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Dependence of the content of starch and reducing sugars on the level of expression of the genes of β-amylases *StBAM1* and *StBAM9* and the amylase inhibitor *StAI* during long-term low-temperature storage of potato tubers

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Abstract. *Solanum tuberosum* L. is the most important non-grain starch crop with a potential yield of 38–48 t/ha and a starch content of 13.2–18.7 %. Potato tubers are stored at a low temperature (2–4 °C) in a state of physiological dormancy. A disadvantage of this type of storage is the degradation of starch and the accumulation of reducing sugars (cold-induced sweetening), including due to an increase in the activity of β-amylases that hydrolyze starch to maltose. In this study, a comparative analysis of the β-amylase (*StBAM1*, *StBAM9*) and amylase inhibitor (*StAI*) gene expression, as well as starch and reducing sugar content in tubers during long-term low-temperature storage (September, February, April) was performed using potato cultivars Nadezhda, Barin, Krasavchik, Severnoe siyanie and Utro. The β-amylase genes, *StBAM9* and one of the two *StBAM1* homologs (with the highest degree of homology with *AtBAM1*), were selected based on phylogenetic analysis data. Evaluation of the expression of these genes and the amylase inhibitor gene showed a tendency to decrease in transcription for all analyzed cultivars. The starch content also significantly decreased during tuber storage. The amount of reducing sugars increased in the September–April period, while in February–April, their content did not change (Krasavchik), decreased (Barin, Severnoe siyanie) or continued to grow (Utro, Nadezhda). It can be assumed that the gene activity of *StBAM1* and *StBAM9* correlates with the amount of starch (positively) and monosaccharides (negatively). The level of *StAI* expression, in turn, may be directly dependent on the level of *StBAM1* expression. At the same time, there is no relationship between the degree of cultivar predisposition to cold-induced sweetening and the expression profile of the *StBAM1*, *StBAM9*, and *StAI* genes.
Key words: *Solanum tuberosum*; potato cultivars; tuber storage; starch catabolism; gene expression; β-amylase.

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Зависимость содержания крахмала и редуцирующих сахаров от уровня экспрессии генов β-амилаз *StBAM1* и *StBAM9* и ингибитора амилаз *StAI* при длительном низкотемпературном хранении клубней картофеля

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Аннотация. Картофель (*Solanum tuberosum* L.) – первая по важности незерновая крахмалоносная культура с уровнем потенциальной урожайности 38–48 т/га и содержанием крахмала 13.2–18.7 %. Клубни картофеля хранятся при низкой температуре (2–4 °C), что обеспечивает состояние физиологического покоя. Недостатком такого хранения являются распад крахмала и, как следствие, накопление редуцирующих сахаров (холодовое осахаривание), в том числе за счет роста активности β-амилаз, гидролизующих крахмал до мальтозы. В настоящем исследовании проведен сравнительный анализ динамики экспрессии генов β-амилаз (*StBAM1*, *StBAM9*) и ингибитора амилаз (*StAI*), а также содержания крахмала и редуцирующих сахаров в процессе длительного низкотемпературного хранения (сентябрь, февраль, апрель) клубней пяти сортов картофеля (Надежда, Барин, Красавчик, Утро и Северное сияние). Гены β-амилаз – *StBAM9* и один из двух гомологов *StBAM1*

(с наибольшей степенью гомологии с *AtBAM1*) – выбраны на основе данных филогенетического анализа. Оценка экспрессии этих генов, а также гена ингибитора амилаз показала тенденцию к снижению уровня транскрипции для всех анализируемых сортов. Обнаружено, что содержание крахмала в процессе хранения клубней также существенно падает. В то же время количество редуцирующих сахаров увеличивается в период сентябрь–апрель, тогда как в период февраль–апрель их содержание не меняется (Красавчик), снижается (Барин, Северное сияние) или продолжает расти (Утро, Надежда). Можно предположить, что активность генов *StBAM1* и *StBAM9* коррелирует с количеством крахмала (положительно) и моносахаридов (отрицательно). А уровень экспрессии *StAI*, в свою очередь, находится в прямой зависимости от уровня экспрессии *StBAM1*. При этом зависимость между степенью предрасположенности сорта к холодовому осахариванию и профилем экспрессии генов *StBAM1*, *StBAM9* и *StAI* отсутствует.
Ключевые слова: *Solanum tuberosum*; сорта картофеля; хранение клубней; катаболизм крахмала; экспрессия гена; β-амилаза.

Introduction

Starch is a polymer of glucose and is one of the three main natural polysaccharides. Unlike cellulose and chitin (structural biopolymers of the cell), starch is the main storage carbohydrate and is found in large quantities in plastids of heterotrophic plant organs: tubers and roots (tuber and root crops), grains (cereals and legumes), mature and/or immature fruits (Benkeblia et al., 2008; Bello-Perez et al., 2020).

The presence of starch in tubers of potato (*Solanum tuberosum* L.), the fourth most important crop in the world (after cereals), determines its universal use as a food, fodder and industrial crop. Despite the fact that cultivated cereals also have a high content of this polysaccharide in grains, the advantage of using potato starch is provided by its physicochemical properties (granule structure, physicochemical properties, the ratio of amylose and amylopectin polysaccharides, the degree of polymerization of molecules, etc.). Potato cultivars differ in the amount of starch in tubers, but varieties with almost any starch content and characteristics are eaten, determining the choice of a cooking method, as well as digestibility and glycemic response (Bello-Perez et al., 2020).

The content of starch in tubers is determined primarily by the genetic component, namely, the activity of more than 70 genes, including genes for key enzymes of biosynthesis (starch synthase, etc.) and degradation (starch phosphorylase, adenylate kinase, amylases, etc.) (Van Harsselaar et al., 2017). Also, the amount of polysaccharide is affected by post-harvest storage of tubers at low positive temperatures (2–4 °C). Thus, a state of physiological dormancy is maintained, while germination, drying out and development of infections are slowed down. At the same time, by the end of the storage period (closer to the planting season), part of the starch is degraded with the formation of glucose, which is necessary to stimulate the growth of shoots (Benkeblia et al., 2008). However, a number of varieties are characterized by the cold-induced sweetening (CIS) – a significant increase in the content of reducing sugars in response to low temperatures (Fischer et al., 2013), which leads to a deterioration in nutritional and dietary qualities, in particular due to the formation of acrylamide during frying (Sonnewald S., Sonnewald U., 2014; Hou et al., 2019; Tai et al., 2020). At the same time, there are CIS-resistant varieties that are used for the production of french fries.

Starch catabolism is important both for plant growth and in terms of consumer properties. The degree of susceptibility of starch to degradation depends on the composition and structure of the granules, which determines the digestibility of starch and the glycemic response (Bello-Perez et al., 2020). Under the action of α-glucan water dikinase (GWD; EC 2.7.9.4) and phosphoglucan, water dikinase (PWD; EC 2.7.9.5), starch is degraded into branched and linear glucans (Fettke et al., 2007; Shoaib et al., 2021). Degradation to oligosaccharides and maltose molecules is catalyzed by phosphorolytic (starch phosphorylases, EC 2.4.1.1) and hydrolytic (α-amylases, or 1,4-α-D-glucan-glucanohydrolases, AMY, EC 3.2.1.1; β-amylases, or 1,4-α-D-glucan maltohydrolases, BAM or Bmy, EC 3.2.1.2) enzymes (Solomos, Mattoo, 2005; Zeeman et al., 2007; Shoaib et al., 2021). AMY hydrolyzes endo-α-1,4-glycosidic bonds, forming oligosaccharides of various lengths, while BAM cleaves off the second from the end α-1,4-glycosidic bond, releasing disaccharides (Zeeman et al., 2007; Shoaib et al., 2021). The release of glucose molecules occurs under the exo-action of α-glucosidases (1,4-α-d-glycan-glucohydrolase, EC 3.2.1.20), which break the extreme α-1,4- and α-1,6-glycosidic bonds (Taylor et al., 2000). The reduced activity of both α-amylases and α-glucosidases significantly reduces the rate of starch hydrolysis, which is a positive effect both for preventing CIS of tubers during storage and for increasing the dietary value of potato (Riyaphan et al., 2018).

According to studies of β-amylases in various plant species, these hydrolases are also highly significant for starch hydrolysis. In the model species *Arabidopsis thaliana* L., a family of β-amylases is characterized, consisting of nine enzymes with different localization and function (Monroe, Storm, 2018). Phylogenetic analysis of the amino acid sequences of β-amylases from 136 different species of algae and land plants showed that modern angiosperms contain eight clades of β-amylases, as well as a clade of inactive BAM10 enzymes, which is absent in *Arabidopsis* (Thalmann et al., 2019). At the same time, *Arabidopsis* BAM4 homologs are absent in many starchy crops, which suggests species-specific regulation of starch digestion (Thalmann et al., 2019).

The functional activity of individual enzymes of the BAM family is elucidated using various approaches and methods. Thus, the importance of the expression level of endosperm-specific β-amylase (*Bmy1*) and constitutive *Bmy2* genes during the development of barley grain for determining the quality of malting is demonstrated (Vinje et al., 2019). A significant role of the *PbrBAM3* gene (birch pear *Pyrus betulaefolia* Bunge) in plant resistance to cold due to an increase in the level of soluble sugars is shown (Zhao et al., 2019). Most of the stu-

Table 1. Potato cultivars used in the study

Cultivar	Cultivar ID*	Starch content*, %	Purpose*
Nadezhda	9463920	13.9–17.9	French fries
Krasavchik	9553926	12.4–17.8	
Severnoe siyanie	8558886	14.7–15.7	
Barin	8854151	13.4–14.6	Table
Utro	9253216	15.0–18.0	

* According to: https://reestr.gossortrf.ru/.

dies (mainly from the 1990s) are published on β-amylases of sweet potato (*Ipomoea batatas* (L.) Lam.), the results of which indicate the importance of this enzyme in modulating the properties of sweet potato starch in order to increase consumer qualities (Guo et al., 2019).

Despite the participation of β-amylases in the breakdown of starch shown in other plants, there are few works on their study in potato. It has been shown that these enzymes are capable of hydrolyzing potato tuber amylose to maltose without residue (Hopkins et al., 1948). The activity of β-amylases increases significantly when the storage temperature of tubers decreases from 20 °C to 3–5 °C (Nielsen et al., 1997), as well as during the germination of tubers at the physiological dormancy release (Vajravijayan et al., 2018). Transcriptomic and proteomic analysis of potato tubers stored at 15, 4, and 0 °C confirmed that the regulation of reducing sugar accumulation is positively associated with the expression of β-amylases (Lin et al., 2019).

The level of *StBAM1* and *StBAM9* gene expression positively correlates with the accumulation of reducing sugars in tubers stored at low temperatures (Zhang et al., 2014a). The StBAM1 enzyme can be inactivated by interaction with the amylase inhibitor SbAI (Zhang et al., 2014b), as well as by ubiquitination and degradation of StBAM1 triggered by the transcription factor SbRFP1 (Zhang et al., 2019).

In this study, the dynamics of the expression of genes for β-amylases *StBAM1*, *StBAM9* and *StAI* amylase inhibitor, as well as changes in the content of starch and reducing sugars were analyzed in tubers of five potato cultivars (Nadezhda, Barin, Krasavchik, Utro, Severnoe siyanie) under long-term low-temperature storage. The choice of cultivars was due to differences in the tuber starch content.

Materials and methods

In the study, tubers of five potato cultivars (Nadezhda, Barin, Krasavchik, Utro, Severnoe siyanie) were used, differing, according to the originators (https://reestr.gossortrf.ru/), in tuber starch content and purpose (Table 1). The plants were grown in 2021 in the field of the Federal Potato Research Center named after A.G. Lorch (Moscow region, Russia); at the end of August, two plants of each cultivar were transferred to the conditions of the experimental climate control facility in the Institute of Bioengineering (Research Center of Biotechnology, Russian Academy of Sciences). In September 2021, the tubers were collected, homogenized and used (peel and pulp together) for subsequent analysis of β-amylase (*StBAM1* and *StBAM9*) and amylase inhibitor (*StAI*) gene expression, as well as for determining the content of starch and reducing sugars (glucose and fructose).

Table 2. Primers used for qRT-PCR

Gene	Primer	Primer sequence (5′→3′)
StBAM1[3]	Forw/Rev	CCGGGAGAGTATAATTGGGG ACAACCCACCTTGGAAGAGG
StBAM9[3]	Forw/Rev	GATGGAAAGACTCCGGTTCAAG ATGGATTGTGATGAGAAGGATAGC
StAI[1]	Forw/Rev	TTGTAACATGGCTCGCGTTC TGTTGGTGAAGCACTTGGAG
ef1[2]	Forw/Rev	ATTGGAAACGGATATGCTCCA TCCTTACCTGAACGCCTGTCA
SEC3A[2]	Forw/Rev	GCTTGCACACGCCATATCAAT TGGATTTTACCACCTTC-CGCA

[1] Dyachenko et al., 2021; [2] Lopez-Pardo et al., 2013; Tang et al., 2017; [3] primers developed in this study.

Total RNA was isolated from 50–100 mg of tuber tissue (RNeasy Plant Mini Kit, QIAGEN, Germany), additionally purified from DNA impurities (RNase free DNasy set, QIAGEN) and used for cDNA synthesis (GoScript™ Reverse Transcription System, Promega, USA), according to manufacturer's protocols. The quality of RNA was checked by electrophoresis in 1.5 % agarose gel. RNA and cDNA concentrations were determined on a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA) using appropriate reagents (Qubit RNA HS Assay Kit and Qubit DS DNA HS Assay Kit, Invitrogen, USA).

Expression of the *StBAM1*, *StBAM9*, and *StAI* genes in potato tubers was analyzed by quantitative real-time PCR (qRT-PCR) with normalization of data using the reference genes *elongation factor 1-alpha* (*elf1*; LOC102600998) and *SEC3A* (LOC102599118) (Lopez-Pardo et al., 2013; Tang et al., 2017) (Table 2). For qRT-PCR, we used 3 ng of the cDNA template, cDNA-specific primers (see Table 2), the Reaction Mixture for RT-PCR in the Presence of SYBR GreenI and ROX kit (Sintol, Russia), and a thermal cycler CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The reactions were carried out in two biological and three technical replicates under the following conditions: 5 min at 9 °C, 40 cycles (15 s at 95 °C, 50 s at 62 °C).

The β-amylase gene sequences from *S. tuberosum* (*BAM1*, gene ID 102598794; *BAM1*, 102584887; *BAM8*, 102598339; *PCT-BMY1*, 102577806; *BAM3*, 102594291; *BAM-like*,

102584563; *BAM7*, 102593066; *BAM9*, 102590483) and model species *A. thaliana* (*BAM1*, 821975; *BAM2*, At2g45880, 827959; *BMY2* (*BAM8*), 834566; *CT-BMY* (*BAM3*), 827419; *BAM4*, AT5G55700, 835664; *BAM5*, 827185; *BAM6*, 817789; *BAM7*, AT2G45880, 819196; *BAM9* (*BMY3*), At5g18670, 831985) were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov). The phylogeny of the β-amylase protein sequences was assessed to determine the *S. tuberosum* homologs of *Arabidopsis* β-amylases, which are most significant in starch degradation. The analysis was performed with MEGA7 (https://www.megasoftware.net/) using the maximum likelihood method based on the JTT model; bootstrap – 1000 replicates. Based on the transcripts of the *S. tuberosum* β-amylase genes, we designed primers for the analysis of *StBAM1* (gene ID 102584887) and *StBAM9* (gene ID 102590483) expression (see Table 2); the forward and reverse primers were separated by at least one intron. The gene specificity of primers was verified by comparing their sequences with *S. tuberosum* transcripts using the NCBI-primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Starch content (mg/g fresh tissue) was determined using an Eppendorf BioSpectrometer® basic (Eppendorf, Germany; λ = 340 nm) and a Starch enzyme test (Boehringer Mannheim/R-Biopharm AG, Switzerland) with some modifications to the manufacturer's protocol.

Briefly, tuber material (together pulp and peel) (~0.02 g; this amount was determined based on known data on the average starch content in potato tubers (13–20 %) and test requirements for the amount of starch in the sample) was homogenized, suspended in the mixture of 1 ml dimethyl sulfoxide (DMSO) and 0.25 ml concentrated hydrochloric acid, and incubated at 60 °C for 60 min with shaking. Then it was cooled to 25 °C, mixed with 2.5 ml of milliQ; the pH was adjusted to 4.5 with 2N sodium hydroxide. The suspension was settled or filtered through Miracloth (Merck, USA). An aliquot of the supernatant was diluted 5, 10, 20 and 100 times; 0.05 ml of the resulting solution was used for the enzyme test and subsequent spectrophotometry. The values corresponding to $\Delta A = 0.115 \pm 0.035$ were considered (based on the manufacturer's recommendations). The analysis was carried out in two biological and three technical replicates.

The content of reducing sugars (glucose and fructose) (mg/g fresh tissue) was measured using high performance liquid chromatography (HPLC) with a Varian ProStar chromatograph (Varian Inc., USA), a 102 M differential refractive index detector for the chromatograph (Stayer model, Khromatek, Russia) and Agilent Pursuit 200Å PFP columns (4.6 × 150 mm, 5 µm HPLC Column, A3050150X046, Agilent, USA). Briefly, 1 g of the tuber material (together the pulp and peel) was ground in liquid nitrogen, suspended in 10 ml of 80 % ethanol, and centrifuged at 16,000 g for 15 min. The supernatant was used for HPLC analysis. Isocratic elution was performed with acetonitrile:water (75:25 v/v) as the mobile phase; flow rate – 1.5 ml/min, temperature – 30 °C. The analysis was carried out in two biological and three technical replicates.

Statistical processing of the qRT-PCR and the starch and sugar content data was performed using the GraphPad Prism v. 8 (GraphPad Software Inc., USA; https://www.graphpad.com/scientific-software/prism/). Data were expressed as mean (M) with standard deviation (± SD) based on two biological and three technical replicates for each measurement option. Welch's *t*-test (unequal variance) was used to assess differences in gene expression and carbohydrate content ($p < 0.05$ indicates statistical significance of differences).

Results

The study was focused on the characterization of the expression of three genes – *StBAM1*, *StBAM9* and *StAI*. The amylase inhibitor gene (*StAI*, gene ID 102591697) is present in the potato genome in one copy (Zhang et al., 2014b; Dyachenko et al., 2021), while the β-amylase family consists of several members (Van Harsselaar et al., 2017). Based on the available NCBI and published data, the available sequences of the *S. tuberosum* and *A. thaliana* β-amylase genes were obtained. Comparative structural-phylogenetic analysis of the encoded enzymes classified *S. tuberosum* β-amylases according to their homology with *A. thaliana* proteins that form nine clades (AtBAM1–AtBAM9) (Fig. 1).

S. tuberosum homologs were found for seven clades of *A. thaliana* β-amylases (with the exception of AtBAM2 and AtBAM4). In particular, two StBAM1 and one StBAM9 (gene ID 102590483) homologs were identified in the potato genome. Based on the obtained dendrogram, StBAM1 (gene ID 102584887) with the highest degree of homology with AtBAM1 was selected from two β-amylases of the BAM1 clade for study (see Fig. 1). We designed primers (see Table 2) for the selected genes *StBAM1* (gene ID 102584887) and *StBAM9* (gene ID 102590483) and used them to analyze their expression.

Tubers of five potato cultivars, Nadezhda, Krasavchik, Severnoe siyanie, Barin, Utro (see Table 1), were collected in September and stored at +3 °C. Tuber tissues were collected for expression and biochemical analyses in September (fresh harvest), February (5–6 months of storage) and April (8 months of storage).

To determine the possible activity of *StBAM1*, *StBAM9* and *StAI*, key enzymes of starch degradation (Zhang et al., 2014a, b), the expression of genes encoding them in tubers was analyzed during low-temperature storage (+3 °C; September, February, April) (Fig. 2). A significant decrease in *StBAM1* expression was shown in April compared to September (most pronounced in cv. Krasavchik and Utro). At the same time, the difference between the February and April data was insignificant: the level of gene expression continued to slightly decrease or did not change (see Fig. 2).

StBAM9 expression also decreased significantly in February compared to September, but not as sharply as *StBAM1* expression. The exception was cv. Krasavchik, where the *StBAM9* transcription has not changed. In April, compared to February, *StBAM9* expression slightly increased (Nadezhda), did not change (Utro, Barin, Severnoe siyanie), or decreased (Krasavchik) (see Fig. 2).

A similar trend was also observed for the *StAI* gene. Its expression sharply decreased in April compared to September in cv. Severnoe siyanie, Barin and Utro. In cv. Nadezhda and Krasavchik, the level of *StAI* transcription decreased smoothly. In April, as compared to February, *StAI* expression slightly increased (Utro), did not change (Nadezhda, Kra-

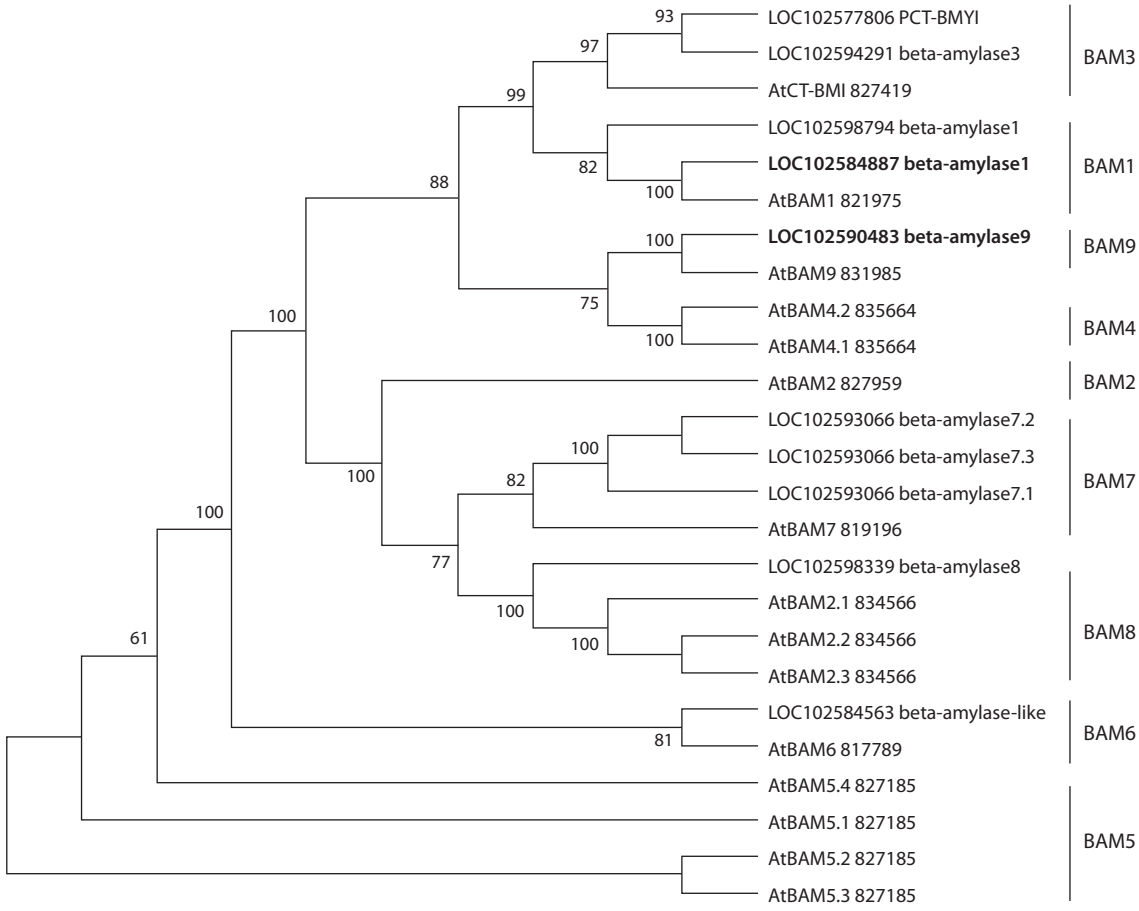

Fig. 1. Unrooted consensus dendrogram based on the alignment of 25 amino acid sequences of β-amylases from *S. tuberosum* (BAM1, gene ID 102598794; BAM1, 102584887; BAM8, 102598339; PCT-BMY1, 102577806; BAM3, 102594291; BAM-like, 102584563; BAM7, 102593066; BAM9, 102590483, including isoforms) and model species *A. thaliana* (BAM1, 821975; At2g45880 BAM2, 827959; BMY2 (BAM8), 834566; CT-BMY (BAM3), 827419; AT5G55700 BAM4, 835664; BAM5, 827185; BAM6, 817789; AT2G45880 BAM7, 819196; At5g18670 BAM9 (BMY3), including isoforms).

The analysis was carried out in the MEGA 7.0 using the maximum likelihood method based on the JTT model. Branches corresponding to clusters replicated in less than 50 % of bootstrap replicates are collapsed. The percentage of repeating trees where related taxa are grouped together in the bootstrap test (1000 replicates) is shown next to the branches.

savchik, and Barin), or sharply decreased (Severnoe siyanie) (see Fig. 2).

Thus, we observed a similar trend towards a decrease in the expression level for all three analyzed genes in potato tubers during low-temperature storage.

To assess the possible correlations between the expression of β-amylase and amylase inhibitor genes with the content of starch and reducing sugars in the tubers, a biochemical analysis of the content of starch, glucose, and fructose was carried out during low-temperature storage of tubers (September, February, April) (Fig. 3).

As expected, compared to September, the starch content in tubers of all cultivars significantly decreased in April (see Fig. 3). At the same time, the content of reducing sugars in February and April in all cultivars was significantly higher than in September. Compared with February, in April the content of glucose and fructose in the tubers of cv. Utro, Nadezhda, and Krasavchik continued to grow, while in cv. Barin and Severnoe siyanie, it sharply decreased (see Fig. 3). At the same time, in February, the tubers of cv. Barin had the highest content of fructose and glucose – 1.5–3.0 and 1.5–4.0 times higher than in the other cultivars. The lowest rates were in the tubers of cv. Nadezhda. In April, there were no significant differences between the cultivars, except for a lower (compared to the other cultivars) glucose content in the cv. Severnoe siyanie tubers.

Thus, during low-temperature storage from September to April, the starch content in tubers of all cultivars decreased to varying degrees, and the content of reducing sugars increased in tubers of cv. Nadezhda and Utro. In cv. Krasavchik, Barin and Severnoe siyanie, the content of reducing sugars increased from September to February, and in April it did not change compared to February (Krasavchik) or significantly decreased (Barin, Severnoe siyanie).

Discussion

Potato tubers stored at a low temperature (+3 °C) were characterized in dynamics (harvest, 5–6 and 8 months of storage) by the expression of β-amylase (*StBAM1*, *StBAM9*) and amylase inhibitor (*StAI*) genes, as well as by the content of starch and

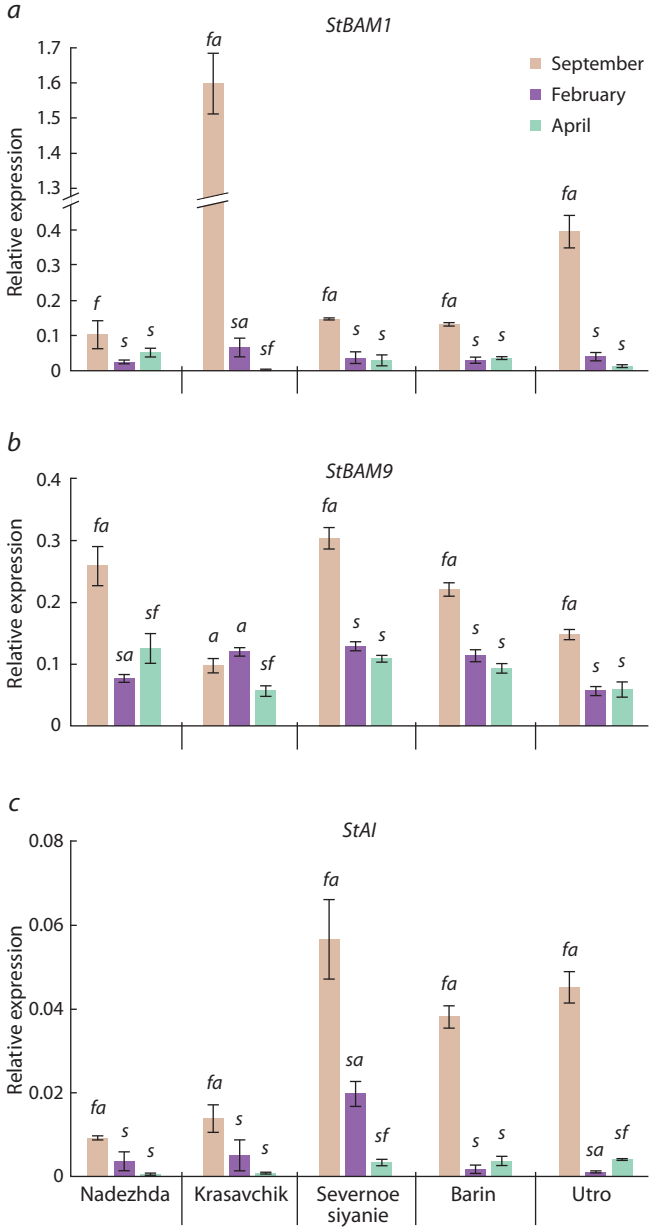

Fig. 2. *StBAM1* (*a*), *StBAM9* (*b*), and *StAI* (*c*) gene expression pattern in tubers of five potato cultivars (Nadezhda, Krasavchik, Severnoe siyanie, Barin, Utro) during low-temperature (+3 °C) storage (September, February, April).

The letters *s*, *f*, and *a* above the columns indicate a significant difference ($p < 0.05$) of a particular value of gene expression from the values for two other months within each sample (*s* – September, *f* – February, *a* – April).

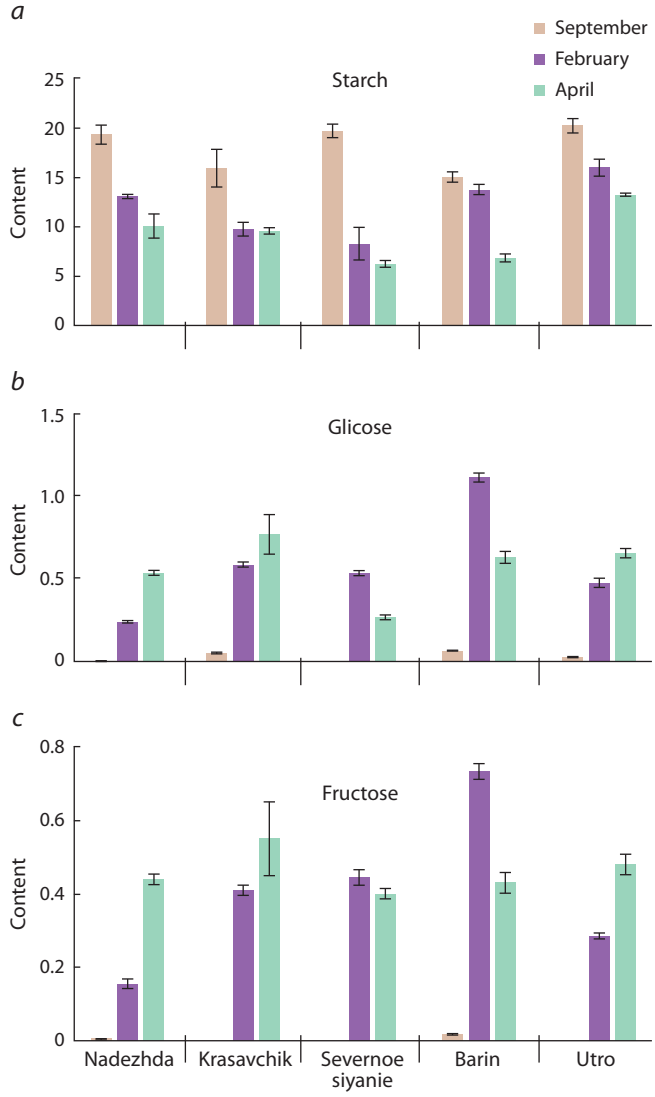

Fig. 3. The content of starch and reducing sugars (glucose, fructose) (mg/g fresh tissue) in potato tubers of five cultivars (Nadezhda, Krasavchik, Severnoe siyanie, Barin, Utro) during low-temperature (+3 °C) storage (September, February, April).

reducing sugars. The five cultivars selected for analysis are divided into two groups depending on the purpose: table (Barin and Utro) and french fries (Nadezhda, Krasavchik, Severnoe siyanie) (see Table 1). This division is related to the degree of sensitivity of each cultivar to cold-induced sweetening of tubers; the higher the resistance, the more suitable the variety for the production of french fries, since in CIS-unstable varieties, frying is accompanied by an increased formation of reducing sugars, leading to the synthesis of acrylamide (Sonnewald S., Sonnewald U., 2014; Hou et al., 2019; Tai et al., 2020).

The accumulation of reducing sugars, which is characteristic of both cold-induced sweetening and the release of tubers from dormancy, positively correlates with the expression of β-amylase genes (Zhang et al., 2014a; Lin et al., 2019). The analyzed *StBAM1* and *StBAM9* are homologs of the *A. thaliana BAM1* and *BAM9* (see Fig. 1), localized in plastid and catalytically active (BAM1) or inactive (BAM9) (Monroe, Storm, 2018). The putative functional similarity of StBAM1 and StBAM9 with the corresponding *A. thaliana* enzymes is supported by other studies. Thus, it was shown that StBAM1 and StBAM9 make different contributions to the cold-induced sweetening of tubers. StBAM1 is localized in the amyloplast stroma and hydrolyzes soluble starch (Hou et al., 2017). StBAM9 is an inactive enzyme (Zhang et al., 2014b), but plays a dominant role in cold-induced sweetening (Hou et al., 2017). Localized on the surface of a starch granule, StBAM9 forms a protein complex with StBAM1, thus attracting catalytically

active StBAM1 to release soluble glucan molecules from the surface of the granules (Hou et al., 2017). The StBAM1 enzyme can be inactivated by interaction with the amylase inhibitor SbAI (Zhang et al., 2014b), as well as by ubiquitination and degradation of StBAM1 triggered by the transcription factor SbRFP1 (Zhang et al., 2019).

Considering the above data, we expected an increase in the expression level of *StBAM1* and *StBAM9* and a decrease in *StAI* transcription in tubers during long-term low-temperature exposure (5–6 and 8 months). However, we observed a significant decrease in the expression of all three genes (see Fig. 2), although the starch content decreased and the amount of reducing sugars increased (see Fig. 3). It can be assumed that the *StBAM1* and *StBAM9* gene activity correlates with the amount of starch (positively) and monosaccharides (negatively). The level of *StAI* expression, in turn, may be directly dependent on the level of expression of the *StBAM1* and α-amylase genes.

In addition, an increase in *StBAM1* and *StBAM9* expression was previously shown after 30 days of low temperature exposure (Zhang et al., 2014a), while in this study, analyses were performed 7 and 9 months after storage. We assume that after 30 days of storage of physiologically dormant tubers, it can be considered as a short-term cold stress, during which the tubers accumulate a sufficient amount of reducing sugars for cold resistance, after which an equilibrium is established between the content of starch/disaccharides and the activity of enzymes for starch degradation. In addition, the participation of α-amylases (hydrolysis) (Zhang et al., 2014a) and plastid starch phosphorylase (phosphorolysis) (Slugina et al., 2020) in starch catabolism should be taken into account.

Conclusion

Considering the data obtained, it can be concluded that there is no relationship between the degree of cultivar predisposition to cold-induced sweetening of tubers and the expression profile of β-amylase (*StBAM1*, *StBAM9*) and amylase inhibitor (*StAI*) genes.

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Combining ability for quantitative traits related to productivity in durum wheat

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Abstract. The present study was to determine the nature of gene action and combining ability of six quantitative traits related to productivity of five varieties and ten hybrid combinations of durum wheat. Five modern durum wheat varieties were used in diallel crosses as parents. The study includes three F_1 and two F_2 generations. The experiments were done in a randomized block design in three replications during three years. Significant differences between the genotypes in both generations was found for all the traits. The general combining ability and specific combining ability showed reliability in both generations. Obtained results suggests that breeding schemes should include both types of genetic effects in order to improve productivity components. The ratio of variances showed that general combining ability has a greater influence on the inheritance of plant height, spike length and thousand kernels weight. For productivity tillering capacity, number of spikelets per spike and kernels weight per spike, specific combining ability has a great impact in inheritance. For thousand kernels weight a redetermination of the genetic formula was established in both generations. Durum wheat varieties Deni, Superdur and Progres were found to be the best general combinators for studied productivity elements. The most valuable cross combinations were Deni × Superdur, Superdur × Predel and Progres × Predel. Parental wheat varieties and progenies from these crosses can be used for improving productivity components and for increasing yields in durum wheat breeding programs.
Key words: gene action; combining ability; quantitative traits; durum wheat; diallel cross.

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Комбинационная способность количественных признаков, связанных с продуктивностью твердой пшеницы

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Аннотация. Настоящее исследование направлено на определение характера действия генов и комбинационной способности твердой пшеницы по шести количественным признакам для пяти сортов и десяти гибридных комбинаций. В исследование были включены пять современных сортов твердой пшеницы в качестве родителей при диаллельном скрещивании. Изучены три поколения F_1 и два поколения F_2. Эксперименты выполнены в рандомизированном блочном дизайне в трех повторениях в течение трех лет. Выявлены достоверные различия между генотипами по всем изучаемым признакам в обоих поколениях. Общая комбинационная способность и специфическая комбинационная способность показали достоверные отличия в обоих поколениях. Полученные результаты позволяют предположить, что селекционная схема должна учитывать оба типа генетических эффектов для улучшения элементов продуктивности. Соотношение дисперсий демонстрирует, что общая комбинационная способность больше влияет на наследуемость признаков «высота растения», «длина колоса» и «масса 1000 зерен». Для признаков «продуктивная кустистость», «число колосков в колосе» и «масса зерен в колосе» большее влияние на наследование оказывает специфическая комбинационная способность. Для массы 1000 зерен в обоих поколениях установлено переопределение генетических формул. Лучшими общими комбинаторами по элементам продуктивности одновременно по нескольким признакам являются сорта Дени, Супердур и Прогрес. Самые ценные комбинации скрещиваний по нескольким признакам – Дени × Супердур, Супердур × Предел и Прогрес × Предел. Эти сорта и комбинации можно использовать для улучшения признаков продуктивности и повышения урожайности в программах селекции твердой пшеницы.
Ключевые слова: действие генов; комбинационная способность; количественные признаки; твердая пшеница; диаллельное скрещивание.

Introduction

Breeding strategy of durum wheat is based on genetic information on the inheritance of the main quantitative traits related to productivity. To obtain such information, it is necessary to apply a genetic model corresponding to the source material to be used. In a regular breeding program, it is important to identify the best parents for hybridization and crosses to select valuable genotypes (Inamullah et al., 2006). Diallel crosses have been used for a long time in genetic research to determine the inheritance of a trait among a set of genotypes and to identify superior parents for hybrid or varieties development.

Information on additive gene effects i. e. general combining ability effects (GCA) is of great importance, because it successfully predicts the genetic potential of parents who give desired results in segregating generations. In determining the specific combining ability effects (SCA), a relationship is established with the non-additive gene effects (dominance and epistasis components). The identification of a good hybrid combination with high SCA on a given trait makes it possible to expect a more probable transgressive form for the trait. Combining ability describes the breeding value of parental varieties to produce better hybrids as well as their crosses (Griffing, 1956).

The importance of combining ability is related to the evaluation of parental lines and their hybrids by their respective additive and non-additive genetic effects in relation to a certain trait. Diallel crosses give a more general view of combining ability, where general and specific combining ability are indicators for nature of gene action (Farooq et al., 2010). Assesment of GCA effects show that it is not possible to choose a good general combiner for all traits of the productivity. This is due to the inability to combine in one genotype high GCA on all traits (Kashif et al., 2008). However, some parents show desired GCA effects for several traits. It is obvious that high-yielding varieties included in crosses are mainly responsible for increasing productivity (Adel, Ali, 2013).

A number of authors, using schemes of full and half diallel crosses, have established the breeding value of a large number of varieties and the gene action for traits related to productivity. In the publications cited below, the authors found that both additive and non-additive gene effects played a role in the inheritance of tested traits. According to J. Yao et al. (2011) and M. Singht et al. (2018) plant height and spike length mainly controlled by additive gene effects. It was reported that in the inheritance of thousand kernels weight the non-additive gene effects play an essential role (Akinci, 2009; Pansuriya et al., 2014), while A. Hannachi et al. (2017) and A. Hassan et al. (2018) establish inverse. Plant height, tillering capacity and number of spikelets per spike were mainly controlled by non-additive gene effects (Adel, Ali, 2013; Pansuriya et al., 2014; Kandil et al., 2016), when A. Hannachi et al. (2017) reported that plant height and productivity tillering capacity were additive. The inheritance of the spike length, number of spikelets per spike, and kernel weight per spike are controlled by non-additive gene effects and they have a major role (Patel et al., 2016; Tiwari et al., 2017), also A. Pansuriya et al. (2014) for these traits and for spike length. Productivity tillering capacity and number of spikelets per spike are controled by non-additive gene effects, on the other hand, for spike length and thousand kernel weight additive gene effects dominate in inheritance (Farooq et al., 2019).

It can be concluded from the published data that parental varieties have a great influence on both types of combining ability. On the other hand, they are carried out in different growing conditions, which gives additional confirms to this statement of diverstity.

The present investigation was undertaken to determine the nature and magnitude of gene action and general and specific combining ability for five modern durum wheat varieties and for six quantitative traits related to productivity in diallel cross of durum wheat.

Materials and methods

Parents and crosses. Five modern durum wheat varieties were included in the study as the parental varieties in the half diallel crosses. The varieties are selected among the new Bulgarian varieties of durum wheats, including the old and the new variety-standard and the Austrian variety Superdur, which has recently become widespread in Bulgaria. Victoria – Bulgaria, Deni – Bulgaria, Superdur – Austria, old variety-standard Progres – Bulgaria and new variety-standard Predel – Bulgaria. The choice of varieties is based on their previous observation. They are created in Field Crops Institute, Chirpan and are genetically distant. Progres and Deni are created by experimental mutagenesis combined with hybridization and Victoria and Predel are created by hybridization. A diallel cross was performed in which all the described varieties were crossed with each other without reciprocal combinations. The crosses was carried out handmade at the beginning of heading time in field condition. The following ten combinations were performed: Victoria × Deni, Victoria × Superdur, Victoria × Progres, Victoria × Predel, Deni × Superdur, Deni × Progres, Deni × Predel, Superdur × Progres, Superdur × Predel, Progres × Predel. From each combination, 30 spikes were castrated and pollinated. From the harvested F_1 plants, the seeds necessary for sowing of F_2 generation were randomly selected.

Management. The parents are sown in each replication in two rows, the F_1 hybrids in two rows, and the F_2 hybrids in five rows. Genotypes are sown handmade in the field in beds. Row length – two meters, row spacing – twenty cm and inside the row – five cm in a randomized block design with three replications. After the full maturity phenophase, the necessary plants from each replication are harvested and collected for biometric research. Twenty plants were selected from the parents and F_1 generation and thirty plants from F_2 generation at random. The diallel cross was performed in three consecutive years. Thus, generation F_1 for three years and F_2 for two years are provided. The experiments was conducted in three harvest years 2014, 2015 and 2016. The experiments was carried out in the breeding field of the Field Crops Institute – Chirpan according to the adopted technology for growing durum wheat. The predecessor is spring peas. The soil type is Chernozems compact Eutric Vertisols (by FAO). The three years meteorological condition are characterized by higher temperatures compared to the multi-year period (Fig. 1). The first year have 18.5 % and the second 58.2 % precipitation over the multi-year period during the growing season, while in the third year precipitation are 17.5 % less than in the multi-year period (Fig. 2).

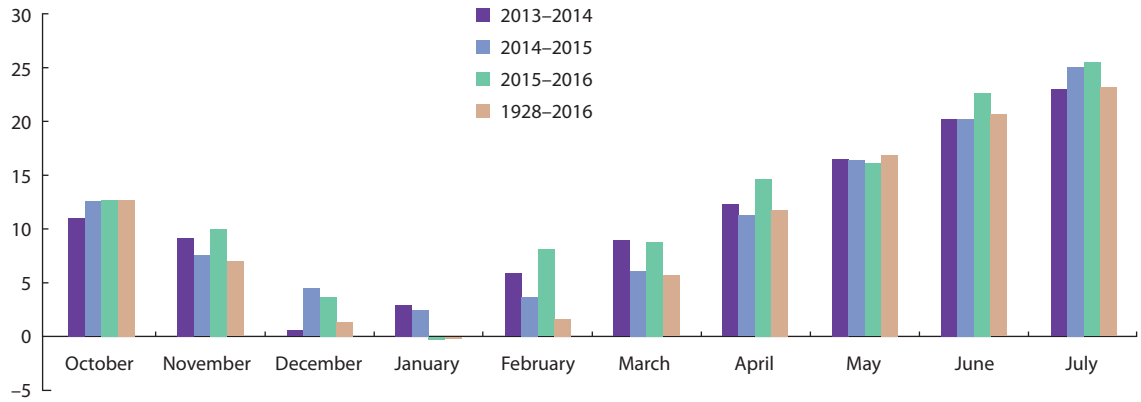

Fig. 1. Average monthly and multiyear air temperature during 2013–2016 harvest years.

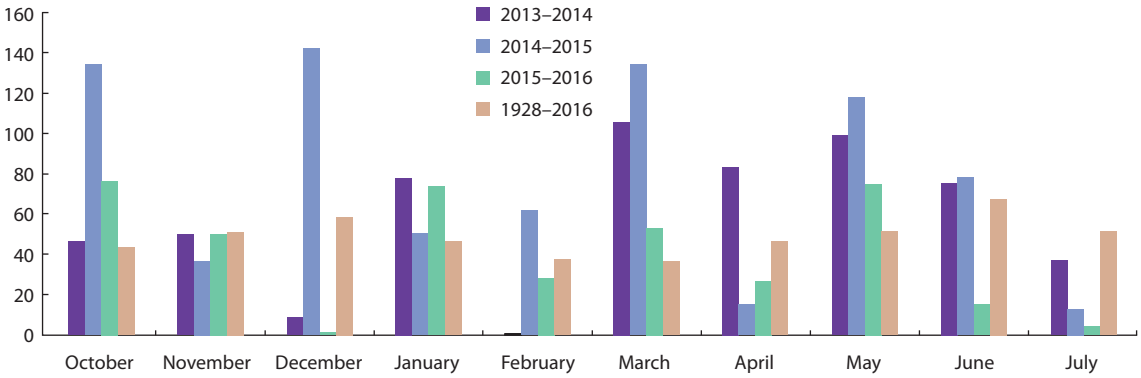

Fig. 2. Average monthly and multiyear amount of precipitation during 2013–2016 harvest years.

The following traits were observed. Plant height (cm) – it is measured from the ground surface to the end of the spike without the awns on the main stem in centimeters. Productivity tillering capacity (pcs.) – the fertile spikes of one plant are counted. Spike length (cm) – measured on the main stem from the base of the spike to the top of the uppermost spikelet. Number of spikelets per spike (pcs.) – the spikelets in the main spike are counted. Number of kernels per spike (pcs.) – all kernels of the main spike are counted after handmade threshing. Thousand kernels weight (g) – five hundred kernels are weighed and multiplied by two. All traits are determined by methodology by Y. Enchev et al. (1976).

Statistical analysis. The data from the three years F_1 and the two years F_2 are averaged and on them are conducted statistical processing. In the processing of the experimental data, mathematical and statistical methods were used on the results according to the set goal of the research. To perform diallel analysis was used combining ability analysis in diallel crosses – by method II model I (Griffing, 1956) with the program software of M. Burow and J. Coors (1994). Analysis of variance (ANOVA) by traits is derived through the same program on M. Burow and J. Coors (1994).

Results

The results of analysis of variance showed statistically significant differences between the genotypes for all studied traits in both generations. The values of the variances for GCA and SCA were significant in both generations (Table 1). Therefore, both additive and non-additive gene effects (dominance and epistasis) were of significant importance in the inheritance of the traits. The studied traits related to durum wheat productivity show that they are controlled by both additive and non-additive gene effects.

The ratio of GCA and SCA variances (σ_g^2/σ_s^2) for F_1 and F_2 are presented in Table 1. For plant height, spike length, number of spikelets per spike and thousand kernel weight, the sum of squares indicates that additive gene effects have a greater influence in inheritance. For the other two traits, the sum of the squares indicates that non-additive gene effects have a greater impact. This is proved by the ratio of the variances of GCA and SCA, respectively. The preponderance of additive gene effects ($\sigma_g^2/\sigma_s^2 > 1$) was found in the inheritance of plant height, spike length and thousand kernel weight. The spike length in F_2 generation showed a significant increase, which indicates that the additivity increases. Domination of additive gene effects allow application of classical breeding methods. For these traits selection can start in early segregating generations (F_2–F_3).

Domination of non-additive gene effects ($\sigma_g^2/\sigma_s^2 < 1$) is observed for the productivity tillering capacity, number of spikelets per spike and kernels weight per spike. Non-additive gene effects (dominance and epistasis) prevalence in their expression.

This analysis does not allow to determine or dominance or epistasis are responsible for the inheritance of the traits. It is well known that when inheritance is determined by non-

Table 1. ANOVA for general combining ability (GCA), specific combining ability (SCA) and relation to variance of GCA and SCA (σ_g^2/σ_s^2) for six traits related to productivity

Traits	Source of variance	F_1			F_2		
		Sum of squares	Mean squares	Significant (*, **, ***)	Sum of squares	Mean squares	Significant (*, **, ***)
Plant height	Genotype	1965.8	140.4	***	2032.0	145.1	***
	GCA	1695.3	423.8	***	1515.8	378.9	***
	SCA	270.4	27.0	***	516.3	51.6	***
	Error	78.9	2.8		92.7	3.3	
	σ_g^2/σ_s^2	2.32			1.08		
Productivity tillering capacity	Genotype	30.299	2.164	***	12.83	0.91	***
	GCA	9.987	2.497	***	2.22	0.55	***
	SCA	20.311	2.031	***	10.6	1.06	***
	Error	2.75	0.098		2.89	0.10	
	σ_g^2/σ_s^2	0.03			0.06		
Spike length	Genotype	20.97	1.49	***	19.19	1.37	***
	GCA	17.26	4.31	***	18.22	4.55	***
	SCA	3.71	0.37	***	0.97	0.09	*
	Error	1.29	0.04		2.2	0.08	
	σ_g^2/σ_s^2	1.8			42		
Number of spikelets per spike	Genotype	27.78	1.98	***	16.15	1.15	**
	GCA	15.12	3.78	***	10.95	2.73	***
	SCA	12.67	1.26	***	5.2	0.52	*
	Error	5.68	0.20	***	7.42	0.26	
	σ_g^2/σ_s^2	0.31			0.12		
Kernels weight per spike	Genotype	2.64	0.189	***	1.80	0.12	***
	GCA	0.08	0.20	*	0.66	0.17	***
	SCA	2.55	0.25	***	1.13	0.11	***
	Error	0.47	0.01		0.33	0.01	
	σ_g^2/σ_s^2	0.14			0.06		
Thousand kernels weight	Genotype	445.8	31.8	***	626.6	44.7	***
	GCA	334.6	83.6	***	456.3	114.1	***
	SCA	111.2	11.1	***	169.3	16.9	***
	Error	82.5	2.94		56.8	2.03	
	σ_g^2/σ_s^2	1.26			1.02		

* $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$; σ_g^2 – GCA variance; σ_s^2 – SCA variance.

additive genetic effects, selection in early segregating generations will be difficult. In this case effective selection must start in the later segregating generations F_4–F_5.

Although the preponderance of additive genetic effects for the thousand kernel weight in the individual years and generations has been established, there is a change in the genetic effects controlling the trait. This is due to the genotype-environment interaction and is explained by the phenomenon of redetermination of the genetic formula. In F_1 in 2014 the non-additive genetic effects preponderance, and in 2015 and 2016 the additive ones. In F_2 in 2015 the non-additive genetic effects preponderance and in 2016 the additive ones (data not shown). In the individual years in both generations, all other traits show a one-way ratio of variances that determine the influence of genetic effects.

The analysis for GCA of parents and SCA of hybrids for the studied traits in F_1 and F_2 is presented in the next two tables (Tables 2 and 3). From a breeding point of view, genotypes with a negative value for plant height due to the connection with lodging are more valuable. For all other traits, positive values are preferable, as their increase will lead to an increase in productivity.

Table 2. General combining ability of parents and specific combining ability of crosses for three traits related to productivity

Genotype	Plant height		Productivity tillering capacity		Spike lenght	
	F_1	F_2	F_1	F_2	F_1	F_2
Parents/error of parents	±0.51	±0.56	±0.096	±0.099	±0.06	±0.08
Victoria	6.69*	5.82*	–0.58*	–0.06 n. s.	–0.79*	–0.79*
Deni	–0.26 n. s.	–1.46*	0.23*	0.19*	0.32*	0.35*
Superdur	–4.8*	–3.55*	0.16*	–0.10*	0.11*	0.15*
Progres	1.58*	2.99*	–0.04 n. s.	0.15*	0.25*	0.29*
Predel	–3.2*	–3.79*	0.23*	–0.17*	0.09*	–0.004 n. s.
Hybrid combinations	F_1	F_2	F_1	F_2	F_1	F_2
Crosses/error of crosses	±1.15	±1.25	±0.216	±0.22	±0.14	±0.19
Victoria × Deni	0.12 n. s.	2.59*	–0.60*	0.23*	0.04 n. s.	0.14 n. s.
Victoria × Superdur	3.30*	5.51*	0.36*	–0.01 n. s.	0.25*	–0.01 n. s.
Victoria × Progres	1.37*	–1.07 n. s.	–0.42*	0.13 n. s.	0.31*	–0.15 n. s.
Victoria × Predel	0.37 n. s.	2.29*	0.03 n. s.	–0.17 n. s.	0.26*	0.07 n. s.
Deni × Superdur	5.42*	3.58*	0.01 n. s.	–0.20 n. s.	0.30*	0.26*
Deni × Progres	–1.40*	0.45 n. s.	1.12*	0.10 n. s.	0.29*	–0.003 n. s.
Deni × Predel	–0.10 n. s.	0.05 n. s.	0.41*	–0.77*	0.31*	–0.27*
Superdur × Progres	–1.59*	4.11*	0.09 n. s.	–0.13 n. s.	–0.15*	0.13 n. s.
Superdur × Predel	0.26 n. s.	1.8*	1.41*	–1.10*	–0.03 n. s.	–0.13 n. s.
Progres × Predel	–3.28*	–1.88*	0.22*	0.33*	0.15*	0.22*

* $p \leq 0.05$; n. s. – no significant.

Table 3. Values for general combining ability of parents and specific combining ability of crosses for three quantitative traits related to productivity

Genotype	Number of spikelets per spike		Kernels weight per spike		Thousand kernels weight	
	F_1	F_2	F_1	F_2	F_1	F_2
Parents/error of parents	±0.13	±0.15	±0.04	±0.03	±0.52	±0.43
Victoria	0.15*	0.23*	0.03 n. s.	0.01 n. s.	1.00*	–0.15 n. s.
Deni	0.64*	0.49*	–0.02 n. s.	–0.01 n. s.	0.86*	–0.49*
Superdur	–0.29*	–0.13 n. s.	–0.03 n. s.	–0.05*	–2.06*	–2.00*
Progres	–0.43*	–0.40*	0.02 n. s.	0.12*	2.32*	3.96*
Predel	–0.06 n. s.	–0.18*	0.01 n. s.	0.04*	–2.12*	–1.3*
Hybrid combinations	F_1	F_2	F_1	F_2	F_1	F_2
Crosses/error of crosses	±0.31	±0.35	±0.08	±0.07	±1.18	±0.98
Victoria × Deni	0.67*	0.62*	0.18*	0.04 n. s.	–0.79 n. s.	0.24 n. s.
Victoria × Superdur	0.44*	0.54*	0.12*	–0.08*	1.87*	–0.47 n. s.
Victoria × Progres	0.89*	0.12 n. s.	0.07 n. s.	0.04 n. s.	–0.28 n. s.	–0.24 n. s.
Victoria × Predel	0.32*	–0.12 n. s.	0.16*	–0.06 n. s.	1.46*	0.79 n. s.
Deni × Superdur	0.42*	0.12 n. s.	0.32*	0.15*	2.61*	–0.70 n. s.
Deni × Progres	–0.16 n. s.	–0.01 n. s.	0.10*	0.01 n. s.	1.22*	0.85 n. s.
Deni × Predel	0.09 n. s.	–0.58*	0.19*	0.16*	0.7 n. s.	2.36*
Superdur × Progres	–0.76*	–0.14 n. s.	0.21*	0.28*	0.29 n. s.	3.83*
Superdur × Predel	–0.36*	–0.22 n. s.	0.16*	0.17*	1.5*	2.71*
Progres × Predel	–0.25 n. s.	0.01 n. s.	0.01 n. s.	0.09*	0.34 n. s.	0.61 n. s.

* $p \leq 0.05$; n. s. – no significant.

Plant height. Table 2 presents the values for plant height. The varieties Victoria and Progres has a significant and positive values for GCA in F_1 and F_2. They increase the plant height in the hybrids in which he participated as parents. The varieties Superdur and Predel have negative significant values of GCA in both generations. These varieties reducing the plant height in the hybrids in which they participate. They can be used successfully in the breeding program for obtaining dwarf durum wheats. In terms of SCA, valuable are the hybrid combination Progres × Predel, with significant negative values in both generations. The other crosses have different values for SCA, and in different generations they are differently significant and change their signs according to the generation.

Productivity tillering capacity. Table 2 presents the values for GCA and SCA for the productivity tillering capacity. Significant values of GCA to increase the trait of productivity tillering capacity have Deni variety in both generations. The varieties Superdur and Predel have positive and significant values in F_1 generation, while in F_2 generation the values are significant but negative. Variety Victoria has a significant and negative GCA in F_1 and a negative and nonsignificant in F_2 and reduces the values of the trait. Of greater interest are hybrid combinations and their SCA values (see Table 2), as non-additive effects have been found to preponderance. The results show that one of the crosses Progres × Predel has significant and positive values in both generations for SCA. The other crosses occupy an intermediate position.

Spike length. Table 2 presents the values for the general and specific combining ability of parents and hybrids for spike length. We define the varieties Deni, Superdur and Progres as good general combinators to increase spike length, as they have positive and significant values for GCA in both generations. Victoria variety has significant and negative values in both generations and it decreases the spike length in the hybrids in which it participates. The SCA values of the hybrids show that two crosses showed significant and positive values in F_1 and F_2 are Deni × Superdur and Progres × Predel.

Number of spikelets per spike. The values for GCA and SCA for the trait number of spikelets per spike are presented in Table 3. The Victoria and Deni varieties in both generations have significant values to increasing number of spikelets per spike and they are good general combinators for this trait. Variety Progres has negative GCA and reducing the values of the trait. The other varieties have nonsignificant values, which shows their insignificant role. The greate interest is in hybrid combinations, as non-additive gene effects have been shown to play a major role in inheritance. The hybrid combinations Victoria × Deni and Victoria × Superdur show significant and positive SCA effects in both generations. With the highest SCA value is the cross Victoria × Deni.

Kernels weight per spike. Table 3 represents the values for parental GCA and hybrid SCA. In F_1 there are no varieties with significant GCA effects. No good general combinators have been reported to increase kernels weight per spike in both generations. In the F_2 generation, the Progres and Predel varieties increase the values of the kernels weight per spike, and the Superdur variety decreases it.

These results are very contradictory and it is difficult to define any of the varieties as a good general combiner on this trait. We can consider that the varieties Progres and Predel are good general combinators on the basis of showing significant and positive GCA effects in F_2 generation. Greater attention should be paid to hybrid combinations, as SCA effects preponderance. Four significant good hybrid combinations are observed in terms of SCA effects. They are positive and significant in both generations. The most promising hybrid combinations are Deni × Superdur, Deni × Predel, Superdur × Progres and Superdur × Predel.

Thousand kernels weight. The variety Progres (see Table 3) is good general combiners for increasing the values of the trait thousand kernels weight. Variety Progres has positive and significant values in both generations. On the other hand, general combiners that have been significant to reduce grain size are the Predel and Superdur varieties in both generations. In Table 3 can be seen that the cross Superdur × Predel, which in both generations shows positive and significant values for the SCA effects, is interesting in terms of breeding. The remaining crosses in most cases have a significant value in only one of the generations. For all traits the values for GCA and SCA in most cases are in one-way direction and can be relied on their reliability.

For the possibilities of heterosis in the breeding of durum wheat and obtaining transgressive forms, it is necessary to consider the crosses with significant SCA effects on several traits. Of the studied hybrid combinations (see Tables 2 and 3) as the most promising with significant SCA effects are Progres × Predel for plant height; Progres × Predel for productivity tillering capacity; Deni × Superdur and Progres × Predel for spike length; Victoria × Deni and Victoria × Superdur for the number of spikelets per spike; Deni × Superdur, Deni × Predel, Superdur × Progres and Superdur × Predel for grains weight per spike; Superdur × Predel for the thousand kernels weight.

Discussion

Development of wheat varieties possessing improved yield related characters had been the major objective of durum wheat breeders. Thus availability of genetically based variation for traits like plant height, productivity tillering capacity, spike length, number of spikelets per spike, kernels weight per spike and thousand grain weight breeding population is essential. Present genetic material used here to generate information on genetic nature of these traits. A number of studies by other investigations are in line with the results obtained by us for GCA and SCA. Many researchers have also found significant GCA and SCA effects for the plant height (Topal et al., 2004; Pansuriya et al., 2014; Ali et al., 2018; Singh et al., 2018; Sharma et al., 2019; Ayoob, 2020); for productivity tillering ability (Topal et al., 2004; Akinci, 2009; Adel, Ali, 2013; Parveen et al., 2018; Talha et al., 2018; Bajaniya et al., 2019; Farooq et al., 2019; Hammam et al., 2020); for spike length (Topal et al., 2004; Yao et al., 2011; Pansuriya et al., 2014; Patel et al., 2016; Rajput, Kandalkar, 2018; Sadeghzadeh-Ahari et al., 2018; Khaled et al., 2020; Shamsabadi et al., 2020); for number of spikelets per spike (Adel, Ali, 2013; Pansuriya et al., 2014; Kandil et al., 2016; Patel et al., 2016; Saeed, Khalil, 2017; Parveen et al., 2018; Khaled et al., 2020); for kernels weight per spike (Topal et al., 2004; Adel, Ali, 2013; Mandal, Madhuri, 2016; Patel et al., 2016; Talha et al., 2018; Amin, Towfiq, 2019; Shamsabadi

et al., 2020); for thousand kernels weight (Topal et al., 2004; Akinci, 2009; Desale, Mehta, 2013; Brahim, Mohamed, 2014; Motawea, 2017; Ali et al., 2018; Hassan et al., 2018; Ali, 2019; Khokhar et al., 2019; Sharma et al., 2019).

The obtained results for the GCA and SCA give a very clear idea of the control in the inheritance of the traits elements of the yield. The impact of additive and non-additive gene action in the inheritance of the structural elements of the yield shows that in order to maximize the productivity of durum wheat, a system should be used that includes both variances in simultaneously.

Plant height and spike length are used for an individual selection by the classical methods. In both generations they are controlled by additive gene effects. In most cases thousand kernels weight is also controlled by additive genetic effects. This shows that it is possible for breeders to obtain better results in improving these traits. It should be noted that in the case of plant height, spike length and in most cases for thousand kernels weight, the selection may start in the earlier segregating generations F_2–F_3. Because they are controlled by additive genetic effects. It should be noted that the main structural elements of yield – spike length and in most cases thousand kernels weight are controlled by additive gene effects. Preponderance of additive gene effects in inheritance of plant height has been reported by a number of other researchers (Yao et al., 2011; Motawea, 2017; Ali et al., 2018; Hassan et al., 2018; Rajput, Kandalkar, 2018; Singh et al., 2018; Talha et al., 2018; Sharma et al., 2019; Ayoob, 2020); for spike length (Kandil et al., 2016; Motawea, 2017; Parveen et al., 2018; Rajput, Kandalkar, 2018; Sadeghzadeh-Ahari et al., 2018; Singh et al., 2018; Farooq et al., 2019; Sharma et al., 2019; Khaled et al., 2020; Shamsabadi et al., 2020); for thousand kernels weight (Hannachi et al., 2017; Motawea, 2017; Ali et al., 2018; Hassan et al., 2018; Ali, 2019; Amin, Towfiq, 2019; Farooq et al., 2019; Khokhar et al., 2019; Sharma et al., 2019).

For other three traits preponderance non-additive gene effect in this investigation was observed. Therefore, selection in early segregating generations will be difficult. In this case, it is recommended that an effective selection must start in the later segregating generations F_4–F_5 when the influence of the non-additive effects (dominance) decreases and the additivity increases. The results from this study for productivity tillering capacity are in line with those obtained by other authors (Desale, Mehta, 2013; Mostafa et al., 2014; Kandil et al., 2016; Ahmad et al., 2017; Saeed, Khalil, 2017; El-Gammaal, Morad, 2018; Parveen et al., 2018; Talha et al., 2018; Amin, Towfiq, 2019; Bajaniya et al., 2019; Farooq et al., 2019; Ayoob, 2020; Hammam et al., 2020); for number of spikelets per spike (Mostafa et al., 2014; Kandil et al., 2016; Ahmad et al., 2017; Saeed, Khalil, 2017; Tiwari et al., 2017; Parveen et al., 2018; Talha et al., 2018; Farooq et al., 2019; Ayoob, 2020; Khaled et al., 2020); for kernels weight per spike (Padhar et al., 2013; Mostafa et al., 2014; Kandil et al., 2016; Mandal, Madhuri, 2016; Tiwari et al., 2017; Talha et al., 2018; Amin, Towfiq, 2019, Shamsabadi et al., 2020).

For thousand kernel weight, a redermination of the genetic formula of the trait was found. This is due to the genotype-environment interaction. Redetermination of the genetic formula is especially evident in the case of quantitative traits that are controlled by a large number of small polygens significantly influenced by environmental conditions (Dragavtsev, Averyyanova, 1983). The presence of this phenomenon makes it difficult to lead an effective selection on the thousand kernel weight in different years and generations and the selection must be conducted longer (Dragavtsev, Averyyanova, 1983; Dragavtsev et al., 1984). When the phenomenon of redetermination of the genetic formula is observed in the individual years, different forms are selected, controlling the trait in the breeding process. This means that in different years valuable forms are selected in which the trait is controlled by both additive and non-additive genetic effects.

The deepening of the research allows to specify the methods of the applied breeding strategy and to optimize and increase the efficiency of the selection. The possibility of evaluating genotypes and their breeding value as a starting material for increasing productivity is also important. With the conducted research it is possible to get information about two of the most important moments in a successful breeding program – choosing parents for hybridization and leading a purposeful selection. The selection on a separate trait can increase the yield, but a more significant increase would be obtained by simultaneously comprehensively improving its elements.

Varieties that have significant GCA effects for more than one trait are of grait interest for breeding. The results for the respective traits are presented in Tables 2 and 3. Tables show which varieties are good combiners on the studied traits. Variety Victoria is a good general combiner on the trait number of spikelets per spike and a bad combiner for the traits plant height and spike length. The Deni variety is a good general combiner in terms of productivity tillering capacity, spike length and number of spikelets per spike. The Superdur variety is defined as a good combiner for plant height and spike length and a bad combiner for thousand kernels weight. Variety Progres shows significant and positive values for GCA for the traits spike length and thousand kernels weight, and is a bad combiner for number of spikelets per spike and plant height. Variety Predel is a good combiner for the trait plant height and a bad combiner for thousand kernels weight. A good general combiner at the same time on three traits is the Deni variety. Good general combinators on two traits at the same time are the varieties Superdur and Progres. Good general combinators on one trait are the varieties Victoria and Predel. Varieties Victoria and Progres are bad combiners on two traits. Varieties Superdur and Predel are bad combiners on one trait. The only exception is the Deni variety, which has no traits like a bad combiner. The varieties Deni, Superdur and Progres emerge as the best general combinators for the elements of the productivity on several traits at the same time. To increase the yield, it is necessary to simultaneously improve several valuable traits. The certain general combining abilities are a prerequisite for the correct selection of parental forms and their crossing for the purposes of the durum wheat breeding program.

As can be seen, the varieties bearing high GCA most often enter the crosses with high SCA. According to the various traits, there are good crosses, such as combined parents with high X high GCA and those who have combined parents with low X low GCA. Some with high SCA values are also

a combinations of high X low GCA. As the most valuable hybrid combination with significant SCA effects on several traits its define Deni × Superdur, Superdur × Predel and Progres× Predel.

Determining the combining ability shows that it is not possible to have one variety can good combinator for all traits. Not all crosses with high SCA effects were obtained from the crosses of a good X good GCA parent (Kumar, Maloo, 2012). Rather, crosses with high SCA effects are obtained from crosses between bad X bad and bad X good combiner. They argue that such manifestations are due to the involvement of dominant or epistatis gene effects. Crosses with high SCA may be more likely to be sources of transgression (Gami et al., 2011; Tiwari et al., 2015). Transgressive lines on a certain traits can be a source for creating highly efficient durum wheat varieties. Evaluations of gene action explain the genetic potential of breeding materials and contribute to the targeted management of breeding progress in durum wheat productivity.

Conclusion

The study found that both additive and non-additive gene effects are of significant importance in the nature of gene action of the productivity traits. This implies a breeding system that includes both gene effects for improving the elements of productivity. Inheritance of plant height, spike length and thousand kernels weight is mainly controlled by additive gene effects and it is possible to start selection of genotypes in the early segregating generations F_2–F_3. Inheritance of productivity tillering capacity, number of spikelets per spike and kernels weight per spike is controlled by non-additive gene effects. Therefore, the selection on these traits should start in the later segregating generations F_4–F_5. There is obtained a change in the genetic effects affecting the expression of the trait thousand kernels weight, which indicates the presence of the redetermination of the genetic formula.

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Naked barley: taxonomy, breeding, and prospects of utilization

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Abstract. This review surveys the current state of taxonomy, origin, and utilization prospects for naked barley. The cultivated barley *Hordeum vulgare* L. incorporates the covered and naked barley groups. Naked barleys are divided into six-row naked barley (convar. *coeleste* (L.) A. Trof.) and two-row naked barley (convar. *nudum* (L.) A. Trof.). The groups include botanical varieties differing in the structural features of spikes, awns, floret and spikelet glumes, and the color of kernels. The centers of morphogenesis for naked barley are scrutinized employing archeological and paleoethnobotanical data, and the diversity of its forms. Hypotheses on the centers of its origin are discussed using DNA marker data. The main areas of its cultivation are shown, along with possible reasons for such a predominating or exclusive distribution of naked barley in highland areas. Inheritance of nakedness and mechanisms of its manifestation are considered in the context of new data in genetics. The biochemical composition of barley grain in protein, some essential and nonessential amino acids, β-glucans, vitamins, and antioxidants is described. Naked barley is shown to be a valuable source of unique combinations of soluble and insoluble dietary fibers and polysaccharides. The parameters limiting wider distribution of naked barley over the world are emphasized, and breeding efforts that could mitigate them are proposed. Pathogen-resistant naked barley accessions are identified to serve as promising sources for increasing grain yield and quality. Main stages and trends of naked barley breeding are considered and the importance of the VIR global germplasm collection as the richest repository of genetic material for the development of breeding is shown.
Key words: naked barley; taxonomy; origin; genetics; grain quality; disease resistance; yield; breeding.

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Голозерный ячмень: систематика, селекция и перспективы использования

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Аннотация. В обзоре освещены вопросы современного состояния систематики, происхождения и перспектив использования голозерного ячменя. Представлены положения систематики культурного ячменя *Hordeum vulgare* L., в которой выделяются группы многорядного (convar. *coeleste* (L.) A. Trof.) и двурядного (convar. *nudum* (L.) A. Trof.) голозерного ячменя. Группы включают разновидности согласно особенностям строения колоса, остей, цветковых и колосковых чешуй и цвету зерновок. Отражены вопросы очагов формообразования голозерного ячменя, по данным археологии, палеоэтноботаники, разнообразию форм и сортов, а также рассмотрены вопросы происхождения голозерного ячменя по результатам ДНК-маркирования. Приведены основные зоны возделывания и возможные причины такого преимущественного или исключительного распространения голозерных ячменей в районах высокогорья. Обсуждается вопрос наследования признака голозерности ячменя и механизмов его проявления в соответствии с новыми данными в области генетики. Представлены характеристики биохимического состава зерна по белку, некоторым заменимым и незаменимым аминокислотам, β-глюканам, витаминам, антиоксидантам. Показано, что голозерный ячмень – ценный источник уникальных комбинаций растворимых и нерастворимых пищевых волокон и полисахаридов. Подчеркнуто значение параметров голозерного ячменя, которые ограничивают широкое распространение этой культуры в мире, и выделены направления селекционной работы для устранения этих недостатков. Приведены примеры образцов голозерного ячменя, устойчивых к вредным организмам, которые являются перспективными источниками для повышения урожайности и качества зерна, а также сохранения экологической чистоты и безопасности продукции. Отражены этапы и направления селекционных работ с голозерным ячменем и показано значение мировой коллекции ВИР как основного источника генетического материала для развития селекции.
Ключевые слова: голозерный ячмень; систематика; происхождение; генетика; качество зерна; устойчивость к болезням; урожайность; селекция.

Introduction

Barley has been one of the most important cereal crops cultivated in all the world's agricultural areas since ancient times. Barley belongs to the genus *Hordeum* of the Triticaceae family and is an obligate self-pollinator with a diploid set of chromosomes ($2n = 14$). It is rightfully recognized as a universal crop in terms of both the distribution scope and use. It is the fourth among the most important cereals in the world after wheat, maize and rice.

The wide area of distribution and a long cultivation history induced the rich intraspecific diversity of *H. vulgare* L. This cultivated species is divided into two subspecies: multi-row (*H. vulgare* L. subsp. *vulgare*) and two-row (*H. vulgare* L. subsp. *distichon* (L.) Koern.). They include groups of covered and naked botanical varieties. Among naked barleys, the groups of multi-row (convar. *coeleste* (L.) A. Trof.) and two-row naked barley (convar. *nudum* (L.) A. Trof.) were identified (Luk'yanova et al., 1990).

A specific feature of the naked barley groups is that their grains are bare and do not adhere to glumes, so kernels are easily separated from them when threshed. Naked barley is a valuable source for breeding for grain quality. Various forms of naked barley are characterized by a high content of protein and essential amino acids, primarily lysine, phenylalanine, methionine and threonine, fats, β-glucans, sterols, tocotrienols, flavonols and phytophenols possessing antioxidant activity (Aniskov et al., 2015; Meints et al., 2021).

Naked barley has its drawbacks. The main disadvantage of naked barley is the protrusion of the central radicle beyond the sphere of the grain surface, which leads to damage to the embryo during threshing. The crop is characterized by low adaptability to changing environmental conditions and low resistance to drought, lodging, and various diseases. It means that breeding work with naked barley should be aimed not only at increasing its positive properties but also at eliminating its major disadvantages. Currently, plant genetic resources are being actively analyzed in order to identify sources and donors for the main breeding trends.

Taxonomy

The history of barley classification dates back to ancient times. In 1747, C. Linnaeus laid the foundations of scientific plant taxonomy, covering a huge botanical diversity, including barleys. The classification was based on the number of fertile spikelets in each joint of the spike and the density of the spike itself. According to the *Hordeum* L. classification, there were four cultivated barley species within the genus, and the botanical varieties of naked barley, var. *nudum* L. (two-row) and var. *coeleste* L. (six-row), were identified within those species, i. e., already at that time the division of barley into covered and naked forms existed (Bakhteev, 1955; Trofimovskaya, 1972).

A great contribution to the development and further evolvement of the intraspecific classifications of barleys was made by such scientists as C.B. von Trinius, J.C. Doll, C. Koch, R.E. Regel, S.A. Nevsky, N.I. Vavilov, A.A. Orlov and F.Kh. Bakhteev. The naked barley was identified as a separate subspecies by J.C. Doll. The classifications by A.A. Orlov and F.Kh. Bakhteev recognized naked and covered barleys as varieties within different species and subspecies (Orlov, 1936; Bakhteev, 1955).

The contemporary classification, used at the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) and based on the works by N.I. Vavilov, R. Mansfeld, S.A. Nevsky, F.Kh. Bakhteev and A.A. Orlov, was presented by A.Ya. Trofimovskaya (Trofimovskaya, 1972). The cultivated species *H. vulgare* L. is divided into two subspecies: multi-row (*H. vulgare* L. subsp. *vulgare*) and two-row (*H. vulgare* L. subsp. *distichon* (L.) Koern.). They include the groups of covered and naked botanical varieties. Among naked barleys, the groups of multi-row naked barley (convar. *coeleste* (L.) A. Trof.) and two-row naked barley (convar. *nudum* (L.) A. Trof.) were identified (Trofimovskaya, 1972; Luk'yanova et al., 1990). Naked barley groups are characterized by a large number of endemic varieties from various foothill and highland areas. This classification mirrors the enormous polymorphism of species and botanical varieties within this genus, including naked barley.

The group of multi-row naked barley, *H. vulgare* L. subsp. *vulgare* convar. *coeleste* (L.) A. Trof., includes 58 botanical varieties (Luk'yanova et al., 1990). Its typical feature is that all three spikelets sitting in the grooves of the spike's stem are fertile in most of the spike and have normally developed grains, while the grains themselves are bare, which means that they are easily separated from the glumes. Botanica varieties within the group are distinguished by the width of glumes, the presence, length and smoothness of awns, the color of the spike, the density of the spike, and also by the color of grains.

The density of the spike differentiates varieties into those with dense and lax spikes. Dense spikes are further subdivided into dense and very dense ones. A very dense spike is characteristic of the following botanical varieties: var. *nudipyramidatum* Koern., var. *uljassutaicum* Vav. et Orl., var. *subnudipyramidatum* Orl., var. *micrurum* Vav. et Orl., and var. *latinudipyramidatum* Vav. et Orl. These botanical varieties belong to the Japanese, Chinese and Mongolo-Tibetan agroecological groups. Their specific feature, in addition to a dense spike, is a stunted growth habit. Botanical varieties with a dense spike are: var. *revelatum* Koern., var. *ancoberense* Vav. et Orl., var. *brevisetum* Regel, var. *nanum* Vav. et Orl., and var. *latirevelatum* Vav. et Orl. These botanical varieties, belonging to the Japanese, Chinese and Abyssinian agroecological groups, are common in Japan, China, and Ethiopia.

Botanical varieties within the group of multi-row naked barley are described as having three-lobed appendages instead of awns (furcae): var. *trifurcatum* (Schlecht.) Wender., var. *pseudotrifurcatum* Langsd., and var. *aethiops* Koern., obtained from China, Mongolia and Ethiopia, but some botanical varieties with the same feature have been identified in hybridization nurseries. This group also includes

botanical varieties with awns or furcae only on the middle spikelets; the lateral ones are awnless or have only small rudiments of awns: var. *cornutum* Schrad., var. *cornutiforme* Aoberg., var. *subaethiops* Koern., var. *nuditransiens* Koern., and var. *nudijaponicum* Vav. et Orl., from Japan, South Africa, Tibet, and hybrids obtained in hybridization nurseries.

Grain color features are the criteria for differentiating many botanical varieties. Naked barley grains are of various colors: yellow (var. *coeleste* L. and var. *brevisetum* Regel), green (var. *himalayense* (Ritt.) Koern., var. *urgaicum* Vav. et Orl. and var. *kobdicum* Vav. et Orl.), purple (var. *violaceum* Koern., var. *gobicum* Vav. et Orl. and var. *uhangaicum* Vav. et Orl.), black (var. *duplinigrum* Koern. and var. *aethiops* Koern.), and brown (var. *tibetanum* Vav. et Orl.); there are also diverse shades of these colors acquired during splitting in crosses. Botanical varieties with yellow grains occur everywhere. Varieties with green grains are also widespread, but they predominantly belong to Asian agroecological groups. The purple grain color is observed in botanical varieties from the Mongolo-Tibetan and Chinese agroecological groups. Varieties with black grains belong to the Abyssinian agroecological group. The variety with brown grains belongs to the Tibetan agroecological group.

The two-row naked barley group, *H. vulgare* L. subsp. *distichon* (L.) Koern. convar. *nudum* (L.) A. Trof., is characterized by the fact that of the three spikelets sitting in the grooves of the spike's stem only one spikelet, the middle one, is always fertile, with a normally developed grain; the grains are bare, i. e., they are not firmly adhered to the glumes, so they are easily separated from them during threshing. This group consists of 38 botanical varieties. Similarly to the multi-row naked barley group, the varieties within this group are differentiated according to the morphological features of the spike and the color of grains. All varieties mainly belong to the Abyssinian, Dagestani, Japanese and Indian agroecological groups; besides, many varieties were obtained in the process of crossing in hybridization nurseries. Botanical varieties with a dense spike (var. *gymnocrithum* Koern., var. *neogenes* Koern., var. *nudimelanocrithum* Giess., etc.) belong to the Abyssinian agroecological group.

Botanical varieties are differentiated within the group according to the presence/absence of awns, their length, and the presence of awn appendages (furcae). The varieties that have three-lobed appendages instead of awns (furcae) are var. *nudifurcatum* Regel, var. *zhukovskii* Chodk., var. *sublaxum* Koern., and var. *gymnospermum* Koern. The awnless varieties are var. *dupliatrum* Koern., var. *duplialbum* Koern., var. *subduplialbum* Koern., and var. *subdupliatrum* Koern. These varieties belong to Asian agroecological groups or resulted from crosses in hybridization nurseries.

The two-row group of naked barley contains varieties that are described to have lateral spikelets completely reduced and represented by only glumes: var. *nudideficiens* Koern., and var. *daghestanicum* Vav. et Orl. They belong to the Dagestani agroecological group.

Color is also a distinctive feature of many botanical varieties. Similarly to the multi-row naked barley, grains can be yellow (var. *nudum* L. and var. *colonicum* Orl.), green (var. *viride* Vav. et Orl., var. *virideinerme* Giess. et al. and var. *daghestanicum* Vav. et Orl.), purple (var. *nudidubium* Koern. and var. *janthinum* Koern.) or black (var. *nigrinudum* Vav. and var. *nudimelanocrithum* Giess. et al.), or manifest various other shades. The distribution of varieties with different grain colors is similar to that of the multi-row naked barley group.

The VIR global collection includes more than 1230 naked barley accessions collected from all over the world. These accessions are valuable genetic material for barley breeding and can serve as a source for the development of high-yielding naked barley cultivars adaptable to the environments of various regions of the Russian Federation.

Origin and distribution of naked barleys

Very little is known about the origin of naked barley, because no targeted studies have been performed on the subject; it has been considered only in the context of the origin of barley in general. The timeframe of the origin of naked barley started to be discussed for the first time when multi-row naked and covered barleys were discovered during the excavations at Ali Kosh. A radioisotope analysis ascertained that naked barley appeared approximately in the period of 7900 B.C. These data indicate that naked barley evolved much later than covered barley which grew in the Pre-Pottery Neolithic (9700–9300 B.C.) (Helback, 1959). The very process of naked barley emergence also remains not quite clear. The most common hypothesis is the occurrence of a mutation in the gene that controls the process of husk formation in grains.

Scientists of VIR found out in their research that naked barley had three main foci of morphogenesis (Vavilov, 1965; Luk'yanova et al., 1990). N.I. Vavilov perceived these foci as "the loci of morphogenesis", "extremely small spaces", where wild plant species were domesticated by man. Such foci are identifiable by the data of archeology and paleoethnobotany, but they are mainly identified according to the modern varietal diversity of cultivated plant species and forms. It is important to understand that ancient foci could appear in different parts of the continents (i. e., polytopically) and at different times (heterochronously); later another phenomenon became known – repeated domestication (redomestication) of plant species against the background of a well-established ancient assortment.

The first focus is Southeast Asia and mountainous Central and Western China with the lowland areas adjacent to it. Naked barley is cultivated there mainly in mountainous areas at an altitude of at least 2000 m. The second focus is Northeast Africa (mountainous regions of Ethiopia), where naked barley varieties are represented by endemic forms. The third focus includes Western Asia: Turkey, Transcaucasia, Iran, and Tajikistan (Vavilov, 1957).

Recent efforts have been intensively associated with DNA markers. The data obtained can shift the concept of

naked barley domestication to a higher level and confirm or refute the existing hypotheses of the origin and distribution of naked barleys.

One of the hypotheses concerning the origin of naked barley is monophyletic origin in the territory of Southwestern Iran, from where it started to migrate to other regions. This assumption is based on the analysis of the dominant SCARsKT7 marker, which is closely associated with the *nud* locus (Taketa et al., 2004). The highlands of the Himalayas can be considered as a possible center of domestication for naked barley (Badr et al., 2000) due to a number of distinctive features (Xifeng et al., 2013). Recent studies, however, manifest the opposite view. Based on the whole genome data and the published exon-swapping resequencing data for 437 accessions, the authors showed that the modern Tibetan barley (*Hordeum vulgare* L., qingke) was derived from domesticated oriental barley and introduced into Southern Tibet, presumably via Northern Pakistan, India, and Nepal, about 3500–4500 years ago. The scant genetic diversity of qingke suggests that Tibet can be ruled out as a center of origin or domestication of barley. The rapid decrease in genetic diversity on the way from domesticated oriental barley to qingke can be explained by the effect of isolation in the Tibet region from 2000 to 4500 years ago (Zeng et al., 2018).

In addition to the hypothesis on monophyletic origin of naked barley from Southwestern Iran, there is also a hypothesis about multiple independent origin of naked barley. It is based on a comparative morphological analysis of varieties from different foci of morphogenesis and consists in the independent emergence of naked barleys in several centers of crop origin (Helback, 1959).

The groups of multi-row and two-row naked barleys occur in all areas of barley cultivation. They are most widespread in Southeast Asia (China, Japan, and the Republic of Korea), Northeast Africa (Ethiopia and Eritrea), and Central Asia – in mountainous regions (Pamir, Tibet, Tajikistan, Mongolia and India) (Luk'yanova et al., 1990). The most common varieties of these groups are var. *coeleste* L., var. *himalayense* (Ritt.) Koern. and var. *nudum* L. Crop areas under naked barley in the abovementioned countries are not uniform: in some countries they reach 95 % of the total barley crop area, while in others only 50 % or less. The areas under naked barley abruptly diminish in the direction from east to west. In Russia, naked barley is cultivated on a small scale due to a number of factors limiting its distribution (Tetyannikov, Bome, 2020).

There are two viewpoints on the reasons for the spread of naked barley mainly in mountainous areas. Some authors attribute this phenomenon to the active use of naked barley for food needs (Helbaek, 1966; Nevo, 1992), while others hypothesize that naked barley is better adapted to such conditions (Harlan, 1979). The second point of view was proved by A.A. Pomortsev et al. (1996) who studied the dynamics in the genotypic composition of barley populations obtained from crosses between the cultivars Moskovsky 121 (two-row, covered) and Dzhau Kabutak (six-row, naked, var. *himalayense*). The hybrids were grown concurrently from F_2 to F_9 in the Pamirs (2600 m above sea level) and from F_2 to F_{10} in Moscow. As a result, it was shown that under the impact of natural selection the dynamics of populations according to marker loci during reproduction in the Pamirs and in Moscow was different and led to the divergence of populations. Under the conditions of the Pamir highlands, the selection was targeted against plants with a covered grain and two-row spike, while in the environments of Moscow the selection was targeted against plants with naked grains, which confirms the second viewpoint: naked barley is more adapted to growing in highland environments than covered barley (Pomortsev et al., 1996).

The gene controlling grain nakedness and the mechanism of its action

It is currently recognized that the difference between covered and naked barleys is controlled by a single locus. Grain covering is classified as a dominant trait, and nakedness as a recessive one. The genetic locus was assigned to the long arm of barley chromosome 7H and named *nud* (from the word *nudum*) (Gerasimova et al., 2020). The *nud* gene is located at a distance of 0.3 cM from the proximal end and 1.2 cM from the distal one in the region (SCAR) of KT 2 and KT 4 (Kikuchi et al., 2003). The structural part of the *nud* gene consists of two exons and one intron.

The *Nud* gene is present in covered barley; it encodes the transcription of the ethylene response factor (ERF) family, which belongs to the group of Wax Inducer 1/Shine 1 (WIN1/SHN1)-like transcription factors. The said factor controls lipid biosynthesis and encodes protein of 227 amino acids (Taketa et al., 2008). There are three allele variations at the *nud* locus, designated as *nud 1.a*, *nud 1.b*, and *nud 1.c*. The *nud 1.a* allele is the result of a *Nud* deletion. The *nud 1.b* allele contains a nucleotide substitution of thymine with adenine in the second exon, which leads to a substitution of valine with aspartic acid at position 134. The *nud 1.c* allele has a 1 bp deletion in the second exon, which causes a reading frameshift and generates a premature stop codon, resulting in a truncated protein sequence of 199 amino acids.

The mechanism of forming hullness or nakedness on barley grains is not yet fully understood. The version most often encountered in publications is that the recessive *nud* gene is in an intact state and does not generate an adhesive lipid layer between the epidermis of the grain pericarp and the glumes, which allows them to be freely separated during threshing. As for the dominant *Nud* allele, it controls the biosynthesis of lipids which contribute to the adhesion of the glumes to the caryopsis and the formation of covered barleys (Taketa et al., 2008; Hoad et al., 2016).

Recent works on the *nud* locus sequencing showed that in all naked barley accessions this gene was characterized by a 17-kb deletion or the presence of the non-synonymous SNP T643A when compared with the functional *Nud* gene (Yu et al., 2016). The analysis of X-ray-induced naked

grain mutants confirmed that the *Nud* gene carried non-synonymous single-nucleotide polymorphisms in all cases. It was also demonstrated that site-directed mutagenesis of the *Nud* gene induced the appearance of naked grains on initial transgenic plants (Gasparis et al., 2018). In addition to the normal *Nud* deletion mutation contributing to hullness, a new *nud* allele, designated *nud 1.g*, was identified in three naked barley varieties collected in Tibet. The *nud 1.g* allele contains the non-synonymous T643A SNP, unlike the functional *Nud* gene. Genetic analysis showed that SNP T643A *nud 1.g* co-segregates with the naked phenotype. Besides, the *in silico* prediction of functionally conservative sites and three-dimensional structures showed that the amino acid substitution (valine with aspartate) induced by SNP T643A could lead to a dramatic structural change in *Nud* that could result in the loss of its function. This study provides evidence of a possible new mechanism underpinning the origin of the naked phenotype of domesticated barley in Tibet (Yu et al., 2016).

More and more research is focused on the work with naked barley. In 2020, an article was published where the authors demonstrated a targeted change in the first exon of the *Nud* gene with the help of the RNA-guided endonuclease Cas9, which led to the conversion of the phenotypic features of grain hullness into nakedness. The covered barley cultivar Golden Promise served as the target research material, while the changes were implemented through the mediated DNA transfer by agrobacteria (Gerasimova et al., 2020).

However, a limited number of studies on this topic and broad variability of the source material that has not yet been fully studied impede the efforts to throw light on the molecular mechanisms that regulate grain nakedness or hullness in barley grains. Perhaps, using a wider diversity of naked barleys from around the world and applying modern methods would help to identify new loci responsible for grain nakedness.

Chemical composition of naked barley grains

The main feature differentiating naked barley from covered barley is the biochemical composition of its grain. Barley grain contains unique combinations of soluble and insoluble dietary fibers and polysaccharides along with low-molecular-weight bioactive components (Madakemohekar et al., 2018). Naked barley exceeds covered barley in the content of nutrients, such as protein, some essential and nonessential amino acids, β-glucans, vitamins, macro- and micronutrients, phenolic and flavonoid compounds. It has been established that all useful components of barley grain are preserved during its processing, including such active antioxidants as proanthocyanidins (Zheleznov et al., 2013; Polonsky et al., 2021).

A physiologically important dietary component in naked barley grain is (1,3;1,4)-β-D-glucans. They help to reduce the risk of cardiovascular disease, maintain or reduce the amount of cholesterol in blood, decrease the risk of hyperglycemic syndrome, improve liver functions, and reduce overweight (Wirkijowska et al., 2012; Bozbulut, Sanlier, 2019). The dry matter of covered barley grain contains 4–8 % of β-glucan, but for naked barley this index can reach 16 %. Its content in barley grain is determined by varietal characteristics and environmental factors (Huth et al., 2002). Naked barley is characterized by a high vitamin E content (Moreau et al., 2007) and is considered a good source of phenolic compounds, such as derivatives of cinnamic and benzoic acids, proanthocyanidins, flavonols, flavanones, and flavones (Shen et al., 2016; Ge et al., 2021), which manifest antioxidant, anti-inflammatory, and antiproliferative effects.

Parameters limiting wider distribution of naked barley

One of the main factors limiting wider distribution of naked barley is its low yield compared with covered barley. This is largely due to its low field germination caused by the protrusion of the radicle beyond the grain. It affects the embryo's resistance to the mechanical impact of threshing equipment, results in injuries, and leads to a decrease in field germination of its seeds.

In order to overcome this disadvantage of naked barley in breeding practice, it is necessary to control the morphology of the grain shape and the nature of the embryo's positioning (Tetyannikov, Bome, 2020). Quite a few lines with oval-shaped and even rounded grains have already been developed by crossing the Canadian naked barley cultivars McGwire and BRL-6 with the covered cultivars Getman, Vakula, Linus and others (Kirdoglo et al., 2013). A very promising naked barley accession, 95683/73 (k-27730) from Germany, was identified in the VIR collection. Its grain is shortened (7.2 mm) but rather wide (3.8 mm) and maximally thick (2.8 mm). Such parameters make this accession uniquely important for practical use in naked barley breeding. Besides, accessions with the optimal grain shape were identified: Alar-Erd-Ene from Mongolia, Hora from the Netherlands, 1218-524 from the Czech Republic, and S-257 from Mexico (Malashkina, 2008).

Long ago it was shown that covered barley cultivars were more productive than naked ones. However, many authors who studied naked barley observed that the glumes, tightly adhered to the caryopsis, accounted for at least 12–14 % of the total bulk of covered barley harvest. Barley glumes themselves are the same straw, so it should be taken into account when measuring the real yield of covered barley (Gryaznov, 2014).

Barley yields are highly variable depending on environmental conditions, especially in arid areas (Gryaznov, 2014). Naked barley yields also vary significantly depending on the characteristics of cultivars. The productivity of naked barley forms was studied in many regions of Russia: in the Tyumen (Tetyannikov, Bome, 2020), Omsk (Aniskov et al., 2015) and Kemerovo (Zaushintsena et al., 2007) regions and in the provinces of North Caucasus (Doroshenko et al., 2019), and others. Accessions with the highest yield and best adaptive properties were identified in those regions.

Besides, one of the most important agronomic traits of naked barley is its resistance to lodging in different environments. T.M. Bogdanova et al. (2001) identified single accessions resistant to lodging under the conditions of Northwestern Russia: KM 280 (k-29419, var. *nudum*, Czech Republic), Nacta (k-20928, var. *nudum*, Germany), and complex hybrids from Mexico (k-28019, var. *nudum*, and k-28083, var. *neogenes*). Later, under the same conditions, accessions resistant to lodging were selected from two-row barleys: k-29863 (var. *neogenes*, Czechoslovakia) and k-28083 (var. *neogenes*, Mexico), and from multi-row barleys: k-28961 (var. *coeleste*, India), k-4365 (var. *coeleste*, Belarus), and k-21319 (var. *subnudupyramidatum*, Japan) (Tyaglyi, 2007). Testing under the conditions of Tyumen Province revealed resistance to lodging scoring 9 points in the two-row accessions: k-22308 (H 2198 Ubamer Baco), k-23450 (H 2866 Coll. Halle EP 80), k-25008 (Local), and k-25855 (Ra 6), and in multi-row ones: k-30663 (C.I.11073) and k-30624 (C.I.10975) (Tetyannikov, Bome, 2020).

An important criterion for increasing the yield of naked barley in the areas of risky farming is its earliness. For example, when studying the earliness of barleys from the countries of Southeast Asia, two ultra-early forms from China were identified: k-15881 (var. *coeleste* L.) and k-15882 (var. *nudipyramidatum* Koern.), with an interval of 30–33 days between the germination and ear emergence phases (Zveinek, Kovaleva, 2017), which makes these accessions promising for cultivation in areas with unfavorable abiotic factors. Besides, a study of the extensive material from the VIR collection resulted in the identification of 16 early-ripening accessions, such as k-25090 (Mexico, var. *nudum*) and k-29820 (Ethiopia, var. *nigrinudum*) from the two-row group, and k-5489 (Ukraine, var. *glabriduplinigrum*) and k-24817 (Ethiopia, var. *tibetanum*) from the multi-row one (Bogdanova et al., 2001).

Practical cultivation of naked barley cultivars, including cv. Nudum 95, attests to the need for the development of measures to adapt such cultivars to local environmental conditions, which will increase their yield to the level of covered barley. Such technologies have already started to appear, but only for certain regions (Gryaznov, 2016; Gladkikh et al., 2019).

Resistance to various diseases

Disease resistance of cultivars is one of the important reserves for increasing the yield and quality of grain as well as for maintaining the ecological cleanliness and safety of products.

Diseases caused by fungi, bacteria and viruses, and pests of barley occur each year in various regions of Russia, resulting in abrupt decreases in grain yield and quality. They affect the normal rhythm of plant development, produce a negative impact on grain formation, reducing grain size and filling, and disrupt plant stands. Therefore, breeding for quality is closely associated with breeding for resistance to diseases and pests. The most profitable and safest way to reduce grain contamination is to develop genetically resistant cultivars. Breeding problems cannot be solved without a constant search for new sources and donors of resistance, because plant resistance genes lose their effectiveness in the process of emergence and accumulation of virulence mutations in pathogen populations (Luk'yanova et al., 1990).

It is known that barley can be infected with a wide range of pathogenic fungi, and many of them can persist in the grain. The genera *Bipolaris*, *Pyrenophora*, *Phaeosphaeria*, *Alternaria*, *Ustilago*, *Puccinia*, *Blumeria* and *Fusarium* are considered the most common fungi infecting barley grain worldwide (Chen et al., 2016). Reduction of losses in harvests and valuable grain qualities requires careful selection of source material and involvement of the most resistant sources and donors in breeding practice to develop new cultivars.

Fusarium is a common disease of cereals, such as wheat, maize or barley, and can lead to an abrupt decrease in yield and product quality (Polisenska et al., 2020) through the formation of mycotoxins. Mycotoxins in the human organism cause anorexia, vomiting, diarrhea and, in high doses, intestinal bleeding; sometimes they produce additional effects, such as impaired immune function. In plants, mycotoxins inhibit protein synthesis, while fungal enzymes lead to protein degradation, thus inducing plant defense mechanisms (Martin et al., 2018).

Plant resistance to *Fusarium* and accumulation of mycotoxins is a complex mechanism. Five major resistance classes have been established for wheat, barley and maize. Resistance of type I acts against initial penetration and infection of plants. Type II restricts the spreading of infection within the plant. Resistance of type III deals with grain infestation, type IV is associated with resistance and the ability to maintain yield, and type V combines all mechanisms of resistance to mycotoxin accumulation (Martin et al., 2018). Resistance of type V is suggested to be divided into two components. The first, called type V-1, is the resistance to toxin accumulation driven by metabolic transformation, including enzyme-catalyzed biochemical modification. The second component (type V-2) corresponds to the resistance obtained through inhibition of mycotoxin biosynthesis by endogenous compounds within the plant itself (Martin et al., 2017).

There is proof that a large number of various plant metabolites play a decisive role in the resistance to *Fusarium*: phenolic acids, flavonoids, carotenoids, tocopherols, benzoxazinoids, fatty acids, amino acids and their derivatives, carbohydrates, amines and polyamines, terpenoids, etc. (Gauthier et al., 2015; Atanasova-Penichon et al., 2016). They suppress reactive oxygen species, scavenge free radicals during lipid peroxidation and contribute to the establishment of a physical barrier against pathogenic infection, while some metabolites can interfere with the biosynthesis of mycotoxins (Siranidou et al., 2002). High β-glucan content in grain was also shown to contribute to the resistance of type V (Martin et al., 2018).

When studying covered and naked barleys, some researchers demonstrated that covered barley turned out to be more resistant (Warzecha et al., 2010), while others found very low content of toxins in naked barley cultivars, justifying this by the fact that a significant amount of toxins remains in glumes (Buerstmayr et al., 2004). The latest data on the specific features of barley resistance to *Fusarium* witness that the advantage of some naked barley forms over covered ones in terms of the content of metabolite groups that enhance the resistance of type V should be taken into account.

A study of *Fusarium* resistance in barley cultivars, conducted by domestic researchers, identified 14 highly resistant accessions. Five of them were naked forms (k-2946, k-11070, k-11073, k-11076 and k-11082) with large grains, but prone to lodging and susceptible to powdery mildew (Gagkaeva, Gavrilova, 2009).

Powdery mildew (causative agent: *Blumeria graminis* (DC.) Golovinex Speerf. sp. *hordei* Marchal), **brown rust** (*Pucciniahordei* G.H. Otth.) and ***Helminthosporium*** are among the most widespread and harmful diseases of barley in Russia (Kusch, Panstruga, 2017). Long-term resistance to the powdery mildew pathogen of barley cultivars is provided almost all over the world by the *mlo11* gene and, to some extent, *mlo9* (Radchenko et al., 2020). A number of naked barley accessions with resistance or low susceptibility to powdery mildew were identified: Dublet (Belarus), Omsky Golozerny 1 (Russia), k-26648 (Pakistan), Buck CDC, CDC VC Gwire and CDC Dawn (Canada), k-3038 (Turkmenistan), Orgeniepetite (France), NB-OWA (Nepal), etc. Resistance to leaf spots caused by *Helminthosporium* was observed in Buck CDC and Bowman (Canada), 84469/70, k-3038 (Turkmenistan), Dublet (Belarus), Brunee (Ethiopia), Orgeniepetite (France) and others. Complex resistance to both pathogens was demonstrated by the accessions: Dublet, Omsky Golozerny 1, Omsky Golozerny 2, Yudinsky 1, k-26648, 84469/70, Orgeniepetite, CDC Dawn, NB-OWA, k-3038, CDC VC Gwire and E.E.B.N.46. They are recommended for use in breeding programs targeted at resistance to fungal diseases (Doroshenko E.S., Doroshenko Ed.S., 2018).

Accessions k-5448, k-8682 and k-17554 with powdery mildew resistance were identified while testing Ethiopian accessions at Pushkin and Pavlovsk Laboratories of VIR as potential sources of the *mlo11* gene allele in the development of cultivars resistant to powdery mildew. Accession k-5448 (Abyn 8, var. *duplinigrum*, Ethiopia) was also resistant to net blotch: the plant damage did not exceed the score of 1 point (Alpateva et al., 2016). A stock of accessions resistant to fungi was obtained during the study of naked barley cultivars from the VIR collection. The best accessions that retain powdery mildew resistance for 30 years are k-2930 (var. *violaceum*, China), k-5983 (var. *coeleste*, Afghanistan) and k-3282 (var. *nigrinudum*, Ethiopia) (Bogdanova et al., 2001).

In addition, wider distribution of naked barley is hampered by its susceptibility to **smut fungi**. Among numerous pathogens of cereals, smut fungi manifest one of the highest levels of harm due to the fact that they are ubiquitous, cause a very significant decrease in yield and worsen the quality of grain. They can also provoke reduction of dry matter accumulation in grain, shortening of the ear length, and a decrease in tillering and the number of grains per ear (Bechtol'd, Orlova, 2018). Phytopathological analysis of 40 naked barley accessions for covered smut showed that only three cultivars had absolute resistance (0.0 %) to the pathogen: Chugokuhadaka N2 (Japan), Buck CDC (Canada), and k-30313 (Ethiopia).

Mid-ripening naked barley accessions were found to be more affected by smut fungi compared to mid-late and early ones. Seeds infected with loose smut have a lower absolute weight (by 10–20 %) and their field germination deteriorates (Zhichkina, Stolpivskaya, 2015). As a result of the studies (2005–2007), 8 covered and 4 naked cultivars were identified for being not affected by false loose smut and covered smut, including the references Omsky 85 and Omsky Golozerny 2. Accessions combining resistance to false loose, covered and loose smuts were identified. They were recommended to breeders for inclusion into crosses in order to obtain immune cultivars (Meshkova, Sabaeva, 2009). Accessions k-23851 (var. *himalayense*, Mountainous Badakhshan) and k-21544 (var. *trifurcatum*, Bolivia) from the VIR collection were resistant to loose smut for 12 years (Bogdanova et al., 2001). They are also of interest as promising sources.

Breeding improvement of naked barley

Historically, the study of naked barley was started by such scientists as N.I. Vavilov, A.A. Orlov and F.Kh. Bakhteev. They drew the attention of plant breeders, geneticists and agronomists to the diversity of naked barley forms available in the global collection. They identified distinctive features and areas of distribution of these forms, assessed their agroecological characteristics, described the scope of their application, and also initiated the collecting of naked barely samples from all over the world (Khod'kov, 1985).

In Russia, the areas where naked barley was grown were very limited. The first information about naked barley cultivation in Eastern Siberia dates back to the beginning of the 19th century. It was mostly in those years that the so-called Himalayan barley appeared among cultivated barleys. This form was borrowed in 1826 by S.I. Gagarin, Vice-President of the Imperial Moscow Agricultural Society, from Archduke John of Austria (Surin, 2011). According to L.E. Khod'kov (1985), in the early period of domestic breeding, only some agricultural institutions were interested in naked barley. For example, Himalayan naked barley was studied for several years at the Zapolskaya Experiment Station in the late 19th century. In 1914, the first cultivar of naked barley, Nudum 155, was released at the Dnepropetrovsk Experiment Station. It was obtained through individual selection.

However, this is not the first information about the cultivation of naked barley in the Russian Federation. An analysis

of fossil plant remains discovered by the expedition of the Dagestan Branch of the USSR Academy of Sciences during excavations of ancient settlements near the village of Gilyar in Southern Dagestan showed that 4.5–5 thousand years ago (in the Ancient Bronze Age) local residents cultivated wheat and barley. With this in view, it is noteworthy that naked barley was the most widespread (Omarov, 1981). Local highlanders even singled out naked barley as an independent crop, different from ordinary cultivated barley. Naked barley was exclusively cultivated in the mountainous and alpine areas of Dagestan, where its grain was used for food purposes.

Naked barley appeared in the global collection of VIR at the very beginning of its establishment. It is interesting that the first (k-1) registered number in the VIR catalogue was given to a sample of naked barley (var. *himalayense*) from Uzbekistan acquired in 1897. The first accessions of naked barley arrived to the VIR collection from completely different parts of the world: Uzbekistan, China, Ukraine, Armenia, Georgia, Germany, Romania, Kazakhstan, Latvia, France, Kyrgyzstan, etc., and from all over Russia: Saratov, Yaroslavl, Tobolsk and Vyatka Provinces, Dagestan, Kuban, Kursk Province, Don and Black Sea regions, Yenisey and Terek basins, etc., which proves the widespread growth of naked barley in Russia.

The State Variety Network has been testing naked barley almost regularly since 1927, while the research into this crop has become systematic. In the 1920–1930s, the naked barley cultivars Nudum Rostovsky 0289, Nudum Rostovsky 3001, Coeleste 086, Nudum 021, Coeleste 08, Byloe, Kolkhozny 7 and Nudum 92 were developed. However, for various reasons, almost all of them were not officially commercialized and zoned. Post-war breeding efforts also did not achieve outstanding results in the development of new naked barley forms. Thus, since the end of the last century, the attention of both individual plant breeders and various domestic breeding institutions has been drawn to the problem of the development of naked barley cultivars and their introduction into agricultural production, but almost all of them were unsuccessful. L.E. Khod'kov (1985) in his publication "Naked and Awnless Barley" (1985) analyzed the experience in the breeding work with naked barleys in the country and showed a number of promising improved forms of his own breeding.

Targeted research on the development of naked barley cultivars is currently being carried out in Canada, Japan, the USA, Sweden and the Czech Republic. Russia, Ukraine and Belarus are also interested in this subject. In Canada, at the end of the 20th century, such cultivars as Scout and Tupper (1980), Condor (1988), Buck and Richard (1990) were released; they currently occupy the area of more than 350,000 hectares (Aniskov et al., 2015). Besides, in 1997, the "waxy" barley was first developed in Canada; it exceeded common barley in the content of β-glucans by 32–41 %. In Belarus, the first studies on the development of naked barley cultivars date back to the 1970s. Those efforts resulted in releasing such cultivars as Golozerny 76, Belorussky 76, Golozerny 94 and Dublet (Trofimovskaya, 1972). Significant progress has been made in Switzerland, where several naked barley cultivars were included in the official catalogue in the late 1980s.

In the Russian Federation, breeding works with naked barley are actively carried out in the Siberian Research Institute of Agriculture, Krasnoyarsk Research Institute of Agriculture, Siberian Research Institute of Plant Production and Breeding, and Kemerovo Research Institute of Agriculture. At present, there are already six cultivars of naked barley listed in the State Register for Selection Achievements: Omsky Golozerny 1 (2004), Omsky Golozerny 2 (2008) and Omsky Golozerny 4 (2020), Oskar (2007), Nudum 95 (2010), and Ergeninsky Golozerny (2020) (State Register…, 2021). However, these cultivars are adapted to the environments of certain regions, and in other regions their quantitative and qualitative traits are not manifested. For example, cv. Ergeninsky Golozerny is adapted to arid areas in the black earth zone, which makes it in demand in the south of Russia and other arid regions (Characteristics of Plant Varieties…, 2020).

Contemporary plant breeding has changed significantly. These changes are associated with the development of molecular marker technologies and the possibilities of sequencing (Khlestkina, 2013). They make selection by genotype possible, which significantly accelerates the breeding process (Jaganathan et al., 2020). Currently, SNP markers are widely used for genotyping (Agarwal et al., 2008; Jaganathan et al., 2020), and they are applied for both covered and naked barleys.

The use of NGS (next-generation sequencing) technologies for studying naked barley is not as widespread as for covered barley. J. Hernandez et al. (2020) associated it with the problem of the absence of model naked barley. X. Chen (2014) studied two local naked barley cultivars XQ754 and Nimubai from Tibet using paired-end RNA sequencing on the Illumina HiSeq 2000 platform and derived their transcriptomes. All in all, 13.1 and 12.9 million 90 bp paired reads were produced from two cultivars. Based on databases, a description of the genes and conservative protein domains in the developing grain of naked barley was presented. Moreover, the sequences and expression levels of the genes associated with coding storage proteins and enzymes for starch synthesis and β-glucans were analyzed. Their temporal and spatial patterns were derived from the transcriptome data of the covered barley cultivar Morex (Chen et al., 2014). These data ensure the genetic potential to improve the qualitative traits of naked barley in future studies.

At present, the results of sequencing jointly with high-throughput genotyping technologies can be used for effective targeted selection of the desired genotypes among breeding lines, which will significantly accelerate the development of new barley cultivars with desired characteristics (Rozanova, Khlestkina, 2020).

The global barley collection held by VIR contains an extensive gene pool of naked barley. The group of multirow naked barleys constitutes a small part of the collection

compared to covered ones and consists of 827 accessions, including 34 varieties. The two-row naked barley group includes only 303 accessions and 21 varieties. Many naked barley varieties in the VIR collection are endemic, very rare, and represented in the collection by single accessions, which makes the VIR collection a unique source of valuable genetic material.

Economic importance of barley

Currently, the trend of food production from various cereals meeting the dietary needs of humans is actively developing. The dietetic, preventive and therapeutic effect of such food products on a human organism is based on the biochemical composition of cereal grains. In recent years, breeding practice has been aimed at developing high-yielding cultivars combining the maximum content of biochemical components and their optimal ratio with other grain quality indicators and resistance to various abiotic and biotic stressors (Loskutov, Khlestkina, 2021). Barley is among such crops: it is an important source of food and feed and a valuable industrial crop in many countries of the world. Barley demonstrates stable grain harvests every year: in 2020, according to FAO, the barley grain harvest amounted to over 151 million tons (http://www.fao.org/faostat/en/#data/QC, accessed June 25, 2021). About 75 % of the world's barley production is used for animal feed, 20 % goes to the production of malt for the brewing industry, and only 5 % for food production (Blake et al., 2011).

Use of barley for food purposes

Barley is highly adaptable to high-altitude climate conditions, drought, and soil salinity, which makes it an important staple food crop in North Africa and the Tibetan Plateau in China where other crops, such as wheat and rice, cannot produce high and stable yields (Moza, Gujral, 2016).

The list of food products from naked barley grain is currently expanding. Barley grain is used to make barley and pearl barley groats. Since the grain of naked barley is easily separated from glumes, the yield of groats from naked barley is higher than from covered barley. Naked barley cultivars meet all requirements for producing high-quality groats. Therefore, it seems advisable to replace a number of covered cultivars with naked ones for the production of groats (Borisonik, 1971).

Naked barley grain is also used to make flour. In highland areas, the flour from naked barley is mainly consumed as satu (flour made from roasted barley grains) and also mixed with flour made from other crops, such as wheat, buckwheat, millet and pea, to cook flatbread, dumplings and thukpa (Tibetan noodle soup). In addition to flour, a special traditional fermented drink (Chhyang) and distilled liquor are prepared from the grain for various cultural and religious events.

In Canada, products made from mixtures of wheat and barley flour in varying proportions are quite common. To preserve the biological value of the grain, whole grain flour is used, without siftings and technological waste (Trofimovskaya, 1972). In Italy, naked barley is widely used for processing into dietary barley flour or such products as quick breakfasts and coffee substitutes. To obtain barley coffee, the grain is roasted to a dark brown color and finely ground, and the resulting powdery mass is used as a coffee substitute.

The work is underway to use whole-grain oat and barley for the preparation of functional drinks, including plant-based milk. Such drinks are rich in B-group vitamins, complex carbohydrates, and various mineral components. Whole grains used in beverages also contain a wide variety of phenolic compounds with antioxidant activity (Fernandes et al., 2018/19).

In Russia, naked barley flour mixed with bread wheat flour is becoming more and more popular for baking purposes in order to enrich products with compounds useful for the human organism and significantly increase the nutritional and consumer value of bakery products.

A large number of experiments helped to identify the optimal ratio of wheat/barley flour (90/10 %) for bread. With such ratio, the characteristics of bread (organoleptic assessment of the surface and color of the crust, porosity and elasticity) are not inferior to those of the products made from pure wheat flour; however, it should be noted that an increase in the share of barley flour for bakery products to 25 % or more worsens the quality of products (Letyago, Belkina, 2019). Research is also underway to obtain bread products with increased antioxidant activity. Similar optimal ratios have been calculated for the addition of flour from the grain of the naked pigmented barley cultivar Granal 32, which shows increased antioxidant activity of 10 % (Gryaznov et al., 2019; Martínez-Subirà et al., 2020).

Including naked barley, rich in β-glucans and anthocyanins, in a balanced diet provides many health benefits, and such low-glycemic and fiber-rich dietetic foods may help to regulate blood glucose levels in healthy individuals and diabetic patients (Shakib, Gabrial, 2010; Martínez-Subirà et al., 2020).

Use of barley for feed purposes

Most of the world's barley production (> 70 %) is used to meet the needs of animal husbandry. Barley as a fodder crop is widely utilized in Russia, CIS countries, Eastern Europe and Canada, where one of the most popular fodder crops, maize, is not so widespread (Avdeichik et al., 2009).

The grain of naked barley is a valuable high-energy feed, rich in protein and a number of essential amino acids, while the content of cellulose is low. Numerous studies on naked barley are aimed at including it in feeds with various enzyme supplements to improve the quality of animals and reduce rearing costs. Active introduction of naked barley grain into animal rations showed a positive effect mainly on laying hens (Dadashko et al., 2010), pigs (Tatarkina, 2019), broiler chickens (Teimouri et al., 2018), and geese (Toropova, Sukhanova, 2013).

Other uses of barley

Actively developed is the trend when not only covered but also naked barley is used for the production of malt for the brewing industry and the sector dealing with other alcoholic drinks. Aqueous extracts from barley malt are also used in medicine, textile and leather industries. The possibility of using naked barley as a crop for brewing has been discussed for a very long time, but it is necessary to make adjustments to the technological process, for example by replacing natural filtration through films with artificial filters (Borisonik, 1971).

There is also a trend to use naked barley to make diastatic malt, which has a composition and enzymatic activity comparable to that of brewing and distilled malts, but superior to that of wheat malt brewed under identical conditions. The first advantage of such malt is a shorter maturation time than that of malting barley or wheat. Another advantage of naked barley malt is that it can be used directly for food purposes without the need to prepare malt extracts and syrups, as is the case with brewing and distillation industries (Bhatty, 1996). Besides, naked barley can serve as basic material for the production of fuel alcohol (Ingledew et al., 1995).

The latest studies on naked barley have shown its versatility for uses both for feed and food as well as for various production needs. Although researches have formed an opinion based on their long-term experience that there are difficulties in their approaches to promoting the use of naked barley, many of them believe in the expediency of at least partial replacement of covered barley with the naked one in order to increase produce quality and possibly reduce production costs.

Conclusion

After a detailed review of published sources on naked barley, we can conclude that such forms of barley undoubtedly have a number of advantages over the traditional covered ones, such as easy separation of glumes during threshing, a more balanced biochemical composition, an increased content of protein, various amino acids, β-glucans and compounds with antioxidant activity, and a lower cellulose content, which increases its value as a fodder crop.

The VIR collection preserves and maintains more than 1230 naked barley accessions collected from all over the world. It can serve as a source for the development of high-yielding cultivars of naked barley with adaptive properties, thus providing an advantage in producing high-quality harvests, while easy separation of the kernel from glumes would facilitate grain processing operations.

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The effect of the 7DL-7Ae#1L·7Ae#1S translocation on the productivity and quality of spring bread wheat grain

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Abstract. The 7DL-7Ae#1L·7Ae#1S translocation with the *Lr29* gene attracts the attention of bread wheat breeders by its effectiveness against *Puccinia triticina*. However, its impact on useful agronomic traits has been little studied. In this report, the prebreeding value of 7DL-7Ae#1L·7Ae#1S was studied in analogue lines (ALs) of spring bread wheat cultivars Saratovskaya 68 and Saratovskaya 70 during 2019–2021. The presence of the *Lr29* gene was confirmed by using molecular marker Lr29F24. The ALs with the *Lr29* gene were highly resistant to *P. triticina* against a natural epiphytotics background and in laboratory conditions. 7DL-7Ae#1L·7Ae#1S in Saratovskaya 68 ALs reduced grain productivity in all years of research. On average, the decrease was 35 and 42 %, or in absolute figures 1163 and 1039 against 1802 kg/ha in the cultivar-recipient. In Saratovskaya 70 ALs, there was a decrease in grain yield in 2019 and 2020, and there were no differences in 2021. On average, the decrease was 18 and 32 %, or in absolute figures 1101 and 912 against 1342 kg/ha in the cultivar-recipient. The analogues of both cultivars showed a significant decrease in the weight of 1000 grains, which ranged from 14 to 20 % for Saratovskaya 68 and 17–18 % for Saratovskaya 70. An increase in the period of germination-earing was noted only in Saratovskaya 68 lines, which averaged 1.3 days. ALs of Saratovskaya 70 had no differences in this trait. 7DL-7Ae#1L·7Ae#1S did not affect plant height and lodging resistance in all ALs. Studies of the bread-making quality in lines with 7DL-7Ae#1L·7Ae#1S revealed a significant increase in grain protein and gluten content. As for the effect on the alveograph indicators, there were differences between ALs of both cultivars. While Saratovskaya 68 ALs had a decrease in elasticity and in the ratio of dough tenacity to the extensibility, Saratovskaya 70 lines had an increase in these indicators. All lines increased the flour strength and the loaves volume, but while Saratovskaya 68 ALs had an increased porosity rating, Saratovskaya 70 ALs had the same rating as the recipient.
Key words: bread wheat; translocation 7DL-7Ae#1L·7Ae#1S; analogue lines; efficiency of the *Lr29* gene; effect on grain productivity and bread-making quality.

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Влияние транслокации 7DL-7Ae#1L·7Ae#1S на продуктивность и качество зерна яровой мягкой пшеницы

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Аннотация. Транслокация 7DL-7Ae#1L·7Ae#1S с геном *Lr29* от хромосомы 7Ae#1 пырея удлиненного (2*n* = 70) привлекает внимание селекционеров мягкой пшеницы эффективностью против возбудителя листовой ржавчины. Однако ее влияние на хозяйственно полезные показатели изучено недостаточно. В представленной статье исследована агрономическая ценность транслокации 7DL-7Ae#1L·7Ae#1S у аналогов яровой мягкой пшеницы сортов Саратовская 68 и Саратовская 70 в течение 2019–2021 гг. Наличие гена *Lr29* у исследуемого материала было подтверждено с помощью ПЦР-анализа с маркером Lr29F24. Линии с геном *Lr29* характеризовались высокой устойчивостью к *Puccinia triticina* как на фоне естественной эпифитотии, так и в лабораторных условиях. Транслокация 7DL-7Ae#1L·7Ae#1S у аналогов сорта Саратовская 68 снижала продуктивность зерна во все годы исследований. В среднем понижение составило 35 и 42 %, или в абсолютных цифрах 1163 и 1039 против 1802 кг/га у сорта-реципиента. В то же время у линий-аналогов сорта Саратовская 70 отмечено понижение урожайности зерна в 2019 и 2020 гг. и не было отличий в 2021 г. В среднем понижение урожайности было 18 и 32 %, или в абсолютных цифрах 1101 и 912 против 1342 кг/га у сорта-реципиента. У аналогов обоих сортов выявлено значимое понижение массы 1000 зерен, которое колебалось от 14 до 20 % на сорте Саратовская 68 и 17–18 % – на сорте Саратовская 70. Увеличение периода «всходы–колошение» обнаружено только у линий-аналогов сорта Саратовская 68, которое составило в среднем 1.3 суток. У аналога

сорта Саратовская 70 различий по этому показателю не было. Транслокация 7DL-7Ae#1L·7Ae#1S не влияла на высоту растений и устойчивость к полеганию у всего набора линий. Исследования качества муки и хлеба у линий с транслокацией 7DL-7Ae#1L·7Ae#1S выявили значимое увеличение содержания зернового белка и клейковины. Однако наблюдались различия между линиями сортов Саратовская 68 и Саратовская 70 по влиянию на показатели альвеографа. Если у линий сорта Саратовская 68 отмечалось понижение упругости, отношения упругости теста к растяжимости, то у линий сорта Саратовская 70 эти показатели повышались. Все линии увеличивали силу муки и объем хлебцев, но если у линий сорта Саратовская 68 была повышенная хлебопекарная оценка, то линии сорта Саратовская 70 не отличались от реципиента.
Ключевые слова: мягкая пшеница; транслокация 7DL-7Ae#1L·7Ae#1S; линии-аналоги; эффективность гена *Lr29*; влияние на продуктивность и качество зерна.

Introduction

The Lower Volga region of the Russian Federation is one of the main regions growing bread wheat. The main crops are in the Saratov and Volgograd regions. In 2020, in the Saratov region, the total area under bread wheat (winter and spring) amounted to 1,380,524 ha (http://srtv.gks.ru/storage/mediabank/f2raAGzs). In the Volgograd region, the total area under bread wheat (winter and spring) amounted to 1,528,000 ha (https://volgastat.gks.ru/storage/mediabank/posev_21.pdf). According to "The State Register of Selection Achievements Authorized for Use for Production Purposes" in 2021, 90 cultivars of winter bread wheat and 27 cultivars of spring bread wheat have been registered in the Lower Volga region (gossortrf.ru/wp-content/uploads/2021/04/Final-register-2021.pdf).

In this region, one of the main fungal diseases of wheat is leaf rust (pathogen *Puccinia triticina* f. sp. *tritici* Erikss.). Despite the fact that some Russian grain-growing regions of the last decade are characterized by a decrease in the importance of this disease, the losses from it are quite large (Gultyaeva et al., 2021). In the Lower Volga region, the disease occurs annually, and strong epiphytoties are observed every three to four years (Gultyaeva et al., 2020). The last strong epiphytoty was in the growing season of 2017 (Sibikeev et al., 2020). Despite the above-mentioned large number of winter and spring bread wheat cultivars registered in this region, a significant part of them are susceptible to *P. triticina* (Gultyaeva et al., 2021). Thus, in the Left Bank zone of the Saratov region, the prevailing cultivars of spring bread wheat are Saratovskaya 42, Saratovskaya 55 and Albidum 32, which are not protected by any resistance genes or they have ineffective *Lr10* (Gultyaeva et al., 2020, 2021).

In general, each region of Russia has its own set of common wheat cultivars with leaf rust resistance genes (*Lr*-genes). However, in general, it is not large and is limited to genes *Lr1*, *Lr3*, *Lr9*, *Lr10*, *Lr19*, *Lr20*, *Lr24*, *Lr26*, *Lr34*, *Lr37* and *Lr6Agi1*, *Lr6Agi2*, *LrSp*. These genes are used in practical breeding in various combinations, but in general, only *LrSp*, *Lr6Agi1*, and *Lr6Agi2* genes have not been overcome (Gultyaeva et al., 2021). Moreover, there is reason to believe that the last two genes are allelic (Sibikeev et al., 2017). In this regard, most breeding centers in Russia are searching for and transferring new unidentified *Lr*-genes from wild relatives into promising material (Davoyan et al., 2017, 2019, 2021; Gultyaeva et al., 2020) or attracting effective previously unused *Lr*-genes (Sibikeev et al., 2019). The latter include the *Lr29* gene, which is highly effective both in Russia (Gultyaeva et al., 2021) and abroad (Labuschagne et al., 2002; Li et al., 2018; Atia et al., 2021).

As is known, the *Lr29* gene is introgressed into the bread wheat cultivar Chinese Spring from the short arm 7Ae#1 of the chromosome *Agropyrum elongatum* (Host) Beauvois =*Thinopyrum ponticum* (Podp.) Backworth and Dewey by homeologous recombination (Sears, 1973). E.R. Sears (1973) identified a 7D/Ag#11 transfer that differed from others in its resistant response to the leaf rust pathogen. Unlike other leaf rust resistance genes (*Lr24*, *Lr19*) introduced from *Ag. elongatum*, the *Lr29* gene is not linked to stem rust resistance genes and yellow flour (McIntosh et al., 1995). The catalog of wheat gene symbols does not list any commercial cultivars with this gene (McIntosh et al., 2013). However, there is information on the presence of *Lr29* in Egyptian varieties (Atia et al., 2021). Based on the research of E.I. Gultyaeva, it is absent in Russian cultivars of winter and spring bread wheat (Gultyaeva et al., 2021). The reason for the limited use of the *Lr29* gene in practical breeding, more precisely the 7DL-7Ae#1L·7Ae#1S translocation, is not known.

The question of whether this is due to the fact that it does not compensate for the absence of wheat chromatin, or contains undesirable linkages, is open, since there is little information on the effect of this translocation on economically useful traits. There are only two studies of the 7DL-7Ae#1L·7Ae#1S translocation available to us: they were conducted in Canada and South Africa, focused mainly on the study of flour and bread quality and were carried out in small plot crops for one or two growing seasons (Dyck, Lukow, 1988; Labuschagne et al., 2002). In Russia, such studies have not been conducted. To identify the effect of the 7DL-7Ae#1L·7Ae#1S translocation with the *Lr29* gene on grain productivity and the quality of bread flour in the laboratory of genetics and cytology of the Federal Center of Agriculture Research of the South-East Region, analogue lines of spring bread wheat were bred using Saratovskaya 68 and Saratovskaya 70 cultivars.

The purpose of our research was to reveal the prospects of the 7DL-7Ae#1L·7Ae#1S translocation with the *Lr29* gene for practical breeding both in terms of effectiveness against *P. triticina* and in terms of its effect on grain productivity and flour and bread quality.

Materials and methods

The material used included the following genotypes: 1) cultivar-recipient of spring bread wheat Saratovskaya 68 (C68) and Saratovskaya 70 (C70); 2) analogue lines of spring bread wheat Saratovskaya 68*4//Tc*Lr29*; 3) analogue lines of spring bread wheat Saratovskaya 70*4//Tc*Lr29*. Analogue lines were obtained by crossing the C68 and C70 cultivars with a near isogenic line of the Thatcher cultivar (Tc*Lr29*, RL-6080)

containing the 7DL-7Ae#1L·7Ae#1S translocation with the *Lr29* gene, followed by fourfold backcrossing with cultivar-recipients. In total, 20 analogue lines were obtained from the C68 cultivar and 11 lines were obtained from the C70 cultivar. For further studies, two lines of analogues for each cultivar were taken. Since both recipient cultivars are susceptible to the leaf rust pathogen, the main criterion for backcross selection was resistance to *P. triticina*.

Two different recipient cultivars were taken into the study to identify the possible influence of the recipient genotype on the studied traits. C68 and C70 cultivars differ from each other. The first cultivar is awned, red-grained, white-ears, tall-growing, mid-ripening, susceptible to the leaf rust pathogen, contains the ineffective *Lr10* gene (Gultyaeva et al., 2020), belongs to the category of valuable wheat in terms of flour and bread quality. The second cultivar is awnless, white-grained, white-ears, tall-growing, early maturing, susceptible to the leaf rust pathogen, does not contain any *Lr*-genes (Gultyaeva et al., 2020); belongs to the category of valuable wheat in terms of flour and bread quality.

The studies included three stages: the first stage was to confirm the presence of alien material in the studied analogue lines C68*4//Tc*Lr29* (C68*Lr29*) and C70*4//Tc*Lr29* (C70*Lr29*), *Lr*-genes were identified using the molecular marker *Lr29* (Lr29F24) (Procunier et al., 1995). DNA was isolated from the leaves of 5-day-old seedlings by the micro method according to the method of D.V. Dorokhov and E. Clocke (Dorokhov, Clocke, 1997). Three plants were taken from each line. The DNA concentration in the standard solution was 50–100 ng/µl. The polymerase chain reaction was carried out in a MyCycler Thermal Cycler (Bio-Rad, USA) under the following conditions: 94 °C – 3 min, 35 cycles (94 °C – 30 s; 60 °C – 30 s; 72 °C – 1 min). The amplified fragments were separated by electrophoresis in 1.5 % agarose gel in 1×TBE buffer; the gels were stained with ethidium bromide and photographed under ultraviolet light. The Tc*Lr29* line was used as a positive control.

The second stage was an evaluation of the lines susceptibility to the pathogen of leaf rust at the juvenile stage and the stage of adult plants. The susceptibility of plant material at the stage of adult plants (milky-wax ripeness phase) was evaluated in the field conditions of the Federal Center of Agriculture Research of the South-East Region during a strong epiphytoty of the pathogen in 2017 (Sibikeev et al., 2020).

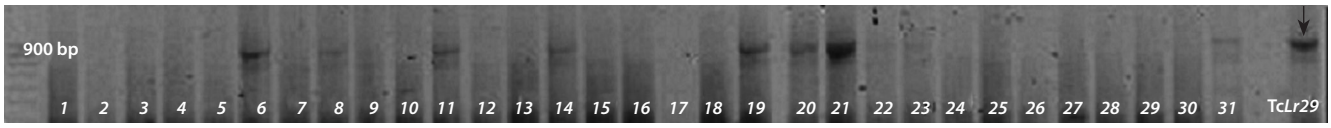
In the field, the resistance degree was determined using the A.P. Roelfs et al. (1992) scale, where *R* is resistant, *MR* is moderately resistant, *MS* is moderately susceptible, and *S* is susceptible, respectively. The percentage degree of rust damage was assessed according to the scale of R.F. Peterson et al. (1948). Lines at the juvenile stage were evaluated in laboratory conditions in the first leaf phase at the All-Russian Institute of Plant Protection in 2018. *P. triticina* clones marked with virulence for genes *Lr9* (K9), *Lr19* (K19), *Lr26* (K26) and the combined Saratov population of the pathogen were used. Test clone K9 was isolated from the Ural population, test clone K19 – from Tambov, K26 – from Krasnodar population, respectively. The Saratov population was collected at the Lysogorsk phytonursery of the Saratov region in 2018. The test clones and population used were avirulent to Thatcher (Tc*Lr*) lines with genes *Lr24*, *Lr23*, *Lr28*, *Lr29*, *Lr39*(= 41), *Lr45*, *Lr47*, *Lr51*, *Lr53* and virulent to those with *Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr14a*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr30*.

Clone *K9* was virulent to Tc*Lr9* and avirulent to Tc*Lr19*, and Tc*Lr26*; clone K19 was virulent to Tc*Lr19* and avirulent to Tc*Lr9*, Tc*Lr26*; clone K26 was virulent to Tc*Lr26*, avirulent to Tc*Lr9*, and Tc*Lr19*. These *P. triticina* test clones were chosen for analysis, since virulence to *Lr9* is common in the Ural region, to *Lr19* – in the Middle and Lower Volga regions, and to *Lr26* – in all regions of the Russian Federation where bread wheat is grown.

The Saratov population of the pathogen was represented by a mixture of two races: virulent to the Tc*Lr19* line, avirulent to Tc*Lr9*, Tc*Lr26* and virulent to the Tc*Lr26* line, avirulent to Tc*Lr9*, Tc*Lr19*. For infection, 10–12 day old seedlings (the first leaf phase) of the studied lines of analogues and recipient cultivars grown in pots with soil were used. They were sprayed with an aqueous suspension of spores of each test clone and a population with the addition of Tween 80 detergent. After infection, the plants were placed in a light installation under controlled conditions (temperature 20 °C, photoperiod 16 h day/8 h night). The type of wheat reaction was determined according to the scale of E.B. Mains, H.S. Jackson (1926), where 0 is the absence of symptoms; 0; – necrosis without pustules; 1 – very small pustules surrounded by necrosis; 2 – pustules of medium size, surrounded by necrosis or chlorosis; 3 – pustules of medium size without necrosis, 4 – large pustules without necrosis, *X* – pustules on the same leaf of different types, chlorosis and necrosis are present. Plants with 0–2 point damage were classified as resistant (*R*), and 3, 4 and *X* (*S*) were classified as susceptible (Mains, Jackson, 1926).

The third stage is the evaluation of grain productivity traits, physical and baking properties of dough and bread in the C68*Lr29* and C70*Lr29* analogue lines in comparison with the recipient cultivars C68 and C70. The studies were carried out in 2019–2021. The hydrothermal coefficient for the growing season of bread wheat in 2019 was 0.6 (very dry conditions), in 2020 – 0.8 (dry conditions) and 0.9 (dry conditions) in 2021. The main differences between the weather conditions in 2019 and 2021 there were high temperatures during the flowering period (above the long-term average by 4.2 and 8.0 °C, respectively) with a reduced amount of precipitation (below the long-term average by 13 mm), which sharply reduced grain productivity. At the same time, in 2020, during the flowering period, a lower temperature was observed (below the long-term average by 1 °C) with an increased amount of precipitation (above the long-term average by 48 mm), which increased the grain yield.

The experimental material was randomly sown in 7 m^2 plots in three replications. The seeding rate was 400 grains per 1 m^2. The bread-making quality was evaluated by the content of crude gluten, gluten strength and the indicators of the IDG-3 device (deformation index of gluten) and the Chopin alveograph with the baking of experimental bread samples. The protein content of grain harvested in 2020 and 2021 was determined on the Infratec™ 1241 Grain Analyzer. The data obtained for each set of lines of analogues and the corresponding recipient cultivars were subjected to one-way ANOVA with multiple comparisons according to Duncan using the Agros-2.10 breeding and genetic software package.



Electrophoregram of the fragments amplification in the presence of the Lr29F24/Lr29R24 marker.

M – length marker of 1000/100–500 (Diam). The arrow indicates a 900 bp diagnostic fragment. Tracks *1–20* – analogue lines C68*Lr29*; *21–31* – analogue lines C70*Lr29*.

Results

Identification of resistance genes

To confirm the presence of the 7DL-7Ae#1L·7Ae#1S translocation, and, accordingly, the *Lr29* gene, PCR analysis with the Lr29F24 marker was performed in the C68*Lr29* and C70*Lr29* analogue lines (Procunier et al., 1995).

Amplification fragments, 900 bp in size, were detected in the entire set of C68*Lr29* and C70*Lr29* analogue lines, as well as in the positive control (Tc*Lr29* line). 31 samples were analyzed; a 900 bp size amplification product was determined in lines No. 6, 8, 11, 14, 19, 20 – C68*Lr29*, No. 21, 31 – C70*Lr29* (see the Figure). Based on the molecular analysis performed using a marker developed to detect the 7DL-7Ae#1L·7Ae#1S translocation with the *Lr29* gene, it was suggested that the C68*Lr29* and C70*Lr29* analogue lines carry this translocation, and hence the *Lr29* gene. To reveal the effect of the 7DL-7Ae#1L·7Ae#1S translocation on economically valuable traits, lines No. 6 and 8 C68*Lr29* and lines No. 21, 31 C70*Lr29* were chosen.

Phytopathological analysis of resistance to the leaf rust causative agent

Under the leaf rust epiphytotics condition of 2017, all lines with the *Lr29* gene had a resistant reaction type (*R*) (infestation 0 %, reaction type – IT = 11+), while the recipient cultivars C68 and C70 had susceptibility to the pathogen (*S*) (infestation 40 and 60 %, reaction type IT = 3). Similar results were obtained during lines inoculation in the seedling phase in laboratory conditions (Table 1).

Thus, phytopathological analysis of resistance to the leaf rust pathogen in the C68*Lr29* and C70*Lr29* analogue lines under field and laboratory conditions evidenced a high level of their resistance and the effectiveness of the *Lr29* gene, compared with the original recipient cultivars.

Effect of the 7DL-7Ae#1L·7Ae#1S translocation on grain productivity and flour and bread quality

The results of studying grain productivity in lines with the 7DL-7Ae#1L·7Ae#1S translocation (*Lr29* gene) showed that, on average, for the period from 2019 to 2021, there are no significant differences in yield in the lines compared to the recipient cultivars C68 and C70 (Table 2). This is expected, since the grain productivity traits in 2020 are three to five times higher than the grain yield in 2019 and 2021. Similar results were obtained when identifying the effect of *Sr22*+*Sr25* and *Sr22*+*Sr35* gene combinations on lines of spring bread wheat compared to the L503 and Favorit cultivars. The grain yield of these cultivars and lines was 2.3–2.7 times higher in 2020 compared to 2019 (Sibikeev et al., 2021). Nevertheless, the analysis of grain productivity separately by years revealed that, in the C68*Lr29* analogue lines, for all three years of study, the grain yield was significantly lower than that of the recipient cultivar C68. Similar conclusions were reached when comparing the grain productivity of the C70*Lr29* lines for two years of study (2019 and 2020), and only in the growing season of 2021 the grain productivity of the lines was at the level of the recipient cultivar C70.

Table 1. Characteristics of lines susceptibility with translocation 7DL-7Ae#1L·7Ae#1S and parental cultivars to the pathogen *P. triticina* in the seedling phase

Cultivar, line	Reaction type (IT), score			
	Clone tests			Saratov population *P. triticina*
	K9	K19	K26	
C68	3	3	3	3
C68*Lr29-6*	0	0	0	0
C68*Lr29-8*	0	0	0	0
C70	3	3	3	3
C70*Lr29-21*	0	0	0	0
C70*Lr29-31*	0	0	0	0

The 2019–2021 seasons were characterized by drought, but the 2020 season was distinguished by precipitation distribution during the growing season. This year, there was moisture excess from germination to flowering, and then there was a drought with high temperatures until full maturity. The main positive moment of the growing season in 2020 was the increased precipitation amount in the third decade of June (the flowering phase of spring bread wheat). At the same time, the excess of long-term indicators was 80 % at low air temperatures, which further contributed to a higher grain yield.

On average for 2019–2021, the analysis of the 1000 grain weight, as one of the important elements of grain productivity, showed a significant decrease in the C68*Lr29* lines – 26.6 and 24.6 g compared to the recipient cultivar – 30.9 g. Similar results were obtained for the C70*Lr29* analogue lines – 29.4 and 29.7 g versus 36.0 g for C70 (see Table 2).

On average for 2019–2021, the effect of the 7DL-7Ae#1L·7Ae#1S (*Lr29* gene) translocation on the duration of the germination-earing period was ambiguous. If significant differences were observed between the C68*Lr29* lines (42.3 days) and the recipient cultivar C68 (41.0 days), then there were no differences between the C70*Lr29* lines (40.7 and 40.0 days) and the cultivar C70 (40.0 days). Thus, the

Table 2. Grain productivity traits in spring bread wheat lines with the translocation 7DL-7Ae#1L·7Ae#1S (*Lr29* gene) in 2019–2021

Cultivar, line	Seedling–earing period, days, average for 2019–2021	Grain yield, kg/ha				1000 grain weight, g, average for 2019–2021	Grain protein content, %, average for 2019–2021
		2019	2020	2021	Average		
C68	41.0	684	3650	1073	1802	30.9	16.6
C68*Lr29-6*	42.3	562	2578	348	1163	26.6	18.9
C68*Lr29-8*	42.3	462	2344	340	1049	24.6	18.5
HCP_{05}	1.0	110	238	121	NS	2.7	0.5
C70	40.0	562	3164	299	1342	36.0	16.7
C70*Lr29-21*	40.7	352	2574	378	1101	29.4	17.5
C70*Lr29-31*	40.0	307	2112	318	912	29.7	17.5
HCP_{05}	NS	152	250	115	NS	5.2	0.5

Table 3. Bread-making quality traits in lines of spring bread wheat with the 7DL-7Ae#1L·7Ae#1S translocation (*Lr29* gene) for 2020 on the average

Cultivar, line	Alveograph*			Bread**		
	P, mm	*P/L*	*W*, units	*V*, cm^3	Porosity, score	Crumb color
C68	97	1.5	281	710	4.2	White
C68*Lr29-6*	79	1.0	268	820	4.9	White
C68*Lr29-8*	88	1.0	314	750	5.0	White
HCP_{05}	9	0.3	20	40	0.3	
C70	91	1.5	222	820	4.9	White
C70*Lr29-21*	103	1.6	327	875	4.8	White
C70*Lr29-31*	110	1.8	280	870	5.0	White
HCP_{05}	9	NS	30	40	NS	

* Indicators of the alveograph: *P* – dough tenacity; *P/L* – tenacity to extensibility ratio; *W* – flour strength.
** Indicators of bread evaluation: *V* – bread volume; porosity.

effect of the 7DL-7Ae#1L·7Ae#1S translocation was not the same in different genotypes of the recipient cultivars: in lines based on the mid-season cultivar C68, the germination-earing period lengthened, and on the early-ripening cultivar C70, it remained at the recipient level. There were no differences in plant height and lodging resistance between the studied lines and the recipient cultivars.

Unfortunately, the involvement of alien genetic variability in the bread wheat gene pool worsens some traits of flour and bread quality. Therefore, when studying the effect of chromosome introgression or translocations from related species into bread wheat, an important step is to determine the quality of the final product – flour and bread. On average for 2020–2021, studies revealed that lines with the 7DL-7Ae#1L·7Ae#1S translocation (*Lr29* gene) significantly exceeded the recipient cultivars in grain protein content (see Table 2). Moreover, the C68*Lr29* analogue lines exceeded the recipient cultivars by 2 %, and C70*Lr29* – by 0.8 %.

According to the indicators of gluten, the following results were obtained: the C68*Lr29* lines significantly exceeded the recipient cultivar C68 in gluten content, namely 41.7 and 41.4 versus 31.4 % in the recipient, LSD_{05} = 2.5. There were no significant differences in gluten strength between the C68*Lr29* lines and the recipient, but it should be noted that, according to IDK-3, the C68*Lr29* lines have weaker gluten – 76 and 80 units, against 72 units in C68. The C70*Lr29* lines showed a significant excess in gluten content of the recipient cultivar C70, namely 37.0 and 38.5 versus 35.0 % in the recipient, LSD_{05} = 1.5. There were no significant differences in gluten strength between the C70*Lr29* lines and the recipient cultivar. In addition, according to the indicators of IDK-3, in the C70*Lr29* lines, the gluten of the first group is 71 and 75 units, in C70 – 69 units, respectively.

When studying the alveograph indicators, it was found that the C68*Lr29* lines differed not only from the recipient cultivar, but also from each other. According to the dough elasticity and the ratio of the dough tenacity to extensibility (*P/L*), there was a decrease, but in one of the C68*Lr29* lines, the decrease in elasticity (*P*) was insignificant. The C68*Lr29* lines showed an ambiguous effect of the 7DL-7Ae#1L·7Ae#1S translocation on the flour strength: one line slightly decreased, and the second one significantly increased this indicator. Crumb porosity and bread volume in the C68*Lr29* lines increased relative to the recipient cultivar C68, but in one of the lines the bread volume increase was insignificant. At the same time, in the C70*Lr29* lines, the effect of the 7DL-7Ae#1L·7Ae#1S translocation on the alveograph parameters was unambiguous: an increase in dough elasticity, equal to the *P/L* ratio, an increase in flour strength, bread volume, and a high score for bread porosity at the level of the recipient cultivar C70 (Table 3).

Discussion

As noted above, the *Lr29* gene in the 7DL-7Ae#1L·7Ae#1S translocation is highly effective against leaf rust pathogen populations in many countries of the world. Only two *P. triticina* pathotypes from Turkey and one from Pakistan are known to be virulent to this gene (Huerta-Espino, 1992, from McIntosh et al., 1995). In our studies, the effectiveness of the *Lr29* gene was confirmed during severe leaf rust epiphytosis in the Saratov region (*R*-type resistance and type of response to the pathogen IT = 1) and in laboratory studies. Lines with the *Lr29* gene were resistant when inoculated with *P. triticina* isolates virulent to *Lr9*, *Lr19*, *Lr26* (IT = 0;). Since, under field conditions, adult plants were evaluated in the phase of the beginning of grain filling, and in laboratory studies, seedlings were evaluated in the one leaf phase, it can be argued that the protective effect of *Lr29* was expressed throughout the growing season.

Analyzing the effect of the 7DL-7Ae#1L·7Ae#1S translocation (gene *Lr29*), it is necessary to note the translocation size. As can be seen from its designation, it includes a part of the long arm and the entire short arm of the chromosome 7Ae#1 of *Thinopyrum ponticum* and a part of the long arm of the 7D chromosome of bread wheat. The break point is indicated at the distal part of 7DL-7Ae#1L of arms (Friebe et al., 1996). Thus, there is reason to expect a large impact on agronomic traits, primarily on grain productivity and the quality of flour and bread.

Unfortunately, there are few studies on the effect of the 7DL-7Ae#1L·7Ae#1S translocation (gene *Lr29*) on economically valuable traits (prebreeding study) (Dyck, Lukow, 1988; Labuschagne et al., 2002). These studies were carried out on near isogenic lines of the Thatcher cultivar (Dyck, Lukow, 1988) and Thatcher and Karee cultivars (Labuschagne et al., 2002). They mainly focused on determining the effect of the 7DL-7Ae#1L·7Ae#1S translocation (*Lr29* gene) on bread-making quality traits. Grain productivity has been studied during one year, and it showed a neutral effect (Dyck, Lukow, 1988).

In our studies, based on the results of three-year field trials under conditions of moisture deficiency (drought of varying degrees), a significant decrease in grain productivity was observed in the C68*Lr29* lines for all three seasons. A similar effect was found in the C70*Lr29* lines: a significant decrease in grain yield for two seasons out of three. P.L. Dyck, O.M. Lukow (1988) and M.T. Labuschagne et al. (2002) found a positive effect on grain protein content (Dyck, Lukow, 1988; Labuschagne et al., 2002). Our results are consistent with this conclusion. The increase in the grain protein content of the analogue lines compared to the recipient cultivars ranged from 0.8 to 2.0 %. The conclusions about a positive effect on the volume of experimental breads also coincided. According to the results of our studies, the excess was from 40 to 110 cm^3. In the ratio of dough tenacity to extensibility (*P/L*), same as in the studies of M.T. Labuschagne et al. (2002), we determined the effect of the recipient variety. Thus, a decrease was noted on the C68*Lr29* lines, and a neutral effect on the C70*Lr29* lines. In studies by P.L. Dyck and O.M. Lukow (1988), a positive or neutral effect on the weight of 1000 grains was noted (Dyck, Lukow, 1988). According to our data, the presence of the 7DL-7Ae#1L·7Ae#1S translocation lowers this parameter, moreover, in two sets of analogue lines it decreases over three years of study. The decrease was from 4.3 to 6.6 g.

For the rest of the studied traits, our studies complement the results of P.L. Dyck, O.M. Lukow (1988) and M.T. Labuschagne et al. (2002). So, in the studies of P.L. Dyck, O.M. Lukow (1988) and M.T. Labuschagne et al. (2002), a positive or neutral effect on water absorption capacity and flour yield, and a negative effect on dough formation time were found. Our studies have established a positive effect on the gluten content and a slight decrease in its strength. In addition, the effect of the recipient cultivar on the dough elasticity was revealed, so in the C68*Lr29* lines the 7DL-7Ae#1L·7Ae#1S translocation reduces this indicator, and in C70*Lr29* it is significantly increased. In terms of the effect on the flour strength, the C70*Lr29* lines showed a significant increase, while in the C68*Lr29* lines, one line slightly decreased, and the second significantly increased this indicator. It is possible that, in addition to the effect of the 7DL-7Ae#1L·7Ae#1S translocation, the set of the C68*Lr29* lines was also affected by selection during line generation. M.T. Labuschagne et al. (2002) also observed selection effects within a set of near isogenic lines of the Karee cultivar with the *Lr29* gene, which had ambiguous flour quality indicators.

In our studies, all analogue lines either increased bread porosity indicators (C68*Lr29* lines) or had high indicators at the level of the recipient cultivar (C70*Lr29* lines). In addition, a different effect (depending on the recipient cultivar) on the duration of the seedling – earing period was revealed. Thus, significant differences were observed between the C68*Lr29* lines (42.3 days) and the recipient cultivar C68 (41 days), while there were no differences between the C70*Lr29* lines (40.7 and 40.0 days) and the variety C70 (40.0 days). No effect of the 7DL-7Ae#1L·7Ae#1S translocation on plant height and lodging resistance was found.

Conclusion

The high efficiency of the *Lr29* gene against the Saratov population of the leaf rust pathogen, as well as pathotypes virulent to *Lr9*, *Lr19*, *Lr26*, was confirmed. For the whole complex of economically valuable traits, analogue lines with the 7DL-7Ae#1L·7Ae#1S translocation (gene *Lr29*) were more promising than the recipient cultivars in terms of flour and bread quality, but were inferior to them in terms of grain productivity. The decrease in grain yield is apparently associated with a decrease in drought resistance compared to the recipient cultivars Saratovskaya 68 and Saratovskaya 70. For further use of the 7DL-7Ae#1L·7Ae#1S translocation (*Lr29* gene) in breeding programs, additional studies are needed to reduce the negative impact on a number of agronomically important traits.

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Molecular genetic detection and differentiation of *Xanthomonas oryzae* pv. *oryzicola*, bacterial leaf streak agents of rice

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Abstract. The genus *Xanthomonas* comprises phytopathogenic bacteria which infect about 400 host species, including a wide variety of economically important plants. *Xanthomonas oryzae* pv. *oryzicola* (Fang et al., 1957) Swings et al., 1990 is the causal agent of bacterial leaf streak (BLS) being one of the most destructive bacterial diseases of rice. BLS symptoms are very similar to those of bacterial blight caused by closely related *Xanthomonas oryzae* pv. *oryzae*. *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* and often occur in rice fields simultaneously, so separate leaves may show symptoms of both diseases. The quarantine status and high severity of the pathogen require a highly efficient, fast and precise diagnostic method. We have developed an assay for *Xanthomonas oryzae* pv. *oryzicola* detection using real-time polymerase chain reaction (qPCR) and PCR amplicon sequencing. The DNA samples of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* were obtained from the collection of CIRM-CFBR (France). To evaluate the analytical sensitivity of the assay, a vector construct based on the pAL2-T plasmid was created through the insertion of *X. o.* pv. *oryzicola* target fragment (290 bp). Primers and a probe for qPCR were selected for the *hpa1* gene site. They allowed identifying all the strains the sequences of which had been loaded in the GenBank NCBI Nucleotide database before November 11, 2021. The SeqX.o.all sequencing primers were selected for the *hrp* gene cluster sequence, namely for the nucleotide sequence encoding the Hpa1 protein, the sequencing of which allows for efficient differentiation of *X. oryzae* species. The analytical specificity of the system was tested using the DNAs of 53 closely related and accompanying microorganisms and comprised 100 % with no false-positive or false-negative results registered. The system's analytical sensitivity was not less than 25 copies per PCR reaction. Its efficacy has been confirmed using five different qPCR detection systems from different manufacturers, so it can be recommended for diagnostic and screening studies.
Key words: *Xanthomonas oryzae* pv. *oryzicola*; *Xanthomonas*; polymerase chain reaction; qPCR; bacterial leaf streak; specificity; sensitivity; species diagnostics.

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Молекулярно-генетическое выявление и дифференциация возбудителей бактериальной полосатости листьев риса *Xanthomonas oryzae* pv. *oryzicola*

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Аннотация. Бактерии рода *Xanthomonas* Dowson, 1939 поражают около 400 видов растений, в том числе важные сельскохозяйственные культуры. Бактериальная полосатость риса – одно из самых разрушительных заболеваний, вызвано бактериями вида *Xanthomonas oryzae* pv. *oryzicola* (Fang et al., 1957) Swings et al., 1990. Сильное сходство симптомов поражения с другим карантинным близкородственным патовариантом – *Xanthomonas oryzae* pv. *oryzae* (Ishiyama, 1922) Swings et al., 1990, а также возможность совместного заражения делают визуальную идентификацию невозможной. Карантинный статус и высокая вредоносность патогена требуют высокоэффективного, быстрого и точного метода его диагностики. Целью исследования были разработка и апробация наборов реагентов для выявления бактерии *Xanthomonas oryzae* pv. *oryzicola*, вызывающей бакте-

риальную полосатость листьев риса, методом полимеразной цепной реакции в реальном времени (ПЦР-РВ), а также ПЦР с последующим секвенированием ампликонов. В работе изучены образцы ДНК *X. o.* pv. *oryzae* и *X. o.* pv. *oryzicola*, полученные из коллекции CIRM-CFBR (Франция). Для проверки аналитической чувствительности была создана конструкция на основе вектора pAL2-T с целевой вставкой 290 п. н. Были подобраны и апробированы праймеры и зонд для специфической амплификации фрагмента гена *hpa1* методом ПЦР-РВ, позволяющие обнаруживать ДНК *X. o.* pv. *oryzicola*. Показана способность с помощью разработанных праймеров обнаруживать все штаммы *X. o.* pv. *oryzicola*, последовательности которых находились в базе данных GenBank NCBI на 11.11.2021. Аналитическая специфичность набора реагентов протестирована на выборке из ДНК, выделенных из 53 близкородственных и сопутствующих организмов, и составила на исследованной выборке 100 %. Ложноположительных и ложноотрицательных результатов не обнаружено. Проверка аналитической чувствительности показала, что стабильный специфичный сигнал ПЦР-РВ наблюдался при разведении контрольной плазмиды до 25 копий на реакцию. Работоспособность полученного набора реагентов была подтверждена тестированием на пяти приборах для ПЦР-РВ разных производителей, что дает возможность рекомендовать его для проведения диагностических и скрининговых исследований. Праймеры для секвенирования seqX.o.all были подобраны на последовательность кластера генов *hrp*, а именно на нуклеотидную последовательность, кодирующую белок Hpa1. Секвенирование выбранного участка позволяет эффективно дифференцировать бактерии вида *X. oryzae*.
Ключевые слова: *Xanthomonas oryzae* pv. *oryzicola*; *Xanthomonas*; полимеразная цепная реакция; ПЦР-РВ; бактериальная полосатость риса; специфичность; чувствительность; видовая диагностика.

Introduction

Bacteria *Xanthomonas* Dowson, 1939 are spread worldwide and able to infect at least 400 kinds of plants including those of high economic importance (Bogdanove et al., 2011; Ryan et al., 2011; Fang et al., 2019). Currently, 27 species of this family have been known, many of which demonstrate high levels of virulence and specificity in certain kinds of plants (Leyns et al., 1984; Ryan et al., 2011; An et al., 2020). Bacterial leaf streak (BLS) is considered to be one of the most devastating diseases caused in rice by *Xanthomonas oryzae* pv. *oryzicola* (Fang et al., 1957), Swings et al., 1990 (Soto Suárez et al., 2010).

The disease results in 8 to 32 % of yield loss and is regarded as a serious problem in rice-producing countries (Liu et al., 2014; Jiang et al., 2020). Since the damage done by BLS can seriously threaten the world's food security (Tang et al., 2000; Lang et al., 2014), *Xanthomonas oryzae* pv. *oryzicola* was included in List 1 of harmful quarantine organisms that are not present in the EEU territory as well as in List A1 of the European and Mediterranean Plant Protection Organization (EPPO) that considers the bacteria as quarantine ones[1]. Despite the fact that BLS is believed to have been detected for the first time in 1918 in the Philippines, its pathogen was identified only in 1957 in China (Nino-Liu et al., 2006). For the time being, BLS spread is limited to the tropical and subtropical parts of Asia, Northern Australia and a part of Western Africa (EPPO, 2007; Xie et al., 2014; Jiang et al., 2020). The pathogen is absent in the Russian Federation despite cases of infection[2] in the southern part of the country and the Russian Far East (EPPO, 2007, 2018). According to the EPPO Reporting Service, no cases of *X. o.* pv. *oryzicola* infection have been registered since 1994[3].

Oryza sativa L., 1753, commonly known as Asian rice, is a typical host plant for *X. o.* pv. *oryzicola.* In addition, it affects some weed cereals and several other cultivated plants such as *Poaceae* including *Leersia* spp., *Leptochloa* spp., *Oryza* spp., *Paspalum scrobiculatum*, *Zizania*, *Zoysia* spp. (Ou, 1985; Saddler, Bradbury, 2005; EPPO, 2007). The bacteria mainly spread through infected seeds as well as due to mechanical damage. In case of small plants, infection occurs through wind, raindrops, watering or after contacting infected plant material (Mew et al., 1993).

In plants, *X. o.* pv. *oryzicola* reproduce in the substomatal cavity where they get through the stomata to affect the intercellular space of the parenchyma. However, they do not get as far as the xylem and their spread is limited by the mesophyll tissue's apoplast (Nino-Liu et al., 2006; Triplett et al., 2011; Jacques et al., 2016). The early stage of infection is characterized by small watery interveinal strokes that later transform into bacterial effusion (Mew et al., 1993). The veins act as barriers preventing the pathogen's further spread and extending a leaf's affected areas along its length, so they can merge later. In case of severe infection, BLS becomes difficult to differ from the bacterial burn caused by *Xanthomonas oryzae* pv. *oryzae*, another quarantine bacterial species. Visual identification can also be complicated by favorable environmental conditions and plant resistance (Swings et al., 1990; Poulin et al., 2014), since both species can infect rice fields at the same time (Mew et al., 1993; Nino-Liu et al., 2006).

The objective of the presented study was to develop and test an assay for genetic detection and diagnostics of the

[1] EPPO for the EU under Contract 90/399003. Data Sheets on Quarantine Pests. https://gd.eppo.int/download/doc/530_ds_XANTOR_en [Accessed: 23.11.2021].

[2] Cabi Invasive Species Compendium. Datasheet *Xanthomonas oryzae* pv. *oryzicola* (bacterial leaf streak of rice). https://www.cabi.org/isc/ [Accessed: 23.11.2021].

[3] EPPO Global Database. https://gd.eppo.int [Accessed: 23.11.2021].

Xanthomonas oryzae pv. *oryzicola* pathogen using real time polymerase chain reaction (qPCR) and PCR amplicon sequencing.

Materials and methods

The presented study was carried out at the Biotechnology Collective Use Center of the All-Russian Research Institute of Agricultural Biotechnology and Syntol LLC. As qPCR positive controls, the DNA samples of the Indian typical strain of *X. o.* pv. *oryzae* (2532) and Malaysian pathotype of *X. o.* pv. *oryzicola* (2286) from the French Collection of Plant Associated Bacteria (CIRM-CFBP, France) were used. For the last pathotype, a draft whole genome assembly was obtained (Wilkins et al., 2015). *X. o.* pv. *oryzae*'s geographic distribution is limited to the territories of Asia, Africa and North America, while that of *X. o.* pv. *oryzicola* – to the countries of Asia and Africa. Selecting the target strains, we relied upon the customs statistics of rice import to Russia and according to their data 31.7 % of imported rice in 2018 was supplied by India, followed by Thailand, Pakistan and Kazakhstan[4].

In design of oligonucleotides for qualitative detection of *X. o.* pv. *oryzicola* DNA, the *hpa1* gene region was used. As many other gram-negative pathogens, *X. o.* pv. *oryzicola* has the type III secretion system (T3SS) being a molecular syringe with which the bacteria deliver effector proteins directly into the host cell cytosol (Zhu et al., 2000; Furutani, 2003; Li et al., 2011). The T3SS and its secreted components promote a hypersensitive response (HR) in resistant plants and plants not being the main host for the pathogen. The system is coded as *hrp*, a hypersensitivity and pathogenicity gene (Cho et al., 2008; Fan et al., 2017), the main operon of which is composed of more than 20 genes in several transcription units that contain the *hrp*, *hrc* and *hpa* genes (Zou et al., 2006; Cho et al., 2008). The oligonucleotides were synthesized by Syntol LLC using their expendables. To design the qPCR and PCR amplicon sequencing reactions, reaction buffer B-009 (Syntol LLC, Russia) was used.

The buffer had the following component concentrations: 3 mmol of $MgCl_2$, 0.25 mmol of dNTP, and 2.5 e. a. of polymerase with antibodies to inhibit ferment activity (Syntol LLC). When designing the oligonucleotides, we made sure the annealing temperature was 60–62 °C for the primers and 64–67 °C – for the probe with 3′-GC-clamp.

The multiparameter analysis of the properties of the selected primers was performed using such online applications as Thermofisher Multiple Primer Analyzer (https://www.thermofisher.com), Promega Biomath Calculator – Tm for Oligos Calculator (https://worldwide.promega.com), Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu). The qPCR fluorescence-labelled probe incorporated a FAM dye attached to the probe's 5′ end. The RTQ-1 dye attached to the probe's 3′ end served as a quencher. The primer concentration in reaction mixture was 800 nM, and 400 nM – in the probe. The qPCR reaction's repeatability and reproducibility was assessed using the following detection systems: ANK-M (IAI RAS, Russia), QuantStudio 5 (Thermo Fisher Scientific, USA), CFX-96 (Bio-Rad, USA), DTprime 5 (DNA-Technology, Russia), Rotor-Gene 6000 (Qiagen, USA). The obtained results were considered positive if the fluorescence signal level exceeded the threshold of 10 % module difference of the lowest and highest signals.

To verify the analytical sensitivity of the assay, a vector pAL2-T-based structure (Eurogen, Russia) with a 290 bps inclusion of *X. oryzae* pv. *oryzicola* was designed. Ligation was carried out after the PCR product was purified using the ColGen DNA purification kit (Syntol LLC). To design the vector-based structure, a T4 DNA ligase buffer (Thermo Fisher Scientific) was used. The plasmid DNA was impregnated into *Escherihia coli* bacteria (Migula 1895) through thermal shock. The vector's presence was attested using the PCR-colony method with the standard M13 primers followed by 1.5 % agarose-gel visualization. Plasmid DNA separation was carried out using a PlasGen reagent kit (Syntol LLC). The obtained circular plasmid was processed with the *NotI* restriction enzyme (ThermoFisher Scientific), its concentration measured in a Quantus fluorometer (Promega Corporation, USA). To test the analytical sensitivity of the designed assay, qPCR to dissolve the plasmid were replicated 2 and 4 times. The analytic specificity of the designed primers and probes was tested using the DNAs of 53 closely related and accompanying microorganisms (Alyapkina et al., 2018).

Bioinformatic analysis and data processing were performed using the UGENE (Unipro, Russia) and AliView (Sweden) software solutions.

To sequence *X. o.* pv. *oryzicola*'*s* DNA, a primer couple including seqX.o.all_F 5′-TCTTTGAACACACAATTC GGCGG-3′ and seqX.o.all_R 5′-TGG AGAATCTCTC CGACGATA-3′ was designed. The amplification program of PCR amplicon sequencing reaction included primary denaturation (5 min at 95 °C); cyclic denaturation (15 s at 95 °C); annihilation (40 s at 60 °C); cyclic elongation (36 cycles of 30 s at 72 °C); final elongation (5 min at 72 °C). The sequencing was carried out using a Nanofor 05 genetic analyzer (IAI RAS).

Results and discussion

The search for nucleotide sequences in GenBank NCBI found 208 of them to belong to the Xanthomonas family including 20 strains of *X. o.* pv. *oryzicola*. During sequence alignment performed in AliView, qPCR oligonucleotides were selected for the regions of the *hpa1* target gene conservative to *X. o.* pv. *oryzicola* in such a way that the selected

[4] Agrobusiness Think Tank. https://ab-centre.ru/articles/analiz-importa-risa-v-rossiyu-v-2001-2019-gg-

Table 1. Primer and probe sequencies selected for the *hpa1* gene region of *X. o.* pv. *oryzicola* and their amplification programs

Name	Sequence	Amplification program
Sva3X.o.cola_F	F 5′-ATTCGAGCCAGGGCRGCAATG-3′	5 min at 95 °C 15 s at 95 °C 40 s at 60 °C 50 cycles
Sva3X.o.cola_R	R 5′-ACCAAAGTCGCCGCCGCTGCT-3′	
Sva3X.o.cola_FAM	Pb 5′-(FAM) AATCAGCAGGCCGGGAAGGAGAA (RTQ1)-3′	

Note. F – forward primer; R – reverse primer; Pb – probe.

primers' attachment sites were strictly specific and enabled the detection of all the target's stains the DNA sequences of which had been loaded in the GenBank NCBI database[5] before 11.11.2021. Table 1 demonstrates the primer and probe sequencies for *X. o.* pv. *oryzicola* diagnostics, selected for its *hpa1* gene region.

The analytic specificity of the assay was tested using 53 DNA samples of closely related and accompanying microorganisms from different collections that included 9 DNA samples of the bacteria belonging to the Xanthomonas family. The samples were obtained from collections:

– of the All-Russian Center of Plant Quarantine and Federal Service for Veterinary and Phytosanitary Surveillance's divisions: *Ralstonia solanacearum* 0023, 0027, 0029, 0030, *Erwinia amylovora*, *Clavibacter michiganensis* subsp. *sepedonicus* 0140, 0028, 0244, *C. m.* subsp. *michiganensis* 0240, 0241, 0242, 0243, *X. o.* pv. *oryzae* 0227, *X. phaseoli*, *Pectobacterium carotovorum* subsp. *carotovorum* 0141, 0168, *P. atrosepticum* 0142, *Dickeya solani*, *Xylophilus ampelinus* 0124, *Pantoea stewarti*, *P. st.* subsp. *indologenes*, *P. aglomerance*, *Candidatus Liberibacter*, *Acidovorax citrulli*;
– CIRM-CFBP collection, France: *P. st.* subsp. *indologenes* CFBP 3614, *C. m.* subsp. *nebraskensis CFBP 2405*, *CFBP 3491*, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens CFBP 3418*, *C.* fl. pv. *poinsettiae CFBP 2403*, *C. fl.* pv. *oortii CFBP 1384*, *X. axonopodis* pv. *phaseoli CFBP 2534*;
– Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany: *X. gardneri* DSM 19127, *X. perforans* DSM 18975, *P. wasabiae* DSM 18074, *X. euvesicatoria* DSM 19128, *X. vesicatoria* DSM 22252, *X. translucens* pv. *translucens* DSM 18974, *P. cacticida* DSM 21821, *P. betavasculorum* DSM 18076, *D. dadantii* subsp. *dieffenbachiae* DSM 18013, *D. d.* subs. *dadantii* DSM 18020, *D. paradisiaca* DSM 18069, *D. chrysanthemi* DSM 4610, *D. zeae* DSM 18068, *P. c.* subsp. *odoriferum* DSM 22556;
– Singerta Company's collection (Russia): *C. m.* subsp. *michiganensis*, *Agrobacteria* spp., *X. campestris* pv. *campestris*, *X. translucens* pv. *translucens*;

[5] National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov [Accessed: 23.11.2021].

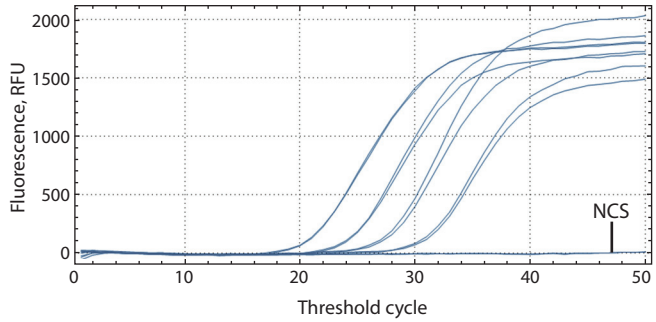

Fig. 1. qPCR results, a series of dilutions of a plasmid containing *X. o.* pv. *oryzicola* DNA target insertion (0376), FAM detection channel.
CFX-96 (Bio-Rad) interface; NCS – negative control sample.

– All-Russian Microorganism Collection of G.K. Skryabin Institute of Microorganism Biochemistry and Physiology (Pushchino, Moscow region, Russia): *C. m.* subsp. *insidiosus* BKM Ac-1402^T, *C. m.* subsp. *nebraskensis* BKM Ac-1404^T, *Pseudomonas savastanoi* BKM B-1546;
– All-Russian Collection of Industrial Microorganisms of Kurchatov National Research Center – GosNIIgenetika (Moscow, Russia): *C. albidum* ВКПМ B-1834.

The primers and probe's analytic specificity for the abovementioned sample set was 100 %. All the samples containing *X. o.* pv. *oryzicola* DNA came positive, which was confirmed by sequencing. No false-positive results were registered including those for the DNA of *X. o.* pv. *oryzae*, which is a closely related variant of the target pathogen.

For testing the assay's analytical sensitivity, the initial concentration of the plasmid with *X. o. pv. oryzicola* insertion of 13 ng/µl or 3×10^9 copies/µl was used. qPCR in a series of seven dilutions was performed as four 10-time dilutions, first in double repeat, and then in quadruple repeat starting from the fifth series (Fig. 1). After the first dilution, the plasmid concentration reduced to 3×10^5 copies/µl. Starting from 150 copies, all the following dilution series were additionally titrated as 2×10^n, 5×10^n, 7×10^n in quadruple repeat. For a series of seven 10-time dilutions, the kinetic curve slope comprised A = –2.671, and the correlation ratio, $R^2 = 0.989$. A stable specific signal was observed down to 25 copies in the reaction mixture. In case of 10-time dilution of *X. o.* pv. *oryzicola* DNA, the

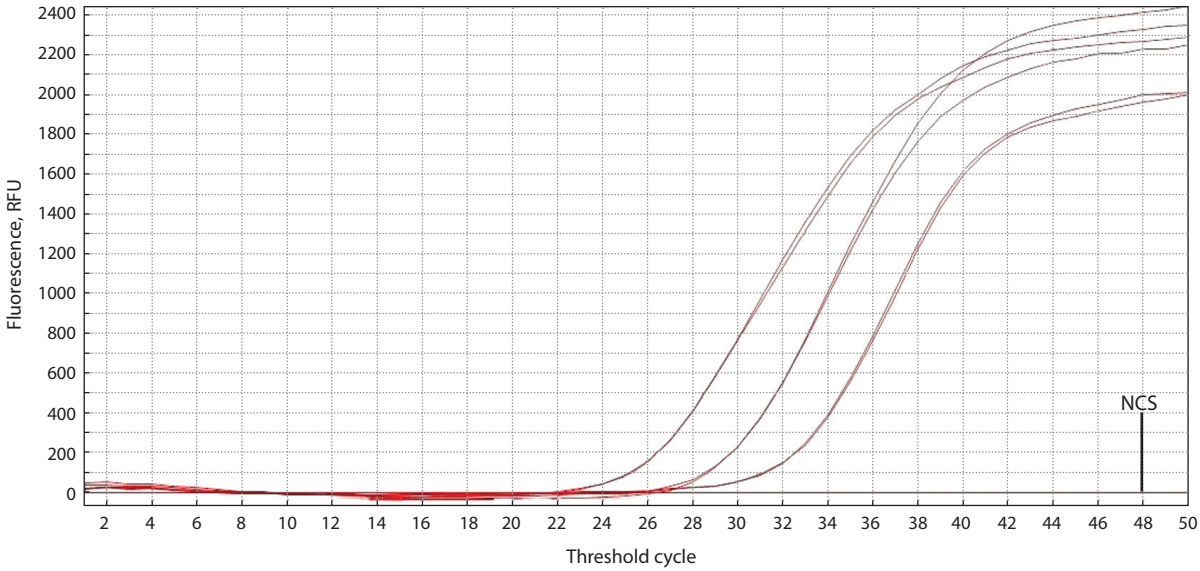

Fig. 2. qPCR results, a series of dilutions of a plasmid containing *X. o.* pv. *oryzicola* DNA target insertion (0376), FAM detection channel. ANK-M (IAI RAS) interface. NCS – negative control sample.

Table 2. qPCR results obtained in different detection systems using the assay to detect *X. o.* pv. *oryzicola*'s *hpa1* gene region

Samples, 10-time dilution	Threshold cycle, Ct				
	ANK-M (IAI RAS)	QuantStudio 5 (Thermo Fisher Scientific)	CFX-96 (Bio-Rad)	DTprime 5 (DNA-Technology)	Rotor-Gene 6000 (Qiagen)
10^{-3}	25.84	25.52	26.43	25.40	25.54
10^{-3}	25.99	25.37	26.43	25.40	25.45
10^{-4}	29.36	29.32	29.48	29.10	29.02
10^{-4}	29.53	29.33	29.43	29.00	28.95
10^{-5}	32.27	32.68	32.56	32.20	32.46
10^{-5}	32.35	32.88	32.29	31.80	32.26
Negative control	Not available	Undetermined	Not available	Not available	Not available
Negative control	Not available	Undetermined	Not available	Not available	Not available
Kinetic curve slope, A	3.20	3.67	3.00	3.30	3.43
Correlation ratio, R^2	0.998	0.999	0.998	0.997	1.000
Efficiency, E %	105	87	116	101	96

assay showed lower sensitivity – down to 43 copies in the reaction mixture.

The designed assay was tested using five qPCR detection systems from Russian and foreign manufacturers (Fig. 2, Table 2). As a matrix, a series of 10-time dilutions of the pathogen's DNA was applied. The kinetic curve slope comprised A = 3.00–3.67, the correlation ratio, $R^2 = 0.997$–1.000, and the efficiency, E = 87–116 %. The threshold value difference comprised ± 1 cycle, which was due to the features of the systems' design and their threshold cycle computation algorithms.

To test the primer pair (seqX.o.all_F and seqX.o.all_R) enabling for Sanger sequencing diagnostics, direct PCR was performed. As a matrix, the DNAs of *X. o.* pv. *oryzae* (2532) and *X. o.* pv. *oryzicola* (2286) were used as well as a 1:1 bacterial DNA mixture to imitate joint infection.

To differentiate the two closely related bacterial species, a region from 2 288 483–2 288 778 bps characterized by a large number of nucleotide changes relative to the reference sequence CP050113.1 from the NCBI GenBank database was used. Comparison of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola*'s nucleotide sequences and their mixture

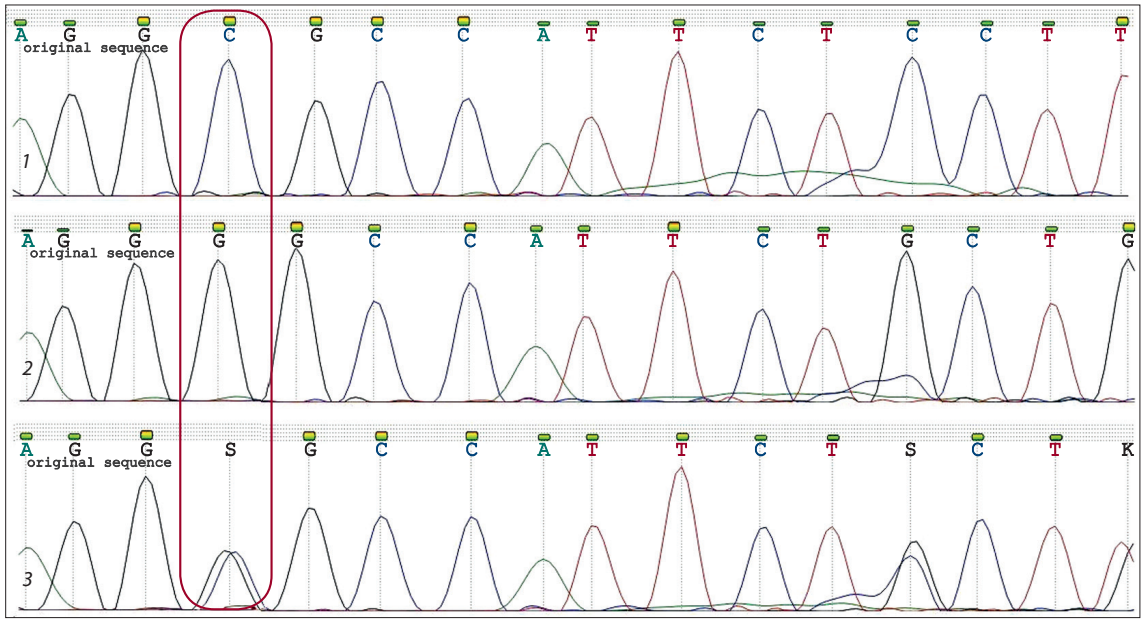

Fig. 3. DNA sequence fragments of *X. oryzae* pv. *oryzicola* (*1*), *X. oryzae* pv. *oryzae* (*2*), and a DNA mixture of the two agents (*3*) that resulted from the sequencing using the seqX.o.all primer pair.

The box marks peak matching. Data processed in UGENE v. 38.1 (Unipro).

can be seen in Fig. 3 where peaks C (*X. o.* pv. *oryzicola*) and G (*X. o.* pv. *oryzae*) match unlike the sequencing results for each of the agents. Bioinformatic analysis of the obtained sequences confirmed they could infect a host both individually and jointly.

Alignment of the obtained nucleotide sequences in the mixture of DNA *X .o.* pv. *oryzae* and *X. o.* pv. *oryzicola* detected 19 nucleotide changes relative to the reference genome CP050113.1 (Table 3).

Apart from the nucleotide changes listed in the Table 3, a three-nucleotide insertion in *X. o.* pv. *oryzicola* (in position 2288667 bps) and a three-nucleotide deletion in *X. o.* pv. *oryzicola* (in position 2288702 bps) were found relative to the reference *X. o.* pv. *oryzae* genome (Fig. 4).

The specific primer placement on *X. oryzae* enables analyzing pathovariant sequences to detect joint infection by two closely related bacteria and indicate their species.

The obtained nucleotide sequences made it possible to confirm the cultures' relation to the collected strains in relation to the genome data base. Alignment of the sequences from the data base enabled us to understand certain intraspecific diversity of the *X. o.* pv. *oryzae* strains that came from Africa. At the same time, *X. o.* pv. *oryzicola*'s diversity was not that high and limited to a single SNP per studied region of the *hpa1* gene cluster. Despite the genetic polymorphism of the population of bacterial infections in rice, the designed sequencing and qPCR primers make it possible to detect all isolates irrespectively of the origin of the material included in the Nucleotide NCBI database.

Most of the procedures to detect *X. o.* pv. *oryzae* are applicable for *X. o.* pv. *oryzicola* as well. Their identifica-

Table 3. Detected nucleotide changes in the amplified fragment resulted from the sequencing using the seqX.o.all primer pair

No.	Nucleotide change's position relative to CP050113.1, bps	Nucleotide change's type	
		X. o. pv. *oryzae*	*X. o.* pv. *oryzicola*
1	2288729	G	C
2	2288720	G	C
3	2288717	T	G
4	2288677	A	C
5	2288656	C	T
6	2288640	C	T
7	2288636	A	G
8	2288622	G	T
9	2288621	A	G
10	2288578	G	A
11	2288571	T	C
12	2288566	G	A
13	2288565	C	T
14	2288564	C	T
15	2288563	C	G
16	2288562	T	C
17	2288561	G	T
18	2288559	T	C
19	2288558	T	C

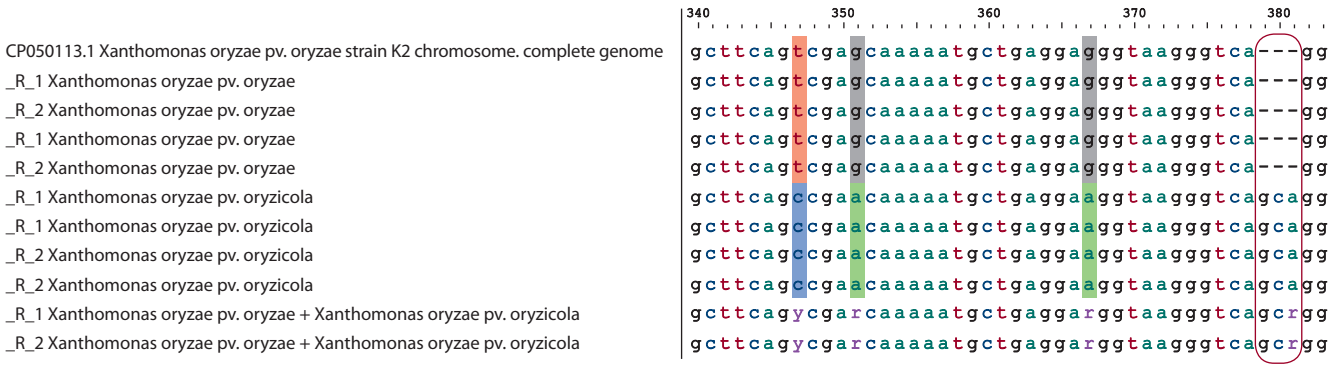

Fig. 4. Alignment of the DNA sequencies of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* and that of their mixture resulted from the sequencing using the seqX.o.all primer pair.

The box marks the deletion. The alignments were obtained in AliView v. 1.27 (Sweden).

tion begins with selecting the samples with clear infection symptoms for further cultivation in a nutrient solution. The method has its drawbacks since the colonies of both bacteria grow slowly in isolation media. Another problem is the presence of dominating kinds of bacteria and bacterial antagonists that prevent proper observation of the target ones. Division of *X. oryzae*'*s* two pathovariants is possible due to their phenotypical features, induction symptoms, serological test, fingerprinting (polyacrylamide gel electrophoresis) and phagotyping results (Vera Cruz et al., 1984; Benedict et al., 1989; EPPO, 2007).

Restriction fragment length polymorphism changes allow one to observe the almost compete genetic diversity of isolates and their origin (Gonzalez et al., 2007). However, this technique has a number of significant disadvantages such as insufficient sensitivity and specificity; high labor intensity that prevents the method from being used in diagnostic and industrial laboratories. For that reason, PCR has become the key method for detection of the *X. oryzae* bacteria (Sakthivel et al., 2001).

Current assays allow for *X. oryzae* detection in general, and further separation into pathovariants requires a standard PCR assay with species-specific primers recommended by the EPPO and All-Russian Center of Plant Quarantine, the results of which are to be sequenced. The EPPO protocol for *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola* identification recommends the TXT/TXT-4R primers (Sakthivel et al., 2001; EPPO, 2007; Lang et al., 2010) to be used. To detect *X. oryzae* DNA using qPCR, it is recommended to use the X.o.F/X.o.R primers devised by the All-Russian Center of Plant Quarantine (Egorova et al., 2014). To separate the two pathovariants, qPCR can be performed using the PF/PR primers and the TaqMan probe that have been specifically designed to detect *X. o.* pv. *oryzae* (Zhao et al., 2007).

In 2021, the All-Russian Center of Plant Quarantine carried out an interlaboratory comparison to detect BLS in rice (21XOO). 16 reference centers and interregional vet laboratories took part and successfully passed a test to detect the disease using the *Xanthomonas oryzae* pv. *oryzicola*-RT assay we have designed.

Conclusion

The designed assay enables the detection of BLS agents in rice. Being a reagent kit for qPCR, is also equipped with the seqX.o.all_F/R primers for PCR amplicon sequencing that detect *X. oryzae* in cases of individual and joint infection. The system allows for robust screening of quarantinable products and confirms obtained qPCR results with DNA sequencing. The assay has been successfully tested using five qPCR diagnostic systems from different manufactures and can be recommended for diagnostic and screening analysis in research and diagnostic laboratories.

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Rare *Wolbachia* genotypes in laboratory *Drosophila melanogaster* strains

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Abstract. Symbiotic bacteria of the genus *Wolbachia* are widespread in *Drosophila melanogaster* populations. Based on the polymorphism of the *Wolbachia* genome, the symbionts' diversity in *D. melanogaster* is presented by two groups: MEL (*w*Mel, *w*Mel2, *w*Mel3 and *w*Mel4) and CS (*w*MelCS and *w*MelCS2). The *w*Mel genotype is predominant in natural *D. melanogaster* populations and is distributed all over the world. The CS genotypes, on the other hand, are of particular interest because it is unclear how they are maintained in the fruit fly populations since they should have been eliminated from them due to their low frequency and genetic drift or been replaced by the *w*Mel genotype. However, this is not what is really observed, which means these genotypes are supported by selection. It is known that the *w*MelPlus strain of the *w*MelCS genotype can increase the lifespan of infected flies at high temperatures. The same genotype also increases the intensity of dopamine metabolism in *Drosophila* compared to the MEL-group genotypes. In the present study, we searched for the rare *Wolbachia w*MelCS and *w*MelCS2 genotypes, as well as for new genotypes in wild-type *D. melanogaster* strains and in several mutant laboratory strains. The symbiont was found in all populations, in 200 out of 385 wild-type strains and in 83 out of 170 mutant strains. *Wolbachia* diversity in *D. melanogaster* wild-type strains was represented by the *w*Mel, *w*MelCS and *w*MelCS2 genotypes. More than 90 % of the infected strains carried *w*Mel; 9 %, *w*MelCS2; and only two strains were found to carry *w*MelCS. No new *Wolbachia* genotypes were found. The northernmost point reported for the *w*MelCS2 genotype was Izhevsk city (Udmurtia, Russia). For the first time the *w*MelCS2 genotype was detected in *D. melanogaster* from the Sakhalin Island, and *w*MelCS, in the flies from Nalchik (the North Caucasus). A comparison of *Wolbachia* genetic diversity between the wild-type laboratory strains and previously obtained data on mutant laboratory strains demonstrated differences in the frequencies of rare CS genotypes, which were more prevalent in mutant strains, apparently due to the breeding history of these *Drosophila* strains.
Key words: *Drosophila melanogaster*; *Wolbachia*; genotypes; laboratory stock.

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Редкие генотипы *Wolbachia* в лабораторных линиях *Drosophila melanogaster*

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Аннотация. Симбиотические бактерии рода *Wolbachia* широко распространены в популяциях *Drosophila melanogaster*. На основе полиморфизма перестроек генома разнообразие *Wolbachia* у *D. melanogaster* подразделяется на две клады: MEL (генотипы *w*Mel, *w*Mel2, *w*Mel3 и *w*Mel4) и CS (*w*MelCS и *w*MelCS2). Генотип *w*Mel доминирует в природных популяциях *D. melanogaster* и распространен по всему миру. Генотипы CS-клады представляют особый интерес, поскольку неизвестно, как они поддерживаются в популяциях *D. melanogaster*. При низкой частоте встречаемости они должны элиминироваться вследствие генетического дрейфа или вытесняться генотипом *w*Mel, чего в действительности не происходит. Следовательно, эти генотипы поддерживаются отбором. Например, штамм *w*MelPlus (генотип *w*MelCS) способен увеличивать продолжительность жизни мух при повышенных температурах. Генотип *w*MelCS также увеличивает интенсивность дофаминового метаболизма у дрозофил по сравнению с генотипами MEL-клады. В настоящей работе проведен поиск редких генотипов *Wolbachia w*MelCS и *w*MelCS2, а также новых генотипов в линиях *D. melanogaster* дикого типа и в отдельных мутантных линиях лабораторного фонда. Симбионт был выявлен во всех популяционных выборках у 200 из 385 линий дикого типа и у 83 из 170 мутантных. Разнообразие *Wolbachia* в линиях *D. melanogaster* дикого типа представлено генотипами *w*Mel, *w*MelCS и *w*MelCS2. Более 90 % инфицированных линий несут *Wolbachia w*Mel генотипа, 9 % – *w*MelCS2, и только в двух линиях обнаружен *w*MelCS. Новых генотипов *Wolbachia* не

зафиксировано. Для генотипа *w*MelCS2 отмечена наиболее северная точка распространения – Ижевск (Удмуртия). Впервые показано присутствие генотипа *w*MelCS2 в линии *D. melanogaster* из популяции о. Сахалин, а в линии из популяции г. Нальчик – генотипа *w*MelCS. Сравнение генетического разнообразия *Wolbachia* между лабораторными линиями дикого типа и ранее полученными данными для мутантных лабораторных линий показало различие в частотах редких генотипов CS-группы, у мутантных линий их больше, что может быть связано с историей поддержания линий *Drosophila*.
Ключевые слова: *Drosophila melanogaster*; *Wolbachia*; генотипы; лабораторный фонд.

Introduction

Symbiotic bacteria of the *Wolbachia* genus are widespread in *Drosophila melanogaster* populations (Riegler et al., 2005; Richardson et al., 2012; Ilinsky, 2013; Bykov et al., 2019). Apart from a number of point mutations, these *Wolbachia* genomes differ by a series of the rearrangements that can be easily detected by polymerase chain reaction (PCR) assay followed by electrophoretic analysis as per M. Reigler et al. (2005). Their polymorphism has enabled one to distinguish MEL (*w*Mel, *w*Mel2, *w*Mel3 and *w*Mel4) and CS (*w*MelCS and *w*MelCS2) group of genotypes (Riegler et al., 2005; Ilinsky, 2013). The *w*Mel genotype, whose name is similar to that of the strain, prevails in *D. melanogaster*, the others have either rare or local spread (Riegler et al., 2005; Ilinsky, Zakharov, 2007a, b; Ilinsky, 2013; Bykov et al., 2019), e. g. while being widely spread in the world, the *w*MelCS genotype is rare, where its prevalence does not exceed 10 % (Riegler et al., 2005; Ilinsky, Zakharov, 2007a, b; Serga et al., 2014; Bykov et al., 2019).

Meanwhile, the *w*MelCS2 genotype is often detected in the *D. melanogaster* populations of Eastern Europe, Central and Northern Asia and Western Siberia, whose prevalence in some samples could reach up to 40 % (Riegler et al., 2005; Ilinsky, Zakharov, 2007a, b; Ilinsky, 2013; Bykov et al., 2019). In the strains of South and South-East Asia, singular cases of *w*Mel2 genotype presence have been detected (Riegler et al., 2005; Bykov et al., 2019), while the *w*Mel4 genotype was first registered in the Sinai Peninsula, and no other data are currently available regarding its spread (Ilinsky, 2013). The *w*Mel3 genotype was found only in a single laboratory strain and is most likely absent in the wild (Riegler et al., 2005).

Detailed genome analysis of the *Wolbachia* bacteria in *D. melanogaster* confirmed the abovementioned subdivision and enabled one to subdivide the MEL and CS groups into several clades (Richardson et al., 2012; Chrostek et al., 2013; Early, Clark, 2013; Ilinsky, 2013). Thus, the most widespread *w*Mel genotype has four (I, II, III and V) clades, and the *w*Mel2 genotype – two (IV and VIII). As for the CS group, it has only one clade (Richardson et al., 2012; Chrostek et al., 2013; Ilinsky, 2013). Analysis of the nucleotide polymorphism of the full genomes of the *w*MelCS and *w*MelCS2 genotypes detected four haplotypes (Bykov et al., 2019). One of which is present in wild-type *D. melanogaster* and the mutant strains of the fruit-fly stock, while the others have only been found in a small number of mutant strains, which confirms the low genetic diversity of the CS group.

For some of the *Wolbachia* genotypes, their effect on the fruit fly's biological features has been described, e. g., clade V of the *w*Mel genotype prevailing in the *D. melanogaster* population of the Palearctic (Bykov et al., 2019), and clade VI of the *w*MelCS genotype induce weak cytoplasmic incompatibility (Ilinsky, Zakharov, 2011; Ilinsky, 2013). Comparing the temperature survivability of flies (Versace et al., 2014; Mazzucco et al., 2020) has shown that those infected with clade V of the *w*Mel genotype withstand cold temperatures better than those infected with clade VI of the *w*MelCS genotype and clades I, II, III of the *w*Mel genotype. *D. melanogaster* also change their temperature preferences depending on the infection status and *Wolbachia* genotype (Arnold et al., 2019; Truitt et al., 2019). It has been demonstrated that *w*MelCS increases dopamine metabolism intensity unlike the *w*Mel, *w*Mel2 and *w*Mel4 genotypes (Gruntenko et al., 2017). The female fruit flies infected with the *w*Mel genotype are more productive than non-infected ones or those infected with the *w*MelCS genotype (Serga et al., 2014). The authors also note the *w*MelCS genotype is able to reduce the fruit fly's fertility.

Many data have been accumulated to describe *Wolbachia*'s spread and variability in the wild *D. melanogaster* populations (Hoffmann et al., 1994, 1998; Riegler et al., 2005; Ilinsky, Zakharov, 2007a, b; Vesprool, Haddrill, 2011; Bykov et al., 2019), while the set of investigations studying the issue in the laboratory strains includes only two reports (Clark et al., 2005; Ilinsky et al., 2014). A study of the flies kept at Bloomington Drosophila Stock Center was carried out only to estimate the infection degree in wild-type strains and the strains containing different mutation groups and *P*-element (Clark et al., 2005). It demonstrated the differences in the number of infected lines for different groups of fruit flies, which were probably related to their breeding history.

The second study was carried out in the stock of Laboratory of Population Genetics of Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, and its objective was not only to detect *Wolbachia* infection frequency but also to estimate its genetic diversity in the mutant strains of the stock (Ilinsky et al., 2014). It has been found the line groups with different mutation differed both in terms of infection frequency and *Wolbachia* genotype composition. In some cases, it could be related to the breeding history, in particular, to using the specific infected balancing strains for maintaining certain mutations.

When it comes to *Wolbachia*'s genetic diversity, the CS group is of particular interest for it is still unknown how these genotypes are maintained in *D. melanogaster* populations. Considering their low frequency, they should be eliminated in the populations either due to genetic drift or being replaced by the *w*Mel genotype, but this is not what happens in reality (Riegler et al., 2005; Ilinsky, 2013; Bykov et al., 2019). It is likely that these genotypes are supported thorough selection.

Recently, new data have been published concerning some phenotypic effects observed in this genotype group, e. g., the *w*MelPlus strain of *w*MelCS genotype increases the flies' survival in presence of thermal stress. However, the mechanism of this phenomenon remains unknown (Burdina et al., 2021). Another strain (*w*MelPop) of the same genotype was detected when observing flies' death due to rampant bacterial proliferation in the host's cells (Min, Benzer, 1997; Woolfit et al., 2013).

Genetic differentiation and comparative analysis of *Wolbachia* isolates will make it possible to detect new effects and understand the mechanisms of host-symbiont interactions, which can later be used for practical applications, e. g., for the *w*Mel and *w*MelCS genotypes, their ability to block mosquito-borne viral infections has been found. In other words, they prevent dengue fever, Zika virus infection and other viral infections when they are transmitted from the fruit fly to the mosquito (Schultz et al., 2017; Xue et al., 2018; Flores et al., 2020).

The aim of the present study was performing a search in the *D. melanogaster* strains of the laboratory stock of Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences to detect the rare *Wolbachia* genotypes such as *w*MelCS and *w*MelCS2 as well as new genotypes. These strains can later be used to investigate the effect *Wolbachia* has on the biological features of *D. melanogaster*, in particular, to analyze its effect on the metabolism of infected fruit-fly strains, their fertility and thermal stress resistance. The results of our study will also complement to the early obtained data on *Wolbachia* diversity in natural and laboratory populations of *D. melanogaster.*

Materials and methods

In the study, 555 strains of *D. melanogaster* from the laboratory stock of Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences were used. The lines were bred from the natural populations collected in different regions of Russia, Ukraine and Kyrgyzstan from 1985 to 2016 as well as in Kenia in 2019 (Tables 1 and 2). For the DNA extraction, pools of five females were used. The flies were homogenized in STE buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) and incubated during an hour at 56 °C. After the incubation, the samples were centrifuged at 13,000 RPM for 10 min for debris removal, and the supernatant was PCR assayed for 1) presence of *Wolbachia* (whole collection); 2) infection frequency and presence of rare *Wolbachia* genotypes (370 wild-type strains (see Table 1)); for the population represented with more than 10 strains, infection frequency was determined and 95 % confidence intervals (CI) was estimated using Clopper–Pearson method; 3) CS-genotype diversity (170 strains containing natural mutations (see Table 2)); 4) possible infection loss (15 strains of wild-type *D. melanogaster* from the Tomsk population (see Table 1) that had been earlier characterized in terms of their *Wolbachia* genotype and infection status (Bykov et al., 2019)).

The *Wolbachia* infection status and genotype were determined according M. Riegler et al. (2005) based on four markers such as insertion in WD_1310 and WD_0516 locus; the number of vntr 105 and vntr 141 minisatellite repeats. For the 170 mutant strains, *Wolbachia* presence was checked only for loci 1310 and 0516/7 to determine whether the bacteria belonged to the MEL or CS group. For the detected CS variants, additional assay for loci vntr 105 and vntr 141 was carried out to distinguish the *w*MelCS and *w*MelCS2 genotypes. These 170 strains were discarded from the analysis of the infection and genotype frequencies since they did not provide information on the symbiont's prevalence in the population. Statistical analysis of the obtained data was performed using the Minitab 17.1.0 software (Minitab Inc., State College, PA, USA).

Results

In the 555 strains of *D. melanogaster* assayed, the *Wolbachia* infection was detected in 51.9 % of wild-type (see Table 1) and 48.8 % of mutant (see Table 2) strains. In the assayed wild-type strains, the infection rate varied from 15.8 to 100 % (see Table 1), 52 % on average (95 % CI 46.8–57.0 %). The symbiont was detected in all population samples. Fifteen strains of the Tomsk population turned out to be infected with *Wolbachia* of expected genotype, i. e., no infection loss after 10 years of breeding was found.

Wolbachia diversity in the assayed wild-type *D. melanogaster* strains was represented by three genotypes *w*Mel, *w*MelCS and *w*MelCS2. More than 90 % of infected strains carried the *w*Mel genotype, that correlated with its dominance in natural populations worldwide (Riegler et al., 2005; Ilinsky, Zakharov, 2007a, b; Bykov et al., 2019). About 9 % of the infected strains obtained from the natural populations of Altai (Gorno-Altaisk, Biysk), Kyrgyzstan (Bishkek) and Udmurtia (Izhevsk) carried the *w*MelCS2 genotype. The only case of *w*MelCS was detected in a strain from a natural population of Ukraine. Rare CS-clade variants were also found in the mutant flies from the populations of Sakhalin and Nalchik (see Table 2). At the same time, the *w*MelCS genotype had never been found in Sakahlin earlier as well as *w*MelCS had never been detected in Nalchik.

Discussion

In the present study, we carried out a search for the *Wolbachia* bacteria of *w*MelCS and *w*MelCS2 genotypes in the *D. melanogaster* strains collected from natural populations and maintained in laboratory stock for 3–36 years. These genetic variants of the symbiont are rare in natural populations but still can be widely spread worldwide (Riegler et al., 2005; Ilinsky, Zakharov, 2007a, b; Bykov et al., 2019). In the majority of cases in this study they were found in the strains from the regions where these genotypes had been registered earlier.

In Udmurtia (Izhevsk), the *w*MelCS2 genotype had never been registered in *D. melanogaster*, which was probably due to the small number of assayed strains (Ilinsky, Zakharov, 2007a). For the time being, this is the northernmost geographical location where this genotype has been registered (Bykov et al., 2019), but one has to keep in mind that we know quite a little about the northern populations of *D. melanogaster* and the boundaries of its spread can be much wider than the ones known to us today. At the same time, accidental delivery

Table 1. *Wolbachia* prevalence and genetic content in the wild-type *D. melanogaster* strains from the laboratory stock of Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences

Region, year	N/N_{w+} (%*w*+; 95 % CI)*	Number of genotypes**
Ukraine, Kiev, 1985	1/1	*w*MelCS2
Crimea, Magarach, 1990	7/4	*w*Mel
Ukraine, Zaporozh'e, 1990	9/1	*w*Mel
Russia, Gorno-Altaisk, 1992	8/5	*w*Mel (1), *w*MelCS2 (4)
Russia, Biysk, 1993	49/20 (40.8 %; 27.0–55.8 %)	*w*Mel (14), *w*MelCS2 (6)
Ukraine, Nikopol, 1997	10/6 (60 %; 26–88 %)	*w*Mel
Russia, Izhevsk, 2000	10/5 (50 %; 19–81 %)	*w*Mel
Russia, Karambay, 2000	19/3 (15.8 %; 33.8–39.6 %)	*w*Mel
Russia, Pychas, 2000	11/7 (63.6 %; 30.8–89.1 %)	*w*Mel
Ukraine, Cherkassy, 2000	30/12 (40 %; 22.7–59.4 %)	*w*Mel
Kyrgyzstan, Bishkek, 2001	25/6 (24 %; 9–45 %)	*w*Mel (4), *w*MelCS2 (2)
Russia, Pychas, 2001	28/19 (67.9 %; 47.6–84.1 %)	*w*Mel
Ukraine, Cherkassy, 2001	46/10 (22 %; 11–36 %)	*w*Mel (9), *w*MelCS (1)
Russia, Adler, 2002	2/1	*w*Mel
Russia, Izhevsk, 2002	22/15 (68.2 %; 45.1–86.1 %)	*w*Mel (14), *w*MelCS2 (1)
Kyrgyzstan, Bishkek, 2006	3/3	*w*Mel (2), *w*MelCS2 (1)
Russia, Krasnodar, 2006	11/8 (73 %; 39–94 %)	*w*Mel
Russia, Tomsk, 2006	3/2	*w*Mel
Ukraine, Nikopol, 2006	17/7 (41.2 %; 18.4–67.1 %)	*w*Mel
Crimea, Magarach, 2008	4/2	*w*Mel
Ukraine, Kiev, 2008	2/2	*w*Mel
Ukraine, Polesskoe, 2008	14/10 (71.4 %; 42–92 %)	*w*Mel
Ukraine, Chernobyl, 2008	10/7 (70 %; 35–93 %)	*w*Mel
Russia, Tomsk, 2011***	15/15 (100 %; 78.2–100 %)	*w*Mel
Kenia, Nairoby, 2019	1/1	*w*Mel
Kenia, Kitale, 2019	10/10 (100 %; 69–100 %)	*w*Mel
Kenia, Kiboko, 2019	8/8	*w*Mel
Kenia, 2019	10/10 (100 %; 69–100 %)	*w*Mel
Total	385/200	*w*Mel (184), *w*MelCS (1), *w*MelCS2 (15)

* N – the number of assayed strains; N_{w+} – the number of infected strains; %*w*+ – proportion of infected samples; 95 % confidence intervals were estimated using the Clopper–Pearson method for samples with $N \geq 10$; ** the number are indicated in cases of several genotypes detected; *** the strains have been earlier characterized (Bykov et al., 2019).

of *D. melanogaster* infected with this *Wolbachia* genotype together with products should not be excluded. So, later it may disappear from the local population due to the death of its hosts in the winter period. A similar case of accidental delivery was probably observed in the mutant strain from the Sakhalin Island. These flies had *w*MelCS-genotype *Wolbachia* that had never been registered in this territory.

Earlier, we characterize in detail the infection rate and genetic diversity of *Wolbachia* in *D. melanogaster* populations from Nalchik collected in 2010–2013, the single cases of

Table 2. *Wolbachia* prevalence in the collection of mutant *D. melanogaster* strains derived from natural populations

Region, year	N/N_{w+}	N_{MEL}	N_{CS} (genotype)
Russia, Sakhalin, 2014–2016	128/53	52	1 (*w*MelCS2)
Russia, Nalchik, 2000	42/30	25	4 (*w*MelCS2), 1 (*w*MelCS)
Total	170/83	77	5 (*w*MelCS2), 1 (*w*MelCS)

Note. N – the number of assayed strains; N_{w+} – the number of infected strains; N_{MEL}, N_{CS} – the number of the strains harboring *Wolbachia* of MEL and CS clades, respectively.

Table 3. Comparison of *Wolbachia*'s genetic compositions in the wild-type, mutant and natural strains of *D. melanogaster*

Strains	N_{wMel}/%*w*Mel	95 % CI	Rare genotypes (*w*MelCS, *w*MelCS2, *w*Mel2, *w*Mel4)	Rare genotypes, %	95 % CI
Wild-type	184/92	87–95	16	8	5–13
Mutant (Ilinsky et al., 2014)	60/43	35–52	78	57	48–65
Natural (Bykov et al., 2019)	852/98	96–99	17	2	1–3

Note. N_{wMel} – the number of strains with *Wolbachia* of the *w*Mel genotype; %*w*Mel – percents of strains with *Wolbachia* of the *w*Mel genotype.

Table 4. *Wolbachia* infection frequencies for the wild-type, mutant and natural strains of *D. melanogaster*

Strains	N	N_{w+}	N_{w-}	Infection frequency, %	95 % CI
Wild-type	385	200	185	52	47–57
Mutant (Ilinsky et al., 2014)	353	138	215	39	34–44
Natural (Bykov et al., 2019)	1505	869	636	57	55–60

Note. N – the number of strains; N_{w+}, N_{w-} – the number of infected and uninfected strains, respectively.

*w*MelCS2 genotype were found (Bykov et al., 2014, 2019). Analysis of the mutant strains bred from the Nalchik population in 2000 demonstrated the presence of both *w*MelCS2 and *w*MelCS genotypes. The available data enable us to conclude that *w*MelCS2 is constantly supported in the populations of this region. The detected case of *w*MelCS genotype confirm our earlier assumption that this variant of bacteria can present in the fly populations of Nalchik (Bykov et al., 2014). The long-term presence of rare *Wolbachia* genotypes in *D. melanogaster* may be due to several reasons, e. g., the flies harboring the *w*MelCS and *w*MelCS2 genotypes may overwinter and produce new generations of infected insects (Kriesner et al., 2016; Bykov et al., 2019). Also, the symbiont itself may provide advantages for infected species (Hedges et al., 2008; Teixeira et al., 2008; Gruntenko et al., 2017) or induce the reproductive abnormalities that sustain infection in the population (Ilinsky, Zakharov, 2011; Ilinsky, 2013).

In the mutant laboratory strains of *D. melanogaster*, the *w*MelCS and *w*MelCS2 genotypes occur much more often, which is due to strains' breeding history that involved using of the balancing strains infected with these *Wolbachia* genotypes (Ilinsky et al., 2014). Comparative analysis of *Wolbachia* genetic diversity in the natural, mutant and wild-type strains demonstrated the presence of statistically significant differences in genotype ratio between the stock's wild-type strains and the natural populations (Fisher's exact test, $p = 7\times10^{-5}$). The symbiont's genetic composition in the mutant strains also differed significantly from that in the natural strains (Fisher's exact test, $p < 1\times10^{-8}$ for both cases) (Table 3).

In general, the *Wolbachia* prevalence in the stock's cultures of *D. melanogaster* was comparable to those in the studies that had been published earlier, which confirms the symbionts is ubiquitous and its occurrence is of high frequency (Ilinsky, Zakharov, 2007a; Vespoor, Haddrill, 2011; Serga et al., 2014; Bykov et al., 2019). Detailed comparison of our data for wild-type strains to those for mutant strains and natural ones showed some differences in *Wolbachia* infection frequency, hence both mutant and wild-type strains were different from the natural ones (Fisher's exact test, $p = 0.043$ and $p < 1\times10^{-8}$ for both cases). They differed from one another as well ($p = 0.0005$) (Table 4).

A possible explanation of the differences in infection frequencies between natural and wild-type strains is to say some of the lines experienced infection loss after many generations. It is known that *Wolbachia* can eventually be lost in maternal lineage due to incomplete maternal transition, and in absence

of any positive effect on its host can be completely eliminated from a population (Hoffmann et al., 1998; Ilinsky et al., 2014). Our analysis demonstrated that the symbiont preserved itself in the 15 lines of fruit flies from Tomsk, whose populations had been maintaining during 10 years. On the other hand, the mutant strains of *D. melanogaster* had demonstrated possible cases of infection loss (Ilinsky et al., 2014). In (Ilinsky, 2013), strain S400 infected with clade III of *w*Mel genotype experienced infection loss (data not shown).

Conclusion

The present study found two strains of *D. melanogaster* infected with the *w*MelCS genotype of *Wolbachia*, and 20 strains – with the *w*MelCS2 genotype. These strains will be further investigated to estimate the effect the symbiont has on the fruit fly's biology. Our study has extended the boundaries of *w*MelCS2 spread, whose northernmost point now is Udmurtia (Izhevsk). Our results confirm *Wolbachia* can be sustained in laboratory strains, which does not exclude the likelihood of infection loss after long-term breeding. The symbiont's infection frequency and genotypic composition are in general comparable to those estimated in natural populations and supplement the available data. When compared against those in the mutant strains, *Wolbachia* infection frequency and genotypic composition in the wild-type strains turned out to be closer to those observed in natural populations.

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Bacillus anthracis strain differentiation based on SNP and VNTR loci

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Abstract. *Bacillus anthracis* is the anthrax causative agent. For its epidemiology, it is important not only to identify the etiological agent but also to determine the patterns of its evolution and spread. Modern methods of molecular biology make it possible to detect a number of genetic markers suitable for indicating and differentiating the strains of *B. anthracis*, including the loci arranged as variable number tandem repeats (VNTRs) and SNPs, one nucleotide-sized differences in the DNA sequence of the loci being compared. The objective of the present study was to examine the effectiveness of SNP analysis and PCR amplification of VNTR loci combined with the high-resolution amplicon melting analysis for identification and differentiation of the anthrax agent strains. In the study, seven strains of *B. anthracis* obtained from soil samples and animal carcasses were investigated using vaccine strain STI-1 as a reference. For molecular genetic characterization of these bacteria, analysis of 12 SNPs and variability analysis of eight VNTR loci were carried out. To detect the differences between the strains, their PCR product melting points were measured in the presence of the EvaGreen (Sintol, Russia) intercalating dye. For SNP detection, a PCR assay with double TaqMan probes was applied. It was found that the studied virulent strains, except for *B. anthracis* No. 1 and 3, could not be attributed to any phylogenetic subgroup of the anthrax agents. The proposed method made it possible to differentiate four out of the seven investigated strains. Strains No. 5–7 had identical SNP and HRM profiles and, as a result, formed a single cluster. Our investigation has confirmed that the proposed method can be successfully used for preliminary analysis of an epizootic situation in the case of anthrax.

Key words: *Bacillus anthracis*; genotyping; VNTR; SNP; HRM-analysis; epidemiology of anthrax.

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Дифференциация штаммов *Bacillus anthracis* на основе SNP- и VNTR-полиморфизма геномов

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Аннотация. Бактерии *Bacillus anthracis* являются возбудителем сибирской язвы. Для эпидемиологии этой инфекции имеет значение не только идентификация этиологического агента, но и выяснение закономерности его эволюции и распространения. Современные методы молекулярной биологии позволяют определить ряд генетических маркеров, пригодных для индикации и дифференциации штаммов *B. anthracis*. К таким маркерам относят VNTR-локусы – последовательности, организованные в геноме в виде тандемных повторов, а также SNP – отличия в последовательности ДНК в сравниваемых локусах размером в один нуклеотид. Целью настоящей работы была оценка эффективности совместного применения SNP-анализа и ПЦР-амплификации VNTR-локусов с анализом температуры плавления ампликонов высокого разрешения для идентификации и дифференциации штаммов возбудителя сибирской язвы. Исследовали семь штаммов *B. anthracis*, полученных из образцов почвы и трупов животных, в качестве референс-микроорганизма был вакцинный штамм *B. anthracis* СТИ-1. Для молекулярно-генетической характеристики данных бактерий проведен анализ 12 однонуклеотидных полиморфизмов, а также вариабельности восьми VNTR-локусов, для определения различий в которых был впервые использован метод определения температур плавления ПЦР-продуктов в присутствии интеркалирующего красителя EvaGreen (ЗАО «Синтол», Россия). Для детекции SNP применен метод полимеразной цепной реакции (ПЦР) с использованием двойных TaqMan-зондов. Обнаружено, что все изучаемые вирулентные штаммы, кроме *B. anthracis* № 1 и 3, по SNP-профилю не могут быть отнесены к какой-либо филогенетической подгруппе возбудителя сибирской

язвы. Методический подход, включающий в себя анализ SNP- и VNTR-последовательностей, позволил дифференцировать между собой штаммы *B. anthracis* № 1–4, в то время как бактерии *B. anthracis* № 5–7 демонстрируют одинаковые SNP- и HRM-профили и, как следствие, формируют один кластер. Таким образом, показана принципиальная возможность использования рассмотренной в этой работе методики для предварительного анализа эпизоотической ситуации при вспышках сибирской язвы.
Ключевые слова: *Bacillus anthracis*; генотипирование; VNTR; SNP; HRM-анализ; эпидемиология сибирской язвы.

Introduction

Bacillus anthracis is the causative agent of anthrax, a particularly hazardous zoonotic infection. Although effective measures to prevent the occurrence and spread of the disease have been developed and rather widely implemented, from 2000 to 20,000 anthrax cases are registered around the world every year (Pisarenko et al., 2019), mostly in Africa, Central Asia, and Latin America (Hugh-Jones, Blackburn, 2009; Kenefic et al., 2009). In Russia, anthrax commonly occurs in Siberia and North Caucasus (Logvin et al., 2017).

All the *B. anthracis* populations known to researchers are extremely monomorphic and have clonal structure (Achtman, 2008; Keim et al., 2009). This high genetic similarity poses a significant hindrance for strain differentiation of anthrax agents using bacteriological and serological methods. The problem, however, may be solved using molecular genetic approaches. The methods detecting sites with variable number tandem repeats (VNTR) and single nucleotide polymorphisms (SNP) in the agent's genome have turned out to be the most promising for *B. anthracis* strain indication and differentiation (Timofeev et al., 2018; Wang et al., 2020).

Compared to VNTR loci, SNPs are more stable in evolutionary perspective and have low mutation frequency, which comes at a cost of lower resolution. That is why polymorphism detection in SNP loci of the anthrax agent is a rather common first stage in the genotyping systems using a combination of SNP and VNTR markers (Timofeev et al., 2018), in which a set of 14 diagnostically significant canonical SNPs (canSNPs) is widely used. The systems make it possible to attribute the microorganism under study to a particular phylogenetic line and, as a result, make an assumption about its geographic origin (Van Ert et al., 2007). Three phylogenetic lines (A, B, and C) are commonly identified in today's research, which in turn form 14 phylogenetic groups as follows: A.Br.Ames, A.Br.Australia 94, A.Br.003/004, A.Br.Vollum, A.Br.005/006, A.Br.001/002, A.Br.Western, A.Br.WNA, A.Br.008/009, A.Br.011/009, B.Br.001/002, B.Br.KrugerB, B.Br.CNEVA, and C.Br.A1055 (Timofeev et al., 2018). According to the literature, the strains isolated in the Russian Federation belong predominantly to group B.Br.001/002 of line B and groups A.Br.001/002 and A.Br.008/009 of line A, and less often to A.BrAust94 (Eremenko et al., 2018; Koteneva et al., 2019).

Multilocus variable number tandem repeat analysis (MLVA) is used for further strain differentiation within each SNP cluster (Timofeev et al., 2018). PCR analysis with further separation of amplification products in agarose or polyacrylamide gel, often combined with capillary electrophoresis, is the most common MLVA strategy (Bondareva et al., 2014). The most accurate results may be obtained from amplicon sequencing, but the duration of the procedure (at least several days) tends to be a major downside of the approach.

In the present study, differences in VNTR loci, specifically tandem repeat numbers, were determined using HRM (high resolution melting), i. e. real-time analysis of amplicon melting points. The EvaGreen (Sintol, Russia) intercalating dye used for HRM analysis inserts itself between two complementary nucleotides in a double-stranded DNA molecule. The dye's fluorescence under light of 490-nm wavelength is registered in FAM detection channel. When DNA denaturation occurs, there is no fluorescence and hydrogen bonds break. Thus, if we gradually increase the temperature in the thermocycler, continuous detection will enable us to determine the repeat number based on amplicon melting point. The latter approach outperforms the classical MLVA methods since it does not require sequencing or fluorescence-labeled probes, and, as a result, makes it possible to detect differences in VNTR loci of *B. anthracis* strains at a lower financial and time cost. HRM analysis of PCR amplification products had been previously suggested for SNP genotyping (Derzelle et al., 2011) but had never been described for analyzing VNTR loci.

The objective of the present study was to evaluate the efficiency of SNP analysis and PCR amplification of VNTR loci in combination with high-resolution amplicon melting point analysis for identification and differentiation of anthrax agent strains.

Materials and methods

In the study, seven *B. anthracis* strains obtained from soil samples and animal carcasses were used (Table 1). *B. anthracis* vaccine strain STI-1 from the microorganism strain collection of the Federal Center for Toxicological, Radiation and Biological Safety (Kazan, Russia) was used as a reference. The strain samples were prepared for further molecular genetic research in compliance with the MUK 4.2.2941-11 methodological protocol (2011).

The genomic DNA was isolated using the DNA-sorb-B kit (Central Research Institute for Epidemiology of Rospotrebnadzor, Moscow) as per manufacturer's instructions.

The SNP analysis was performed using real-time polymerase chain reaction (qPCR) with the double TaqMan probes and primers described earlier (Van Ert et al., 2007). The master mix volume of 15 μl included 125 μM of each dNTP, 2.5 мМ $MgCl_2$, 5 pM of each primer and probe, 10 ng DNA matrix, 1.0 U Taq polymerase (Evrogen, Russia), ddH_2O (up to 15 μl). The qPCR procedure was run on a Real-Time C1000 thermocycler with CFX96 optical reaction module (Bio-Rad, USA) under the following protocol: initial DNA denaturation at 95 °C for 3 minutes followed by 39 cycles as follows:

Table 1. Studied strains of *B. anthracis*

Strain, No.	Collection site and year of isolation	Source
1	Checheno-Ingush ASSR, 1971	Sheep carcass
2	Tajik SSR, 1971	Cattle carcass
3	Kurgan region, 1971	
4	Ulyanovsk, 2004	Soil (epizootic site)
5		Pig carcass
6	Republic of Tatarstan, 2004	Cattle carcass
7	Republic of Tatarstan, 2008	
STI-1	Russian State Center for Animal Feed and Drug Standardization and Quality, 1980	Vaccine strain

Table 2. Synthetic oligonucleotides used for amplification of VNTR loci

Locus	ID: genomic location, bp	Primer nucleotide sequence, 5′→3′		Expected amplicon size (repeat-free), bp
		Forward	Reverse	
VrrA	CP076222.1: 4103374–4103699	CACAACTACCACCGATGGCAC	GCGCGTTTCGTTTGATTCATAC	266
VrrB1	CP054800.1: 3380187–3380415	ATAGGTGGTTTTCCGCAAGTTATTC	GATGAGTTTGATAAAGAATAGCCTGTG	211
VrrB2	CP054800.1: 3380389–3380541	CACAGGCTATTCTTTATCAAACTCATC	CCCAAGGTGAAGATTGTTGTTGA	135
VrrC1	CP054816.1: 1937943–1938522	GAAGCAAGAAAGTGATGTAGTGGAC	CATTTCCTCAAGTGCTACAGGTTC	544
VrrC2	CP054816.1: 1937447–1937978	CCAGAAGAAGTGGAACCTGTAGCAC	GTCTTTCCATTAATCGCGCTCTATC	460
CG3	CP054816.1: 4931009–4931169	CCATGTCGTTTTACTTCTCTCTCCAATAC	AGTCATTGTTCTGTATAAAGGGCAT	151
pXO1	FR872876	CAATTTATTAACGATCAGATTAAGTTCA	TCTAGAATTAGTTGCTTCATAATGGC	108
pXO2	FR872886	TCATCCTCTTTTAAGTCTTGGGT	GTGTGATGAACTCCGACGACA	123

denaturation at 95 °C for 10 s, annealing oligonucleotides at 50 °C for 30 s (detection in R6G/ROX channel), extension at 72 °C for 5 s. Point nucleotide changes in each locus were identified based on fluorescence intensity in each channel. Variability of SNP loci was numerically estimated based on allelic polymorphism index (h) (Selander et al., 1986).

MLVA amplicon melting points were determined using the EvaGreen intercalating dye. The primer set for amplification of VNTR loci is presented in Table 2.

The qPCR master mix volume of 15 µl included 1.5 µl 10×PCR-buffer with the EvaGreen dye (Syntol), 2.5 мМ $MgCl_2$ solution (Syntol), 1.0 U Taq polymerase (Syntol), 125 µM of each dNTP, 5 pM forward and reverse primers, 10 ng DNA matrix, ddH_2O (up to 15 µl). DNA amplification with further HRM analysis was run on a Real-Time C1000 thermocycler with CFX96 optical reaction module (Bio-Rad) under the following protocol: initial DNA denaturation at 95 °C for 3 min followed by 39 cycles as follows: denaturation at 95 °C for 10 s, annealing oligonucleotides at 60 °C for 30 s (detection in FAM channel), extension at 72 °C for 10 s. Melting parameters were as follows: temperature range from 65 to 95 °C with 0.2 °C increments, 5 s dwell time. Melting curves for amplification products were graphically analyzed using CFX ManagerTM (Bio-Rad). The amplicons were separated in native 8 % polyacrylamide gel (PAAG) (Sambrook et al., 1989).

Bioinformation analysis of *B. anthracis* genomes was performed using the Vector NTI 9.1 software and NCBI databases (https://www.ncbi.gov).

Results

Design and validation

Molecular typing of the strains was performed using the extended protocol including the detection of 12 SNP loci referred to as A.Br.001, A.Br.003, A.Br.004, A.Br.006, A.Br.007, A.Br.008, A.Br.009, B.Br.001, B.Br.002, B.Br.003, B.Br.004, and A/B.Br.001 (Van Ert et al., 2007) and analysis of eight VNTR loci (VrrA, VrrB1, VrrB2, VrrC1, VrrC2, CG3, pX01, and pX02) (Keim et al., 2000). The genotyping was validated using *B. anthracis* vaccine strain STI-1.

The results of SNP analysis presented in Table 3 allowed us to draw conclusions on the configuration of point nucleotide changes in the 12 investigated loci of *B. anthracis* STI-1. It was found that, except for two loci (ABr003 and ABr008), the

Table 3. Results of SNP analysis of investigated *B. anthracis* strains

Strain, No.	Locus												Phylogenetic group
	A.Br.001	A.Br.003	A.Br.004	A.Br.006	A.Br.007	A.Br.008	A.Br.009	B.Br.001	B.Br.002	B.Br.003	B.Br.004	A/B.Br.001	
STI-1	T	G	T	A	T	T	A	T	G	G	T	A	A.Br.008/009
1	T	–	C	A	T	–	A	T	G	G	T	A	A.Br.003/004
2	T	–	C	A	C	–	A	T	G	G	T	A	–
3	T	–	T	A	T	–	A	T	G	G	T	A	A.Br.008/009
4	T	–	C	A	C	–	A	T	G	G	T	A	–
5	T	–	C	C	T	–	A	T	T	A	T	A	–
6	T	–	C	C	T	–	A	T	T	A	T	A	–
7	T	–	C	C	T	–	A	T	T	A	T	A	–

Table 4. VNTR locus characteristics of *B. anthracis* STI-1

Locus	Locus size, bp	Repeat size, bp	Repeat nucleotide sequence	Repeat number	Amplicon melting point, °C
VrrA	314	12	tatcaacaacaa	4	84.5
VrrB1	229	9	caaggtcac	2	86.2
VrrB2	162	9	caatatcaa	3	85.7
VrrC1	616	36	cttcttctgactcttctgtttccgcaattacttcta	2	82
VrrC2	604	72	ctacgaccggtgcttcttctgcaactggttgttcttctacaatcggtgtttcttctacaactgattgttcct	2	85
CG3	156	5	tatta	1	75.2
pX01	156	3	aat	16	74.2
pX02	–	2	at	–	–

SNP profile obtained matched the data on single nucleotide polymorphisms available in the literature for the same strain (Afanas'ev et al., 2014; Eremenko et al., 2018). It should be noted that canSNPs are rather conservative and known for low mutation rate (Timofeev et al., 2018). Therefore, the validity of atypical single nucleotide changes in loci ABr003 and ABr008 detected for *B. anthracis* STI-1 required further confirmation, particularly by sequencing. Thus, ABr003 and ABr008 loci were discarded from the canSNP panel applicable for strain differentiation at the current research stage.

MLVA was performed using classical PCR with further amplicon separation in PAAG that only allowed us to determine the approximate sizes of seven investigated VNTR loci of *B. anthracis* STI-1 as follows: VrrA – 300 bp, VrrB1 – 250 bp, VrrB2 – 190 bp, VrrC1 – 700 bp, VrrC2 – 600 bp, CG3 – 160 bp, pX01 – 160 bp. Amplification products for the locus localized in capsule-encoding plasmid *pX02* were not detected. The absence of plasmid *pXO2* is characteristic for *B. anthracis* STI-1 (Afanas'ev et al., 2014).

The accurate size of the amplified VNTR fragments of the *B. anthracis* STI-1 DNA as well as nucleotide repeat sizes were determined *in silico*. Chromosomal DNA nucleotide sequence of *B. anthracis* STI-1 (GenBank CP066168) was limited by the respective primers (see Table 2) using the Vector NTI 9.1 software. The results obtained after bioinformation analysis of the *B. anthracis* STI-1 genome are presented in Table 4. The complete nucleotide sequence of plasmid *pX01* for the investigated strain is not available in the GenBank database. As a result, the tandem repeat number in the locus of interest was determined as a difference between the amplicon molecular mass and the repeat-free size of the amplified fragment divided by the number of nucleotides in the variable site. Electrophoresis showed that CG3 and pX01 locus sizes for *B. anthracis* STI-1 were identical. Thus, to calculate the repeat number for plasmid *pX01*, the molecular mass of the CG3 locus was used.

The melting curve peaks for qPCR amplification products for MLVA loci of *B. anthracis* STI-1 were also identified (see Table 4). The qPCR procedure was performed using the EvaGreen dye in eight replicas. It was found that the difference between replicas for most loci under study was 0.2 °C. Thus, the values of at least 0.2 °C were considered an acceptable parameter difference for the further differentiation of anthrax strains based on VNTR loci melting point differences.

Table 5. Melting points of the PCR products obtained after amplification in the VNTR loci of the investigated *B. anthracis* strains

Strain, No.	Locus							
	VrrA1	VrrB1	VrrB2	VrrC1	VrrC2	CG3	pXO1	pXO2
1	84.6	86.0	85.4	84.2	83.6	75.6	74.6	78.2
2	84.6	86.0	83.0	84.0	83.4	75.0	74.2	77.8
3	84.2	85.8	85.4	84.2	83.4	75.0	74.2	78.2
4	85.2	86.0	85.4	84.2	83.6	75.2	74.6	78.4
5	85.0	85.8	85.8	84.0	83.4	75.2	74.6	78.2
6	85.0	86.0	85.8	84.0	83.6	75.2	74.6	78.4
7	85.0	86.0	86.0	84.0	83.6	75.2	74.8	78.4

Single nucleotide polymorphism analysis

The results of molecular genetic analysis of SNP loci for the studied virulent strains of *B. anthracis* are shown in Table 2. It turned out that SNP analysis did not show differences in five loci including A.Br001, A.Br005, A.Br009, B.Br001, A/B. Br001. The values of variability index (h) for the remaining seven SNP loci ranged from 0.12 to 0.4.

The results obtained allowed us to divide the investigated strains into four clusters. The largest cluster was formed by the three strains isolated in the Republic of Tatarstan (*B. anthracis* No. 6 and 7) in 2008 and 2014 and in Ulyanovsk (*B. anthracis* No. 5) in 2004. The second cluster included strains No. 2 and 4 collected in the Tajik SSR in 1972 and Ulyanovsk in 2004, respectively. Two remaining clusters were formed by strains No. 1 and 3 found in the Checheno-Ingush ASSR in 1971 and in the Kurgan region in 1972, respectively.

The results of SNP typing allowed us to attribute strain No. 1 to the phylogenetic subgroup A.Br.003/004. It was found that *B. anthracis* bacteria of strain No. 3 could be attributed to the phylogenetic line A.Br.011/009, similarly to the STI-1 reference strain. The SNP profiles obtained for the rest of the investigated microorganisms were not characteristic for any of the previously identified phylogenetic subgroups of *B. anthracis* strains.

Multilocus variable number tandem repeat analysis

The obtained amplicon melting point values were used to perform VNTR strain differentiation (Table 5). The melting points of the obtained PCR fragments after variable-locus amplification depended on their nucleotide compositions, specifically the tandem repeat numbers, i. e., the higher the latter, the higher the melting point.

It was found that the melting curves of strains No. 1 and 2 obtained after amplification in the VrrA1 locus were identical to the melting curve obtained for the reference strain at the same locus and probably included four tandem repeats in VrrA1. Four strains (No. 4–7) showed higher melting points compared to the reference strain, which implies a higher tandem repeat number in the VrrA1 locus. On top of that, strain No. 3 showed the lowest melting point in the VrrA1 locus among the investigated strains, and as a result it was characterized by the lowest repeat number in the VrrA1 locus.

Strains No. 5–7 and STI-1 showed identical amplicon melting curves at VrrB2 and, thus, included three tandem repeats in the VrrB2 locus, similarly to *B. anthracis* STI-1. The data from Table 5 demonstrate that the rest of the investigated strains had lower repeat numbers in this locus, i. e., probably two in strains No. 1, 3, 4, 6 and one – in strain No. 2.

The amplicon melting curves in the CG3 locus indicate that all the strains of interest, except for No. 1, included one tandem repeat, similarly to STI-1, so strain No. 1 probably carries several repeats in this locus.

It was observed that strains No. 2 and 3 were characterized by amplicon temperatures in pX01 identical to those of the reference strain. Thus, a conclusion was made that these bacteria had 16 tandem repeats in plasmid *pX01*, whereas the remaining strains had over 16 repeats.

The melting points of PCR products obtained after amplification in loci VrrB1, VrrC1, and VrrC2 were the same for all investigated strains, and, as a result, their repeat numbers fragments were the same in these DNAs as well. STI-1 had two tandem repeats in these loci. The investigated strains showed lower amplicon melting points in VrrB1 and VrrC2 compared to the reference strain and probably carried one tandem repeat in these loci. With regards to the VrrC1 locus, the melting points of PCR products obtained after amplification for all the investigated strains exceeded the respective value of the reference strain. Thus, a conclusion can be made that the investigated strains carry three or more tandem repeats in the VrrC1 locus. The number of repeats in the pX02 locus could not be determined due to the lack of this marker in the reference strain.

Thus, it was found that strain No. 1 had a different tandem repeat number in the CG3 locus, strain No. 2 – in the VrrB2 and pXO2 loci, and strain No. 3 – in the VrrA1 locus. A unique melting point profile of PCR amplification products in VNTR loci was identified for strains No. 1–3, making it possible to differentiate between them. The remaining three strains (No. 5–7) showed the same melting point profiles and could therefore be combined into one cluster. A similar melting

point profile was detected for strain No. 4, its only difference from strains No. 5–7 being the tandem repeat number in the VrrB2 locus.

Discussion

In recent years, Russian and foreign authors have published a substantial number of papers on the design of viable approaches to genotyping anthrax agent strains (Le Flèche et al., 2001; Keim et al., 2004; Van Ert et al., 2007; Gierczynski et al., 2009; Eremenko et al., 2012; Afanas'ev et al., 2014). Most of the available *B. anthracis* genotyping methods are based on polymorphism of tandem repeats or point mutations in their genome. Some authors believe that the test systems based on a combination of genetic markers with different discriminating power and stability are the most efficient way to differentiate between *B. anthracis* strains (Keim et al., 2004; Chang et al., 2007; Afanas'ev et al., 2014). SNP loci are more stable but have a low variability index compared to VNTR ones. That is why, when these loci are used in combination, it is recommended to first use canSNPs to attribute the investigated strains to a specific phylogenetic group, and then use MLVA to differentiate the strains within each canSNP cluster (Timofeev et al., 2018).

PCR amplification followed by visualization of the obtained amplicons in agarose or polyacrylamide gel is still considered a universal approach to the analysis of VNTR loci (Jackson et al., 1997, 1999; Keim et al., 2000). This method may be effective for differentiation of nucleotide repeats of over 10 bp in size (VrrA1 – 12 bp, VrrC1 – 36 bp, VrrC2 – 72 bp) but is not suited for differentiation of repeats of 2–3 nucleotides in size (VrrB1 and VrrB2 – 9 bp, CG3 – 5 bp, pX01 – 3 bp, pX02 – 2 bp). In our study, the sizes of most repeats did not exceed 10 bp, which made electrophoresis unviable for strain differentiation, so HRM analysis was applied to differentiate between the allelic variants of the VNTR loci based on tandem repeat numbers. This method is widely used in genotyping, specifically to detect mutations, polymorphisms, and epigenetic differences in double-stranded DNA samples (Graham et al., 2005; Margraf et al., 2006). According to the literature, HRM analysis is also applicable for indication and differentiation of Brucella strains (Winchell et al., 2010).

According to the literature, the *B. anthracis* strains circulating in the Russian territories of high anthrax risk are predominantly attributed to the A.Br.001/002, A.Br.008/009, B.Br.001/002, and A.BrAust94 genotypes (Eremenko et al., 2018; Kravets et al., 2018; Koteneva et al., 2019). In particular, the strains isolated in North Caucasus generally fall into canSNP clusters A.Br.008/009 and A.BrAust94. Genotype B.Br.001/002 also occurs in the Republic of Dagestan (Koteneva et al., 2019). The strains attributed to phylogenetic groups A.Br.001/002, A.Br.008/009, and B.Br.001/002 are the most common in Siberia and the Russian Far East (Eremenko et al., 2018; Kravets et al., 2018).

It should be noted that, according to some authors, the variety of canSNP genotypes is probably not restricted to the 14 that are currently described (Afanas'ev et al., 2014; Timofeev et al., 2018). For example, M.V. Afanas'ev et al. (2014) have identified three additional phylogenetic subgroups. Indeed, the results of genetic typing of the seven *B. anthracis* cultures performed in the present study using SNP and MLVA analysis showed that all the studied microorganisms, except for strains No. 1 and 3, could not be attributed to the main phylogenetic canSNP subgroups based on their SNP profiles. Phylogenetic line A.Br.003/004, to which strain No. 1 was attributed, mostly includes strains from the American continents (Eremenko et al., 2018). Among all the investigated strains, strain No. 3 isolated in the Kurgan region showed the most characteristic SNP profile for Russian isolates.

The studied anthrax agent strains are typically organized into SNP clusters based on their geographic origin, the exception being strains No. 2 and 4 isolated in the former Tajik SSR in 1972 and Ulyanovsk in 2004 sharing the same SNP profile. HRM analysis showed that these bacteria had different repeat numbers in loci VrrA1, VrrA2, pXO1, and pXO2 and, as a result, may be differentiated from one another. A reasonable assumption would be that these microorganisms had a common geographic origin but diverged with time affected by trade and migration flows. According to the literature, VNTR loci are characterized by a high mutation rate (10^{-5} to 10^{-4} per generation) (Keim et al., 2004; Birdsell et al., 2012; Thierry et al., 2014) and, compared to SNP loci, are in fact the markers of the later evolution of *B. anthracis* strains.

The HRM profiles obtained for the remaining strains of interest had the patterns matching well with the SNP profiles. Thus, the extended protocol combining SNP and VNTR analyses makes it possible to differentiate between four *B. anthracis* strains. Strains No. 5–7 demonstrated the same SNP and HRM profiles and were therefore combined into the same cluster.

The use of HRM analysis has made it possible to differentiate the strains of interest from one another and attribute them to the respective clusters. We have also determined repeat sizes in the loci, the PCR-product melting points of which were identical to amplicon melting points in the same VNTR loci for the reference strain. The state of the art is that the tandem repeat size may only be accurately determined by sequencing, which means VNTR locus sequencing is to be performed for the strains of interest in the future. We believe that combining the two methods may allow us to create a database of melting curves for VNTR loci, in which the curves will be related to the locus size.

Conclusion

Applying HRM to analyze PCR products in VNTR loci has a high application value. In particular, this approach may be used for a rapid preliminary differentiation of *B. anthracis* strains within the same outbreak. To achieve the most efficient and informative indication and differentiation of anthrax agent strains, we propose the following algorithm: 1) attribute the strains of interest to phylogenetic subgroups using SNP typing; 2) differentiate the strains within each SNP cluster using MLVA and HRM analysis; 3) perform MLVA typing for the differentiated strains using classical PCR, electrophoresis, and sequencing. However, further research is required to investigate the capabilities and limits of this genotyping strategy.

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Effect of colchicine on physiological and biochemical properties of *Rhodococcus qingshengii*

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Abstract. The genus *Rhodococcus* includes polymorphic non-spore-forming gram-positive bacteria belonging to the class Actinobacteria. Together with *Mycobacterium* and *Corynebacterium*, *Rhodococcus* belongs to the Mycolata group. Due to their relatively high growth rate and ability to form biofilms, *Rhodococcus* are a convenient model for studying the effect of biologically active compounds on pathogenic *Mycolata*. Colchicine was previously found to reduce biofilm formation by *P. carotovorum* VKM B-1247 and *R. qingshengii* VKM Ac-2784D. To understand the mechanism of action of this alkaloid on the bacterial cell, we have studied the change in the fatty acid composition and microviscosity of the *R. qingshengii* VKM Ac-2784D membrane. Nystatin, which is known to reduce membrane microviscosity, is used as a positive control. It has been found that colchicine at concentrations of 0.01 and 0.03 g/l and nystatin (0.03 g/l) have no significant effect on the survival of *R. qingshengii* VKM Ac-2784D cultivated in a buffered saline solution with 0.5 % glucose (GBSS). However, colchicine (0.03 g/l) significantly inhibits biofilm formation. *Rhodococcus* cells cultivated for 24 hours in GBSS with colchicine acquire a rounded shape. Colchicine at 0.01 g/l concentration increases C16:1(n-7), C17:0, C20:1(n-9) and C21:0 fatty acids. The microviscosity of the membrane of individual cells was distributed from the lowest to the highest values of the generalized laurdan fluorescence polarization index (GP), which indicates a variety of adaptive responses to this alkaloid. At a higher concentration of colchicine (0.03 g/l) in the membranes of *R. qingshengii* VKM Ac-2784D cells, the content of saturated fatty acids increases and the content of branched fatty acids decreases. This contributes to an increase in membrane microviscosity, which is confirmed by the data on the GP fluorescence of laurdan. All of the above indicates that colchicine induces a rearrangement of the *Rhodococcus* cell membrane, probably in the direction of increasing its microviscosity. This may be one of the reasons for the negative effect of colchicine on the formation of *R. qingshengii* VKM Ac-2784D biofilms.
Key words: *Rhodococcus qingshengii*; colchicine; biofilms; fatty acids; membrane microviscosity.

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Влияние колхицина на физиолого-биохимические свойства *Rhodococcus qingshengii*

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Аннотация. Род *Rhodococcus* объединяет полиморфные неспорообразующие грамположительные бактерии, относящиеся к классу Actinobacteria. *Rhodococcus* вместе с *Mycobacterium* и *Corynebacterium* входят в группу Mycolata. Благодаря относительно высокой скорости роста и способности к образованию биопленок, *Rhodococcus* являются удобной моделью для изучения действия биологически активных соединений на патогенные *Mycolata*. Ранее было показано, что колхицин угнетал образование биопленок у *P. carotovorum* ВКМ B-1247 и *R. quingshengii* ВКМ Ac-2784D. Целью настоящей работы было изучение действия колхицина на жирнокислотный состав и микровязкость мембран *Rhodococcus qingshengii* ВКМ Ac-2784D для понимания механизма действия этого алкалоида на бактериальную клетку. В качестве положительного контроля использовали нистатин, снижающий микровязкость мембран. Установлено, что колхицин в концентрациях 0.01 и 0.03 г/л и нистатин (0.03 г/л) не оказали существенного влияния на выживаемость *R. qingshengii* ВКМ Ac-2784D, культивируемого

в забуференном физиологическом растворе глюкозы (ЗФРГ). Однако колхицин (0.03 г/л) значительно угнетал образование биопленки. Клетки *Rhodococcus*, культивируемые в течение суток в ЗФРГ с колхицином, приобретали округлую форму. Колхицин в концентрации 0.01 г/л вызывал увеличение жирных кислот C16:1(n-7), C17:0, C20:1(n-9) и C21:0. Микровязкость мембраны отдельных клеток распределялась от максимально низких до максимально высоких значений показателя обобщенной поляризации флуоресценции лаурдана (GP), что свидетельствует о разнообразии адаптационных ответов на этот алкалоид. При более высокой концентрации колхицина (0.03 г/л) в мембранах клеток *R. qingshengii* ВКМ Ac-2784D увеличивалось содержание насыщенных жирных кислот и падало – разветвленных. Это способствовало увеличению микровязкости мембраны, что подтверждается данными по GP. Таким образом, колхицин индуцирует перестройку клеточной мембраны *Rhodococcus*, вероятно, в сторону увеличения ее микровязкости, что может быть одной из причин негативного действия колхицина на образование биопленок *R. qingshengii* ВКМ Ac-2784D.
Ключевые слова: *Rhodococcus qingshengii*; колхицин; биопленки; жирные кислоты; микровязкость мембран.

Introduction

The *Rhodococcus* genus includes polymorphic non-sporeforming gram-positive bacteria that belong to the Actinobacteria class. *Rhodococcus* are frequently met in nature, in particular, in living organisms. Among the key features of these microorganisms is their ability to decompose different organic compounds, including pollutants (PAC, biphenyls, alkanes, etc.) (Szőköl et al., 2014; Li et al., 2016). For this reason, *Rhodococcus* continue to attract the growing interest as valuable biotech species.

Along with *Mycobacterium* and *Corynebacterium*, *Rhodococcus* relates to the Mycolata group, which is characterized by the presence of mycolic acids on the cell walls (Sutcliffe, 1998). This makes these bacteria more resistant to the toxic compounds such as disinfectants, antibiotics or PAC. Unlike myco- and corynebacteria, *Rhodococcus* species are mostly non-pathogenic. Therefore, owing to relatively high growth rate and propensity to biofilm formation, the *Rhodococcus* represent a convenient model to examine the effect of biologically active compounds on pathogenic *Mycolata*.

The integrity of a microbial cell drastically depends on the membrane. In order to survive in ever-changing environmental conditions and to maintain optimal membrane fluidity, the bacteria change the fatty acid composition of membrane lipids (Dubois-Brissonnet et al., 2016). The cell membrane is the major target of non-polar organic solvent toxicity (De Carvalho et al., 2005). Plant metabolites also affect the membrane via inhibition of the efflux channels activity (Tegos et al., 2002), the content of porin proteins (Abreu et al., 2012), etc.

Previously, we found that the alkaloid colchicine at a concentration of 0.25 g/l suppressed the formation of a biofilm by *Pectobacterium carotovorum* VKM B-1247 and *Rhodococcus qingshengii* VKM Ac-2784D species (Bybin et al., 2018). Moreover, no negative effect on the viability of these bacteria was revealed. Colchicine is widely known as an alkaloid that interrupts the tubulin polymerization in eukaryotic cells (Zhang et al., 2018). It is likely that colchicine exhibits a similar effect on the microorganisms, affecting the cytoskeleton and preventing the adhesion of microbial cells (Dubey et al., 2011). However, its influence on microbial cells was poorly studied. All of the above sparked our interest in this compound.

In the present work, we have examined the effect of colchicine on the fatty acid composition and microviscosity of *R. qingshengii* VKM Ac-2784D membranes.

Materials and methods

R. qingshengii VKM Ac-2784D strain isolated from the rhizosphere of couch grass (*Elytrigia repens* (L.) Nevski) growing in the oil-contaminated territory of the Irkutsk region (Russia) was used in the work (Petrushin et al., 2021). The *Rhodococcus* strain features a good formation of biofilms, and therefore represents a convenient model for their study.

The bacteria were cultivated on BTN-agar (Biotekhnovatsiya, Russia) for 48 h at 26 °C. Then they were transferred to a 0.5 % glucose buffered saline solution (GBSS) and the density of the suspension was adjusted to OD_{595} 0.26–0.33.

The minimum inhibitory concentration (MIC) of colchicine for *R. qingshengii* VKM Ac-2784D was determined by the limiting dilution method (Guidelines..., 2000).

To evaluate the effect of nystatin and colchicine on growth kinetics and biofilm formation, 150 µl of bacterial suspension was added to the wells of sterile flat-bottom 96-well plates and the optical density was measured on the first, third, and eighth days of cultivation using an iMark plate reader (Bio-Rad, USA) , λ = 595 nm. The plate was washed from loosely attached cells. The precipitate was stained with 1 % crystal violet solution for 45 min. After washing (3 times) to extract the dye, 200 µl of 96 % ethanol was added to the wells. The level of extraction (absorption) of crystalline violet with ethanol was measured using an iMark plate reader (Bio-Rad) at a wavelength of 595 nm in optical density units (OD_{595}). The degree of biofilm formation corresponded to the intensity of dye staining of the wells content (Shaginyan et al., 2007).

Two controls were employed in the work. The first one involved the bacteria cultivated in GBSS without the addition of colchicine. The second control used the bacteria grown in a medium with 0.03 g/l of nystatin (Biosintez, Russia), since nystatin can reduce microviscosity of the cell membranes. Colchicine (Sigma-Aldrich, USA) was applied at concentrations of 0.01 and 0.03 g/l. When plotting the diagrams, the relative optical density in % to the control was used. Cell sizes were assessed using the AxioVision Rel 4.8 software.

To determine the fatty acid composition of the bacterial membrane and the orderliness (microviscosity, fluidity) of its lipid phase, the bacteria were cultivated in the aforementioned media for a day. The membrane lipids orderliness was evaluated by the generalized polarization (GP) of laurdan lipophilic probe fluorescence in each pixel corresponding to the luminescent image domain. To stain the bacteria, 10 μM of a methanolic solution of laurdan (2-(dimethylamino)-6-dodecanoylnaphthalene) (Sigma-Aldrich) was added to each vial. Live stained bacteria were observed using a microscope (laser scanning confocal fluorescent microscope MicroTime 200; PicoQuant GmbH, Germany).

The distribution of GP values was analyzed by visualization with histograms. For each histogram, a theoretical multimodal distribution as a superposition of several normal distributions was plotted (Nurminsky et al., 2017). Next, the parameter fitting of the experimental distributions of bacterial membrane GP values was estimated. The model distribution was a normal distribution or a mixture of distributions and thus consisted of one or more components. Finally, the optimal parameters of the components that were closest to the experimental distribution were selected.

To determine the composition of fatty acids (FA), the bacteria were cultivated similarly without the addition of laurdan. The lipids were extracted according to the published procedure (Bligh, Dyer, 1959). After removal of the solvent, a 1 % methanol solution of H_2SO_4 was added to the lipid extract and heated on a water bath at 60 °C for 30 min. After cooling, the solution was extracted (3 times) with hexane (Christie, 1993). Fatty acids methyl esters were analyzed using an Agilent technology 5973N/6890N MSD/DS chromato-mass spectrometer (USA). Detector (mass spectrometer) was quadrupole, ionization method was electron impact (EI), ionization energy was 70 eV, the mode of the total ion current registration was used for the analysis. Separation was performed on an HP-INNOWAX capillary column (30 m × 250 μm × 0.50 μm). The stationary phase was polyethylene glycol. The mobile phase was helium; gas flow rate was 1 ml/min. Temperature of the evaporator was 250 °C, temperature of the ion source was 230 °C, temperature of the detector was 150 °C, and temperature of the line connecting the chromatograph with the mass spectrometer was 280 °C. Scan range was 41–450 amu. The volume of the injected sample was 1 μl, the flow separation was 5:1. Chromatography was carried out in isothermal mode at 200 °C. To identify the peaks of FA methyl esters, methyl ester standards (Sigma-Aldrich) and mass spectrometry using the NIST 05 mass spectrum library (Ozolina et al., 2017) were used. The content of individual fatty acids was calculated as a percentage of the total amount of fatty acids and divided into groups: saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), saturated *iso*- and *anteiso*-methyl branched fatty acids (BFA) (Rodrigues, de Carvalho, 2015).

The significance of differences in biofilm formation and the quantitative content of fatty acids were assessed using the nonparametric Kruskal–Wallis test with Dunnett's correction (Glantz, 1991). All calculations were performed using the RStudio software.

Results and discussion

It was found that the MIC of colchicine for *R. qingshengii* VKM Ac-2784D is 0.02 g/l. Therefore, in further experiments, the concentrations below and above this value, i. e. 0.01 and 0.03 g/l, respectively, were used. The selected concentrations of colchicine and nystatin did not strongly affect the growth of *Rhodococcus* (Fig. 1). At the same time, it was established that colchicine at a concentration of 0.03 g/l significantly inhibited the formation of a biofilm at all stages of the experiment, while at a concentration of 0.01 g/l a divergent effect was observed. On the first day, nystatin stimulated the formation of a biofilm, whilst on the third and eighth days of cultivation, its effect was comparable to the control (Fig. 2).

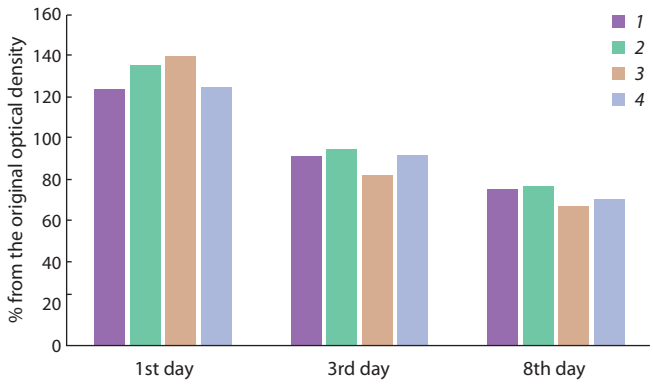

Fig. 1. Optical density of *R. qingshengii* VKM Ac-2784D cell suspension relative to control, %, on the first, third and eighth days of cultivation.

Here and in the Fig. 2: *1* – GBSS; *2* – GBSS with 0.03 g/l colchicine; *3* – GBSS with 0.01 g/l colchicine; *4* – GBSS with 0.03 g/l nystatin.

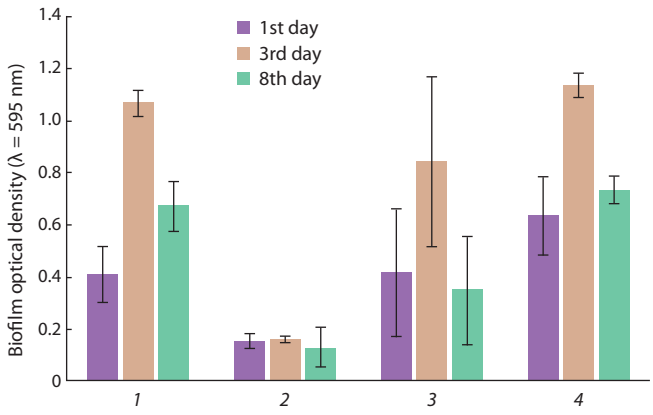

Fig. 2. Optical density of biofilm *R. qingshengii* VKM Ac-2784D on the first, third and eighth days of cultivation.

Table 1. Cell sizes of *R. qingshengii* VKM Ac-2784D after cultivation for a day under control conditions and in the presence of the studied compounds

Experiment	Length, nm	Width, nm	Length/width
Control	1.35 ± 0.29	0.56 ± 0.08	2.42
Colchicine, 0.01 г/л	1.04 ± 0.23*	0.58 ± 0.1	1.78*
Nystatin, 0.03 г/л	1.46 ± 0.36	0.68 ± 0.14	2.12

* $p < 0.05$.

The cultivation of *R. qingshengii* VKM Ac-2784D in the presence of colchicine for a day essentially changed the cell morphology: the cells acquired a more rounded shape (Table 1). Moreover, the intracellular content became heterogeneous (Fig. 3), which is consistent with the results obtained for *Bacillus megaterium* (Dubey et al., 2011). The shape of *Rhodococcus* cells under the action of nystatin remained intact.

The changes in cell morphology are usually accompanied by structural and functional rearrangement of their cell membranes (de Carvalho et al., 2014). Specifically, the degree of saturation of FA, their length, as well as the amount of branched fatty acids are altered.

Under the control conditions, the *Rhodococcus* cell membranes mainly contained palmitic, stearic, and oleic acids (Table 2). The ratio of saturated to monounsaturated fatty acids was 1.64. The content of polyunsaturated and branched acids was small (4.41 and 0.84 %, respectively). Colchicine at a concentration of 0.01 g/l changed the ratio of saturated and monounsaturated FA (1.29) in favor of the latter, and simultaneously reduced the amount of polyunsaturated FA. At the same time, the number of long-chain FA C20:1(n-9), C21:0 and C22:0 increased. With 0.03 g/l of colchicine, the relative content of saturated and branched FA increased (Fig. 4), while the ratio of UFA to MUFA reached 1.89. All this indicates the rearrangement of *R. qingshengii* VKM Ac-2784D membrane after the introduction of colchicine into the cultivation medium. Interestingly, different concentrations of colchicine had an opposite effect on the composition of cell membrane FA. This is probably due to various degrees of regulatory systems disorder. The addition of nystatin, a compound that fairly increases the membrane fluidity, led to a higher content of unsaturated and branched FA, substantially lower amount of palmitic FA, higher concentration of oleic FA.

The fluidity or microviscosity of membranes is an integral index that depends on lipid saturation and content of sterols or proteins. Therefore, further we focused our efforts on the evaluation of the colchicine and nystatin effect on the orderliness of the lipid phase of *R. qingshengii* VKM Ac-2784D membrane. For this purpose, the laurdan fluorescence GP index was used, which can vary from –1 to +1. Its negative values correspond to lower microviscosity (higherfluidity) of the cell membrane (Nurminsky et al., 2015) (see Materials and Methods).

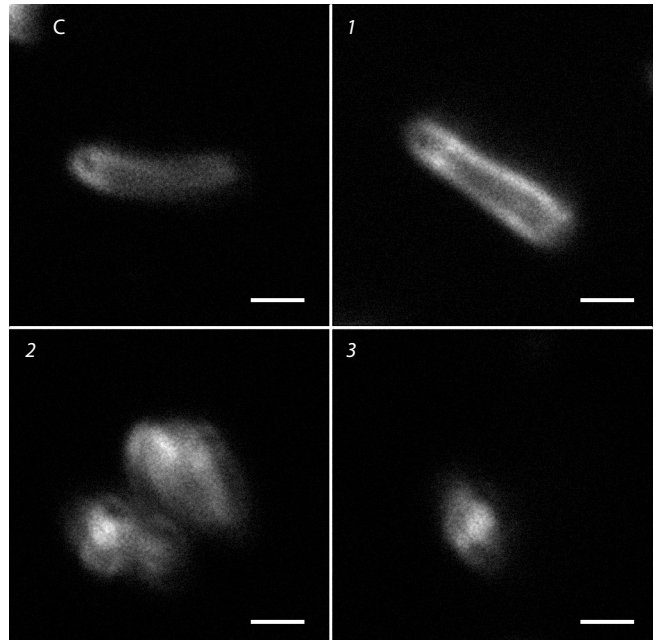

Fig. 3. Cell morphology of *R. qingshengii* VKM Ac-2784D, cultivated for 24 hours in buffered saline with glucose (5 g/L) (C) and after adding 0.03 g/l nystatin (*1*), 0.01 g/l colchicine (*2*), 0.03 g/l colchicine (*3*).
Stained with laurdan. Magnification ×600.

The fitting of experimental distributions of bacterial membrane GP values permitted to find from one to four components under the action of nystatin and colchicine (Fig. 5). In all variants, the most significant component characterizes the liquid-disordered regions of the membrane (α (average GP values): –0.16–0.04, contribution: 73.9–100 %). The sterol-binding agent nystatin shifted GP towards a decrease in the orderliness of the membranes (α: –0.16, contribution: 100 %), which corresponds to the known mechanism of action of this antibiotic on the membranes of eu- and prokaryotes (Efimova et al., 2014). Colchicine, on the contrary, increased the orderliness of the membranes: a of the most significant components shifted, although slightly, towards positive values compared to the control in both concentrations (α: 0.04, contribution: 73.9–89.4 %), which agrees with the observed increase in the amount of saturated FA. However, this significantly expanded the data scattering, and the number of components reached 2 (in the variant with

Table 2. The composition of FA in the membrane cells of *R. qingshengii* VKM Ac-2784D cultivated for a day in GBSS with colchicine (0.03 and 0.01 g/l) and nystatin (0.03 g/l)

Fatty acid	Control	Concentration of alkaloids, g/l		
		Colchicine		Nystatin 0.03
		0.03	0.01	
Lauric C12:0	–	–	–	0.76 ± 0.04
Myristic C14:0	4.76 ± 0.38	6.09 ± 0.02	5.45 ± 0.26	4.89 ± 0.02
Pentadecane C15:0	1.99 ± 0.22	2.36 ± 0.32	2.25 ± 0.01	1.60 ± 0.06
Anteiso-pentadecane C15:0-a	–	–	–	1.08 ± 0.03
Palmitic C16:0	36.76 ± 3.52	34.58 ± 0.33	32.27 ± 2.10	**27.98 ± 0.48***
Isopalmitic C16:0-i	–	–	–	1.63 ± 0.04
Palmitoleic C16:1(n-9)	5.16 ± 1.05	3.78 ± 0.35	4.50 ± 0.14	3.53 ± 0.21
C16:1(n-7)	1.31 ± 0.14	2.33 ± 0.35	**3.11 ± 0.36***	2.21 ± 0.28
C16:1(n-9)	–	–	–	4.78 ± 0.17
C16:1(n-5)	2.30 ± 0.26	2.06 ± 0.09	3.79 ± 1.04	–
Heptadecane C17:0	1.26 ± 0.14	1.35 ± 0.03	**1.60 ± 0.11***	1.28 ± 0.05
Anteiso-heptadecane C17:0-a	0.84 ± 0.23	**1.23 ± 0.03***	0.72 ± 0.16	0.83 ± 0.09
Stearic C18:0	12.09 ± 0.19	14.80 ± 0.25	10.15 ± 0.21	13.91 ± 0.29
Oleic C18:1(n-9)	25.79 ± 2.42	24.24 ± 0.24	28.46 ± 1.10	**30.29 ± 0.09***
C18:1(n-7)	1.36 ± 0.11	0.89 ± 0.02	2.33 ± 0.13	0.59 ± 0.13
Linolic C18:2(n-6)	4.41 ± 0.61	1.54 ± 0.01	2.01 ± 0.49	2.33 ± 0.05
Arachic C20:0	0.99 ± 0.08	**1.55 ± 0.01***	1.16 ± 0.01	0.86 ± 0.00
C20:1(n-9)	–	**0.46 ± 0.04**	**0.36 ± 0.01**	**0.27 ± 0.00**
Heneicosanic C21:0	–	**0.48 ± 0.09**	**0.44 ± 0.09**	**0.26 ± 0.03**
Behenic C22:0	1.10 ± 0.13	**2.28 ± 0.19**	1.40 ± 0.03	0.92 ± 0.17

* $p < 0.05$.

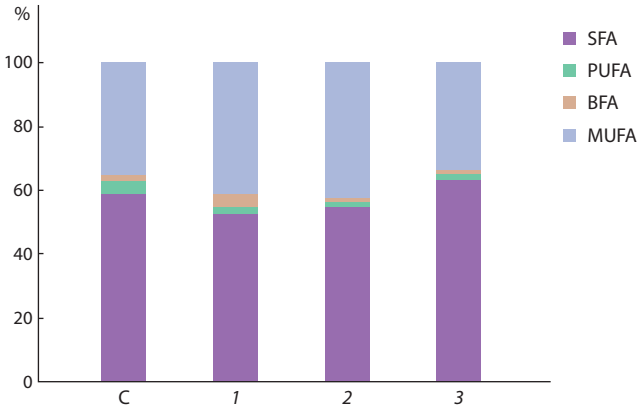

Fig. 4. Relative content of the main groups of fatty acids during cultivation for a day in buffered saline with glucose (5 g/l) (C) and with the introduction of 0.03 g/l nystatin (*1*), 0.01 g/l colchicine (*2*), 0.03 g/l colchicine (*3*).

SFA – saturated fatty acids; PUFA – polyunsaturated fatty acids; MUFA – monounsaturated fatty acids; BFA –saturated iso- and anteiso-methyl branched fatty acids.

0.01 mg/ml) and 4 (in the variant with 0.03 mg/ml). Minor components corresponded to more densely packed regions of the membranes (α: 0.29, contribution: 1.8 %) or, conversely, to less densely packed ones (α: –0.29, contribution: 6.7 %).

Conclusion

In conclusion, colchicine in the composition of GBSS at concentrations of 0.01 and 0.03 g/l did not significantly affect the survival of *R. qingshengii* VKM Ac-2784D, but strongly inhibited the formation of a biofilm. *Rhodococcus* cells cultivated for 24 hours in GBSS with colchicine acquired a rounded shape. With 0.01 g/l of colchicine, the content of C16:1(n-7), C17:0, C20:1(n-9) and C21:0 FA acids increased.

The membrane microviscosity of individual cells is distributed from the lowest to the highest GP values, which indicates a variety of adaptive responses to this alkaloid. At a higher concentration of colchicine (0.03 g/l) in the cell membranes of *R. qingshengii* VKM Ac-2784D, the

a

Variant	Component	Average GP, α	Std. deviation, σ	Contribution, %
Control	1	0.01	0.16	100
Nystatin, 0.03 g/l	1	–0.16	0.17	100
Colchicine, 0.01 g/l	1	–0.08	0.24	26.1
	2	0.04	0.16	73.9
Colchicine, 0.03 g/l	1	–0.19	0.03	2.1
	2	0.04	0.18	89.4
	3	0.29	0.02	1.8
	4	–0.29	0.11	6.7

b

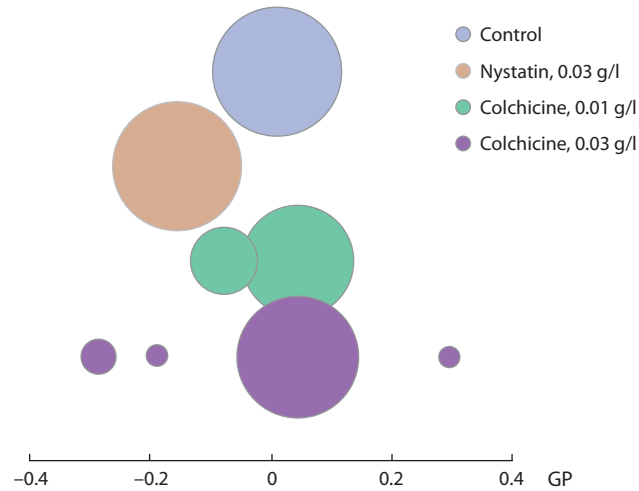

Fig. 5. Influence of nystatin and colchicine on the parameters of the components of the distributions of GP values of *R. qingshengii* VKM Ac-2784D membranes (*a*) and comparison of the components on the bubble diagram (*b*); the area of the circle reflects the contribution of each component, $n = 10–26$.

content of saturated fatty acids increased, while the amount of branched fatty acids reduced. This enhanced the membrane microviscosity that was confirmed by the values of laurdan fluorescence GP. These data testify to an adaptive rearrangement of the cell membrane under the action of the studied alkaloid, which is consistent with the results obtained by other authors (Wang et al., 2020). This may be a reason of the negative effect of colchicine on the formation of *R. qingshengii* VKM Ac-2784D biofilms.

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Activity of alkanmonooxygenase *alkB* gene in strains of hydrocarbon-oxidizing bacteria isolated from petroleum products

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Abstract. Alkanmonooxygenase enzymes AlkB and Cyp153 are responsible for the aerobic degradation of *n*-alkanes of petroleum and petroleum products. To prove the usage of *n*-alkanes from oil and petroleum products by hydrocarbon-oxidizing bacteria isolated from aviation kerosene TS-1 and automobile gasoline AI-95, the detection of the key genes *alkB*, *Alk1*, *Alk2*, *Alk3* and *Cyp153* encoding alkanmonooxygenases AlkB and Cyp153 (responsible for the oxidation of hydrocarbons with a certain chain length) was carried out. It was found that bacterial strains isolated from TS-1 jet fuel, except *Deinococcus* sp. Bi7, had at least one of the studied *n*-alkane degradation genes. The strains *Sphingobacterium multivorum* Bi2; *Alcaligenes faecalis* Bi3; *Rhodococcus* sp. Bi4; *Sphingobacterium* sp. Bi5; *Rhodococcus erythropolis* Bi6 contained the *alkB* gene. In the strains of hydrocarbon-oxidizing bacteria isolated from gasoline AI-95, this alkanmonooxygenase gene was not detected. Using the real-time PCR method, the activity of the *alkB* gene in all bacterial strains isolated from petroleum products was analyzed and the number of its copies was determined. By real-time PCR using a primer with a different sequence of nucleotides to detect the *alkB* gene, its activity was established in all bacterial strains isolated from gasoline AI-95; besides, the strain *Paenibacillus agaridevorans* Bi11 was assigned to the group with a high level of its activity (1290 copies/ml). According to the assessment of the growth of isolated hydrocarbon-oxidizing bacteria on a solid Evans mineral medium with the addition of the model mixture of hydrocarbons, the strains were divided into three groups. The distributions of strains of hydrocarbon-oxidizing bacteria in the groups based on the activity of the *alkB* gene and groups formed based on the growth ability and use of the model mixture of hydrocarbons and petroleum products were found to be consistent. The results obtained indicate that we need to use a complex of molecular and physiological methods for a comprehensive analysis of the distribution of the studied genes in bacteria and to assess their activity in the strains of hydrocarbon-oxidizing bacteria capable of biodegradation of petroleum hydrocarbons.
Key words: biodamage; petroleum products; hydrocarbon-oxidizing bacteria; biodegradation; alkanmonooxygenase; *alkB* gene; real-time PCR.

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Активность гена алканмонооксигеназы *alkB* у штаммов углеводородокисляющих бактерий, выделенных из нефтепродуктов

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Аннотация. Ферменты алканмонооксигеназы AlkB и Cyp153 ответственны за аэробную деградацию *н*-алканов нефти и нефтепродуктов. Для доказательства использования штаммами углеводородокисляющих бактерий, выделенных из авиационного керосина ТС-1 и автомобильного бензина АИ-95, *н*-алканов нефти и нефтепродуктов, проведена детекция ключевых генов *alkB*, *Alk1*, *Alk2*, *Alk3* и *Cyp153*, кодирующих алканмонооксигеназы AlkB и Cyp153, ответственных за окисление углеводородов с определенной длиной цепи. Установлено, что штаммы бактерий, изолированные из реактивного топлива ТС-1, за исключением *Deinococcus* sp. Bi7, имели как минимум один из исследованных генов деградации *н*-алканов. Штаммы *Sphingobacterium multivorum* Bi2, *Alcaligenes faecalis* Bi3, *Rhodococcus* sp. Bi4, *Sphingobacterium* sp. Bi5, *Rhodococcus erythropolis* Bi6 содержали

ген *alkB*. У штаммов углеводородокисляющих бактерий, выделенных из бензина АИ-95, этот ген алканмонооксигеназы не был детектирован. С помощью метода ПЦР в реальном времени проанализирована активность гена *alkB* у всех полученных из нефтепродуктов штаммов бактерий и определено число его копий. Методом ПЦР в реальном времени с использованием праймера с другой последовательностью нуклеотидов для детекции гена *alkB* установлена его активность у всех штаммов бактерий, выделенных из бензина АИ-95, причем штамм *Paenibacillus agaridevorans* Bi11 отнесен к группе с высоким уровнем его активности (1290 копий/мл). По оценке роста исследованных углеводородокисляющих бактерий на плотной минеральной среде Эванса с модельной смесью углеводородов штаммы были разделены на три группы. Отмечены совпадения результатов по распределению штаммов углеводородокисляющих бактерий в группах по активности гена *alkB* и группах, сформированных на основе способности роста и использования модельной смеси углеводородов и нефтепродуктов. Полученные результаты свидетельствуют о необходимости применения комплекса молекулярно-генетических и физиологических методов для всестороннего анализа распространения исследуемых генов у бактерий и оценки их активности в штаммах углеводородокисляющих бактерий, способных к биодеградации углеводородов нефтепродуктов.
Ключевые слова: биоповреждение; нефтепродукты; углеводородокисляющие бактерии; биодеградация; алканмонооксигеназы; ген *alkB*; ПЦР в реальном времени.

Introduction

Petroleum products are the main source of energy from the economical point of view and in human life. Data about biological contamination of petroleum products and, first of all, various types of fuels, especially aviation kerosene, has recently increased significantly in the open press (Martin-Sanchez et al., 2018). Direct and indirect losses from microbiological corrosion of petroleum products in industrialized countries range from 2 to 5 % of the annual gross domestic product (Karimova, 2007). The study of the ability of strains of hydrocarbon-oxidizing bacteria isolated from petroleum products to use *n*-alkanes plays an important role both for protecting petroleum products from bio-damage and in the application of these strains for the disposal of emergency oil spills in water areas and on land (Dedov et al., 2017). In addition, the ability of bacteria to assimilate petroleum hydrocarbons can be the reason for the loss of their quality during transportation, storage and usage of equipment (Martin-Sanchez et al., 2018).

As a rule, microorganisms are capable of selective assimilation of certain types of hydrocarbons, which is determined by the number of carbon atoms and the peculiarity of the structure of the hydrocarbon. In natural conditions, microorganisms form communities in which a single chain of oxidation of hydrocarbons of oil and petroleum products is formed by the type of metabiosis. Each microorganism of the community, having specific enzyme systems aimed at using a certain type of hydrocarbons, uses this substrate in its metabolism. Therefore, with the joint action of microorganisms of the community, not only a larger amount, but also a wider range of hydrocarbons of oil and petroleum products is used (Timergazina, Perekhodova, 2012).

It is known that the vast majority of bacterial transformations of hydrocarbons are oxidative reactions that occur most actively in aerobic conditions. There are data on the molecular mechanisms and ways of aerobic biodegradation of hydrocarbons, which are as follows: 1) many multi-purpose oxygenase systems forming active complexes with hydrocarbon substrates and molecular oxygen have been discovered; 2) several enzymes involved in the initial stage of aerobic biodegradation of alkanes have been characterized (Coon, 2005; Funhoff et al., 2006; Van Beilen, Funhoff, 2007); 3) the metagenomic approach has made it possible to describe new metabolic pathways of hydrocarbon degradation, different from those previously characterized in cultured pure bacterial strains (Sierra-García et al., 2014) and 4) new phylotypes of alkanmonooxygenase (*alkB*) genes encoding alkanmonooxygenases have been found in marine ecosystems (Wasmund et al., 2009; Smith et al., 2013).

Aerobic degradation of alkanes can be carried out by two main types of enzymes: alkanmonooxygenase AlkB (also known as alkanhydroxylase) and some cytochrome P450 systems (Van Beilen et al., 2006) found in bacteria of the genera *Pseudomonas* (Johnson, Hyman, 2006), *Rhodococcus* (Sameshima et al., 2008), *Acinetobacter* (Throne-Holst et al., 2007), Alcanivorax (Liu, Shao, 2005), *Burkholderia* (Mohanty, Mukherji, 2008), *Geobacillus* (Vomberg, Klinner, 2000) and *Gordonia* (Kato et al., 2009). Genes encoding the protein complex of alkanmonooxygenase CYP 153 P450 have been studied by several authors (Whyte et al., 1998; Smits et al., 1999; Kloos et al., 2006; Powell et al., 2006), molecular methods for their identification have been proposed not only in pure cultures, but also at the level of the microbial community (Wang et al., 2010).

However, the regulation of the expression of genes encoding the degradation pathways of alkanes still has many unresolved issues, due to the fact that in many cases genes of central metabolism also participate in these processes (Paisse et al., 2011). In addition, since these genes and their products are adaptive, many of them are often located in plasmids, which can contribute to their variability and horizontal transfer (Korshunova et al., 2011).

The cytochrome P450 Cyp153 family is a type of alkanmonooxygenases used for the degradation of short-chain and medium-chain *n*-alkanes and are commonly found in hydrocarbon-oxidizing bacteria lacking AlkB monooxygenases (Van Beilen, Funhoff, 2007). Oxygen-activated systems lacking this cytochrome are characteristic of prokaryotes and are formed by another integral membrane-bound monooxygenase encoded in most bacteria by the *alkB* gene, and electron transport proteins such as rubredoxin and NADH-dependent reductase encoded by the *alkG* and *alkT* genes, respectively (Van Beilen et al., 2006; Cappelletti et al., 2011). AlkB monooxygenase has been detected in bacteria of various systematic

groups and is used by them for oxidation of *n*-alkanes with a chain length up to C_{16} (Wasmund et al., 2009). Thus, *Alk*-like genes have been studied in Gram-positive bacteria such as *Rhodococcus*, *Mycobacterium*, *Nocardia* and *Praserella* (Andreoni et al., 2000; Vomberg, Klinner, 2000; Van Beilen et al., 2002; Whyte et al., 2002).

To confirm the presence of a specific *n*-alkane oxidation system and the homology degree of its sequence with the previously studied sequences of the *alkB* gene, the method of amplification of fragments of the *alkB* gene using specific primers for this gene was mainly used. Studies on the genetic and structural organization of *n*-alkane oxidation systems, regulation of their genes and the spectrum of utilized substrates were carried out only for individual strains. It should be considered that each microorganism has a certain set of inducible oxygenase systems and the ability to degrade some hydrocarbons depends on the expression of the corresponding oxygenase (Redmond et al., 2010).

The detection and determination of the activity of key genes responsible for the oxidation of certain types of hydrocarbons in oil and petroleum products is a direct proof of the use of hydrocarbons by hydrocarbon-oxidizing bacteria, and can also serve as a measure of the assessment of the metabolic activity of a particular microorganism.

The aim of the work was to detect *alkB*, *Alk1*, *Alk2*, *Alk3* and *Cyp153* genes encoding AlkB and Cyp153 alkanmonooxygenases in strains of hydrocarbon-oxidizing bacteria isolated from samples of TS-1 jet fuel and AI-95 gasoline, and to study the activity of the *alkB* gene by real-time PCR.

Materials and methods

Objects of research. In the current study, 13 strains of hydrocarbon-oxidizing bacteria isolated from TS-1 jet fuel and AI-95 gasoline (Shapiro et al., 2021) were used. The sequences of the fragment of the 16S rRNA gene of isolated strains of hydrocarbon-oxidizing bacteria are deposited in the Genbank international database (Table 1). Bacterial strains are stored in the collection of the Department of Bioengineering of the Faculty of Biology of Moscow State University. The cultures were maintained on a solid organic Rich medium containing peptone, yeast extract, casein hydrolysate, and glucose (Lysak et al., 2003), the growth of isolated strains in the presence of petroleum products was analyzed on an Evans mineral medium (Evans et al., 1970) with the addition of hydrocarbons as the only carbon source.

Isolation of bacterial DNA. DNA isolation was carried out after 7 days of cultivation of hydrocarbon-oxidizing bacteria strains on the Rich medium. To isolate bacterial DNA, the Thermo Scientific™ MagJET™ Plant Genomic DNA Kit was used as described earlier (Shapiro et al., 2021).

Assessment of the growth of pure cultures of hydrocarbon-oxidizing bacteria on a medium with model hydrocarbons. The growth of isolated cultures of hydrocarbon-oxidizing bacteria in the presence of hydrocarbons was compared using the M.V. Zhurina et al. (2008) method. 0.025 µl of culture suspension of a hydrocarbon-oxidizing bacteria strain with an optical density (OD) of 0.2 was added onto the solid EM medium containing 1.96 % by volume of a mixture of hydrocarbons No. 1 ($C_{15}H_{32}$, $C_{16}H_{34}$, $C_{18}H_{38}$ and C_9H_{12}-pseudocumol) and distributed over the surface of the Petri dish with a spatula. After 7 days, the grown colonies of microorganisms were washed off with a 1 % NaCl solution in two portions of 5 ml. In the combined sample, the optical density of the obtained cell suspension was measured using the spectrophotometer KFK-2-UHL 4.2 at $\lambda = 540$ nm and the thickness of the optical layer l = 10 mm.

Table 1. Strains of hydrocarbon-oxidizing bacteria isolated from petroleum products samples

Type of petroleum product	Bacterial strain	Genbank ID
TS-1 kerosene	*Sphingobacterium multivorum* Bi2	MG812313.1
	Alcaligenes faecalis Bi3	MG812316.1
	Rhodococcus sp. Bi4	MK951703
	Sphingobacterium sp. Bi5	MK968142
	R. erythropolis Bi6	MG871403.1
	Deinococcus sp. Bi7	MG812379.1
	Rhodococcus sp. Bi10	MG871414.1
	Sphingobacterium sp. Bi8	MK968144
	S. mizutaii Bi9	MK968143
AI-95 gasoline	*Paenibacillus agaridevorans* Bi11	MK951751
	Bacillus pumilus Bi12	MK951709
	B. safensis Bi13	MK951740
	Bacillus sp. Bi14	MK951752

Detection of alkanmonooxygenase genes *alkB*, *Alk1*, *Alk2*, *Alk3* and Cyp153. To obtain the PCR products of genes encoding various alkanmonooxygenases (Kohno et al., 2002; Ivanova et al., 2014) (the sequences of the used primers are shown in Table 2), PCR was performed with the genomic DNA of the isolated strains using the following parameters: initiation – 94 °C × 3 min, subsequent 35 cycles – 94 ° C × 30 s, 55 °C (*Cyp153*) or 60 °C (*alkB*) × 40 C, 72 ° C – 1 min; final polymerization – 72 ° C × 7 min. (Ivanova et al., 2014). For genes *Alk1-3* PCR was performed in the following mode: initial initiation – 94 °C × 3 min, subsequent 30 cycles – 94 °C × 60 s, 40 °C × 30 s, 72 °C – 30 s; final polymerization – 72 °C × 7 min (Kohno et al., 2002).

PCR was performed on a Mastercycler Gradient DNA amplifier (Eppendorf, Germany). The volume of the amplification mixture was 50 µl and had the following composition: 10 ml of 1× Taq polymerase buffer (Evrogen, Russia), 1 ml of forward and reverse primers, 1 ml of DNA of the sample and 37 ml of water. The amplification results were recorded using electrophoresis. The PCR purification of the product was carried out using the Cleanup Standard kit (Eurogen).

Real-time PCR. The real-time PCR method was used to quantify the number of DNA copies containing the functional *alkB* gene responsible for the degradation of *n*-alkanes. The measurement was carried out on a DTLite4 (DNA Technology, Russia) amplifier after 7 days of cultivation of hydrocarbon-oxidizing bacteria strains on Rich medium (Lysak et al., 2003), according to the method described in (Manucharova et al., 2021). Sequences of primers used to identify hydrocarbon-oxidizing bacteria strains with the functional gene *alkB* were as follows: F(TGGCCGGCTACTCCGATGATCGGAATCTGG); R(CGCGTGGTGATCCGAGTGCCGCTGAAGGTG) (Whyte et al., 2002).

Table 2. Sequences of primers used for the detection of *alkB*, *Alk1*, *Alk2*, *Alk3* and *Cyp153* genes encoding alkanmonooxygenases

Gene	Sequence, 5′→3′	Length of the PCR fragment, bp	Reference
Cyp153	F-GATCCGCTCGCGTGTC R-GGGAGTGAGGCGAACCA	870	Ivanova et al., 2014
alkB	F-AGAACSCRCCSGAYGAGG R-ATRTCRCCGYCRTAGTGC	960	
Alk1	F-CATAATAAAGGGCATCACCGT R-GATTTCATTCTCGAAACTCCAAAC	185	Kohno et al., 2002
Alk2	F-GAGACAAATCGTCTAAAACGTAA R-TTGTTATTATTCCAACTATGCTC	271	
Alk3	F-TCGAGCACATCCGCGGCCACCA R-CCGTAGTGCTGACGTAGTT	330	

The amount of DNA under study was expressed in absolute or relative units. Quantitative determination of the DNA matrix was carried out in the presence of three standards and negative control (a sample without a DNA matrix).

Results and discussion

Previously, strains of hydrocarbon-oxidizing bacteria were isolated from contaminated samples of petroleum products (TS-1 jet fuel and AI-95 gasoline), identified and characterized (Shapiro et al., 2021). 9 strains of hydrocarbon-oxidizing bacteria were isolated, described and identified from TS-1 fuel, and 4 strains were isolated from AI-95 gasoline.

All isolated strains of hydrocarbon-oxidizing bacteria were analyzed for the presence of genes encoding alkanmonooxygenases: *alkB*, *Cyp153*, *Alk1*, *Alk2* and *Alk3* (Table 3). The *Alk1* gene encodes alkanmonooxygenase AlkB, which catalyzes the reactions of terminal oxidation of *n*-alkanes with a chain length of C_6–C_{12} in representatives of the *Pseudomonas* genus. The *Alk2* gene encodes alkanmonooxygenase AlkB in representatives of the *Acinetobacter* genus, which catalyzes the reactions of terminal oxidation of *n*-alkanes with a chain length > C_{12} using monooxygenases or dioxygenases. The *Alk3* gene encodes alkanmonooxygenase AlkB, which has substrate specificity to *n*-alkanes and oxidase systems (Kohno et al., 2002).

It was found that the alkanmonooxygenase *Alk2* gene, typical mainly for *Acinetobacter* bacteria (Kohno et al., 2002), is absent in all bacterial strains studied. Among the strains isolated from TS-1 jet fuel, the strain *Deinococcus* sp. Bi7 did not contain the studied alkanmonooxygenase genes. All the other strains isolated from TS-1 fuel had at least one of the studied *n*-alkane degradation genes. Five strains (*Sphingobacterium multivorum* Bi2, *Alcaligenes faecalis* Bi3, *Rhodococcus* sp. Bi4, *Sphingobacterium* sp. Bi5, *Rhodococcus erythropolis* Bi6) had the *alkB* gene. In the strains of hydrocarbon-oxidizing bacteria isolated from gasoline AI-95, this alkanmonooxygenase gene was not detected (Fig. 1, see Table 3).

All the studied alkanmonooxygenase genes – *alkB*, *Cyp153*, *Alk1* and *Alk3* were identified in the strains *A. faecalis* Bi3, *Rhodococcus* sp. Bi4 and *R. erythropolis* Bi6. It is interesting that different isoforms of the *alkB* gene and the *Cyp153* gene were simultaneously present in these bacteria, and genes *alkB*, *Cyp153* – in the strains of *Sphingobacterium multivorum* Bi2 and *S. mizutaii* Bi9. According to the resent data, the enzyme Cyp153 is a type of alkanmonooxygenase involved in the degradation of short-chain and medium-chain *n*-alkanes in hydrocarbon-oxidizing bacteria that do not have alkB alkanmonooxygenases (Van Beilen, Funhoff, 2007).

n-alkanes account for up to 88 % of the volume in natural oil and petroleum products and can serve as an energy source for microorganisms capable of decomposing them (Van Beilen et al., 2003; Dedov et al., 2017). The detection of alkanmonooxygenase group genes was previously carried out for bacterial communities isolated from petroleum products, and the activity of strains against the degradation of various hydrocarbons, including *n*-alkanes, was shown (Likhoshvay et al., 2014; Lomakina et al., 2014).

alkB family genes are usually present in the genomes of both gram-positive and gram-negative bacteria in several variants (Van Beilen et al., 2003). This is consistent with the data obtained by us on the presence of several *alkB* family genes in isolated strains of gram-negative bacteria of the *Sphingobacterium* genus and gram-positive bacteria of the *Rhodococcus* genus.

The ability to degrade *n*-alkanes in strains for which this has not been described in the literature before may be evidence of the gene localization in the plasmid and its horizontal transfer between community members, which was shown in the works of T.P. Turova et al. (2008), where bacteria of the *Geobacillus* genus could acquire *alkB* genes from bacteria of the *Rhodococcus* genus.

Among the strains of hydrocarbon-oxidizing bacteria isolated from AI-95 gasoline, only the *Cyp153* gene was detected in *P. agaridevorans* Bi11.

The data on the presence of *alkB* family genes in the studied bacterial strains only partially agreed with the data on their ability to grow on liquid and solid media in the presence of 1 % *n*-alkanes with different carbon chains length (Shapiro et al., 2021). Thus, strains *Sphingobacterium mizutaii* Bi9, *Bacillus pumilus* Bi12; *Bacillus safensis* Bi13; *Bacillus* sp. Bi14; *Paenibacillus agaridevorans* Bi11 grew on a model mixture of hydrocarbons containing alkanes with different chain lengths, TS-1 fuel and oil (Fig. 2 and 3). Also, in some cases, the ability to grow and the high activity of the isolated

Table 3. The presence of the studied alkanmonooxygenase genes for the oxidation of hydrocarbons *alkB*, *Cyp153*, *Alk1*, *Alk2* and *Alk3* in strains of hydrocarbon-oxidizing bacteria isolated from petroleum products

Strain/gene	Bi2*	Bi3	Bi4	Bi5	Bi6	Bi7	Bi8	Bi9	Bi10	Bi11	Bi12	Bi13	Bi14
alkB	+	+	+	–	+	–	–	+	–	–	–	–	–
Cyp153	+	+	+	+	+	–	+	+	+	+	–	–	–
Alk1	–	+	+	–	+	–	–	–	–	–	–	–	–
Alk3	–	+	+	–	+	–	–	–	–	–	–	–	–

* Full names of the strains are given in Table 1.

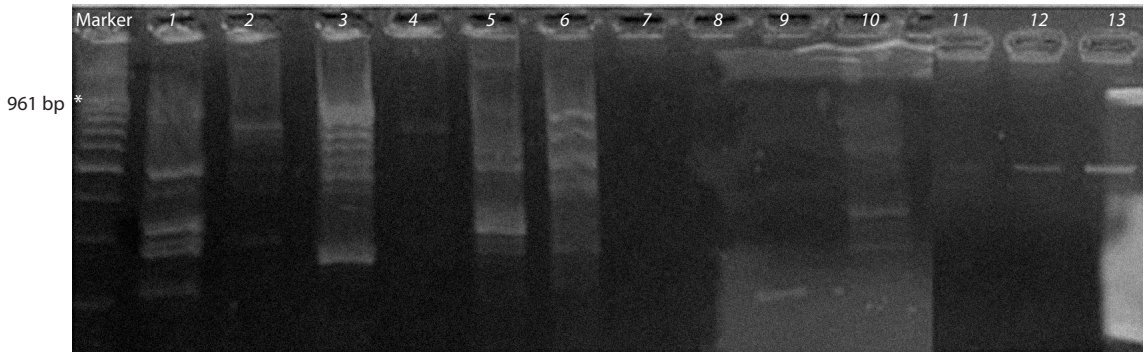

Fig. 1. Electrophoresis in agarose gel of the PCR product of the *alkB* gene.

1 – Sphingobacterium multivorum Bi2; *2 – Alcaligenes faecalis* Bi3; *3 – Rhodococcus* sp. Bi4; *4 – Sphingobacterium* sp. Bi5; *5 – Rhodococcus erythropolis* Bi6; *6 – Deinococcus* sp. Bi7; *7 – Sphingobacterium* sp. Bi8; *8 – Sphingobacterium mizutaii*; *9 – Rhodococcus* sp. Bi10; *10 – Bacillus pumilus* Bi12; *11 – Bacillus safensis* Bi13; *12 – Bacillus* sp. Bi14; *13 – Paenibacillus agaridevorans* Bi11. DNA length marker (100 + bp DNA Ladder). * – is the expected length of the target PCR product.

strains in degrading *n*-alkanes of the model hydrocarbon mixture in the absence of this gene were established (see Fig. 3).

Growth evaluation of hydrocarbon-oxidizing bacteria pure cultures on a solid EM medium with a model mixture of hydrocarbons (see Fig. 3) allowed us to divide the strains by growth rate into three groups. This division was proposed by us and is based on the following: group 1 (active cultures) – the value of the optical density of the cell suspension after cultivation for 7 days from 3 units and above; group 2 (medium activity) – from 2 to 3 units; group 3 (low activity) – the value of the optical density of the cell suspension less than 2 units.

It was found that the most active group of strains capable of using a model mixture of hydrocarbons included strains *R. erythropolis* Bi6, *Rhodococcus* sp. Bi10. The average growth rate is typical for strains *Deinococcus* sp. Bi7, *Sphingobacterium* sp. Bi5, *S. multivorum* Bi2 and *Sphingobacterium* sp. Bi8. At the same time, the tested alkanmonooxygenase genes were not detected in the strain *Deinococcus* sp. Bi7. The strains *Rhodococcus* sp. Bi4, *S. mizutaii* Bi9, *Ochrobactrum* sp. Bi1 and *A. faecalis* Bi3 and all strains isolated from gasoline had the slowest growth in the presence of a model mixture of hydrocarbons. At the same time, bacterial strains isolated from gasoline AI-95 – *Bacillus safensis* Bi13; *Bacillus* sp. Bi14, in which the *alkB* gene was not detected, used pentadecane, octadecane and hexadecane of a model mixture by more than 80 % (Shapiro et al., 2021). In this regard, a quantitative analysis of the number of DNA copies containing the functional alkanmonooxygenase gene in all isolated strains of hydrocarbon-oxidizing bacteria was carried out. Based on the results of real-time PCR, it was found that the *alkB* gene is present and active in all bacterial strains isolated from petroleum products.

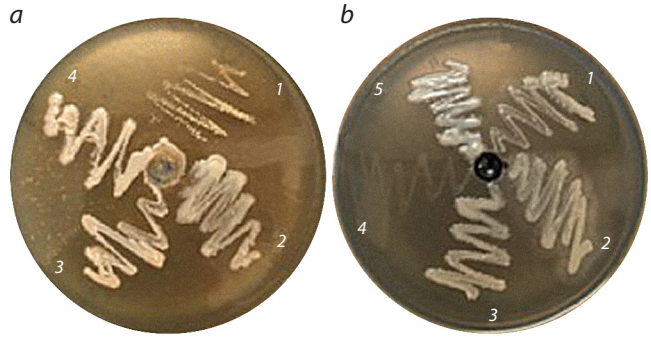

Fig. 2. Growth of the several studied cultures on the Evans mineral medium in the presence of TS-1 jet fuel (*a*) and oil (*b*).

(*a*) *1 – Sphingobacterium multivorum* Bi2; *2 – Sphingobacterium* sp. Bi5; *3 – Rhodococcus* sp. Bi4; *4 – Alcaligenes faecalis* Bi3; (*b*) *1 – Rhodococcus* sp. Bi4; *2 – Sphingobacterium mizutaii* Bi9; *3 – Sphingobacterium* sp. Bi8; *4 – Deinococcus* sp. Bi7; *5 – Rhodococcus erythropolis* Bi6.

According to the number of copies of the gene, all bacterial strains were divided into two groups: the first group with the highest activity of the *alkB* gene, for which the concentration

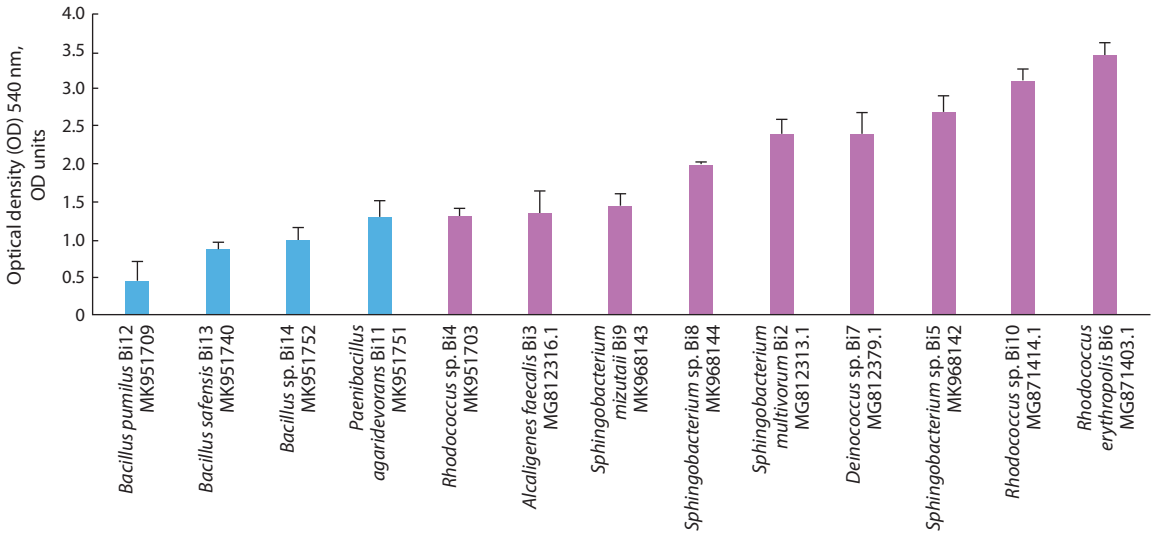

Fig. 3. Growth of isolated bacterial strains from AI-95 gasoline (blue columns) and TS-1 jet fuel (pink columns) on a solid medium with a mixture of hydrocarbons No. 1 for seven days.

values ranged from 1290 to 8060 DNA copies/ml, and the second group, where the concentration values were from 10.4 to 786 DNA copies/ml:

I group	II group
Alcaligenes faecalis Bi3	*Rhodococcus* sp. Bi4
Sphingobacterium multivorum Bi2	*Rhodococcus erythropolis* Bi6
Sphingobacterium mizutaii Bi9	*Rhodococcus* sp. Bi10
Sphingobacterium sp. Bi5	*Sphingobacterium* sp. Bi8
Paenibacillus agaridevorans Bi11	*Deinococcus* sp. Bi7
	Bacillus pumilus Bi12
	Bacillus safensis Bi13
	Bacillus sp. Bi14

It was found that all strains of hydrocarbon-oxidizing bacteria isolated from gasoline AI-95 showed the activity of the *alkB* gene, and the strain *Paenibacillus agaridevorans* Bi11 was assigned to the first group of strains with a high level of its activity (1290 DNA copies/ml). The results obtained were consistent with the data on the ability of strains isolated from petroleum products to grow (see Fig. 3) and use hydrocarbons of a model mixture of hydrocarbons (Shapiro et al., 2021). There were also coincidences of the results on the distribution of strains of hydrocarbon-oxidizing bacteria in groups based on the activity of the *alkB* gene (see Table 2) and groups formed on the basis of their growth ability and the use of a model mixture of hydrocarbons and petroleum products (Shapiro et al., 2021).

In bacteria growing on petroleum products, including both short-chain and long-chain *n*-alkanes, their oxidation system includes several isoenzymes of the key protein alkanmonooxygenase. The strains of bacteria isolated from TS-1 jet fuel and AI-95 gasoline are capable of using a wide range of substrates, which suggests that they have a complex alkanmonooxygenase system. It has been established that representatives of different groups of hydrocarbon destructor microorganisms may have several evolutionary variants of alkanmonooxygenase enzymes, which requires the selection of primer sets for different hydrocarbon-oxidizing bacteria that allow the identification of all variants of hydrocarbon oxygenase genes. In such cases, it is proposed to apply several variants of primers to different groups of isoenzymes (Kohno et al., 2002; Heiss-Blanquet et al., 2005). In our work, two types of primers were used to detect the presence and activity of the *alkB* gene. The detection of the *alkB* gene with primers proposed in the article by A.E. Ivanova and co-authors (2014) showed the presence of this gene in five bacterial strains, and with primers by L.G. Whyte and co-authors (2002) – in all studied strains of petroleum products destructors. This may indicate the greater versatility of the primers proposed by L.G. Whyte and co-author (2002), on the one hand, or the presence of a specific isoform of the enzyme, on the other.

Conclusion

Thus, real-time PCR revealed the activity of the *alkB* gene in all strains of hydrocarbon-oxidizing bacteria isolated from TS-1 jet fuel and AI-95 gasoline. A significant quantitative difference in the activity of this gene in the isolated strains was shown. For strains isolated from gasoline, the activity data correspond to physiological and biochemical data on bacterial growth in the presence of a model mixture of hydrocarbons and the efficiency of their degradation (Shapiro et al., 2021). The results obtained indicate the need to use a set of methods (a polyphase approach) for a comprehensive assessment of the ability of hydrocarbon-oxidizing bacteria strains to degrade petroleum hydrocarbons, including the usage of molecular (in particular, PCR) and physiological methods to analyze the distribution and homology of the specific studied gene in bacteria.

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A characteristic of the species composition of pathogenic fungi of the genus *Fusarium* in corn biocenoses of the Voronezh region

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Abstract. Corn is one of the main crops of modern world agriculture. It ranks first in terms of gross grain harvests and second in terms of acreage, ceding only to the main grain crop of the globe, wheat. The problem of increasing the production of grain and green mass of corn remains one of the urgent tasks of agricultural production. High potential yields very often remain untapped due to diseases, direct losses from which are estimated at 20–50 %. The purpose of this work was to study the species composition of micromycetes on corn collected in different phases of vegetation in May-July 2020 in the Voronezh region, to identify phytopathogenic genus *Fusarium* fungi, to study pathogenic and phytotoxic strains of the fungi to replenish the collection of the All-Russian Scientific Research Institute of a Phytopathology. Preservation of infectious material of fungi from the genus *Fusarium* is of no small importance for phytopathological, immunological, breeding, genetic and toxicological studies. As a result of the mycological studies carried out, a lot of fungi isolates from the genera *Fusarium*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhizopus*, *Periconia*, *Pythium*, *Trichothecium*, etc., isolated from the affected roots, stems and ears of corn in the Voronezh region in 2020 were identified. Fungi isolates from seven taxonomic groups: *Fusarium fujikuroi* Nirenberg (*F. moniliforme*, *F. verticillioides*), *Fusarium oxysporum* Schltdl., *Fusarium culmorum* (Wm.G. Sm.) Sacc., *Fusarium graminearum* Schwabe, *Fusarium heterosporum* Nees & T. Nees (*F. lolii*), *Fusarium roseum* Link (*F. sambucinum*), *Fusarium sporotrichioides* Sherb. were tested for pathogenicity and phytotoxicity on seedlings of plant-testers. It has been shown that pathogenic and phytotoxic activity in fungi varies significantly between *Fusarium* species and within the same species. The greatest danger to corn is represented by the species *F. sporotrichioides*, *F. graminearum*, *F. culmorum*, *F. fujikuroi*, *F. oxysporum*, *F. heterosporum*, which have a high intensity of phytotoxic activity associated with the fact that they contribute to the synthesis and accumulation of dangerous toxins in plant tissues. As a result of the conducted studies, 55 strains of fungi from the genus *Fusarium* belonging to seven species were selected. The isolates, stable in morphological and cultural characteristics and studied for pathogenicity and toxicity, were placed for long-term storage in the Russian State Collection of Plant Pathogenic Microorganisms and Cultivars for Identification of Phytopathogenic Microbial Strains at the All-Russian Scientific Research Institute of a Phytopathology.
Key words: collections of microorganism; micromycetes; genetic diversity; corn; plant pathogens; *Fusarium*.

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Особенности видового состава патогенных грибов рода *Fusarium* в биоценозах кукурузы Воронежской области

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Аннотация. Кукуруза относится к основным культурам современного мирового земледелия. Она стоит на первом месте по валовым сборам зерна и на втором – по посевным площадям, уступая лишь основной хлебной культуре земного шара – пшенице. Одна из актуальных задач аграрного производства – проблема увеличения валового сбора зерна и зеленой массы кукурузы. Высокая потенциальная урожайность очень часто остается нереализованной вследствие развития болезней, прямые потери от которых оцениваются в 20–50 %. Цель настоящей работы – изучение видового состава микромицетов на растениях кукурузы, собранных в разные фазы вегетации в мае-июле 2020 г. в Воронежской области, идентификация фитопатогенных грибов из рода *Fusarium*, выявление патогенных и фитотоксичных штаммов грибов рода *Fusarium* для пополнения коллекции Всероссийского научно-исследовательского института фитопатологии. Сохранение инфекционного материала грибов из рода *Fusarium* имеет немаловажное значение для фитопатологических, иммунологических, селекционных, генетических и токсикологических исследований. В результате проведенного микологического анализа обнаружено большое количество изолятов грибов из родов *Fusarium*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhizopus*, *Periconia*, *Pythium*, *Trichothecium* и др., выделенных из пораженных корней, стеблей и початков кукурузы

в Воронежской области в 2020 г. Изоляты грибов из семи таксономических групп: *Fusarium fujikuroi* Nirenberg (*F. moniliforme*, *F. verticillioides*), *Fusarium oxysporum* Schltdl., *Fusarium culmorum* (Wm.G. Sm.) Sacc., *Fusarium graminearum* Schwabe, *Fusarium heterosporum* Nees & T. Nees (*F. lolii*), *Fusarium roseum* Link (*F. sambucinum*), *Fusarium sporotrichioides* Sherb., были проверены по патогенности и фитотоксичности на всходах растений-тестеров. Показано, что патогенная и фитотоксичная активность у грибов как между видами *Fusarium*, так и в пределах одного вида существенно различается. Наибольшую опасность для кукурузы представляют виды *F. sporotrichioides*, *F. graminearum*, *F. culmorum*, *F. fujikuroi*, *F. oxysporum*, *F. heterosporum*, обладающие высокой интенсивностью проявления фитотоксической активности, связанной с тем, что они способствуют синтезу и накоплению опасных токсинов в тканях растений. В результате проведенных исследований отобрано 55 штаммов грибов из рода *Fusarium*, относящихся к семи видам. Стабильные по морфолого-культуральным признакам и изученные по патогенности и фитотоксичности изоляты помещены на длительное хранение в Государственную коллекцию фитопатогенных микроорганизмов и сортов растений-идентификаторов патогенных штаммов микроорганизмов, созданную на базе Всероссийского научно-исследовательского института фитопатологии.
Ключевые слова: коллекции микроорганизмов; микромицеты; генетическое разнообразие; кукуруза; фитопатогены; *Fusarium*.

Introduction

Along with the fact that sugar corn (lat. *Zéa máys* L. ssp. *mays*) is the only cultural representative of the genus Corn (Zea) of the Cereal family (Poaceae) and the oldest bread plant in the world, it remains one of the most popular in the realities of modern agriculture (Sotchenko, 2005). Corn ranks first in terms of gross grain harvests and second in terms of acreage, second only to the main grain crop of the globe – wheat. The USA (about half of the world harvest), China, Brazil, Mexico, France, Argentina, India, Indonesia, Italy and Romania are the largest producers of corn (Babich, 1986; Berezkin, Malko, 1998; Elmore, Abendroth, 2008). Corn is cultivated mainly in the southern regions of Russia (Suprunov, 2009).

Due to the high yield and useful qualities of corn, its importance for versatile human use can hardly be overestimated. More than 20 % of corn grain is used for food purposes in the countries of the world, 15–20 % – for technical purposes, and about two thirds – for livestock feed (Sotchenko, 2009).

As a food crop, corn ranks third in the world in terms of acreage giving way only to wheat and rice. And in terms of grain yield, it has a leading position. Corn grain contains 65–70 % carbohydrates, 9–12 % protein, 4–8 % vegetable oil (up to 40 % in the embryo) and only about 2 % fiber. Corn grain contains vitamins A, B1, B2, B6, E, C, D, F, essential amino acids, mineral salts and trace elements. Corn grain is used for food and medical purposes (Sotchenko, 2002).

In the modern world, fodder corn yields large harvests and highly nutritious feed which makes it crucial in the development of husbandry. Corn plays the main role in the feed balance because of its caloric characteristics and the possibility of using both corn grains and its green mass – silage (https//universityagro.ru/растениеводство/кукуруза/; Ivashchenko, Sotchenko, 2006; Sotchenko, Gorbacheva, 2011).

Corn is also of great importance for industry. Corn oil is a raw material for the production of expensive paints, soaps and rubber substitutes. Corn starch is used for dressing fabrics and leather, increasing the density and smoothness of paper, in the production of viscose fiber, explosives, dextrin glue. Construction and packaging materials, paper, soil improving additives, explosives are obtained from stems and other vegetative parts of plants. Furfural, a raw material for the production of plastics, nylon and other synthetic substances, is isolated from the stalks of corn cobs (https//universityagro.ru/растениеводство/кукуруза/).

The problem of increasing corn grain production remains one of the urgent tasks of agricultural production (Sotchenko, 2005). In Russia, high potential yield of corn often remains unrealized due to the development of diseases, among which the main role belongs to micromycetes from the genera *Fusarium*, *Bipolaris*, *Alternaria*, etc. Direct grain losses from *Fusarium* root and ear rot in Corn at 20–50 % (Ivashchenko, 2007, 2012).

Fusarium root and ear rot are widespread corn diseases, especially in areas with high humidity. Up to 50–60 % of corn crops are affected. A large group of maize diseases are fungi from the genus *Fusarium*: *Fusarium acuminatum* Ellis & Everh, *F. culmorum* (W.G. Sm.) Sacc., *F. equiseti* (Corda) Sacc., *F. gibbosum* Appel. et Wollenw., *F. graminearum* Schwabe, *F. heterosporum* Nees & T. Nees, *F. oxysporum* f. *conglutinans* (Wollenw.) W.C. Snyder & H.N. Hansen, *F. oxysporum* f. *cucumerinum* Berk. & Broome, *F. poae* (Peck) Wollenw., *F. roseum* Link, *F. solani* (Mart.) Sacc., *F. sporotrichioides* Sherb. and others (Ali et al., 2005; Eller et al., 2008).

Fusarium ear rot in Corn caused by the hemibiotrophic fungus *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *Fusarium moniliforme* J. Sheld., marsupial stage – *Gibberella fujikuroi*) leads to a decrease in yield and deterioration of its quality (Miller et al., 2007; Murillo-Williams, Munkvold, 2008; Mesterhasy, Lemmens, Reid, 2012). The fungus produces fumonisins when storing cobs in conditions of high humidity and insufficient aeration. These toxins are carcinogenic to humans and animals (Clements, White, 2004; Robertson-Hoyt et al., 2007).

As for the species that cause root rot, low temperature during seed germination, increased humidity and soil acidity increase the development of the disease (Suprunov, 2009). At the same time, a weak pink or white fungus bloom forms on the surface of the germinating grain. Soon after the corn plants come to the surface, the sprout turns brown and dies. If the sprout survives, then it has a poorly developed root system, plants are delayed in growth, leaves dry up, often lie down (Ivashchenko et al., 2006).

Since the pathogens of *Fusarium* root and ear rot reside in the soil and grain, the question of studying the range of

the most pathogenic micromycetes, including those from the genus *Fusarium*, is relevant for the development of environmentally friendly methods of combating them, including the creation of disease-resistant varieties and hybrids of corn (Hooker, 1967; Ivashchenko, 2009a; Ivashchenko, Matveeva, 2010). Particular attention is paid to the creation of infectious backgrounds, where measures are carried out to assess and select resistant forms (Ivashchenko, 2007, 2009b). The preparations of compositions of infectious backgrounds, which includes the study of the species composition of corn micromycetes, the identification of the most pathogenic isolates of fungi of the genus *Fusarium* and the creation of conditions for their long-term storage without loss of pathogenic properties, are also of importance.

Mycological analysis of maize samples and analysis of the scientific literature related to the issue under development indicates that monitoring the species composition of fungi on the cultivated crop is currently very relevant both for taking urgent preventive and health measures and for developing a strategy to prevent negative consequences from the development of diseases. Researches aimed at studying the species composition of micromycetes that cause *Fusarium* root and ear rot ultimately determine the possibility of obtaining environmentally friendly and stable corn crops.

Preservation of infectious material of fungi from the genus *Fusarium* is important for phytopathological, immunological, breeding, genetic and toxicological studies (Dubovoy et al., 2016; Kolomiets et al., 2018; Kolomiets, Zhemchuzhina, 2018). The State Collection of Phytopathogenic Microorganisms and Plant Varieties, identifiers of pathogenic strains of microorganisms of the All-Russian Scientific Research Institute of Phytopathology (GKFM VNIIF), was created to solve the tasks set in accordance with Federal Law No. 7-F3 of 10.01.02 (ed. of 24.11.2014, with amendments dated 29.12.2014) "On Environmental Protection", Decree of the Government of the Russian Federation No. 725-47 dated 24.06.1996 "On Measures for the Conservation and Rational Use of Collections of Microorganisms, Cultivated Cells of Higher Plants, Transplanted Somatic Cells of Vertebrates", as well as taking into account the provisions of the Convention on Biological Diversity (1992) and the recommendations of the European Organization for Economic and Social Development (GENERAL GUIDELINES FOR ALL BRCS, 2006; GUIDANCE FOR THE OPERATION OF BIOLOGICAL RESOURCE CENTERS (Part 2: Micro-Organization Domain), 2006; OECD Best Practice Guidelines for BRCs 2007). It is a State Depository of phytopathogenic microorganisms.

As for the creation of a collection of fungi from the genus *Fusarium*, its main tasks were not only to preserve the viability and genetic stability of strains of these fungi according to morphological and cultural characteristics for a long time, but also to replenish the fund with new species with a different spectrum of pathogenicity and phytotoxicity properties, as well as to expand the range of geographical areas for collecting affected maize samples (Gagkaeva, Levitin, 2005; Gagkaeva et al., 2008). To fulfill these tasks, samples of infected plants received annually from various regions of the country are subjected to mycological studies, and based on the data of the analysis of the material, the most pathogenic and phytotoxic samples are selected for the collection.

The purpose of this work was to study the species composition of micromycetes on corn plants collected in different phases of vegetation in May–July 2020 in the Voronezh region, to identify pathogenic and phytotoxic strains of fungi of the genus *Fusarium* to replenish the collection of the ARSRIP.

Materials and methods

Maize plants with various signs of fungal infections on leaves, stems and roots served as the material for research. Samples of zoned varieties of corn (Ajaks, Donskaya visokoroslaya, Zernogradsky) were collected in different phases of vegetation: the formation of 5–6 leaves – f.2, according to the classification of phenological development according to the system of BBCH, tubing, or the formation of 8–10 leaves – f.32, filling – milk ripeness – f.75 (Large, 1954; Lancashire et al., 1991). Research was carried out using the equipment of the Collective Use Center of the Russian State Collection of Plant Pathogenic Microorganisms and Cultivars for Identification of Phytopathogenic Microbial Strains at the All-Russian Scientific Research Institute of a Phytopathology (http://www.vniif.ru/vniif/page/ckp-gkmf/1373).

The phytosanitary condition of the samples was assessed according to methods generally accepted in phytopathology (Gerlach, Nirenberg, 1982; Leslie, Summerell, 2006; Dictionary..., 2008; Watanabe, 2010). Fungal species were determined by the morphology of spores under a microscope ×400 (Bilai, 1977; Bilai, Ellanskaya, 1982; Gagkaeva et al., 2008).

The isolation of hemibiotrophic and saprophytic micromycetes from the affected plants was carried out using potato-glucose and potato-carrot agar-agars. Fungi from plant samples were isolated according to the standard method (Bilai, 1977; Bilai, Ellanskaya, 1982). The diseased plants of each sample were washed with tap water and then were cut into fragments 5–10 mm in size, sterilized in 50 % alcohol for 20–30 seconds and, under aseptic conditions, were laid out on the surface of 2 % potato-glucose agar-agar in Petri dishes (4–6 fragments in each). Each sample was represented by at least 150–200 fragments of the affected tissue. Petri dishes were placed in a thermostat with a temperature of 22–24 °C. The development of fungi was monitored daily. As the colonies of fungi grew, a piece of mycelium was sifted onto the nutrient medium in the center of the Petri dish. Cultures of fungi were viewed under a microscope. Fungal species were identified by the main morphological features of colonies and spores: by growth rate, mycelium color and structure, pigmentation; by shape, size of apical and basal cells of macroconidia, by the presence of microconidia. An average microscopy index of 300 conidia was taken to estimate the size of macroconidia.

Determinants were used as reference literature when determining the species of the fungus (Gerlach, Nirenberg, 1982; Bilai, Kurbatskaya, 1990; Leslie, Summerell, 2006; Dictionary..., 2008; Watanabe, 2010). The current taxonomic status of the selected *Fusarium* species was clarified at http://www.indexfungorum.org.

The frequency of occurrence of original *Fusarium* species in samples of affected plants as a percentage was determined by the formula

$$P = (100 \times n) / N,$$

where P is the frequency of occurrence of the species in the population (in %); N is the total number of isolates of fungi of

the genus *Fusarium* in the sample; *n* is the number of isolates of a certain type of *Fusarium* in the sample.

Isolates of fungi isolated from the affected corn samples were placed for storage in the laboratory of the Russian State Collection of Plant Pathogenic Microorganisms and Cultivars for Identification of Phytopathogenic Microbial Strains at the All-Russian Scientific Research Institute of a Phytopathology. Isolates have been stored in refrigerators at a temperature of 7–10 °C in biological test tubes on slants of nutrient medium – potato-glucose agar-agar (Bilai, Ellanskaya, 1982).

Pathogenic and toxic properties of strains were studied using the method of bioassay on seeds. The pathogenicity of spore suspensions and phytotoxicity of filtrates of culture fluids (FCF) of fungi were tested on wheat seeds (cv. Mironovskaya 808). The degree of pathogenicity and toxicity of strains was judged by the effect of suspensions of conidia and FCF on seed germination, the development of germ and primary roots of wheat, but the main indicator was the length of the roots.

The degree of pathogenicity and toxicity was determined on the 5th day from the beginning of seed germination. If the length of seedlings and roots (in mm) in the experimental version was 0–30 % of the length of the control, then this indicated a strong pathogenic (P) and strong toxic (T) activity of the fungus; 31–50 % – moderate pathogenicity (MP) and moderate toxicity (MT); 51–70 % – weak pathogenicity (WP) and weak toxicity (WT); 71–100 % – non-pathogenic (NP) and non-toxic (NT) properties of isolates. The length of the sprouts and primary roots of seeds germinated in water was considered as a control and was taken as 100 % (Parfenova, Alekseeva, 1995).

Results and discussion

Mycological studies of the analyzed maize plants collected in different phases of vegetation (formation of 5–6 leaves, tube formation, milk ripeness) showed the presence of micromycetes on them, related to both phytopathogens and saprotrophs. In total, more than 30 species of micromycetes were isolated and identified from corn samples.

Saprotrophic species of fungi from the genera *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhizopus*, *Periconia*, *Pythium*, *Trichothecium*, etc. prevailed on the tissues of the roots and basal areas of corn stalks (Table 1). Heterotrophic species of fungi were more often found on the leaves of the samples. Almost half (1600 units) of the fungi isolates identified from the leaves and roots belonged to the genera *Alternaria*, *Bipolaris*, *Exserohilum* and *Fusarium*. It should be noted that the frequency of occurrence of fungi *Alternaria* spp. depended on the phenological phase of corn plants. So, in the phase of formation of 5–6 leaves, fungi of this genus were significantly more often isolated from the tissues of corn roots, in the phase of milk ripeness – from the leaves.

Symptomatic analysis of maize samples revealed signs of infection with the pathogen *Exserohilum turcicum* (Pass.) K.J. Leonard & E.G. Suggs (=*Setosphaeria turcica*). On the leaves of the corn of the lower tier, large spots were noted, gray in the center and with darker edges with a sooty coating. Samples with such signs were found in the phase of tube formation and milk ripeness, the intensity of their lesion was low and ranged from 1 to 20 % of the leaf area of the lower tier.

Isolates of *Bipolaris sorokiniana* Shoemaker (*Cochliobolus sativus*) were mainly found on the roots and basal part of corn stalks during the 5–6 leaf formation phase. The fungus was not identified on the leaves during this and later phenophases.

The manifestation of diseases caused by fungi from the genus *Fusarium* had similar symptoms. As a rule, brown or yellow areas were noted on the leaves, stems, basal neck and roots of corn, often with signs of maceration or rottenness. The study of samples of the affected tissues of maize plants in culture allowed to isolate more than 900 isolates of the genus *Fusarium* into a culture and identify by morphological characteristics (colony growth rate, mycelium color and structure), the presence, shape and size of macroconidia and microconidia (if present) the following 11 species of this genus: *F. culmorum*, *F. gibbosum*, *F. graminearum*, *F. heterosporum*, *F. fujikuroi*, *F. incarnatum*, *F. oxysporum*, *F. poae*, *F. roseum*, *F. sporotrichioides*, *F. solani* (Table 2). In some cases, more than one or two micromycetes from the genus *Fusarium* were isolated from one sample of affected corn tissue. This was especially often noted when *F. oxysporum* was isolated into culture, which, as a rule, was accompanied by the species *F. roseum*, *F. poae*, *F. solani*, etc.

The frequency of occurrence of fungi from the genus *Fusarium* was ambiguous and varied markedly depending on the phase of the growing season of corn and possibly the prevailing weather conditions of the season. The species *F. heterosporum* and *F. oxysporum* were most often found in the complex of micromycetes from the genus *Fusarium* on corn crops in the Voronezh region. The total share of these two species was half of all identified isolates belonging to other species of this genus. Nevertheless, fluctuations in the frequency of occurrence of these types of fungi were observed in all phenological phases of corn development. When assessing the frequency of occurrence of species from the genus *Fusarium* in the phase of milk ripeness, it was noticed that the proportion of *F. heterosporum* and *F. oxysporum* isolates decreased by 1.5–2 times (see Table 2). It should also be pointed out that *F. heterosporum* isolates were more often isolated from affected corn roots, and *F. oxysporum*, from stems.

In mycological studies of corn tissues, isolates of *F. fujikuroi* were found in all variants of the experiment. The frequency of occurrence of the fungus gradually changed from low (6.5 %) in the phase of formation of 5–6 leaves to high (19.4 %) in the phase of milk ripeness. Probably, over time, more favorable conditions for the accumulation of *F. fujikuroi* in the soil and on maize plants had been created. A similar pattern was observed for the species *F. poae* and *F. sporotrichioides*, the frequency of occurrence of which varied significantly from the phase of formation of 5–6 leaves to the phase of milk ripeness, respectively, from low (0 and 5.6 %) to high (11.7 and 15.1 %).

As for *F. culmorum*, there were no significant fluctuations in the frequency of occurrence of the fungus on maize samples in different phenological phases. This indicates a sufficiently high viability of the micromycete, which occupies a certain niche in the *Fusarium* spp. pathocomplex. As a rule, macroconidia of the fungus were detected on the affected samples from the roots and leaves of the lower tier.

Species of *F. roseum*, *F. solani*, *F. graminearum*, *F. gibbosum*, *F. incarnatum* in the pathogenic complex of the Fusarium

Table 1. Micromycetes found on corn crops in the Voronezh region in 2020

Species	Phenological phases of development		
	Formation of 5–6 leaves	Tube formation	Milk ripeness
Acremonium sp.	++	++	+++
Alternaria alternata (Fr.) Keissl.	+++	+++	+++
Alternaria tenuissima (Kunze) Wiltshire	+++	+++	+++
Aspergillus ustus (Bainier) Thom & Church	++	+++	++
Aspergillus flavus Link	+	+++	++
Aspergillus niger Tiegh.	+++	+++	++
Bipolaris sorokiniana Shoemaker	+++	+	++
Botrytis cinerea Pers.	++	++	+++
Cladosporium herbarum (Pers.) Link	++	+	++
Cladosporium cladosporioides (Fresen.) G.A. de Vries	+++	++	+++
Cephalosporium sp.	+	+	++
Chaetomium murorum Corda	+++	+++	+++
Curvularia sp.	+	+	+
Gliocladium sp.	+	+	++
Exserohilum turcicum (Pass.) K.J. Leonard & E.G. Suggs	–	+	++
Fusarium culmorum (Wm.G. Sm.) Sacc.	++	++	+++
Fusarium gibbosum Appel & Wollenw.	+	+	++
Fusarium graminearum Schwabe	+	++	+
Fusarium heterosporum Nees & T. Nees	+++	+++	+++
Fusarium fujikuroi Nirenberg	++	+++	+++
Fusarium incarnatum (Desm.) Sacc.	–	–	+
Fusarium oxysporum Schltdl.	+++	+++	+++
Fusarium poae (Peck) Wollenw.	–	+	+++
Fusarium roseum Link	+	+++	++
Fusarium solani (Mart.) Sacc.	–	++	++
Fusarium sporotrichioides Sherb.	++	++	+++
Mucor mucedo Fresen.	+++	+++	+++
Nigrospora sp.	++	++	+++
Penicillium sp.	+++	+++	+++
Periconia sp.	++	++	++
Pythium sp.	++	++	+++
Rhizopus stolonifer (Ehrenb.) Vuill	+++	+++	++
Talaromyces luteus (Zukal) C.R. Benj.	+	+	+
Trichoderma sp.	++	+	+
Trichothecium roseum (Pers.) Link	++	+++	+++
Sterile mycelium	++	+++	+++

Note. "+" – from 1 to 10 fungus isolates; "++" – from 11 to 20 fungus isolates; "+++" – above 20 fungus isolates.

Table 2. Frequency of occurrence of *Fusarium* species detected on the affected maize samples from the Voronezh region in 2020

Species	Phenological phases of development						Frequency of occurrence	
	Formation of 5–6 leaves		Tube formation		Milk ripeness			
	Units	%	Units	%	Units	%	Units	%
F. culmorum	28	13.0	22	6.9	31	8.2	81	8.9
F. gibbosum	7	3.3	0	0	12	3.2	19	2.1
F. graminearum	5	2.3	12	3.8	5	1.3	22	2.4
F. heterosporum	66	30.7	108	33.7	68	18.6	242	26.5
F. fujikuroi	14	6.5	32	10.0	73	19.9	119	13.0
F. incarnatum	0	0	0	0	7	1.8	7	0.8
F. oxysporum	81	37.7	76	23.8	51	13.5	208	22.8
F. poae	0	0	7	2.2	44	11.7	51	5.6
F. roseum	2	0.9	25	7.8	12	3.2	39	4.3
F. solani	0	0	20	6.2	17	4.5	37	4.0
F. sporotrichioides	12	5.6	18	5.6	57	15.1	87	9.6
Number of isolates	215	100	320	100	377	100	912	100

fungi on corn were quite rare. Basically, isolates of these micromycetes were determined on the affected roots and the root zone of the stems. It is possible that either these types of fungi do not play a significant role in the pathogenesis of corn, or there were no conditions for their development.

As a result of mycological studies, biological material was obtained represented by a large number of fungal isolates: 11 species from the genus *Fusarium*. Of them, 55 isolates of fungi from 7 taxonomic groups (*F. fujikuroi*, *F. oxysporum*, *F. culmorum*, *F. graminearum*, *F. heterosporum*, *F. roseum*, *F. sporotrichioides*) were tested for pathogenicity and phytotoxicity on seedlings of testers.

Table 3 shows the results of assessing the effect of metabolites of spore suspensions and filtrates of culture fluids of fungal isolates of the most pathogenic and phytotoxic species of the genus *Fusarium* on the development of wheat seedlings of cv. Mironovskaya 808 (seed germination, length of the sprout and roots). It was shown that isolates of fungi from the genus *Fusarium* represented by different species had a wide intraspecific diversity in the studied characteristics. Within the same species, there were strains of the fungus belonging to different categories – from pathogenic/toxic to non-pathogenic/non-toxic (see the Figure).

The species *F. sporotrichioides* and *F. graminearum* showed high phytotoxic and pathogenic properties. Culture fluid filtrates and spore suspensions of the isolates of these species almost completely suppressed the development of seedlings of plants of the tester variety.

The species of fungi *F. culmorum*, *F. fujikuroi*, *F. oxysporum*, *F. heterosporum* had stronger phytotoxic properties than pathogenic, showing a moderately toxic and toxic reaction to the seedlings of the tester variety. The species *F. roseum* was characterized by weak pathogenicity and weak phytotoxicity.

Conclusion

As a result of mycological analysis of the composition of micromycetes on affected maize plants in different phenological phases of plant development, more than 30 species of micromycetes were identified. Saprotrophic species of fungi from the genera *Aspergillus*, *Cladosporium*, *Curvularia*, *Penicillium*, *Rhizopus*, *Periconia*, *Pythium*, *Trichothecium*, etc. prevailed on the roots and root zone of corn. Heterotrophic species of fungi belonging to the genera *Alternaria*, *Bipolaris*, *Exserohilum* and *Fusarium* were found more often on the leaves. It should be noted that the frequency of occurrence of fungi *Alternaria* spp. depended on the phenological phase of corn plants. The pathogen *Exserohilum turcicum* was identified on the leaves of corn of the lower tier. The causative agent *Bipolaris sorokiniana* mainly infected the roots and the basal part of the corn stalk during the formation phase of 5–6 leaves.

During ontogenesis, 11 species of fungi from the genus *Fusarium* were found on corn crops in the Voronezh region: *F. culmorum*, *F. gibbosum*, *F. graminearum*, *F. heterosporum*, *F. fujikuroi*, *F. incarnatum*, *F. oxysporum*, *F. poae*, *F. roseum*, *F. sporotrichioides*, *F. solani*. Among them, two species, *F. heterosporum* and *F. oxysporum*, were noted with high frequency. Similar types of pathogens on corn have been identified by foreign scientists (Ali et al., 2005; Eller et al., 2008). During many years of research, V.G. Ivashchenko and colleagues identified 15 species of fungi of fusarium etiology on corn crops in Russia (Ivashchenko, 2012).

It has been shown that pathogenic and phytotoxic activity in fungi varies significantly between *Fusarium* species and within the same species. The greatest danger to corn is represented by fusarium fungi of the following species: *F. sporotrichioides*, *F. graminearum*, *F. culmorum*, *F. fujikuroi*, *F. oxysporum*, *F. heterosporum*, which have a high intensity of phytotoxic activity associated with the ability to synthesize and

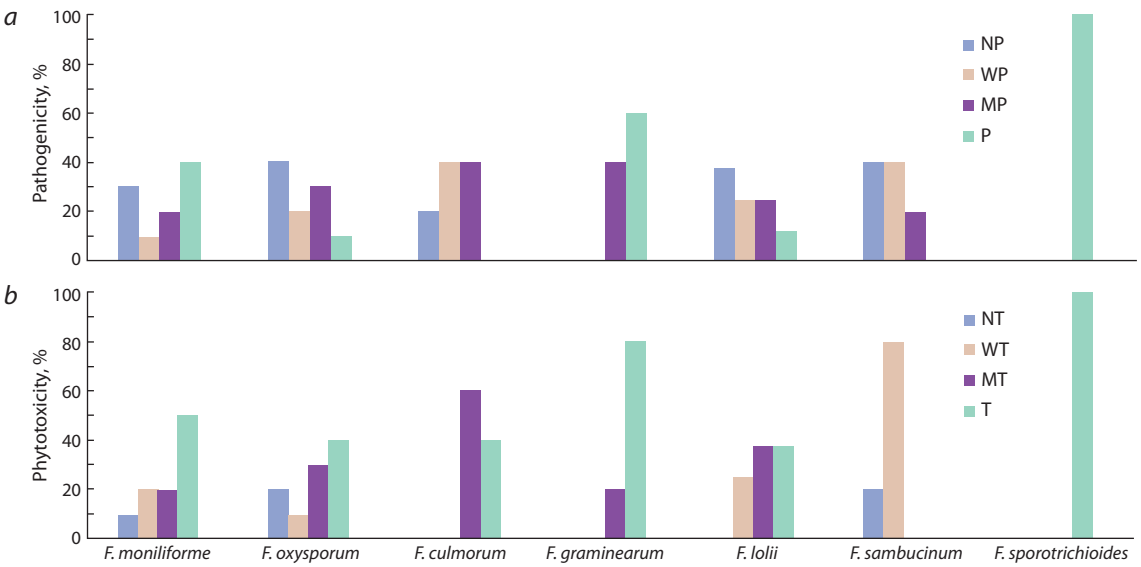
Table 3. Characteristics of strains of fungi of seven species from the genus *Fusarium* by pathogenicity of spore suspensions and phytotoxicity of culture fluid on wheat seedlings of cv. Mironovskaya 808 (in % of control)

Code of strain	Pathogenicity (spore suspension)				Toxicity (culture fluid)			
	Seed germination, %	Sprout length, %	Root length, %	Degree of influence	Seed germination, %	Sprout length, %	Root length, %	Degree of influence
F. fujikuroi Nirenberg								
ZM-FF-4z	100.0	109.9±3.2	103.9±4.0	NP	100.0	96.1±3.5	61.0±2.6	WT
ZM-FF-2z	100.0	113.7±5.2	99.5±4.0	NP	100.0	117.6±2.9	76.4±2.4	NT
ZM-FF-4z-1	96.7	113.0±4.2	104.8±3.1	NP	100.0	66.7±4.3	58.2±3.3	MT
ZM-FF-2l-1	100.0	87.3±4.6	65.6±3.6	WP	100.0	92.1±2.6	59.1±2.6	WT
ZM-FF-2ksh	100.0	75.8±5.3	45.2±3.6	MP	100.0	87.7±4.5	50.7±3.3	MT
ZM-FF-5z	96.7	80.1±3.3	44.0±2.7	MP	100.0	73.3±2.6	86.1±1.7	T
ZM-FF-5k-1	83.3	52.4±4.3	18.7±2.2	P	95.1	68.4±3.7	17.7±4.1	T
ZM-FF-1p	95.5	47.5±2.7	27.5±2.3	P	100.0	67.7±4.4	22.2±2.2	T
ZM-FF-5p	90.0	64.4±3.3	25.5±3.4	P	96.7	65.7±5.1	28.3±4.6	T
ZM-FF-6z	87.7	62.3±4.7	21.1±3.7	P	100.0	83.3±3.3	25.1±3.5	T
F. oxysporum Schlecht.								
ZM-FO-1l	100.0	114.1±4.4	109.5±2.2	NP	100.0	101.5±3.2	77.6±2.4	NT
ZM-FO-1st	100.0	111.1±3.1	101.9±1.5	NP	100.0	83.5±5.2	39.5±2.8	MT
ZM-FO-2k	100.0	108.6±2.3	85.4±1.7	NP	100.0	91.1±4.4	43.1±4.3	MT
ZM-FO-8l	100.0	106.5±4.2	88.0±2.5	NP	96.7	74.1±2.8	26.6±1.6	T
ZM-FO-2l	100.0	101.2±4.1	54.5±2.0	WP	96.7	30.7±1.9	6.6±1.1	T
ZM-FO-4z-2	96.7	105.5±3.5	60.8±2.2	WP	100.0	104.0±3.7	80.3±2.2	NT
ZM-FO-4l-2	100.0	76.7±5.2	42.2±2.5	MP	96.7	96.5±3.1	43.6±2.5	MT
ZM-FO-4st	96.7	78.2±5.8	46.2±2.4	MP	100.0	86.7±3.1	45.3±4.5	MT
ZM-FO-3z	100.0	84.1±3.3	40.3±3.7	MP	100.0	51.8±5.0	25.8±2.4	T
ZM-FO-8z	95.0	43.3±4.8	21.7±3.5	P	100.0	55.8±5.4	18.5±3.7	T
F. culmorum (Sm.) Sacc.								
ZM-FC-1z	100.0	70.4±5.7	40.8±2.4	MP	96.7	96.5±3.1	43.6±2.5	MT
ZM-FC-2k	96.7	78.2±5.8	56.2±4.4	WP	100.0	51.8±5.0	25.8±2.4	T
ZM-FC-3st	100.0	83.5±5.8	75.7±4.7	NP	100.0	86.7±3.3	49.5±3.5	MT
ZM-FC-6l	100.0	83.1±3.5	53.3±5.5	WP	100.0	56.6±3.5	21.1±1.9	T
ZM-FC-4k-1	100.0	75.5±4.3	43.7±4.9	MP	96.7	95.5±3.7	45.5±4.2	MT
F. graminearum Schwabe.								
ZM-FG-3p	83.3	49.4±4.7	29.5±3.2	P	96.7	86.7±3.0	44.8±3.3	MT
ZM-FG-3ksh	96.7	63.3±5.1	56.7±4.7	MP	90.0	23.3±2.7	15.7±2.5	T
ZM-FG-2p	95.0	55.4±4.3	17.5±3.3	P	93.7	76.7±3.5	46.7±3.7	MT
ZM-FG-1ksh	100.0	33.3±3.4	12.5±3.7	P	100.0	64.3±3.2	49.7±3.3	MT
ZM-FG-1l-2	83.3	66.7±4.5	29.1±3.7	P	91.1	65.7±3.0	45.4±3.5	MT
ZM-FG-5p-1	100.0	67.3±4.2	64.7±3.5	MP	100.0	40.3±3.8	15.3±3.3	T
ZM-FG-6ksh	96.3	63.3±4.2	44.7±5.1	MP	90.0	33.3±1.5	8.7±2.5	T
ZM-FG-4l	100.0	81.1±4.4	58.9±3.5	MP	100.0	27.3±3.5	16.7±2.7	T
ZM-FG-1k-2	98.5	53.4±4.7	22.5±3.2	P	100.0	75.6±4.3	44.4±3.0	MT
ZM-FG-3p-1	100.0	63.7±4.3	27.7±5.2	P	95.0	33.3±2.4	19.1±2.5	T

Table 3 (end)

Code of strain	Pathogenicity (spore suspension)				Toxicity (culture fluid)			
	Seed germination, %	Sprout length, %	Root length, %	Degree of influence	Seed germination, %	Sprout length, %	Root length, %	Degree of influence
F. heterosporum Nees & T. Nees								
ZM-FL-1k	100.0	103.5 ± 1.8	77.0 ± 1.6	NP	100.0	84.4 ± 3.0	40.7 ± 1.9	MT
ZM-FL-2l	100.0	109.3 ± 2.5	87.8 ± 2.3	NP	100.0	86.2 ± 2.2	42.3 ± 1.2	MT
ZM-FL-1k-1	100.0	106.9 ± 2.1	81.3 ± 3.0	NP	86.5	32.1 ± 2.7	15.7 ± 3.5	T
ZM-FL-3l	100.0	97.2 ± 2.4	64.9 ± 1.6	WP	96.7	84.3 ± 3.8	52.8 ± 2.9	WT
ZM-FL-3l-1	96.7	75.5 ± 4.3	66.7 ± 2.7	WP	96.7	76.3 ± 3.2	57.3 ± 4.2	WT
ZM-FL-2ksh	98.6	66.5 ± 5.4	50.7 ± 4.7	MP	100.0	88.7 ± 3.7	46.1 ± 3.5	MT
ZM-FL-3k	96.7	86.7 ± 3.0	44.9 ± 3.3	MP	93.3	43.4 ± 2.5	19.7 ± 3.5	T
ZM-FL-3l-2	83.3	47.7 ± 4.3	23.3 ± 3.2	P	90.0	36.7 ± 4.7	25.7 ± 1.5	T
F. roseum Link								
ZM-FR-5k	100.0	101.3 ± 1.8	93.8 ± 3.2	NP	100.0	96.3 ± 1.7	93.9 ± 2.1	NT
ZM-FR-1k-1	100.0	99.1 ± 2.8	95.3 ± 4.1	NP	100.0	87.3 ± 4.6	65.6 ± 3.6	WT
ZM-FR-4p	100.0	90.9 ± 1.8	68.7 ± 3.8	WP	100.0	92.3 ± 2.5	65.4 ± 2.7	WT
ZM-FR-4l-1	100.0	92.3 ± 1.5	65.5 ± 3.4	WP	96.7	84.3 ± 3.8	62.8 ± 3.9	WT
ZM-FR-6k	96.7	88.8 ± 2.3	48.8 ± 2.5	MP	100.0	90.6 ± 4.0	61.4 ± 2.5	WT
F. sporotrichioides Swerb.								
ZM-FS-4k	100.0	34.5 ± 4.5	15.9 ± 1.7	P	100.0	29.8 ± 1.9	4.2 ± 0.8	T
ZM-FS-8k	90.0	53.6 ± 7.1	21.5 ± 2.6	P	90.0	43.3 ± 2.3	15.7 ± 2.5	T
ZM-FS-2st	88.3	53.6 ± 3.3	10.5 ± 3.3	P	98.2	51.1 ± 4.3	25.1 ± 3.6	T
ZM-FS-2l-2	85.7	44.7 ± 3.4	8.5 ± 3.5	P	100.0	44.1 ± 3.5	22.5 ± 3.3	T
ZM-FS-6st	90.0	47.7 ± 3.8	11.1 ± 3.5	P	95.0	43.3 ± 2.3	11.1 ± 2.5	T
ZM-FS-1k-1	90.0	57.3 ± 3.6	22.3 ± 2.4	P	85.3	23.5 ± 2.3	4.7 ± 2.5	T
ZM-FS-4k-2	100.0	55.7 ± 4.1	15.1 ± 3.5	P	83.7	23.7 ± 2.1	5.5 ± 2.5	T

Note. NP/NT is non-pathogenic/non-toxic; WP/WT – weakly pathogenic/weakly toxic; MP/MT – moderately pathogenic/moderately toxic; P/T – pathogenic/toxic.



Distribution of fungal species from the genus *Fusarium* by pathogenicity (*a*) and phytotoxicity (*b*), %.

accumulate dangerous toxins in plant tissues. The results of similar studies were previously obtained by us when detecting pathogenic and phytotoxic activity of fungi from the genus *Fusarium* isolated from affected wheat plants. Isolates of *F. culmorum*, *F. graminearum*, *F. heterosporum*, *F. oxysporum* isolated from wheat had a wide intraspecific diversity according to these characteristics. Among them, as well as on corn, isolates of pathogens with different levels of pathogenic and phytotoxic activity were found (Zhemchuzhina et al., 2021).

The nature of the effect of FCF strains of fungi *F. graminearum*, *F. heterosporum*, *F. fujikuroi*, *F. solani* and *F. redolens* on barley seedlings is characterized by high phytotoxicity, and *F. avenaceum*, *F. poae*, by weak phytotoxicity. Of all the listed species, *F. sporotrichioides* and *F. sambucinum* isolates turned out to be the most pathogenic and phytotoxic on barley. In *F. culmorum* and *F. oxysporum* species, in contrast to those isolated from corn, the frequency distribution of all categories of pathogenicity and phytotoxicity was approximately the same (Kolomiets et al., 2018).

Thus, as a result of mycological studies conducted on affected maize samples from the Voronezh region, the State Collection of phytopathogenic microorganisms of ARRIP was replenished with 55 strains of fungi belonging to seven types of pathogens from the genus *Fusarium.* The selected strains of phytopathogens, stable in morphological and cultural characteristics, characterized by pathogenicity and phytotoxicity, have been stored for long-term storage using lyophilization and cryopreservation methods.

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