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Obtaining Temperature-Resistant Sugar Beet Lines (*Beta vulgaris* L.)

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ABSTRACT
This study aimed to investigate the sugar beet genotypes for resistance to hyperthermia and obtain temperature-resistant lines. Nine hybrids and one variety of sugar beet were screened. Cotyledonary leaves and hypocotyls isolated from aseptic seedlings were used for induction of callus and subsequent subcultivation. To create hyperthermic conditions, the callus lines were maintained in thermostats at temperatures of +27 °C, +41 °C, +45 °C, and +47 °C. The effect of high temperatures on the callus tissue was assessed by the specific callus diameter index. The free proline was detected with chromatography. As result of callus tissue exposed to temperatures of +41 °C, 45 °C and 47 °C, on the 9th day of cultivation at high temperatures, significant differences were observed in the size and colouration of the callus tissues. At a moderate temperature (+41 °C), the growth of the callus mass was somewhat higher compared to the control. At a high temperature (+45 °C), the intensity of the growth processes decreased and ceased at a temperature of +47 °C. After transfer and subsequent cultivation of callus tissues in regeneration medium MSR – Murashige and Skoog medium for regeneration, all genotypes demonstrated the formation of morphological structures that initiated the formation of regenerated plants. The number of regenerated plants largely fluctuated over temperatures and almost was not related to genotypes. Consequently, the temperature-resistant lines obtained as a result of extreme heat treatment, differ in terms of the specific diameter of callus.

Keywords: specific diameter of calluses, proline, hypothermia, cell selection, in virto.

INTRODUCTION

Plants are affected by various environmental stresses, both biotic and abiotic, during their growth and development (Taleghani et al., 2022). The increasing scale of environmental problems, anthropogenic impact and aridization of climate leads to a narrowing of the limits of tolerance and a decrease in the resistance of crop plants to abiotic and biotic stress factors. Abiotic stress is considered one of the major causes of yield loss in most crops worldwide. The loss can reach 50% and more (Philanim et al., 2022). Therefore, in recent years, the development of crop production is aimed at creating agroecosystems capable of rapid response to stressful factors and subsequent self-regulation (Zhuchenko, 2008).

World sugar production presently reached about 175 Mt. Based on projections of per capita sugar consumption and population increase, one million additional tons per year would be required in order to meet the demands of 230 Mt in 2050. Currently, around 20% of the world's sugar production comes from sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) (Stevanato et al., 2019). When this crop is under high temperature, various molecular, biochemical, physiological, morphological, and ecological processes are disrupted which causes a quantitative and qualitative decline in its yield. Therefore, this issue should be at the center of attention to maintain its quantitative and qualitative performance against stress (Arrieta et al., 2021).

The complexity of the genetically determined nature of plant resistance to stress factors requires

new approaches (Checheneva, 2006). One of the promising ways to develop breeding genotypes of sugar beet resistant to extreme weather conditions and increase breeding efficiency is the use of cell selection methods. At the same time, *in vitro* selection is carried out for those signs that can be detected at the cellular level, in particular, an increase in the expression of the genes controlling metabolic pathways that ensure tolerance to stress factors (Dubrovna and Morhun, 2009; Golovko and Tabalenkova, 2014; Taleghani et al., 2022).

The objective of the research is to screen sugar beet genotypes for resistance to hyperthermia and obtain temperature-resistant lines.

MATERIALS AND METHODS

The following genotypes of sugar beet were used in the research: hybrids Yaltushkivskyi ChS 72, Ukrainskyi ChS 72, Ivanivsko-Veselopodilskyi ChS 84, Ivanivskyi ChS 33, Katiusha, Uladovo-Verkhniatskyi ChS 37, Bilotserkivskyi ChS 57, Ukrainskyi ChS 70, Oleksandriia and variety Yaltushkivskyi Odnonasinnyi 64. The seeds were kindly provided by the Institute of Bioenergy Crops and Sugar Beet of the National Academy of Agrarian Sciences of Ukraine. The research was carried out at the Laboratory of Biotechnology and Cell Engineering of the National University of Life and Environmental Sciences of Ukraine in the years 2018−2021.

To obtain aseptic seedlings of sugar beet the seeds were sterilized with the use of concentrated sulfuric acid (7−8 min) and three times washed with sterile distilled water (10 min). Then the seeds were cultivated on agarized Murashige and Skoog hormonal-free nutrient medium (MS) (Murashige and Skoog, 1962) at a temperature of 24 °C.

Cotyledonary leaves and hypocotyls isolated from aseptic seedlings were used for induction of callus and subsequent subcultivation: they were transplanted onto modified MS nutrient media supplemented with 500 mg of casein hydrolysate, 2% sucrose and growth regulators of auxin and cytokinin action at different concentrations. The explants were incubated in a thermostat at a controlled temperature of 25−26 °C, with a relative humidity of 70−80%, in the dark. The replanting of the formed callus tissue on the surface of the medium of the same composition was carried out every 21 days. The increase in callus mass and the rate of induction of callusogenesis (the ratio of the number of explants that formed the callus to their initial number) were determined according to the generally accepted technique (Kucherenko et al., 1981). The morphological diversity of callus tissues was determined according to (Butenko, 1999).

To create hyperthermic conditions, the callus lines were maintained in thermostats at temperatures of +27 °C, +41 °C, +45 °C, and +47 °C. The effect of high temperatures on the callus tissue was assessed by the specific callus diameter index, which was calculated as the arithmetic mean of the diameter of one callus at a certain culture temperature.

Chromatographic determination of free proline was carried out by taking samples of raw substance in an amount of 1 g (regenerated plants or leaf blades of sugar beet) and homogenized in the tricin buffer (0.02 M tricin, 0.1% glycerol, 0.01 M $MgCl_2$, 0.001 M EDTA, 0.05% Triton X-100, pH 3.5). The homogenate was centrifuged at 6,000 rpm for 10 min. In the supernatant fluid, the content of free proline was determined by the chromatographic method on a thin layer of cellulose according to the technique described by Belan and Belan (1969). Isopropyl alcohol-formic acid-water (3:1:1) solution was used for the separation. The area occupied by proline on the chromatographic plate was determined by the following formula at a cellulose layer width of 0.2–0.4 mm:

$$S = (0.252X_1 + 0.398X_2 - 12.21 \quad (1)$$

where: S – the area occupied by the proline (cm^2);
X_1 – the average length of the area occupied by proline (mm);
X_2 – the average width of the area occupied by the proline (mm).

The calibration curve was constructed according to Stahl (Shevyreva et al., 2007) by the area occupied by proline in the chromatogram. Statistical processing of the obtained experimental data was carried out using the software Analysis of Microsoft Excel Spreadsheets and Statistica 6.0. The results of the experiment are presented as m±s, where m is the arithmetic mean of the results obtained from several replications; s is the standard error.

RESULTS AND DISCUSSION

Extreme temperatures are one of the most common environmental stressors that lead to disruption of the water regime, growth slowdown

and decreased crop performance. To obtain temperature-resistant sugar beet lines, we used dense primary callus from leaf explants of the original sugar beet genotypes obtained by us in previous studies. According to our genetic cluster analysis, the genotypes under study were divided into two groups (Kliachenko and Prysiazhniuk, 2014; Kliachenko and Prysiazhniuk, 2016). The first group included variety Yaltushkivskyi Odnonasinnyi 64 and hybrids Yaltushkivskyi Odnonasinnyi 64, Ukrainskyi ChS 72, Ivanivskyi ChS 33 and Katiusha. The second group included hybrids Uladovo-Verkhniatskyi ChS 37, Bilotserkivskyi ChS 57, Ukrainskyi ChS 70, Ivanivsko-Veselopodilskyi ChS 84 and Oleksandriia. The obtained callus tissue was exposed to temperatures of +41 °C, 45 °C and 47 °C for 16 h in the dark, followed by cultivation at a temperature of +22 °C and a 16 h light period. The callus cultured at a temperature of +27 °C served as the control.

On the 9th day of cultivation at high temperatures, significant differences were observed in the size and colouration of the callus tissues of the initial sugar beet genotypes. At a moderate temperature (+41 °C), the growth of the callus mass was somewhat higher compared to the control. At a high temperature (+45 °C), the intensity of the growth processes decreased and ceased at a temperature of +47 °C. We determined the increase in the specific diameter of the calluses under the action of different temperatures in both groups of sugar beet genotypes (Fig. 1). Shown in Table 1 are the data on the specific increase in the diameter of calluses for 9 days under the action of different temperatures for all studied genotypes, which reflect the morphological diversity and increase in the diameter of callus under the action of high-temperature stress.

Under the action of temperature +41 °C, changes in the specific diameter of callus and the formation of callus morphotypes were close to control and varied only over the studied genotypes. In both groups of sugar beet genotypes, callus morphotypes of dense consistency were formed. They were pale milky, light yellow, or light green, without necrosis. All of them were characterized by a significant increase in diameter.

When analyzing the indicators of callus tissue cultivation under the influence of temperatures of +45 °C and +47 °C, a general trend of reducing viability can be seen, which manifests in a variation in the colouration of callus morphotypes from dark green to light grey and grey. Although under these conditions there was a slight increase in the specific diameter of callus, some of the studied genotypes manifested plump structure and dark colouration already at this stage of the experiment, which indicates that they were not morphogenic. On the 20th day of cultivation, the control treatment maintained the tendency to increase the size of the calluses, which in the case of a temperature of +41 °C. Sharp inhibition of the intensity of callus proliferation occurred at a temperature of +45 °C and ceased at a temperature of +47 °C. In all the studied genotypes, callus morphotypes of pale-milky, light-yellow, or light-green colouration, without necrosis, were formed in the control (+27 °C) and at a temperature of +41 °C. In the case of high temperatures of +45 °C

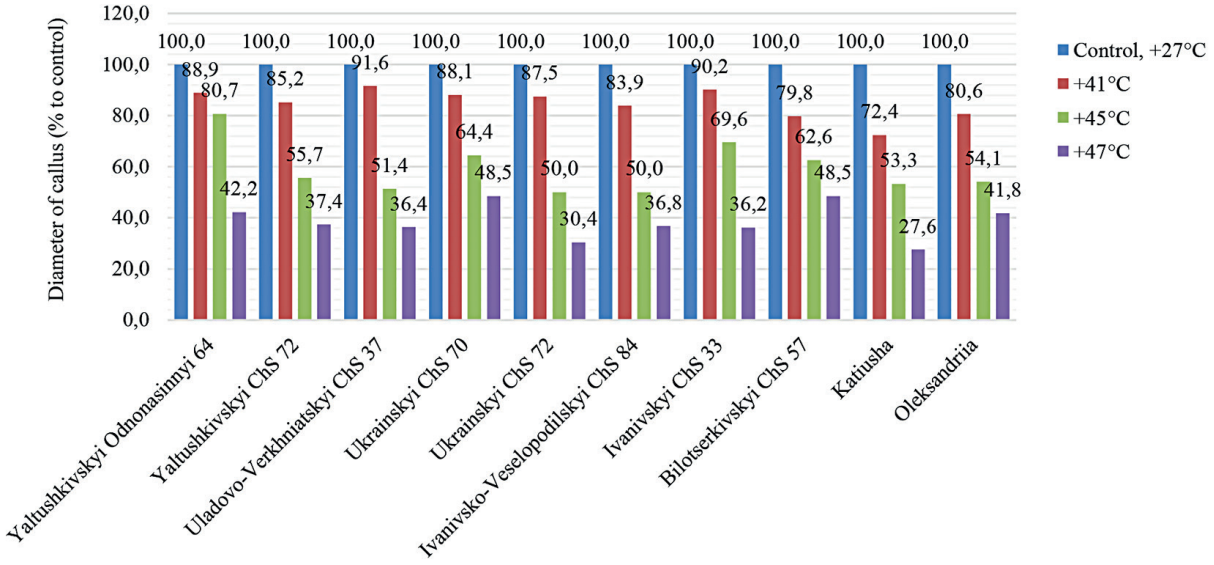

Figure 1. Diameter of callus (% to control)

Table 1. Effect of high temperatures on the growth and formation of callus morphotypes of 9-day sugar beet culture

Genotype	Treatment	Diameter of callus (mm)	Callus morphotypes
Yaltushkivskyi Odnonasinnyi 64	Control (+27°C)	10.9±0.20*	pale milky
	+41°C	9.7±0.17	light yellow
	+45°C	8.8±0.16	dark green
	+47°C	4.6±0.12*	light grey
Yaltushkivskyi ChS 72	Control (+27°C)	11.5±0.21*	light green
	+41°C	9.8±0.18	light green
	+45°C	6.4±0.17*	dark green
	+47°C	4.3±0.09*	grey
Uladovo-Verkhniatskyi ChS 37	Control (+27°C)	10.7±0.22*	pale dairy
	+41°C	9.8±0.17	pale dairy
	+45°C	5.5±0.15*	light grey
	+47°C	3.9±0.16*	grey
Ukrainskyi ChS 70	Control (+27°C)	10.1±0.27*	light yellow
	+41°C	8.9±0.22	light yellow
	+45°C	6.5±0.16*	dark green
	+47°C	4.9±0.12*	light grey
Ukrainskyi ChS 72	Control (+27°C)	11.2±0.22*	light yellow
	41°C	9.8±0.23	light yellow
	+45°C	5.6±0.15*	dark green
	+47°C	3.4±0.10*	light grey
Ivanivsko-Veselopodilskyi ChS 84	Control (+27°C)	10.6±0.18*	light green
	+41°C	8.9±0.15	light green
	+45°C	5.3±0.13*	dark green
	+47°C	3.9±0.11*	grey
Ivanivskyi ChS 33	Control (+27°C)	10.2±0.20*	light yellow
	+41°C	9.2±0.17	light yellow
	+45°C	7.1±0.15*	dark green
	+47°C	3.7±0.09*	light grey
Bilotserkivskyi ChS 57	Control (+27°C)	9.9±0.22*	light green
	+41°C	7.9±0.17	light yellow
	+45°C	6.2±0.15*	dark green
	+47°C	4.8±0.11*	light grey
Katiusha	Control (+27°C)	10.5±0.19*	light yellow
	+41°C	7.6±0.16	light yellow
	+45°C	5.6±0.15*	dark green
	+47°C	2.9±0.11*	light grey
Oleksandriia	Control (+27°C)	9.8±0.19*	pale milky
	+41°C	7.9±0.18	light yellow
	+45°C	5.3±0.14*	dark green
	+47°C	4.1±0.10*	grey

Note: * Significant differences compared to control at $p < 0.05$.

and +47 °C, the colouration of the callus issues became dark green, light grey and grey with distinct signs of non-morphogenic callus.

After transfer and subsequent 20−25-day cultivation of callus tissues in regeneration medium MSR supplemented with 1 mg/mL of vitamin B_1, 1 mg/L of ascorbic acid, 10 mg/mL of glutamine, 0.2 mg/mL of 6-BAP (6-benzylaminopurine), 0.1 mg/mL of IAA (indoleacetic acid), 0.5 mg/mL of NAA (naphthylacetic acid), 30 g/L of sucrose at a temperature +25 °C to 26 °C, all genotypes demonstrated the formation of morphological structures that initiated the formation of regenerated plants. The number of regenerated plants largely fluctuated over temperatures and almost was not related to genotypes (Table 2).

Table 2. Formation of regenerated plants of different sugar beet genotypes

Genotype	Treatment	The number of planted calluses	The number of regenerated plants	Regenerated plants (%)
Yaltushkivskyi Odnonasinnyi 64	Control (+27°C)	27	25±1.08*	93±4.25
	+41°C	25	19±0.74	76±3.65
	+45°C	25	10±0.45*	40±1.80
	+47°C	26	1±0.04*	4±0.18
Yaltushkivskyi ChS 72	Control (+27°C)	24	22±1.02*	92±4.23
	+41°C	27	22±1.02	82±0.39
	+45°C	25	6±0.27*	24±1.14
	+47°C	25	1±0.04*	4±0.17
Uladovo-Verkhniatskyi ChS 37	Control (+27°C)	25	24±1.13*	96±4.54
	+41°C	26	20±1.02	77±3.67
	+45°C	26	11±0.45*	42±1.90
	+47°C	24	-	0
Ukrainskyi ChS 70	Control (+27°C)	29	28±0.36*	97±4.58
	+41°C	26	21±0.96	81±3.95
	+45°C	25	9±0.35*	36±1.60
	+47°C	27	3±0.09*	11±0.45
Ukrainskyi ChS 72	Control (+27°C)	28	27±1.30*	96±4.56
	+41°C	27	24±1.08	89±4.15
	+45°C	26	5±0.15*	19±0.65
	+47°C	26	4±0.17*	15±0.62
Ivanivsko-Veselopodilskyi ChS 84	Control (+27°C)	28	26±0.27*	97±3.28
	+41°C	26	16±0.51	78±4.53
	+45°C	24	5±0.13*	16±0.57
	+47°C	26	1.9±0.07*	3±0.08
Ivanivskyi ChS 33	Control (+27°C)	28	27±1.27*	96±4.25
	+41°C	24	18±0.61	75±3.64
	+45°C	28	4±0.16*	14±0.65
	+47°C	27	1±0.03*	4±0.16
Bilotserkivskyi ChS 57	Control (+27°C)	26	25±1.20*	96±4.40
	+41°C	27	20±0.93	74±3.34
	+45°C	27	10±0.41*	37±1.64
	+47°C	28	4±0.17*	14±0.64
Katiusha	Control (+27°C)	28	27±1.21*	96±4.35
	+41°C	27	22±0.97	81±3.84
	+45°C	26	5±0.19*	19±0.84
	+47°C	26	3±0.09*	12±0.45
Oleksandriia	Control (+27°C)	27	26±1.18*	96±4.26
	+41°C	26	17±0.65	65±3.10
	+45°C	26	6±0.22*	23±1.05
	+47°C	25	-	0

Note: * Significant differences compared to the control at $p < 0.05$.

The resistance of regenerated plants to high temperatures was assessed at the level of test-tube plants. The obtained regenerated plants were re-transferred to a thermostat where they were maintained for 24 h at a temperature of +47 °C, 4000 lux illumination and 75% air humidity. At the same time, regenerated plants obtained from the callus tissues in the control treatment were not viable. The same result was observed in explants whose primary culture temperature was +45 °C and +47 °C. Under prolonged cultivation (40 days) at a temperature of +25 °C to 26 °C in the culture chamber, 3−7 regenerated plants (depending on the genotype) obtained from callus

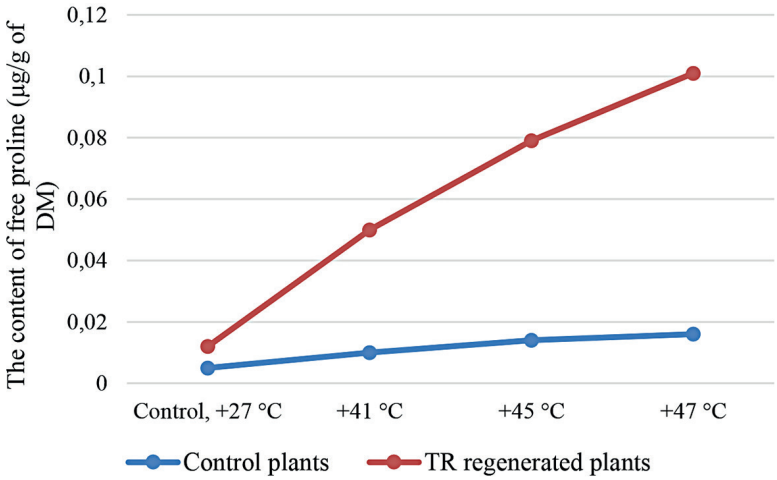

Figure 2. The content of free proline in leaf blades of sugar beet exposed to high temperatures (DM – dry mass)

lines under the action of the initial experimental temperature of +41 °C manifested greening in the lower part of the stem at the level of the upper agar layer. These plant parts were carefully removed and replanted to a fresh medium. On the 30th−32nd day of cultivation, they formed micro rosettes on stems, from which new plants were regenerated.

In temperature-resistant regenerated sugar beet plants obtained under the conditions of the short-term action of extreme temperatures, a significant increase (almost 5−7 times) in free proline content in leaf blades was found, while in the control treatment, prolin content increased only 2−3 times (Fig. 2), which is compliant with the available data on the accumulation of proline in temperature-resistant lines of other plant species (Pomeroy and Mudd, 1987; Kolupaev et al., 2014). The scientists found that free proline as a polyfunctional acid involved in complex integral processes of plant adaptation and resistance. Also, it was developed the concept of proline as a signaling/regulatory molecule in the process of cell growth and differentiation and their programmed death (Butenko et al., 1972; Titok, 2008).

Thus, the intense accumulation of free proline in the leaves of temperature-resistant sugar beet lines is probably associated with increased osmoregulation and other adaptive reactions to the stress action of high temperatures.

To root the regenerated plants, we used two types of nutrient media developed on the basis of MS: MS7 supplemented with 0.5 mg/l NAA and MS8 supplemented with 0.2 mg/L NAA and 5 mg/L IBA (indolebutyric acid). MS8 proved to be the most effective for the rhizogenesis of sugar beet. Among the studied genotypes, the highest percentage (88% and 87%, respectively) of rooted regenerated plants was observed in hybrids Yaltushkivskyi ChS 72 and Bilotserkivskyi ChS 57 (Table 3) for the duration of rooting 10–11 days.

Table 3. Effect of nutrient medium composition on *in vitro* rooting of sugar beet shoots

Genotype	Rooting rate (%)	
	MS7	MS8
Yaltushkivskyi Odnonasinnyi 64	56±0.46	82±0.48
Yaltushkivskyi ChS 72	54±0.43	88±0.57
Uladovo-Verkhniatskyi ChS 37	48±0.35	67±0.36
Ukrainskyi ChS 70	58±0.49	80±0.44
Ukrainskyi ChS 72	51±0.39	76±0.35
Ivanivsko-Veselopodilskyi ChS 84	56±0.44	72±0.33
Ivanivskyi ChS 33	52±0.41	71±0.29
Katiusha	55±0.45	69±0.27
Bilotserkivskyi ChS 57	59±0.53	87±0.58
Oleksandriia	61±0.57	81±0.50

CONCLUSIONS

Consequently, the temperature-resistant lines obtained as a result of extreme heat treatment, differ in terms of the specific diameter of callus. Therefore, they can be used in the process of breeding high-performance hybrids resistant to adverse environmental factors, although in practice this is complicated by the existence of certain contradictions between these signs, which are due to the peculiarities of the energy balance of plant organisms. Obviously, the more resources the plant spends on the processes that

ensure its stability (especially constitutional), the less remains for yield formation under normal conditions. Therefore, to fully realize the genetic program of plant development, breeders' efforts should be directed towards the development of heterozygous sugar beet hybrids with high adaptive potential.

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