JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

POLYMORPHISM IN SUGAR BEET VARIETIES AND HYBRIDS IN CELL SELECTION FOR RESISTANCE TO ABIOTIC FACTORS

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doi: 10.15414/jmbfs.2018.7.6.602-606

ARTICLE INFO

Received 12. 3. 2018
Revised 17. 5. 2018
Accepted 21. 5. 2018
Published 1. 6. 2018

Regular article

OPEN ACCESS

ABSTRACT

In the article, the results on the development of cell selection scheme for sugar beet genotypes resistant to high temperature are presented. In terms of increase in the callus diameter and the ability to regenerate plants, lines of high-temperature resistant genotypes Yaltushkivskyi MS 72, Ukrainian MS 70, Ukrainian MS 72 and Katiusha found to be promising for breeding. Experiments confirmed that 5-7 times increased proline content is typical of the temperature resistant lines. We investigated the molecular and genetic polymorphism in sugar beet genotypes using RAPD and SSR analyses. Fourteen alleles were identified and analyzed by four RAPD markers and five alleles by GZM 086 marker. Cluster analysis using DNA markers shows that Ukrainian MS 72 and Ivanivskyi MS 33 are the most similar genotypes, and they are found in the same cluster. It was determined that the temperature resistant sugar beet genotypes Yaltushkivskyi MS 72, Ukrainian MS 70, Ukrainian MS 72 and Katiusha are genetically distant and therefore can be used to create heterotic hybrids.

Keywords: proline, high temperature, sugar beet, DNA markers

INTRODUCTION

One of the most important ways to increase the productivity of sugar beet (*Beta vulgaris* L.), which provides a significant contribution to the Ukrainian and world economies, is the creation of new hybrids of optimal response to environmental changes that can be adjusted by a cultivation technology and genetically determined resistance to abiotic factors at certain ontogenetic stages (**Roik *et al.*, 2010; Sytnik, 2007; Husiev, 2011; Loel and Hoffmann, 2014**). Advances in DNA technology made identification and differentiation of sugar beet genotypes using DNA markers a topical area of research, with the RAPD-PCR (**Roik *et al.*, 2010; Fedulova *et al.*, 2010; Fedulova, 2010; Fedulova *et al.*, 2015**) and SSR-PCR (**Smulders *et al.*, 2010**) being commonly used. Recent studies show that using DNA markers is an effective mechanism to create breeding material resistant to biotic and abiotic environmental factors (**Izzatullayeva *et al.*, 2014; Stevanato *et al.*, 2014; Norouzi *et al.*, 2017**). In particular, at the 21st Conference of the United Nations Framework Convention on Climate Change in 2015, it was reported that observations proved the negative impact of temperature increase on crop yield (**Prokopenko and Udova, 20**17). It was found out that the physiological approach to the estimation of breeding material improves the efficiency of genetic engineering technologies, expands and deepens their tasks and provides a new experimental basis (**Titok, 2003; Morgun *et al.*, 2010; Shadchyna, 2010**). Particularly, under the conditions of climate change that tends to increase temperature and reduce rainfall, it is important to create hybrids with signs of temperature resistance (**Deryng *et al.*, 2014; Rosenzweig *et al.*, 2014; Ohama *et al.*, 2017, Prasad *et al.* 2017**). The purpose of this study was to determine the polymorphism in different sugar beet genotypes in the process of developing technologies of cell selection *in vitro* in order to create a promising material with signs of temperature resistance.

MATERIAL AND METHODS

We used seeds and callus tissue of the following sugar beet cultivars: variety Yaltushkivskyi monogerm 64 (Yalt. mg. 64); six diploid hybrids: Yaltushkivskyi MS 72 (Yalt. MS 72), Uladovo-Verhniatskyi MS 37 (Ul-Ver. MS 37), Ukrainian MS 70 (Ukr. MS 70), Ukrainian MS 72 (Ukr. MS 70), Ivanivskyi MS 33 (Ivan. MS 33), Katiusha (Kat.); two triploid hybrids: Bilotserkivskyi MS 57 (Bilots. MS 57), Oleksandria (Oleks.). The Institute of Bioenergy Crops and Sugar Beet NAAN (Kiev, Ukraine) provided the cultivars of domestic and foreign breeding. Variety Yaltushkivskyi monogerm 64 is the standard of resistant to disease and has productive-saccharіferous direction of growing. Same direction is characterized for hybrids Yaltushkivskyi MS 72, Uladovo-Verhniatskyi MS 37, Ivanivskyi MS 33, Bilotserkivskyi MS 57 and Oleksandria. All genotypes of sugar beet are differenced of tolerance to disease and pests. Hybrid Katiusha has feature for tolerance to a drought resistance.

Cultivation *in vitro*

Aseptic sprouts of sugar beet were obtained by superficial sterilization of seeds with concentrated sulfuric acid (7-8 min) followed by three-times rinsing with sterile distilled water (10 min) and then cultivated on the non-hormonal agar nutrient medium MS (**Murrashige and Skoog, 1962**) at a temperature of +24°C. To induce callus tissue, we used cotyledonous leaves and hypocotyls isolated from aseptic seedlings. They were transplanted onto nutrient medium supplemented with casein hydrolyzate (500 mg), sucrose (2%), NOC (2 mg/L), 6-BAP (0.4 mg/L), vitamin C (2.5 mg/L), vitamins for MS (1 mg/L). The explants were incubated in a thermostat in the darkness at a temperature of 25-26°C and relative humidity of 70-80% (**Belinskaya, 2007; Klyachenko, 2017**). To provide hyperthermic conditions, the callus lines were maintained in thermostats at temperatures of +27°C, +41°C, +45°C, and +47°C for 16 hours in darkness, after that they were grown at a temperature of +22°C and 16-hour light cycle. The callus cultivated at a temperature of +27°C was used as a reference. The effect of high temperature on callus tissue was estimated by the specific diameter index calculated as the arithmetic mean value of the diameter of one callus at a certain temperature of cultivation. To obtain regenerated plants, callus tissue was transplanted into the MS medium with the addition of vitamin B_1 (1 mg/mL), ascorbic acid (1 mg/L), glutamine (10 mg/mL), 6-BAP (0.2 mg/mL), IAA (0.1 mg/mL), NOC (0.5 mg/mL), sucrose (30 g/L) and cultivated at a temperature of +25-26°C.

Determination of free proline

Determination of the free proline content in 1-gram assays of green matter (regenerants or sugar beet leaves) was carried out by chromatographic analysis on a thin cellulose layer (**Belan and Abdurahmanova, 1969; Lawlor, 2011**).

DNA isolation and PCR

The sampling for each genotype numbered 30 assays. Genomic DNA was isolated from 100 mg of green leaves of 5-day sprouts using cationic detergent CTAB and then dissolved in TE buffer (**Velikov, 2013; Roik *et al*., 2007**). We used four RAPD-markers (GEN 2-80-7: GCAGGTCGCG; GEN 1-80-5: ACCCCAGCCG; GEN 1-70-9/2: TGCAGCACCG; GEN 4-70-2: GGACCGACTG) and the microsatellite marker GZM 086 (f-ACTTCTAATGGAGTAAGAATGG, r-ACGGCTACAGGAGAATATTA) (**Dörnte, 2002; Lučić *et al*., 2011; Smulders *et al.*, 2010**). In the investigation of RAPD loci, multiplex PCR with combination of GEN 2-80-7, GEN 1-80-5, GEN 1-70-9/2 and GEN 4-70-2 primers was carried out in 25-μL reaction mixture (100-250 μM dNTP, 1xPCR buffer, 1.5-3 mM $MgCl_2$, 1 unit Taq polymerase) using 1 μM of each primer and 20 ng of genomic DNA under study. Temperature regimes: +94°C initial denaturation (2 min), +94°C denaturation (30 s), +50°C annealing of primers (45 s), +72°C elongation (1 min), +72°C final elongation (7 min); the number of cycles: 45. The amplification reaction for studying the microsatellite loci was carried out in the 25-μL reaction mixture (200 μM dNTP, 1xPCR buffer, 2.5 mM $MgCl_2$, 1 unit Taq polymerase) using 0.5 μM of each primer and 20 ng of DNA with the aid of Creacon device (USA). Temperature regimes: 94°C initial denaturation (3 min), 93°C denaturation (30 s), 55°C annealing of primers (30 s), 72°C elongation (1 min), 72°C final elongation (5 min); the number of cycles: 33 (**Ćurčić *et al*., 2017**).
The DNA amplification products for determination of the microsatellite loci were separated with the aid of horizontal electrophoresis in 2% agar gel; 3% agar gel was used for RAPD analysis at an electric field potential difference of 5 V/cm (2.5 - 4 h). The results of electrophoretic separation were visualized using bromide ethidium in ultraviolet light with wavelengths in the range of 254-310 nm (**Roik *et al*., 2007; Ghasemi *et al*., 2014**). The size of the alleles obtained by electrophoretic spectra was analyzed in nucleotide pairs using the computer program TotalLab v.2.01. We determined genetic distances, the frequency of detected alleles, and the index of locus polymorphism (PIC) using the matrix of presence/ absence of an allele (**Roik *et al*., 2007; Fedulova, 2014**).

Statistical analysis of data

We applied dispersion analysis to estimate the effect of the high-temperature factor on the callus growth rate and the number of regenerats. To do this, we calculated the F-ratio and the corresponding level of significance.
The clustering of sugar beet genotypes by DNA markers was carried out using the method of unweighted average bonds, in which the average value of the genetic proximity between the members of the cluster and a candidate to it was used as a criterion for determining the degree of genetic proximity (**Ermantraut *et al*., 2007**). Calculations were carried out using software Excel and Statistica 6.0 (**Roik, 2010; Izzatullayeva *et al*., 2014**).

RESULTS AND DISCUSSION

Obtaining temperature resistant lines

To create temperature resistant lines, the obtained calluses of different sugar beet genotypes were exposed to high temperature. On the 9th day of cultivation after the exposure to high temperature, there were significant differences in the size and colour of the callus tissues of the original sugar beet genotypes (Fig. 1).
Moderate heat treatment (+41°C) slightly stimulated callus growth as compared to the reference treatment, while high-temperature (+45°C) slowed down growth processes, with a full stop at a temperature of +47°C. We measured the callus diameters at various temperatures.
At a temperature of +41°C, the changes in the callus diameter were recorded; the morphotypes of the calluses were close to the reference and varied only as affected by genotype. It was noted that the hybrid lines Katiusha, Bila Tserkva MS 57 and Oleksandria showed the smallest increase in the callus diameter. Sugar beet genotypes formed dense calluses of pale-milk, light yellow or light green colour, without necrosis.

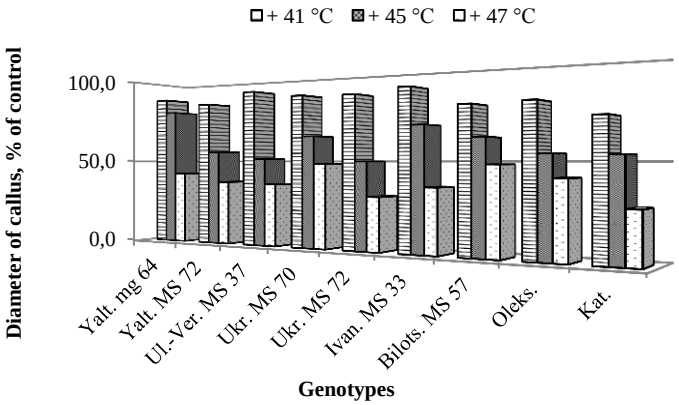

Figure 1 Callus diameter in different sugar beet genotypes

Analysis of the growth rates of the callus tissues under the effect of temperature values of +45°C and +47°C revealed a general tendency to decrease their viability, which was manifested in the variation of the callus pigmentation from dark green to light grey and grey. Although under these conditions, a slight increase in the callus diameter in some genotypes was observed; loose structure and dark colour of the callus and that stage of the experiment indicated that callus was not morphogenic. A significant increase in the callus diameter in comparison with other genotypes at a temperature of +45°C was observed only in the lines of Yaltushkivskyi monogerm 64 and Ivanivskyi 33. The drastic inhibition of the proliferation of the callus tissue was observed at +45°C with the full stop at +47°C. In all the genotypes under study, in the reference treatment (+47°C) and at the experimental temperature of +41°C, morphotypes of calluses were pale-milk, light yellow or light green, without necrosis. Consequently, the increase in the callus diameter was affected by temperature and also by genotype.

Table 1 Formation of plant regenerants in different sugar beet genotypes (% to planted calluses)

Temperature	Yalt. mg. 64	Yalt. MS 72	Ul.-Ver. MS 37	Ukr. MS 70	Ukr. MS 72	Ivan. MS 33	Bilots. MS 57	Oleks.	Kat.
	Number of plant regenerants, %								
Control	93±4,25	92±4,23	96±4,54	97±4,58	96±4,56	96±4,25	96±4,40	96±4,26	96±4,35
+41°C	76±3,65	82±0,39	77±3,67	81±3,95	89±4,15	75±3,64	74±3,34	65±3,10	81±3,84
+45°C	40±1,80	24±1,14	42±1,90	36±1,60	19±0,65	14±0,65	37±1,64	23±1,05	19±0,84
+47°C	4±0,18	4±0,17	0	11±0,45	15±0,62	4±0,16	14±0,64	0	12±0,45

Note: reliable differences as compared to control at $p < 0.05$

In the case of high temperature of +45°C and +47°C, the colour of callus tissue became dark green, light grey and grey, with clear signs of lacking morphogenic callus. After transplantation and further cultivation of the callus tissues on the regenerating medium, in 20-25 days all genotypes marked the formation of morphological structures that initiated the formation of regenerants. The number of regenerants fluctuated to a great extent as affected by the temperature treatment of the calluses and mostly was affected by genotype of sugar beet at by a temperature of + 45°C (Table 1).
In this case, regenerants from the callus tissues of the reference assay were nonviable. The same result was observed in explants, which primary cultivation

temperature was +45°C and +47°C. The smallest number of regenerants under the temperature of +45°C was obtained from hybrid Ivano-Frankivsk 33 (19% of the total number of planted calluses). Thus, according to the ability to regenerate under the effect of temperature +41°C, it is possible to distinguish genotype lines Yaltushkivskyi MS 72, Ukrainian MS 70, Ukrainian MS 72, and Katiusha having 82, 81, 89, and 81% their plants regenerated from calluses. Noticeably, the lines of the Katiusha hybrid demonstrated a rather low value of increase in the callus diameter, but the number of regenerants was significant.

During prolonged cultivation (40 days) in the culture room at a temperature of +25-26°C, in the regenerants obtained from the callus lines under the effect of the initial experimental temperature +41°C, 3 to 7 plants (depending on the genotype) demonstrated green pigmentation in the lower part of the stem at the level of the upper agar layer. These parts of the plants were removed and transplanted to a freshly prepared rooting medium. At the 30th day of cultivation, these parts of stem formed micro-rosettes, later on forming plant regenerants.

When studying the accumulation of free proline in heat-resistant plant regenerants of sugar beet obtained under short exposure to extreme temperatures, there has been a significant (5-7 times) increase in the proline content in leaf blades, whereas in plants of the reference treatment it increased only 2-3 times (Fig. 2).

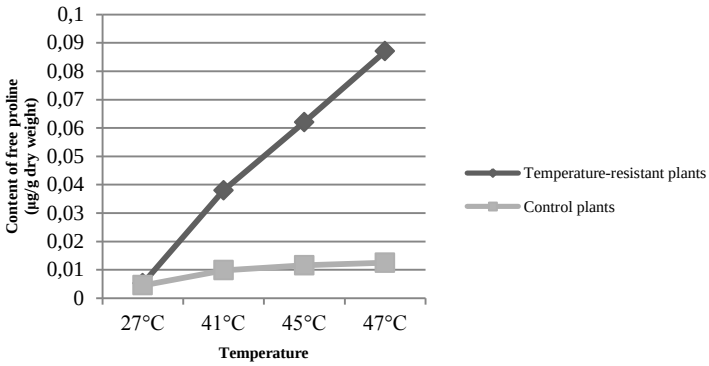

Figure 2 The content of free proline in the leaf blades of sugar beet at high temperature

The obtained data is confirmed by other researchers studied proline accumulation in temperature resistant lines of other plant species (**Trolinder and Shang, 1991; Prins *et al.*, 2008; Kolupaev *et al.*, 2014**). They found that being a polyfunctional acid, free proline participates in complex integral processes of plant adaptation and resistance.

There is growing literature on the development of proline as a signaling (regulatory) molecule in the process of growth and differentiation of cells and their programmed death (**Thebud and Santarius, 1982; Cecchini *et al.*, 2011; Tischenko, 2013**). Consequently, the intensive accumulation of free proline in the leaves of temperature resistant sugar beet lines probably occurs due to increased osmoregulation and other adaptive reactions of the organism to high-temperature stress.

Subsequently, the temperature-resistant lines resulted from high-temperature treatment differed in terms of callus diameter and can be used in breeding aimed at creating high-productive hybrids resistant to adverse environmental factors, although in practice this is complicated by the existence of certain contradictions between these signs caused by the specifics of the energy balance of the plant organism.

Obviously, the more resources a plant spends on providing mechanisms (especially constitutional) that determine its stability, the less remains for the formation of yield under normal conditions (**Iyer and Caplan, 1998; Ribarits et al., 2007; Hossain *et al.*, 2017; Taški-Ajduković *et al.*, 2017**). Therefore, in order to fully implement the plant genetic program of development, the efforts of breeders should be directed to creating heterotic sugar beet hybrids with high adaptive potential.

Estimation of genotypes of sugar beet by DNA markers

In order to select a promising material for the creation of heterotic hybrids, genotypes of sugar beet under study were estimated in terms of polymorphism index using DNA markers. The RAPD and SSR markers were used for this purpose.

The amplification of genomic DNA to determine the polymorphism in sugar beet materials using RAPD analysis was performed using a multiplex approach exploiting two primers for one reaction as following: variant 1: GEN 2-80-7 and GEN 1-80-5; variant 2: GEN 1-70-9/2 and GEN 4-70-2. Amplification results showed that all nine sugar beet genotypes feature high DNA polymorphism when using our proposed variants. At the same time, 14 alleles were found (Fig. 3), which allows genotyping of the variety and hybrids and determining the degree of genetic proximity between them.

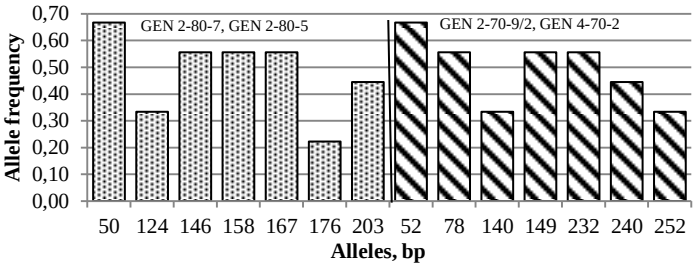

Figure 3 The alleles revealed among various sugar beet genotypes using RAPD markers

Research data shows that majority of genotypes have alleles of 50 and 52 bp with the frequency of 0.67. It should be noted that the allele of 176 bp (frequency 0.22) was identified only in variety Yaltushkivskyi monogerm 64 and diploid hybrid Yaltushkivskyi MS 72. All other identified alleles were distributed among genotypes with a relatively stable frequency of 0.33-0.56. The average PIC value for the applied markers is quite high (0.46). It is known that when there are two alleles detected using dominant markers, the maximum PIC value can reach 0.5. A comparative analysis of the allele composition in the genomic DNA of the sugar beet cultivars using four RAPD primers indicates the heterogeneity of the materials both by the number of identified alleles and by their frequency.

Resulted from PCR analysis of genomic DNA with the GZM 086 marker, it was found that only five cultivars out of nine examined sugar beet genotypes were polymorphs (Uladovo-Verkhniatskyi MS 37, Katiusha, Bila Tserkva MS 57, Yaltushkivskyi monogerm 64, and Ukrainian MS 72). According to the obtained data, five alleles of 157, 177, 192, 202 and 210 bp were identified and analyzed with the frequencies of 0.11, 0.28, 0.11, 0.17 and 0.17, respectively. The value of PIC was 0.89, which indicates their even distribution.

Based on the matrix constructed using RAPD and SSR marker data, genetic distances were calculated and genetic similarity was assessed using cluster analysis of sugar beet genotypes. According to the data on genetic distances resulted from RAPD and SSR analysis, the genotypes under study were grouped into four clusters: Yaltushkivskyi monogerm 64 and Yaltushkivskyi MS 72, Ukrainian MS 72 and Ivanivskyi MS 33, Uladovo-Verhniatskyi MS 37 and Bilotserkivskyi MS 57, Ukrainian MS 70 and Oleksandria (Fig. 4).

The most similar genotypes revealed to be Ukrainian MS 72 and Ivanivskyi MS 33 and they were found in the same cluster. They also have the least value of genetic distance and it is 1.73. It was found that hybrid Katiusha cannot be attributed any cluster in terms of investigated markers and has the greatest value of genetic distances (3.16-3.87) in regard to other genotypes under study.

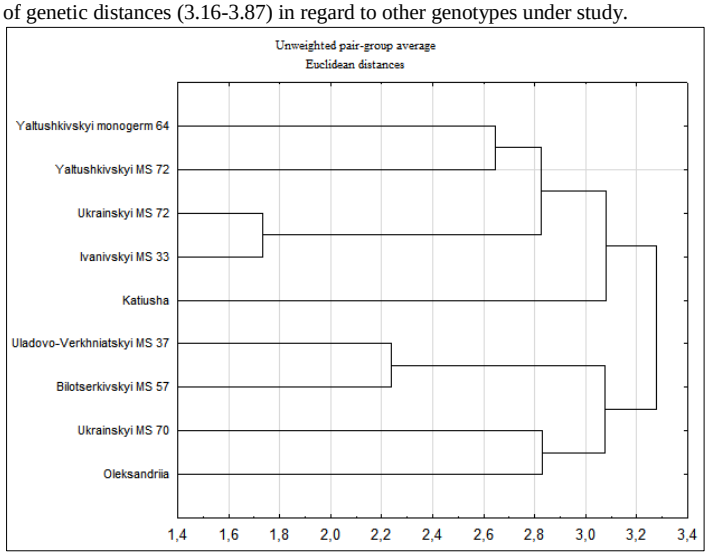

Figure 3 Dendrogram of sugar beet genotypes (by RAPD and SSR analysis)

It was noted that genotypes Yaltushkivskyi monogerm 64 and Yaltushkivskyi MS 72, Ukrainian MS 72 and Ivanivskyi MS 33 are included to some group of clusters, and genotypes Uladovo-Verhniatskyi MS 37 and Bilotserkivskyi MS 57, Ukrainian MS 70 and Oleksandri to another. Genotypes, which are in in the same cluster, Yaltushkivskyi monogerm 64 and Yaltushkivskyi MS 72, Ukrainian MS 70 and Oleksandria are genetically distant and have the follow genetic distances 2.65 and 2.83. The average degree of genetic proximity is found between genotypes Uladovo-Verhniatskyi MS 37 and Bilotserkivskyi MS 57 and comprised 2.24.

Given that the genotypes studied in accordance with the marker system, which involved four RAPD and SSR markers, differed by at least one allele, it can be stated that this marker system has a sufficient level of resolution.

According to the preliminary results on temperature resistance of the callus lines of sugar beet genotypes and their ability to regenerate, we identified promising for breeding genotypes. According to the results of the cluster analysis by DNA markers, cultivars Yaltushkinskyi MS 72, Ukrainian MS 70, Ukrainian MS 72 and Katiusha are found in different clusters and they are sufficiently genetically distant. Thus, based on temperature resistance sign and the results of the analysis using DNA markers, it was possible to obtain promising material for breeding heterotic hybrids, namely Yaltushkivskyi MS 72, Ukrainian MS 70, Ukrainian MS 72 and Katiusha.

CONCLUSION

The proposed scheme of cell selection of adapted to high temperatures sugar beet cells *in vitro* can be used in breeding. Temperature resistant plant regenerants obtained under the short exposure to extreme temperature showed 5-7 times increase in the proline content of leaf blades, which may indicate characteristic adaptive reaction of the plant organism to high-temperature stress. Genotypes Yaltushkivskyi MS 72, Ukrainian MS 70, Ukrainian MS 72, and Katiusha were found promising for the ability to regenerate from callus tissues at a temperature of +41°C. A system of four RAPD and SSR markers allows dividing all the genotypes under investigation into clusters in terms of genetic proximity. The most genetically distant are the genotypes found in different clusters and showing the greatest value of genetic distances.

To conclude, we obtained temperature resistant lines of genetically distant genotypes Yaltushkivskyi MS 72, Ukrainian MS 70, Ukrainian MS 72 and Katiusha which will be used in the further breeding of heterotic sugar beet hybrids.

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