT 4936)x TOp Kp.22	96	54.1	19.7	10.7	93.3	0.5
	Standard, Kubansky MC 95	0	60.2	19.3	11.6	92.8	0.4
	LSD_{05}	-	5.1	0.5	-	-	-

Their resistance to glyphosate ranged between 81 and 100 %. The reference in all surveys was commercial hybrid Kubansky MC 95, which was not resistant to glyphosate. The highest yield was obtained by crossing TMC forms with pollinators TOp2-94 and TOp 3-99; in terms of sugar content, hybrids with catalog numbers 891 and 892 significantly exceeded the reference.

The effect of mother components for crossing lines mm TMC 8-93 and mm TMC 3-127 with the participation of MM pollinators TOp 2-94, TOp 2-110, TOp 3-99, and TOp Kp 22 by the main indicators was as follows:

– yield rate 61.3 t/ha and 58.5 t/ha;

– sugar content 19.3 % and 19.5 %; and

– sugar yield 11.8 t/ha and 11.4 t/ha.

The quality of the purified juice in the hybrids varied in a narrow range between 92.7 and 93.8 %. Veracious exceedances in the yield were noted in the hybrids with catalog numbers 875 and 876; in terms of sugar content — in hybrids with catalog numbers 891 and 892.

Table 5 shows the methods of protecting conventional and glyphosate-tolerant experimental hybrids of sugar beet from weeds. For clarity, comparative data about the number of herbicides and the cost per one hectare of crops with the use of alternative technologies are shown.

Table V. Cost of purchasing herbicides for protecting sugar beet from weeds in 2018

Plant protection product	Rate per 1 ha	Price per 1 l/kg	Costs per 1 ha. rubles
I. Promising technology of protecting glyphosate-resistant sugar beet (environmental test)			
The first treatment, the first third of May			
Total of 480 BP, l	2.5	404.7	1,012
The second treatment, the third ten days of May			
Total of 480 BP, l	2.5	404.7	1,012
Total			2,024
II. The technology used at sugar beet farms (tank mixes)			
The first treatment, the third ten days of April			
Betanal Expert OF (l)	1.0	944.3	944,3
Frontier Optima KE (l)	0.2	1,689.1	338
Arbitr (kg)	0.03	19,950	599
Trend – 90 ZH (l)	0.3	416	125
Dexter KE (l)	0.15	1,529.5	229
Total			2,235
The second treatment, the first third of May			
Betanal 22 (l)	1.2	598.5	718
Frontier Optima (l)	0.5	16,891	845
Kari – Max, SP (kg)	0.03	7,315	220
Agron, BP (l)	0.2	2,660	532
Legion (l)	0.2	1,529.5	306
Bit 90 (l)	0.2	416	83
Total			2,704

The third treatment, the second or third ten days in May			
Betanal 22 (l)	1.3	598.5	778
Kari – Max, SP (kg)	0.03	7,315	220
Frontier Optima (l)	0.5	1,689.1	845
Dexter KE (l)	0.15	1,529.5	229
Legion Combi (l)	0.3	1,529.5	459
Bit 90 (l)	0.2	416	83
Total			2,614
Total			7,553

The cost of purchasing herbicides for protecting sugar beet from weeds when growing glyphosate-tolerant hybrids was significantly lower, compared to the recommended methods for conventional hybrids.

One of the reasons for the different stability of T-hybrids to glyphosate is incomplete synchronism of crossing components flowering, and, consequently, insufficient saturation of the site with pollen grains (pollen) of father T lines (pollinators). For instance, flowering in MC forms was observed 5 – 10 days earlier than in T lines of pollinators. Some pollinator plants continued the process of ripening and seed maturation even when seeds from TMC plants were ready for harvesting. The work in ensuring synchronism of development of the components will be intensified in the subsequent years.

There is also a presumption that some mother plants were capable of partial pollen formation, and it could, to some extent, reduce resistance to glyphosate. In any case, it all adds up to increasing the accuracy in working with sterility fixers, lines of O type, on properties of the genotype of which the manifestation of complete sterility of CMS mother lines depends.

It was found that in the breeding and genetic work, cultivation of root crops of crossing components using the same technology and creating equal conditions for passing the organogenesis phase during storage played an important role for synchronizing the processes. As shown in practice, combining "spring" and "summer" steckling root crops sown in August in the same plot or under the same isolator results in desynchronization and stretching the time of flowering, and reducing seed production both from a single plant and from an area unit. Pollinator lines (T form) are particularly noteworthy. Survival rate of the plants obtained from the seeds of pollinator lines after spraying with glyphosate was in the range between 81 and 100 %, which indicated the absence of many foreign pollen grains, which could significantly reduce expression of the glyphosate resistance gene, and possible predisposition of T forms to self-fertility, which prevented germination of "foreign" pollen grains.

With that, various resistance of hybrids to glyphosate as a result of crossing with these pollinators indicated varying quality and heterozygosity of pollinator plants by the gene of stability and possibly incomplete sterility TMC plants.

There is a good chance that the genome of some pollinator plants was represented by heterozygote Rr; therefore, treatment with glyphosate should be repeated for more accurate genetic assessment of the seed progeny.

IV. CONCLUSION

Based on the results of the experiment, it appears that further breeding and genetic work should be focused on:

– increasing the homozygosity of pollinator lines (T forms) by the trait of sustainability by further self-pollination and selecting specimens with high expression of the resistance gene RR;

– the use of T forms Op3-99, Op Kp-22, Op 2-94, and Op Kp-24;

– choosing components of crossing by the synchronism of flowering, pollen-formation ability, self-fertility and cross-compatibility;

– creating at least two lines of O type that fix the full MC at the level of 100 %, and obtaining their TMC analogs, and single-crosses in the long term; and

– selection of combination-valuable T lines in the process of single-crosses formation, given the resistance to Cercospora blight and other diseases.

During the research, special attention will be given to the energy of growth, uniformity, embedment of root crops relative to the soil level, the habitus of the rosettes and the root crops, the absence of bolters, seed productivity, and, in general, the suitability of biotechnological methods of cultivating profitable hybrids. Thus, it can be assumed that the first fertile syncarpous donors were obtained with the resistance to glyphosate of 81 – 100 %, and sterile to pollen with the resistance of 88 – 100 % and sterility of 95 – 100 %. Separate trial (experimental) hybrids in the preliminary tests were not inferior in terms of their productivity to the standard commercial hybrid Kubansky MC 95. The obtained results of the staged studies allow considering the methods of classical breeding in the process of creating biologically-inspired glyphosate-tolerant hybrids of sugar beet as an efficient tool for practical breeders.

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