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ALLELIC STATE OF WX-GENES AND THEIR RELATIONSHIP WITH GRANULOMETRIC STRUCTURE OF GRAIN STARCH IN COLLECTIVE SAMPLES OF WINTER TRITICALE

SUMMARY

The study was conducted to evaluate the collection of winter triticale (*Triticosecale*) by the polymorphism of alleles of *Wx* genes and the size of starch granules, as well as to establish the relationship between these traits. During research work were used PCR analysis, infrared spectrometry, light microscopy and different statistic methods. As result of study, it was found the presence of a wild-type allele of the *Wx*-B1 gene in all studied samples of winter triticale, eight of which had a null-allele of the *Wx*-A1 gene. The collection samples differed significantly in the size of the starch granules – the limits of variation ranged from 15.4 to 20.0 μm. It was determined that samples with low content of average sizes of starch granules were characterized by greater homogenity of granulometric structure. However, no significant correlations were found between the size of the starch granules, the starch content and the allelic state of the *Wx*-A1 gene. After processing obtained data was selected collection specimens, that could be sources of valuable traits for new varieties suitable for processing into bioethanol.

Key words: bioethanol, amylopectin starch, sizes of starch granules, correlation analysis, sources of valuable traits.

INTRODUCTION

The **aim** of our study was to evaluate the collection of winter triticale (*Triticosecale*) by the polymorphism of *Wx* gene alleles and the size of starch granules of grain, to establish links between these traits. Main **significance** of this research is to identify collection specimens, that will be sources of valuable traits to create new varieties suitable for processing into bioethanol.

The main type of biofuel in the world is ethanol, the production of which is environmentally friendly and is relatively cheap (Chen-Guang *et al.*, 2019;

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Deepak end Vijay, 2019; Schneider *et al.*, 2018; Shreyas *et al.*, 2018; Sujit *et al.*, 2019; Zabed *et al.*, 2017). In Ukraine, cereals are mainly used as raw material for bioethanol production (Brei end Shchutskyi, 2016). Researchers have proven the prospects for the use of triticale grain for biofuel production, the advantages of which are primarily due to high yields and unpretentiousness to growing conditions (Cristina *et al.*, 2016; Habtamu *et al.*, 2018; McGoverin *et al.*, 2011). In addition, triticale grain contains a lot of starch, which is characterized by increased enzymatic activity, which generally provides better cost-effectiveness of bioethanol production compared to other crops (Abdul *et al.*, 2020; Klikocka *et al.*, 2019; McKenzie *et al.*, 2014; Rybalka, 2012). To further increase the volume and efficiency of biofuel production from triticale grains, it is necessary to create and introduce new varieties that will meet the modern requirements for suitability for processing into bioethanol (Dumbravăa *et al.*, 2016; Grabovec *et al.*, 2015; Kour. *et al.*, 2019).

The yield of alcohol depends not only on the starch content in the grain, but also on the efficiency of its transformation into bioethanol, which is determined by the qualitative characteristics of starch. Amylopectin or waxy starch is characterized by high enzymeability, so cereals with starch of this type provide increased total yield of alcohol per unit mass and have advantages over use in the alcohol distillate industry (Yancheng *et al.*, 2013). There are currently no triticale wax varieties among those introduced into production. Also, for triticale varieties suitable for processing into bioethanol, the granulometric structure of starch is an important indicator, because grain with small and aligned starch granules due to the larger area of reaction with enzymes has an accelerated fermentation process (Rybalka *et al.*, 2013; Yaeel end Francisco, 2015; Yaeel, 2018). Thus, in the selection of triticale it is relevant and promising to create varieties with high content of amylopectin starch and with a fine and homogeneous composition of starch granules, which will increase the yield of bioethanol in grain processing (Rybalka *et al.*, 2018; Zhao *et al.*, 2009).

MATERIAL AND METHODS

The research was conducted during 2017–2019 at the National Scientific Center "Institute of Agriculture of NAAS", Ukraine. The soils of the fields belong to sod-medium-podzolic sandy loams. During the years of research, the temperature regime generally exceeded the value of the average long-term norm, and the amount of precipitation was characterized as arid.

The object of the study were 43 collection samples of winter triticale, which are represented by breeding numbers and varieties of selection of the Institute of Agriculture and varieties of foreign origin (Poland, Russia).

To determine the allelic state of the *Wx* gene by polymerase chain reaction (PCR), total DNA was isolated by CTAB + PVP. PCR was performed in an Applied Biosystems 2720 Termal Cycler using a set of GenPak®PCRCore reagents in 20 μl of the reaction mixture containing 1 unit. Taq DNA polymerase

(Somma *et al.*, 2006; Stewart *et al.*, 1993). When performing PCR analysis used the following primers:

gene Wx-A1 – Wx-A1F, 5`-ccccaaagcaaagcaggaaac-3`.
Wx-A1R, 5`-cggcgtcgggtccatagatc-3`.
gene Wx-B1 – Wx-BDFL, 5`-ctggcctgctacctcaagagcaact-3`.
Wx-BRC1, 5`-ggttgcggttggggtcgatgac-3`.
Wx-BFC, 5`-cgtagtaaggtgcaaaaaagtgccacg-3`.
Wx-BRC2, 5`-acagccttattgtaccaagacccatgtgtg-3`.

Detection of PCR results was performed by horizontal electrophoresis in 1.2% agarose gel in TBE buffer in the presence of ethidium bromide, followed by visualization of the results in an ultraviolet transilluminator (Brody end Kern, 2004). The size of DNA fragments was evaluated for motility compared to control DNA markers.

For analysis to determine the size of starch granules, several grains from each sample were ground in a laboratory grinder and 20–30 mg of obtained flour was taken, followed by staining in 2 ml of Lugol's iodine solution. The particle size distribution of the starch was examined by light microscopy in combination with digital analysis of the obtained images via a USB camera and *ImageJ* software (Wilson *et al.*, 2006; Starychenko end Levchenko, 2019).

Determination of starch content in the grain was performed by infrared spectrometry on the device Infratec 1241. To confirm the statistical reliability of the obtained data were used various statistical methods using computer programs *Excel 2007* and *STATISTICA 8*.

RESULTS

The collection of winter triticale was analyzed by the allelic state of wax genes by PCR. The controls were soft winter wheat varieties: Sofiyka wax variety and Oksana variety with ordinary starch (wild type).

According to the results of molecular genetic analysis of 16 samples the amplicon 652 bp, which corresponds to the null-allele of the *Wx*-A1 gene, was found in breeding numbers triticale 229, 223 and 201 (tracks 7, 9 and 10) and in the control variety Sofiyka (15). Amplicons 495 and 176 bp were found in all other samples of winter triticale and wheat control variety Oksana, which confirms the presence of a wild-type allele of the *Wx*-A1 gene (Fig. 1). Among the following analyzed 29 collection samples, the presence of amplicon 652 bp (null-allele *Wx*-A1) was found only in varieties Lubomyr, Petrol, Poliskyi 7 and breeding numbers 141 and 153.

The results of the analysis of the winter triticale collection by the allelic state of the *Wx*-B1 gene showed that all triticale samples have an amplicon of 778 bp, which indicates the presence of a wild-type allele of the *Wx*-B1 gene.

To evaluate the collection of winter triticale by the granulometric size distribution of starch in the samples, the maximum, minimum and average sizes of starch granules were determined. The largest and smallest granules are usually present in small quantities, so an important characteristic of the particle size distribution is the average size of starch granules. It was found that the collection samples differed significantly in this indicator. Thus, in the variety Yasha the average grain size of starch was 15.4 μm, and in the variety Almaz – 20.0 μm (Fig. 2).

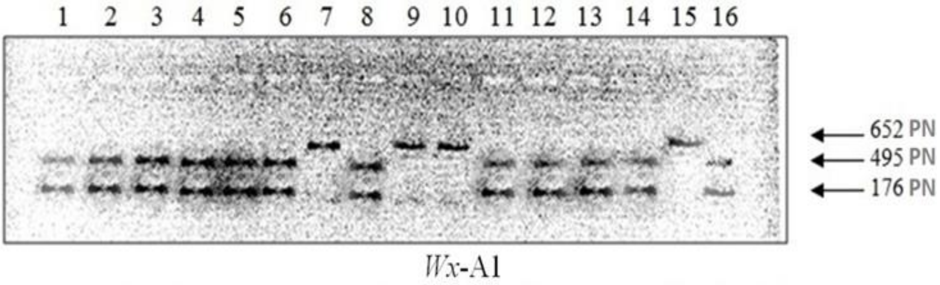
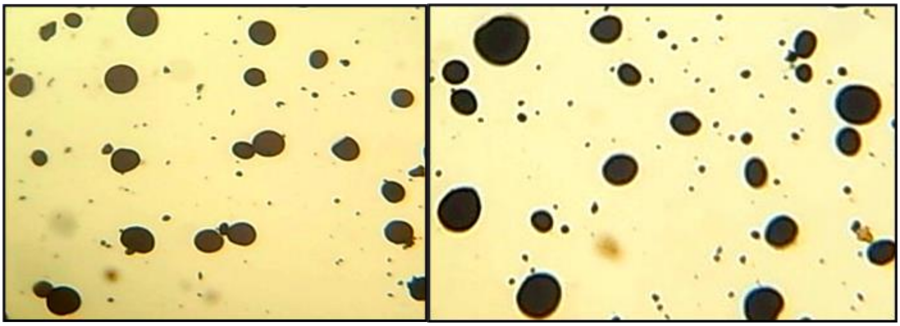


Figure 1. Electrophoregram of PCR of *Wx*-A1 gene products in collection samples of winter triticale and control varieties of winter wheat



Variety Yasha variety Almaz

Figure 2. Granules of starch grain of winter triticale, microphoto at magnification x 45

The largest average sizes of starch granules were determined in such samples as Maetok Polissya, 215, Dokuchaevske, Almaz (18.1–20.0 μm), and the smallest – in samples Yasha, Mundo, 199, 141, 123 (15.4–15.9 μm). The number of samples with average sizes of starch granules up to 16.0 μm was 11.6% of the total collection, with a size from 16.1 to 17.0 μm – 41.8%, from 17.1 to 18.0 μm – 32, 6%. Groups of samples with average granule sizes of 18.1–19.0 μm and 19.1–20.0 μm accounted for 9.3 and 4.7% of the total collection.

According to the results of comparative evaluation of collection samples of triticale, no relationship was found between the size of starch granules and the content of starch in the grain. Such pairs of samples as Yasha and Mundo, 201 and 185, Petrol and Lubomyr, which were characterized by a close-sized particle size distribution, differed significantly in starch content (Fig. 3). The use of correlation analysis confirmed the lack of reliable relationships between these traits, which proves the possibility of selection work in the direction of simultaneously increasing the starch content and reducing the size of starch granules.

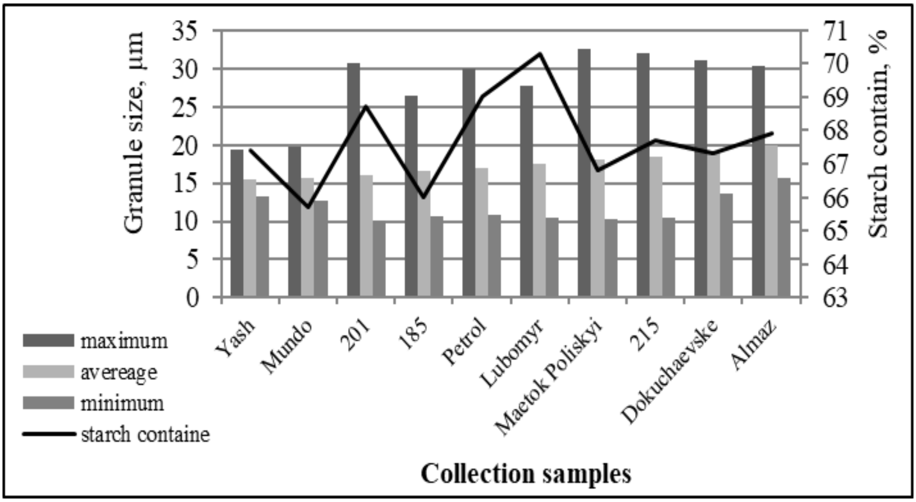


Figure 3. The size of starch granules and starch content in the grain of winter triticale collection samples, 2017–2019

Grain, the starch of which is homogeneous in granule size distribution, is more suitable for processing into bioethanol. Therefore, the analysis of starch samples is relevant not only for the size of its granules, but also for the alignment of the granule size distribution. It was found that in the varieties Yasha and Mundo starch consisted only of small granules up to 20.0 µm. Also, in the variety Poliskyi 7 and numbers 221, 201 and 199 revealed a relatively uniform particle size distribution of starch, where such granules ranged from 85.7 to 86.8%. In general, the collection shows a tendency to link the alignment of the granule size distribution and the average size of starch granules. Samples with large indicators of medium size differed in their greater heterogenity compared to samples with small starch granules.

In the collection samples of winter triticale with null allele of the *Wx*-A1 gene, a relatively small starch grain size was found, which averaged 16.5 µm in these samples. In sample 141 the average size of starch granules was 15.9 µm, in samples 201, Poliskyi 7, 229, 223 and 153 it varied from 16.1 to 16.6 µm and only in varieties Petrol and Lubomyr was 17.0 and 17.5 µm, respectively (Table 1). In varieties with the usual type of starch, such as Aristokrat, Solodyuk, Almaz and Dokuchaevske, the average granule size ranged from 18.0 to 20.0 µm, and the average for the collection of samples of triticale with wild-type *Wx*-A1 – 17.3µm. Therefore, an assumption was made about the tendency to decrease the size of starch granules in partially amylopectin forms of triticale with a null allele of the *Wx*-A1 gene.

To confirm the association of the allelic state of the *Wx*-A1 gene with such quantitative traits as starch content and size of starch granules, a nonparametric method of calculating the Kendall correlation force was used (Khalafian, 2010).

Table 1. The average size of starch granules in collection samples of winter triticale with different allelic state of the *Wx* genes, 2017–2019

Collection sample	Country of origin	Allelic state of the *Wx* genes		Average size of starch granules, μm
		Wx-A1	*Wx*-B1	
Samples with partially amylopectin type of starch				
141	UKR	null-allele	wild type	15,9
201	UKR	null-allele	wild type	16,1
Poliskyi 7	UKR	null-allele	wild type	16,3
229	UKR	null-allele	wild type	16,4
223	UKR	null-allele	wild type	16,5
153	UKR	null-allele	wild type	16,6
Petrol	UKR	null-allele	wild type	17,0
Lubomyr	UKR	null-allele	wild type	17,5
Samples with the usual type of starch				
Arystokrat	UKR	null-allele	wild type	18,0
Soloduk	UKR	null-allele	wild type	18,0
Dokuchaevske	RUS	null-allele	wild type	19,1
Almaz	RUS	null-allele	wild type	20,0

Table 2. Correlation of amylopectin type of starch controlled by the *Wx*-A1 gene with starch content and size of starch granules in winter triticale

Quantitative signs		Year of the study	*Wx*-A1 alleles
The starch content in the grain		2017	-0,07
		2018	0,00
		2019	0,02
		average for 3 years	-0,01
The size of starch granules	maximum	2017	-0,15
		2018	-0,09
		2019	0,01
		average for 3 years	-0,08
	average	2017	-0,09
		2018	-0,03
		2019	-0,10
		average for 3 years	-0,06
	minimum	2017	-0,06
		2018	-0,20*
		2019	-0,21*
		average for 3 years	-0,19*

* Correlations are significant at $p < 0.1$

There are no significant correlations between amylopectin-type starch and starch content, which is confirmed by the results of studies separately for 2017–2019 and on average for three years (Table 2). There was also no significant correlation between the allelic state of the *Wx*-A1 gene and the maximum and minimum sizes of starch granules. Weak negative significant correlation (at p <0.1) was observed with the average size of starch granules of the samples in 2018, 2019 and on average over three years. The revealed regularities prove the possibility of creating amylopectin varieties of triticale with small starch granules.

The graphic image clearly showed a different range of variation of the average sizes of starch granules in the collection samples of winter triticale in groups with wild-type and null-allele gene *Wx*-A1 (Fig. 4).

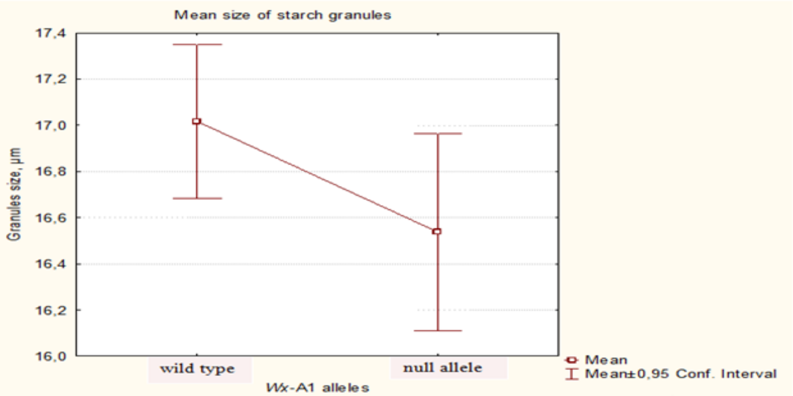


Figure 4. Ranges of distribution of average size of starch granules in winter triticale samples depending on the allelic state of the *Wx*-A1 gene.

Null-allele genotypes had a smaller average granule size than samples with the wild-type allele of the *Wx*-A1 gene, which was confirmed by a downward shift in the confidence interval in the group of samples with a partially amylopectival type of starch. The range of variation in the samples of the first group was 16.7–17.3 µm, and that of the second group was 16.1–16.9 µm. A larger range of variation was found in samples with a zero allele. Therefore, the dependence of the average size of starch granules on the allelic state of the *Wx*-A1 gene was confirmed.

DISCUSSION

The consumption of fuel and energy is increasing in the world. But fossil sources are being depleted and not renewed. Therefore, it is important to use renewable biological fuels, one of which is bioethanol. The source for the production of bioethanol can be triticale grain (Mupondwa *et al.*, 2018). The triticale varieties, that used for processing into bioethanol, should meet certain quality traits, including the content of starch and its quality.

Researchers have shown that the efficiency of processing grain into bioethanol also depends on the particle size distribution of starch (Cornejo-Ramirez *et al.*, 2015; Litvyak *et al.*, 2018). Thus, according to Rybalka (Rybalka *et al.*, 2013), Litvyak (Litvyak *et al.*, 2018) and others, it is established that the best efficiency of transformation of starch into alcohol have varieties of crops with the highest percentage of fine granules. It was found that the starch granules of different cultures differ in size, shape and surface morphology. Starch grains of regular and irregular oval, round and multifaceted shape, the sizes of which vary from 0.5 to 60.0 μm (Lindeboom *et al.*, 2004), have been identified. A number of scientists from the United States, China, Ukraine and other countries have shown that the size of starch granules differs not only within genera and species, but also different varieties of the same culture (Seok-Ho *et al.*, 2009; Zabolotets *et al.*, 2018). There is evidence that the size of the granules and starch content in different crops are interdependent: at high values of the average size of starch grains, the starch content decreases, and at lower values, the percentage of starch increases (Zabolotets *et al.*, 2018). According to the results of our research, a different genetically determined granulometric structure of starch in varieties and selection numbers of winter triticale was established. However, no relationship was found between the starch content in the grain and the size of the granules: collection samples with both high and low starch content were characterized by different particle size distribution.

Physico-chemical properties of amylopectin starch determine its advantages over conventional types of starch and the prospects for use in various industries, especially for the production of bioethanol (Chunyan *et al.*, 2020a; Chunyan *et al.*, 2020b; Fan, 2018; Yongfeng end Jay-lin, 2018).Varieties of wheat, corn, millet, sorghum and other crops with high amylopectin content have been obtained in the world, in which the synthesis of amylose is completely or partially blocked (Evžen end Dvořáček, 2017; Gago *et al.*, 2014). To identify new valuable sources of starch wax, scientists are widely conducting research to identify polymorphism of *Wx* genes in different cultures using polymerase chain reaction (Morhun *et al.*, 2015; Yuangen *et al.*, 2013; Zhirnova *et al.*, 2019). Three *Wx* genes have been identified in common wheat, each with several alleles (Juan and Carlos, 2016; Maningat *et al.*, 2009; Zhao *et al.*, 2009) – 77, 171, 185. According to Rybalka O. (Rybalka, 2015), in the triticale two recessive alleles of the genes *Wx*-A1 and *Wx*-B1 are localized in genome A, in genome B *Wx*-alleles are not detected. According to the results of the analysis of the winter triticale collection, we isolated 8 samples with the presence of the *Wx*-A1 gene in the genome of null-alleles.

According to the results of research by a number of scientists in determining the granule size distribution of different types of starch revealed a tendency to reduce the size of the granules in amylopectin varieties of wheat. Amylose content has been shown to be negatively correlated with the percentage of fine granules in the starch and positively correlated with the percentage of large ones (Wenyang *et al.*, 2016; Xurun *et al.*, 2015). Our studies found a decrease in the size of starch granules in partially amylopectin samples of winter triticale and found a significant negative correlation between the presence of the *Wx*-A1 allele in the samples and the average size of starch granules. In general, we can say that the identification of patterns of variation in the size of starch granules and polymorphism of the gene pool of cultures by allelic state of *Wx* genes require further in-depth study to use the results in creating a new source material with a combination of genotypes biofuels.

CONCLUSIONS

Eight samples (varieties Lubomyr, Petrol, Poliskyi 7 and breeding numbers 141, 153, 201, 223, 229) with a null-allele by the *Wx*-A1 gene were isolated, which indicates a sufficient frequency of this allele in the gene pool of triticale common in Ukraine. It was found that all collection samples of winter triticale had a wild-type allele by the *Wx*-B1 gene.

It was proven the genetic diversity of the winter triticale collection by the granulometric structure of starch has been brought. Limits of variability for the average size of the granules were set from 15.4 µm in the variety Yasha to 20.0 µm in the variety Almaz. It has been established that from among the high indicators of the average size of starchy grains, a large heterogeneous stock of starch was grown.

A weak negative significant correlation (at $p < 0.1$, according to Kendall) was found between the presence of *Wx*-A1 allele samples and the average size of starch granules, which confirmed the observed tendency of decreasing starch grain size in partially amylopectin samples of triticale.

Selected varieties Yasha and Mundo, characterized by fine and homogeneous granulometric structure of starch (average granule size 15.4 and 15.6 µm) and samples 141, 201, 229, 223, 153, Poliskyi 7 with partially amylopectin starch and medium granule size from 15.9 to 16.6 µm are valuable sources for creating varieties for alcohol-distillate use.

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