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STUDY OF THE GENETIC DIVERSITY AND SYMBIOTIC EFFICIENCY OF MICROSYMBIONTS ISOLATED FROM *Lathyrus palustris* L. AND *Vicia cracca* L. GROWING IN ARCTIC YAKUTIA

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Abstract

The formation of highly productive pasture phytocenoses, based on legumes that form nitrogen-fixing symbiosis with nodule bacteria, is a necessary condition for the spread and sustainable growth of herbivorous farm animals under climate change and radical restructuring of plant ecosystems in the Arctic. At the same time, the issues of biodiversity of nodule bacteria of Arctic territories and the efficiency of their symbiotic interaction with legumes are currently almost unstudied in Russia. In this work 12 strains isolated from *Lathyrus palustris* and *Vicia cracca* nodules growing in Arctic Yakutia were described for the first time. The taxonomic position of the strains was studied and their ability to form an effective symbiosis with both traditional legumes and wild plants, which are more adapted to the conditions of the Far North and can be used to create highly productive pasture phytocenoses, was shown. The aim of the work was to isolate and study the genetic diversity of nodule bacteria of various populations of wild legume plants of *Lathyrus palustris* L. and *Vicia cracca* L. growing in Arctic Yakutia. The ability of the obtained isolates to form nitrogen-fixing nodules on the roots of different species of forage legume crops was evaluated under the conditions of sterile test-tube experiments. Root nodules of *V. cracca* and *L. palustris* were collected on Samoilovsky Island and in the settlement of Tiksi during the Russian-German expedition to the Lena River Delta. Rhizobial strains from legume nodules were isolated according to the standard method using mannitol-yeast YMA nutrient media. The taxonomic position of 12 isolates was determined by 16S rDNA (*rrs*) sequencing. Seeds of *V. cracca*, *V. sativa*, *L. sativus*, and *L. pratensis* were used to set up of test-tube experiments. Plants were cultivated in sterile 300 ml glass vessels containing 50 ml of Krasilnikov-Korenyako agar medium. The seedlings were inoculated with suspensions of individual strains in the amount of 10^6 cells/vessel. Commercial strains of *Rhizobium leguminosarum* bv. *viciae* RCAM2802, RCAM2806 and RCAM0626 from the Russian Collection of Agricultural Microorganisms (RCAM, ARRIAM, St. Petersburg) were

used as a positive control. Uninoculated plants served as negative controls. The number of nodules formed on the plant roots was counted and described at the end of cultivation. The nitrogen-fixing activity of nodules was determined by the acetylene method using a GC-2014 gas chromatograph (Shimadzu, Japan). Seeds of *V. cracca* and *L. pratensis* were additionally inoculated under the conditions of a separate test-tube experiment with a soil extract from a sample taken from Kotelny Island (Novosibirsk Islands, Arctic Yakutia). A total of twelve rhizobial isolates assigned to the genera *Rhizobium*, *Mesorhizobium* and *Bosea* were isolated from root nodules of *L. palustris* and *V. cracca* populations. Strains of *Mesorhizobium* sp. 33-3/1 and 32-2/1 were isolated only from populations growing in Tiksi. *Rhizobium* sp. 32-5/1 strain showed a low similarity of the *rrs* gene with the closest type strain (less than 98.0 %), which suggests it belonging to the new species of microorganisms. As a result of test-tube experiments, nodules were formed only in the inoculation variants with strains of *Rhizobium* sp. 19-1/1, 20-1/1, 33-1/1 and *Mesorhizobium* sp. 32-2/1. *Rhizobium* sp. 19-1/1 strain formed inactive nodules on the roots of three legume species, except *V. cracca*. *Rhizobium* sp. 20-1/1 strain in the inoculation variant with *V. cracca* formed a greater number of nodules and showed a higher level of nitrogen-fixing activity compared with the commercial strain *Rhizobium leguminosarum* bv. *viciae* RCAM0626 for treatment of *V. sativa*, but the variants did not differ significantly from each other in the number of nodules.

Keywords: Arctic Yakutia, Lena River Delta, legumes, *Lathyrus palustris*, *Vicia cracca*, nitrogen-fixing nodule bacteria

Global climate change causes a significant restructuring of the entire Arctic ecosystem with the active migration of plant communities northwards, the filling of new ecological niches and the displacement of native flora [1, 2]. In such areas, pasture phytocenoses can form a significant part of which are leguminous plants that enter into symbiosis with nitrogen-fixing nodule bacteria (rhizobia). This mutually beneficial strategy allows legumes to expand into new territories due to their broad ecological plasticity and tolerance to environmental stressors, including low soil nitrogen. Legumes are a major source of protein for both herbivorous farm animals and wild reindeer and musk oxen [3, 4].

The first and only description of nodule bacteria isolated from the root nodules of wild leguminous plants *Oxytropis nigrescens* (Pall.) Fisch., *O. maydelliana* Trautv., *Astragalus alpinus* L., *A. umbellatus* Bunge and *Hedysarum obscurum* L. which grow in the Asian part of the Arctic in the tundra of the Chukotka Peninsula, Kolyuchin and Wrangel Islands, is given in the work of A.E. Criss et al. [5]. The authors isolated eight bacterial strains that were not capable of forming nodules on the roots of agricultural legume plants clover, sweet clover, alfalfa, peas and vetch in pot trials. The ability of some strains to form nodules on legume plants *H. alpinum* L., *H. sibiricum* Poir. and *A. trautvetteri* Bunge. was observed but the nitrogen-fixing activity of nodules has not been studied.

The species *Lathyrus palustris* L. and *Vicia cracca* L. belong to the vetch tribe *Vicieae* (Adans.) Bronn of the *Fabaceae* family. Many mambers of the genera *Lathyrus* and *Vicia* are valuable forage pasture and hay crops in the diet of large and small ruminants, horses [6, 7], and wild herbivores of Central Yakutia [8]. In particular, the species *L. palustris* has high feeding qualities and is considered a particularly promising crop for introduction into pasture agrophytocenoses of Eastern Siberia [6, 9], while *V. cracca* is included in the State Register of Breeding Achievements approved for use and is actively cultivated as valuable forage grass in pasture and hay phytocenoses [10].

The main microsymbionts of leguminous plants of the genera *Vicia* and *Lathyrus* are strains of *Rhizobium leguminosarum* bv. *viciae* [11-13]. Symbionts that are not typical for these genera and related to both other *Rhizobium* species [14, 15] and the genus *Phyllobacterium* members [11] are also isolated from the root nodules of various *Vicia* and *Lathyrus* species. It was shown that *Mesorhizobium alhagi* CCNWXJ12-2T isolated from *Alhagi sparsifolia* native to northeastern China could form nodules on the *V. cracca* roots [16].

Arctic rhizobia are of interest for studying the evolutionary development

of nitrogen-fixing bacteria and their adaptation to low temperatures. Arctic rhizobia also provide an opportunity to analyze the functional relationships between rhizobia and leguminous plants in isolated aboriginal populations of the North [17]. It is promising to use Arctic strains of nodule bacteria in the production of microbial preparations to create highly productive pasture phytocenoses in the Arctic [18]. However, the issues of biodiversity of nodule bacteria in Arctic territories and the effectiveness of their symbiotic interaction with leguminous plants remain practically unstudied in Russia.

This paper is the first to report about the taxonomic position of 12 innovative strains from the nodules of the swamp lathyrus and mouse pea grown in Arctic Yakutia and their effective symbiotic nodulation of both traditional legumes and wild plants that are more adapted to the Far North conditions. These strains may be of interest for creating highly productive pasture phytocenoses.

The work aimed at studying the genetic diversity of rhizobial isolates from populations of arctic wild legumes *Lathyrus palustris* L. and *Vicia cracca* L. and to assess their ability to form nitrogen-fixing nodules on the roots of forage pasture legumes in micro-pot trials.

Materials and methods. Root nodules of wild populations of leguminous plants *V. cracca* and *L. palustris* were collected in 2021 on Samoilovsky Island and in the village of Tiksi during a Russian-German expedition to the Lena River delta (Arctic Yakutia).

Rhizobial strains were isolated according to a standard procedure using YMA mannitol-yeast nutrient medium [19] after sterilizing the nodules for 1 min in 96% ethanol. rDNA isolation from pure cultures was performed using the DNeasy Blood & Tissue kit (QIAGEN, Germany) and Monarch® (New England Biolabs, USA). Primary identification of strains was carried out by PCR followed by the 16S rRNA marker gene sequencing. Primer pairs fD1 5'-AGAGTTTGATCCTGGCTCAG-3' and rD1 5'-AAGGAGGTGATCCAGCC-3' [20] were used for amplification. The 16S rRNA gene amplification protocol was 3 min 30 s at 95 °C (primary denaturation); 1 min 10 s at 94 °C (denaturation), 40 s at 56 °C (primer annealing), 2 min 10 s at 72 °C (elongation) (35 cycles); 6 min 10 s at 72 °C (final elongation) (a T100 Thermal Cycler, Bio-Rad, USA). The reaction mixture was 38 μl milli-Q H_2O (Evrogen, Russia), 5 μl buffer (Helikon, Russia), 5 μl dNTP kit (Promega, USA), 0.5 μl primers (Evrogen, Russia), 0.5 μl Taq polymerase (Helicon, Russia) and 1 μl (50-100 ng) mDNA. The amount of DNA was assessed visually using electrophoresis in a 1.0% agarose gel in 0.5× TAE buffer with a MassRuler molecular weight marker (Fermentas, Lithuania). The PCR product was purified from an agarose gel using the Cleanup S-Cap kit (Evrogen, Russia). The purified DNA was sequenced (an ABI PRISM 3500xl genetic analyzer, Life Technologies, USA) at the Center for Collective Use “Genomic Technologies, Proteomics and Cell Biology” of the All-Russian Research Institute of Agricultural Microbiology (CCU GTPCB ARRIAM).

The obtained DNA sequences were analyzed using the Chromas Lite 2.6.4 program (https://technelysium.com.au/wp/chromas/). For multiple alignment and comparison of nucleotide sequences, the ClustalOmega program (https://www.ebi.ac.uk/Tools/msa/clu-stalo/) was used. Sequences of closely related type strains were searched in the GenBank database (https://www.ncbi.nlm.nih.gov/). Nucleotide sequences have been deposited in the GenBank database (ac. nos OQ685989-OQ686000.

For micro-pot experiments (MPEs), seeds of *V. cracca*, *V. sativa*, *L. sativus* and *L. pratensis* were scarified and surface sterilized in 98% H_2SO_4 for 10 minutes, thoroughly washed with sterile tap water and germinated on filter paper in Petri dishes at 25 °C in the dark for 3-5 days (depending on the plant species). Seeds of *V. sativa* and *L. sativus* were kindly provided by employees of the Vavilov

All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg). *L. pratensis* was used instead of *L. palustris* as the inoculation target due to the lack of seeds.

Plants were grown in 300-ml sterile glass vessels with 50 ml of Krasilnikov-Korenyako agar medium (K_2HPO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 1.0 g/l, $Ca_3(PO_4)_2$ 0.2 g/l, $FeSO_4$ 0.02 g/l, Fedorov's microelement mixture of H_3BO_3_0.05 g/l, $(NH_4)_2MoO_4$ 0.05 g/l, KCl 0.005 g/l, NaBr 0.005 g/l, $ZnSO_4 \cdot 7H_2O$ 0.003 g/l, $MnSO_4$ 0.002 g/l). Each MPE was performed in triplicate (3 glass vessels with 1 seedling for each inoculation treatment).

Seedlings were inoculated with suspensions of individual strains (10^6 cells per vessel). Commercial strains of *Rhizobium leguminosarum* bv. *viciae* RCAM2802, RCAM2806 and RCAM0626 from the Network Bioresource Collection for agriculture genetic technologies (ARRIAM, St. Petersburg) served as a positive control, uninoculated plants were negative controls. Plants were grown in a phytotron at 18-22 °C for 30 days at 50% relative humidity and a four-level lighting/temperature regime: night (18 °C, 8 hours), morning (200 μmol · $m^{-2} \cdot s^{-1}$, 20 °C, 2 hours), day (400 μmol · $m^{-2} \cdot s^{-1}$, 23 °C, 12 hours), evening (200 μmol · $m^{-2} \cdot s^{-1}$, 20 °C, 2 hours). Lighting was provided by L36W/77 FLUORA lamps (Osram, Germany).

At the end of cultivation, the nodules formed on the roots were counted and described. The nitrogen-fixing activity of nodules was determined by the acetylene method using a gas chromatograph GC-2014 (Shimadzu, Japan).

In a separate MPE, the seeds of *V. cracca* and *L. pratensis* were inoculated with a soil extract from a sample taken from Kotelny Island (New Siberian Islands, Arctic Yakutia). To prepare the soil extract, 1 g of sample was added to a sterile flask with 50 ml of liquid Krasilnikov-Korenyako medium. The flask was placed on a shaker and incubated for 1 hour at room temperature. The seedlings were inoculated with 1 ml soil extract per vessel.

Statistical analysis was performed using the STATISTICA 10 program (StatSoft, Inc., USA). For each inoculation treatment, means (*M*) and standard deviations (±SD) were calculated. To assess the significance of differences between mean values, one-way analysis of variance and Fisher's LSD test were used.

Results. Samoilovsky Island (72°22′00″N, 126°30′01″E) is located in the southern part of the Lena River delta in the subzone of typical tundras (Fig. 1).

Fig. 1. Sites for *Vicia cracca* L. and *Lathyrus palustris* L. plants collection on the Samoilovsky Island (the Lena River delta) and the village Tiksi (marked with white dots).

Geomorphologically, the island is clearly divided into two parts, different in age and genesis. Two-thirds of the island is occupied by the surface of the first terrace, and one-third in the western part of the island is the surface of a high floodplain, subject to seasonal flooding by river waters (21). The village of Tiksi (71°38′12″N, 128v52′04″ E) is located on the shore of the bay of the same name, confined to the southern part of the Laptev Sea (see Fig. 1). The predominant elements of the relief in the vicinity of the village are low hills and intermountain swampy saddles. The basis of the vegetation cover contains various variants of tundra and low-lying shallow swamps. The soils are frozen,

gravelly-stony. Flora is mountainous, temperate arctic [22].

The boreal species *Lathyrus palustris* L. and mouse pea *Vicia cracca* L. were found on the territory of the village Tiksi relatively recently [23]. The species *V. cracca* (Fig. 2, B) on Samoilovsky Island was first discovered by N.N. Lashchinsky (personal communication) in 2017, and *L. palustris* plants (Fig. 2, A) by him during the Russian-German expedition to the Lena River delta in 2021. Populations of *V. cracca* and *L. palustris* grow mainly in the northwestern part of the island which is sandy and subject to seasonal flooding by Lena River [24].

FIg. 2. Plants *Lathyrus palustris* L. (a) **and *Vicia cracca* L.** (b) **on the Samoilovsky Island (the Lena River delta)** (photo by I.A. Alyokhina).

From the root nodules of *L. palustris* and *V. cracca*, we cultured 8 and 4 rhizobial isolates, respectively (Table 1), 4 isolates from each of two *L. palustris* populations, and 2 isolates from each of *V. cracca* populations. Most isolates formed colonies on days 3 and 4, with the exception of strain 33-5/1, which formed colonies on day 5. Based on analysis of the *rrs* gene, the isolates were assigned to the genera *Rhizobium* (family *Rhizobiaceae*), *Mesorhizobium* (family *Phyllobacteriaceae*), and *Bosea* (family *Boseaceae*) of the order *Rhizobiales* (*Hyphomicrobiales*). Note that from the nodules of the *L. palustris* population growing on the island. Samoilovsky, only representatives of *Rhizobium* were isolated while the population from the village Tiksi turned out to be represented by a wider composition of microsymbionts (*Rhizobium*, *Mesorhizobium* and *Bosea*), which is apparently associated with different soil and climatic features of these areas (see Table 1).

1. Isolates from nodules of *Lathyrus palustris* L. and *Vicia cracca* L. collected on the Samoilovsky Island (the Lena River delta) and in the village Tiksi (2021)

Site of plant collection	Strain number	Similarity of the *rrs* gene, %	Closest type strain	Identification
		Lathyrus palustris L.		
Samoilovsky islanf	19-1/1	100	*Rhizobium leguminosarum* bv. *viceae* LMG 14904, *R. sophorae* LMG 27901, *R. anhuiense* CCBAU 23252, *R. laguerreae* FB206	*Rhizobium* sp.
	19-3/1			
	19-4/1			
	19-5/1			
Tiksi village	33-1/1			
	33-3/1	100	*Mesorhizobium norvegicum* 10.2.2, *M. loti* LMG 6125	*Mesorhizobium* sp.
	33-4/1	99,72	*Bosea lathyri* R-46060	*Bosea lathyri*
	33-5/1	99,21	*B. lathyri* R-46060	*Bosea* sp.
		Vicia cracca L.		
Samoilovsky islanf	20-1/1	100	*R. leguminosarum* bv. *viceae* LMG 14904, *R. sophorae* LMG 27901, *R. anhuiense* CCBAU 23252, *R. laguerreae* FB206	*Rhizobium* sp.
	20-5/1	99,21	*B. lathyri* R-46060	*Bosea* sp.
пос. Тикси	32-2/1	100	*M. norvegicum* 10.2.2, *M. loti* LMG 6125	*Mesorhizobium* sp.
	32-5/1	97,85	*R. giardinii* H152	*Rhizobium* sp.

Isolates from nodules of *L. palustris* (19-1/1, 19-3/1, 19-4/1, 19-5/1, 33-1/1) and V. *cracca* (20-1/1) showed 100% similarity of the *rrs* gene to four type strains at once, the *R. leguminosarum* bv. *viciae* LMG 14904T, *R. anhuiense* CCBAU 23252T, *R. sophorae* LMG 27901T and *R. laguerreae* FB206T (see Table 1). Therefore, the resulting isolates were not identified to species. To clarify their species identity, it is necessary to sequence and analyze the housekeeping genes

(*recA*, *atpD*, *dnaK*, *gyrB* and *rpoB*).

It is known that the strain *R. leguminosarum* bv. *viciae* LMG 14904T is able to form an effective symbiosis with leguminous plants from the genera *Pisum*, *Vicia*, *Lathyrus*, *Lens* and *Vavilovia* from the tribe *Fabeae* [25, 26]. *R. anhuiense* strain CCBAU 23252T was isolated from the legume plant *V. faba*, native to China. It formed nitrogen-fixing nodules on the roots of *V. faba* and P*isum sativum* [27]. In cross-nodulation experiments, this strain formed an ineffective symbiosis with *Phaseolus vulgaris* and was unable to form nodules on food and forage legumes *Glycine max*, *Arachis hypogaea*, *Medicago sativa*, *Trifolium repens*, *Lablab purpureu*s [27].

Members of the species *R. anhuiense* are known to serve as the main microsymbents of the species *Lathyrus maritimus*, which grows along the marine coastline in China [15]. The *R. sophorae* LMG 27901T was isolated from the nodule of the medicinal legume *Sophora flavescens*, also collected in China [28]. Strain LMG 27901T was shown to be able to form active nodules on the roots of *S. flavescens* and *P. vulgaris*, while other *R. sophorae* strains could form effective symbioses with *V. sativa* and *P. sativum* plants growing in Northern China [29]. *R. laguerreae* FB206T was isolated from the effective nodule of *V. faba* in Tunisia [30], and members of this rhizobial species serve as effective symbionts of many *Pisum*, *Vicia*, *Lens*, and *Phaseolus* species found in various regions of the world [31-34], and have growth-promoting properties, improving crop productivity [35, 36].

Isolates 33-3/1 and 32-2/1 were found in populations of *L. palustris* and *V. cracca* from the village Tiksi, respectively. The isolates showed 100% similarity of the *rrs* gene with the type strains *Mesorhizobium norvegicum* 10.2.2T and *M. loti* LMG 6125T isolated from root nodules of the legume plant *Lotus corniculatus* in New Zealand [37] and Norway [38], respectively. Note that we failed to isolate bacteria of the genus *Mesorhizobium* from the populations of *L. palustris* and *V. cracca* of the Samoilovsky Island.

Isolates 33-4/1, 33-5/1 and 20-5/1 showed the highest similarity of the *rrs* gene (33-4/1 - 99.72%; 33-5/1 and 20-5/1 - 99.21%) with the type strain *B. lathyri* R-46060T from *Lathyrus latifolius* native to Belgium [39]. Isolates related to *Bosea* had different origins: strains 33-4/1 and 33-5/1 were isolated from *L. palustris* plants in the village Tiksi, while 20-5/1 was isolated from *V. cracca* on the Samoilovsky Island. It should be noted that the genus *Bosea* is currently represented by 12 species, of which only 6 species (*B. lupini*, *B. lathyri*, *B. robiniae*, *B. caraganae*, *B. vaviloviae* and *B. spartocytisi*) were isolated from the nodules of leguminous plants of the genus *Lupinus*, *Lathyrus*, *Robinia*, *Caragana*, *Vavilovia* and *Spartocytisus* [39-42]). However, the ability of these strains to independently form nodules has not yet been described. Strain *Rhizobium* sp. 32-5/1 showed low similarity in the *rrs* gene with the nearest type strain (less than 98.0%), which suggests that it belongs to new types of microorganisms.

The ability of isolates to nodulate legumes was studied under two sterile MPE conditions using 9 rhizobial strains of different taxonomic positions (three genera *Rhizobium*, *Mesorhizobium* and *Bosea*) and 4 species of agricultural forage legumes (*V. cracca*, *V. sativa*, *L. sativus* and *L pratensis*) (Tables 2, 3). In the first MPE, nodules on *L. pratensis* were formed only upon inoculation with strains of Rhizobium sp. 19-1/1 and 33-1/1, although the symbiosis in both cases was not nitrogen-fixing (see Table 2). Nodules on *V. cracca* were formed only in the variant with inoculation with a strain of *Rhizobium* sp. 20-1/1, which formed an effective symbiosis with higher nitrogen-fixing activity than when inoculated with a commercial strain of *R. leguminosarum* bv. *viciae* RCAM0626 (differences between treatments in the nitrogen fixation parameter were statistically significant at $p < 0.05$). In inoculation of *V. cracca* with strains of *Mesorhizobium* sp. 32-2/1 and *Rhizobium*

sp. 33-1/1, tumor-like nodule-like formations occurred on the roots (see Table 2).

2. The effect of *Lathyrus palustris* L. and *Vicia cracca* L. plant inoculation with commercial strains of *Rhizobium leguminosarum* bv. *viciae* RCAM2806, RCAM0626 and arctic isolates (sterile micro-pot tests, $n = 3$, $M \pm SD$)

Inoculation	Number of nodules per pot	Acetylene reductase activity, μmol $C_2H_4 \cdot pot^{-1} \cdot day^{-1}$
	Lathyrus palustris L.	
R. leguminosarum RCAM2806	5.5±2.1[a]	0.37±0.08
Без инокуляции	0	0
Rhizobium sp. 19-1/1*	2.6±1.1[a]	0
Rhizobium sp. 20-1/1	0	Not detected
Bosea sp. 20-5/1	0	Not detected
Mesorhizobium sp. 32-2/1	0	Not detected
Rhizobium sp. 32-5/1	0	Not detected
Rhizobium sp. 33-1/1*	4.0±2.0[a]	0
Mesorhizobium sp. 33-3/1*	0	Not detected
Bosea lathyri 33-4/1*	0	Not detected
Bosea sp. 33-5/1*	0	Not detected
	Vicia cracca L.	
R. leguminosarum RCAM0626	5.3±1.5[a]	0.05±0.01[a]
Без инокуляции	0	0
Rhizobium sp. 19-1/1*	0	Not detected
Rhizobium sp. 20-1/1	7.3±0.5[a]	0.68±0.35[b]
Bosea sp. 20-5/1	0	Not detected
Mesorhizobium sp. 32-2/1	NS	0
Rhizobium sp. 32-5/1	0	Not detected
Rhizobium sp. 33-1/1*	NS	0
Mesorhizobium sp. 33-3/1*	0	Not detected
Bosea lathyri 33-4/1*	0	Not detected
Bosea sp. 33-5/1*	0	Not detected

N o t e. NS — nodule-like srucures. Seeds of *L. pratensis* were collected in the Irkutsk Province, seeds of *V. cracca* on the island Samoilovsky (the Lena River delta). An asterisk (*) indicates strains isolated from *L. palustris* nodules; other strains were isolated from *V. cracca*.

[a, b] Variants are marked with different Latin letters, the differences between which are statistically significant (Fisher's LSD test, $p < 0.05$).

3. The effect of *Lathyrus palustris* L. and *Vicia cracca* L. plant inoculation with commercial strains of *Rhizobium leguminosarum* bv. *viciae* RCAM2806, RCAM0626 and arctic isolates (sterile micro-pot tests, $n = 3$, $M \pm SD$)

Inoculation	Number of nodules per pot	Acetylene reductase activity, μmol $C_2H_4 \cdot pot^{-1} \cdot day^{-1}$
	Lathyrus sativus L.	
R. leguminosarum RCAM2802	21.0±2.8[a]	5.20±0.03
Без инокуляции	0	0
Rhizobium sp. 19-1/1*	29.3±9.5[a]	0
Rhizobium sp. 20-1/1	0	Not detected
Bosea sp. 20-5/1	0	Not detected
Mesorhizobium sp. 32-2/1	0	Not detected
Rhizobium sp. 32-5/1	0	Not detected
Rhizobium sp. 33-1/1*	0	Not detected
Mesorhizobium sp. 33-3/1*	0	Not detected
Bosea lathyri 33-4/1*	0	Not detected
Bosea sp. 33-5/1*	0	Not detected
	Vicia sativa L.	
R. leguminosarum RCAM0626	110.0±41.0[ab]	3.70±2.00[a]
Без инокуляции	0	0
Rhizobium sp. 19-1/1*	122.0±13.0[b]	0
Rhizobium sp. 20-1/1	0	0
Bosea sp. 20-5/1	0	н/о
Mesorhizobium sp. 32-2/1	68.0±30.0[a]	0
Rhizobium sp. 32-5/1	0	Not detected
Rhizobium sp. 33-1/1*	107.0±8.0[ab]	0.20±0.10[b]
Mesorhizobium sp. 33-3/1*	0	Not detected
Bosea lathyri 33-4/1*	0	Not detected
Bosea sp. 33-5/1*	0	Not detected

N o t e. Seeds of *L. pratensis* were collected in the Irkutsk Province, seeds of *V. cracca* on the island Samoilovsky (the Lena River delta). An asterisk (*) indicates strains isolated from *L. palustris* nodules; other strains were isolated from *V. cracca*.

[a, b] Variants are marked with different Latin letters, the differences between which are statistically significant (Fisher's LSD test, $p < 0.05$).

In the second MPE, when *L. sativus* was inoculated with Rhizobium sp.

19-1/1, round inactive nodules were formed on the roots of plants, as in the case of *L. pratensis* (see Tables 2, 3). Nodules on *V. sativa* were formed in three inoculation variants, but only with *Rhizobium* sp. 33-1/1 symbiosis was effective: insignificant nitrogen-fixing activity was shown in comparison with the commercial strain *R. leguminosarum* bv. *viciae* RCAM0626 (see Table 3). Interestingly, strains *Mesorhizobium* sp. 32-2/1 and *Rhizobium* sp. 33-1/1 from the nodules of *V. cracca* and *L. palustris*, respectively, formed only nodule-like structures on the roots of *V. cracca*, while on the roots of *V. sativa* both strains formed a determinate type of rounded nodules. The reason for this is probably due to the fairly high degree of specificity of the host plant to the microsymbiont. The resulting nodules were mostly round, white, ineffective, and determinate, with the exception of inoculation of *V. cracca* with *Rhizobium* sp. 20-1/1 and *V. sativa* with *Rhizobium* sp. 33-1/1, where some of the formed nodules were distinguished by their oblong shape and pinkish tint, indicating their nitrogen-fixing activity.

As a result of inoculation of *V. cracca* and *L. pratensis* plants with a soil extract from a sample taken on the island Kotelny, nodules on the roots were not found, which is most likely due to the absence of the corresponding microsymbionts in the soil, since at present the presence of legumes on the island. Boiler room not identified.

Thus, 12 rhizobial strians belonging to the genera *Rhizobium*, *Mesorhizobium*, and *Bosea* were isolated from the nodules of Arctic wild leguminous plants *Lathyrus palustris* and *Vicia cracca*. Most isolates belonged to the genus *Rhizobium*. Strains *Mesorhizobium* sp. 33-3/1 and 32-2/1 were isolated from the village Tiksi populations of *L. palustris* and *V. cracca*, respectively, but in the Samoilovsky Island populations bacteria of this genus were not found. The *L. palustris* on the Samoilovsky Island has been described for the first time. Strain *Rhizobium* sp. 32-5/1 showed low similarity in the *rrs* gene with the nearest type strain (less than 98.0%) which suggests that it belongs to new species of microorganisms. In micro-pot experiments to study cross-nodulation for agricultural forage legumes *Vicia cracca*, *Vicia sativa*, *Lathyrus sativus* and *Lathyrus pratensis*, nodules were formed only upon inoculation with *Rhizobium* sp. 19-1/1, 20-1/1, 33-1/1 and *Mesorhizobium* sp. 32-2/1. Strain *Rhizobium* sp. 19-1/1 was the only one that formed ineffective nodules on the roots of three legume species at once, except for *V. cracca*, which may indicate its wide specificity to various genera and species of host plants. Note that *Rhizobium* sp. 20-1/1 in inoculation of *V. cracca* formed a larger number of nodules (the differences between the experimental variants were not significant due to significant variation in this parameter) and showed higher nitrogen-fixing activity compared to the commercial strain *R. leguminosarum* bv. *viciae* RCAM0626, which makes it promising for the development of new highly effective microbial preparations with the aim of creating productive pasture phytocenoses in the Arctic territories of Russia. To further study the host specificity and symbiotic efficiency of the obtained strains, micro-pot experiments will be carried out with an expanded selection of forage agricultural and wild Arctic legumes.

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MODIFIED SEMISYNTHETIC MEDIUM MMBt FOR PRODUCTION OF PREPARATIONS BASED ON *Bacillus thuringiensis*

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Abstract

One of the trends in the biological control of pests is the use of bacteria belonging to the genus *Bacillus* and, first of all, entomopathogenic strains of *Bacillus thuringiensis*. Of great interest to industrial biotechnology are studies related to the search for optimal cultivation conditions that can improve the manufacturability of the production of microbiological preparations and their effectiveness. Previously, the nutrient media for the production of microbiological preparations based on *B. thuringiensis* which include natural organic components have been developed. Nevertheless, during the production of biopreparations based on this bacterium, the foaming of the culture frequently occurs and expensive filters of bioreactors have to be replaced. Also, during the treatment of plants, working solutions containing organic components of the liquid medium can clog the nozzles. This effect complicates the treatment process. In addition, organic cultural media components are not standard and depend on the quality and source origin. In this regard, it is important to carry out the screening of optimal synthetic media that could eliminate these shortcomings. Our study was aimed at selecting the optimal synthetic media and evaluating the effectiveness of the obtained preparation samples in laboratory and field conditions. The objects of study were the cultures of *B. thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH_{10} 25). The composition of the culture media was as follows: CCY medium — 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 0.05 mM $ZnCl_2$, 0.2 mM $CaCl_2 \cdot 6H_2O$, 13 mM KH_2PO_4, 26 mM K_2HPO_4, 20 mg /l glutamine, 1 g/l casein hydrolysate, 0.4 g/l yeast extract, 0.6 g/l glycerol; MBt medium: 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02 g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$; LB medium: 10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl; modified semi-synthetic medium MMBt (modified MBt): 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02 g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$ (25), glucose (1.0 %), Na citrate (2 g/l). Yeast polysaccharide media (YPM) for BtH_1 and BtH_{10} served as a reference. Bt strains were cultivated in 750 ml Erlenmeyer flasks filled with 40-50 ml of medium on a shaker at 220 rpm and 29 °C for 48-72 h until the maturation of culture, accompanied by the formation of spores and crystalline endotoxin. On the basis of BtH_1 800/15 and BtH_{10} 25 strains, batches of liquid preparations were obtained, the effectiveness of which was evaluated in 2020 and 2021 on potatoes (*Solanum tuberosum* L.) of the Yantar variety in the Far East (Ussuri district of Primorsky Krai) against *Henoseplachna vigintioctomaculata* Motsch

and on potatoes of the Emelya variety in the Tambov region against *Leptinotarsa decemleniata* Say. In the experiments, liquid preparations obtained on YPM and MMBt were used, which were used at consumption rates of 15 and 20 l/ha. The biological effectiveness of the preparations was calculated according to the formula W.S. Abbot. The antifungal activity of the preparation BtH_{10} 25, obtained on MMBt and YPM, was determined by the method of agar blocks in vitro in Petri dishes. The control medium was used without the addition of drugs. Fungi *Botrytis cinerea* Pers (strain C-5) and *Bipolaris sorokiniana* (Sacc.) Shoemaker (strain C-20) served as test cultures. The inhibitory activity was calculated according to the W.S. Abbot. Cultivation of BtH_1 800/15 and BtH_{10} 25 strains on different nutrient media showed that on semi-synthetic media MBt and LB CFU titers were 2 times lower than on YPM, while on CCY medium they were 10 times lower. Their activity, determined by the content of exotoxin, was also lower, but on the MBt medium it was slightly inferior to YPM for BtH_1 800/15. Therefore, MBt medium was chosen for further studies, and the composition of this medium was modified by adding glucose (1.0 %) and Na citrate (2 g/l). The resulting MMBt medium made it possible to achieve a significant increase in titers, activity, and the rate of culture development compared to the initial MBt. In 2020 in the Tambov region, the effectiveness of the preparation based on BtH_1 800/15 obtained on YPM was high against the Colorado potato beetle and on the 5th day was 95.3 %, slightly inferior to the chemical standard. In the case of preparation obtained on MMBt, it was slightly lower (83.3 %), but the protective effect lasted longer, and on day 15 the efficiency was 73.7 %. In 2021, the efficacy of BtH_1 800/15 was lower than in 2020. In the preparation obtained on MMBt, it was slightly inferior to the effectiveness of the preparation obtained on YPM, amounting to 75.3 and 67.7 %, respectively, on the 5th day after treatment. The effect of the BtH_{10} 25 preparation obtained on MMBt was weaker than in the variant with BtH_1 800/15 (47.7 % on day 5). In Primorsky Krai, the high efficacy of liquid preparations against *H. vigintioctomaculata* was also noted. In 2020, at a rate of application of the BtH_1 800/15 preparation of 15 l/ha, the effectiveness in the YPM and MMBt variants was 60.5 and 63.9 %, respectively, on day 5. Similar data was obtained in 2021. The inhibitory activity of the BtH_{10} 25 preparation obtained on MM*Bt* was 12 % higher on day 5 than that of the preparation obtained on YPM, and was 72.3 and 60.8 % for *B. sorokiniana* and 78.9 and 67.4 % for *B. cinerea*. On day 10, this trend persisted, but for the preparation produced on YPM, a decrease in the inhibition of the growth of *B. sorokiniana* and *B. cinerea* colonies, respectively, to 57.3 and 44.3 % was noted. Thus, preparations based on *Bacillus thuringiensis* obtained on the MMBt medium were only slightly inferior in terms of effectiveness against pests to preparations obtained on YPM, while their effectiveness against phytopathogens was higher than that of preparations with YPM. The MM*Bt* medium is promising for agricultural biotechnology, since its use reduces the time required for the formation of spores and crystalline protein endotoxin by increasing the growth rate of the *B. thuringiensis* culture. Thus, on the MMBt medium, this process ends after 48 h, and on the YPM medium, after 72 h, which makes it possible to reduce energy consumption.

Keywords: biopreparation, *Bacillus thuringiensis* var. *thuringiensis*, *Bacillus thuringiensis* var. *darmstadiensis*, *Bipolaris sorokiniana*, *Botrytis cinerea*, Colorado potato beetle, potato ladybug, inhibitory activity, cultural medium

Biologicals against plant pests and pathogens are extremaly relevant for modern agrobiotechnologies. Optimization of cuture to ensure higher efficiency and manufacturability of microbes-based drugs is of particular importance. Environmentally friendly pest control products that are an alternative to chemical pesticides are in the focus. Microorganisms, primarily entomopathogenic bacteria of the genus *Bacillus* are the most promising for creating insect control agents [1]. *Bacillus thuringiensis* is the basis for 90% of biopesticides used commercially to control pests [2]. These drugs are active against insects of the orders *Lepidoptera*, *Diptera*, *Coleoptera*, *Hymenoptera* and nematodes [3, 4]. For biocontrol, three pathovars are most widely used, i.e., A (var. *thuringiensis*, var. *dendrolimus*, var. *galleria*, var. *kurstaki*) mainly against *Lepidoptera*, B (var. *israelensis*) as a producer of larvicidal drugs, and C (var. *tenebrionis*, var. *darmstadiensis*) against beetles [5]. Microbiologicals based on these bacteria are highly toxic to certain groups of insects, safe for humans and have minimal impact on the environment [6-9], reducing the chemical load on the environment.

B. thuringiensis are multifunctional. Along with entomocidal activity, they possess antifungal activity against numerous pathogens that cause dangerous diseases in crops [10-12]. The wide range of *B. thuringiensis* properties is due to a repertoire of genes encoding the synthesis of various protein toxins, minor protein

virulence factors, and various low-molecular metabolites [13].

Preparations active against beetles are widely used against the most common and dangerous pests of crops, such as the Colorado potato beetle (*Leptinotarsa decemleniata* Say) and the 28-spot potato ladybug (*Henoseplachna vigintioctomaculata* Motsch). The Colorado potato beetle lives everywhere. It has high fertility, easily adapts to a variety of external conditions and biotic factors. Different stages of the beetle feed throughout the growing season. Crop losses can reach 30-50% or higher, up to complete loss, if the pest is not controlled and its population increases significantly [14].

In the Far East, where about 5% of potatoes in the Russian Federation are produced, along with the Colorado potato beetle, the 28-spotted potato ladybug (*Henoseplachna vigintioctomaculata* Motsch, 1857) causes significant damage to the crop [15]. It can damage 20 to 100% of the leaf surface, resulting in significant yield losses [16].

For the production of *B. thuringiensis* based preparations, nutrient media have been developed, which include corn and soy flour, starch, protein-vitamin concentrate (PVC) and other natural organic ingredients. Some ingredients may be deficient. Good results were obtained when using pea, barley and oat flour as a source of nitrogen [17]. Preparations obtained in these media are highly active, but there are some disadvantages. Foaming of the culture often occurs and as a result expensive filters in fermenters become clogged and require replacement. Difficulties also arise during treatments, since solutions containing organic components of the medium clog the nozzles. In addition, nutrient media of natural organic composition depend on the quality and origin of the raw materials. Hence, a request arose to develop novel media based on relatively inexpensive and standard, mainly synthetic, components to ensure the best growth and biosynthesis of metabolites which in turn would reduce the cost of biopesticide production.

It is known that culture media containing predominantly synthetic components include carbon and nitrogen sources, the derivatives of natural components that increase spore formation and production of protein crystalline endotoxin toxic to insects [18, 19]. Yeast extract [20] and casein hydrolysate as a source of protein [21] are the most commonly used nitrogen sources. Glycerol and glucose predominate as carbon sources [22, 23].

In this work, for the first time, we submit the improved optimal modified semi-synthetic medium MMBt (Modified MBt). The improved medium ensures the production of effective and technologically advanced biologicals based on various serovars of *B. thuringiensis*.

The purpose of the work is to search for optimal nutrient media for *Bacillus thuringiensis* (Bt) to produce and use Bt-based biologials.

Materials and methods. The study involved gram-positive bacteria *B. thuringiensis* var. *thuringiensis* 800/15 (BtH1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH10 25).

The culture media was as follows. CCY contained 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 0.05 mM $ZnCl_2$, 0.2 mM $CaCl_2 \cdot 6H_2O$, 13 mM KH_2PO_4, 26 mM K_2HPO_4, 20 mg/l glutamine, 1 g/l casein hydrolysate, 0.4 g/l yeast extract, 0.6 g/l glycerol [24]. MWt medium contained 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02 g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$ [25]. LB medium was 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl [26]. Modified semi-synthetic medium MMBt was 7 g/l casein hydrolyzate, 6.8 g/l KH_2PO_4, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.0022 g/l $MnSO_4 \cdot 4H_2O$, 0.014 g/l $ZnSO_4 \cdot 7H_2O$, 0.02

g/l $Fe_2(SO_4)_3$, 0.18 g/l $CaCl_2 \cdot 4H_2O$ [25] + glucose (0.5, 1.0 or 2.0%) and Na citrate (2 g/l). Yeast polysaccharide media (YPS) for BtH1 [27] and BtH10 [28] served as a standard.

Bt strains were cultured on a shaker (220 rpm) at 29 °C in 750 ml Erlenmeyer flasks with 40-50 ml of medium for 48-72 h until spores and crystalline endotoxin complete formation. The cell number and exotoxin amount, expressed in LC_{50} for the housefly *Musca domestica* Linn. Larvae were determined as described by S.D. Grishechkina et al. [29]. The experiment was arranged in triplicate.

Based on strains BtH1 800/15 and BtH10 25, batches of liquid preparations were produced in fermenters at the Ecos branch of the All-Russian Research Institute of Agricultural Microbiology (St. Petersburg-Kolpino). The drug field effectiveness was assessed on potato (*Solanum tuberosum* L.) in 2020 and 2021, on Yantar variety in the Far East (Primorsky Territory, Ussuriysk Province) against *H. vigintioctomaculata* and on Emelya variety in the Tambov Province against *L. decemleniata*. Liquid preparations produced using YPM and MMBt were tested. The experiments were carried out in 4 replicates on 10 m^2 plots. No treatment was carried out in the control; the chemical standard against the Colorado potato beetle was the drug Borey (JSC August, Russia; 0.1 l/ha). The pests were counted on days 5, 10 and 15 after treatment. The rates of liquid preparations against Colorado potato beetle was 20 l/ha, against potato ladybugs 15 and 20 l/ha. The number of pests per plant was calculated (4 replicates of 10 plants).

Biological effectiveness (BE) of drugs was calculated according to Abbot'd formula [30]:

$$BE = (C - T)/C \times 100\%,$$

where C is the number of the pest before treatment, T is the number of the pest after treatment.

The antifungal activity of the BtH10 25-based drug produced in MMBt and YPM was determined in Petri dishes by the in vitro agar block method [31]. The preparations (10% concentration) were added to a molten and cooled to 40 °C potato agar. Blocks 0.8 cm in size cut from a 7-day culture of fungi, were placed on the solidified agar. In the control, the medium was without the drugs. Test fungi cultures were *Botrytis cinerea* Pers. (strain C-5) and *Bipolaris sorokiniana* (Sacc.) Shoemaker (strain C-20). There were 4 dishies for each treatment in 4-fold biological repetitions.

Inhibitory activity was calculated by the Abbot's formula [30]: SI (the degree of fungal colony growth inhibition) = $(D_c - D_t)/D_c \times 100\%$, where D_c and D_t are the diameter of the fungal colony in the control and the test, respectively.

The statistical significance of differences was assessed using two-way analysis of variance with Bonferroni correction for multiple comparisons. Means (*M*), standard deviations (±SD) and standard errors of the means (±SEM) were calculated. Data on the effectiveness of drugs against pests were processed by analysis of variance with a 95% confidence interval [32].

Results. BtH1 800/15 and BtH10 25 culturing showed that in semi-synthetic media MBt and LB the titers were 2 times lower than in YPM, and in CCY medium the titers were 10 times lower. The exotoxin concenteration also turned out to be lower, but for BtH1 800/15, in the MBt medium it was inferior to YPM (Table 1), so the MBt was chosen for further studies. According to K.W. Nikkerson et al. [33], glucose and sodium citrate added to the nutrient medium promotes microbial growth and crystal formation. In this regard, we studied the effect of different glucose concentrations (0.5, 1.0 and 2.0%) on the development of *B. thuringiensis* cultures. It was found that at 0.5 and 1.0% glucose the development of the culture ended after 48 hours, but the titers at a 0.5% concentration were lower

than at 1.0%. When 2.0% glucose was added, the development of the culture was inhibited and the sporulation ended after 72 hours. Having determined the optimal glucose concentration of 1.0%, we added sodium citrate (2 g/l) to the medium as an additional source of carbon. The titers in the MMBt medium increased in BtH1 800/15 to 1.95 CFU/ml (by 21.8%), in BtH10 25 to 1.8 CFU/ml (by 12%) (see Table 1). Activity rates also increased. In the MMBt medium, culture growth accelerated and sporulation and the crystalline protein endotoxin formation ended after 48 hours, whereas in YPM it ended after 72 hours.

1. Characterization of preparations based on *Bacillus thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH_{10} 25), gown on different nutrient media ($M\pm SD$)

Strain	Medium									
	YPM		MBt		MMBt		CCY		LB	
	1	2	1	2	1	2	1	2	1	2
BtH_1 800/15	3.35±0.10	2.4±0.2	1.60±0.10	3.0±0.1	1.95±0.20	2.8±0.2	0.38±0.10	6.9±0.1	1.30±0.10	6.3±0.2
BtH_{10} 25	3.0±0.2	2.5±0.2	1.5±0.1	4.2±0.1	1.8±0.1	3.4±0.2	0.3±0.1	7.9±0.2	1.1±0.1	6.7±0.2

N o t e. 1 — titer (CFU/ml), 2 — activity by LC_{50} for 2nd instar larvae *Musca domestica*. For a description of the media composition and sample sizes, see the Materials and methods section.

Previously, when culturing *B. thuringiensis* in yeast-polysaccharide media, we selected the doses of inoculum. It was found that doses from 0.2 to 1.0% did not significantly affect the strain productivity [17]. The same range of inoculation (0.2; 0.5; 0.8; 1.0%) turned out to be insufficient for MMBt, and the Bt titers were low. An increased doses of inoculum (4.0; 6.0; 10.0%) showed the best results for 10% inoculum from the nutrient medium volume, which is consistent with the data of V.V. Biryukova [34].

An assessment of the liquid preparation effeciacy against the Colorado potato beetles on the Emelya variety potatoes (the Tambov Province) showed a significant dependence on the number of the pest, which, in turn, depended on weather conditions and averaged 9 bettles per bush in 2020 and 33 bettles per bush in 2021.

2. Bioeffectiveness of liquid preparations based on *Bacillus thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH_{10} 25) cultures in different nutrient media against Colorado beetle (*Leptinotarsa decemlineata* Say) on potato (*Solanum tuberosum* L.) cv. Emelya ($N = 4$, $n = 10$; Tambov Province, 2020-2021)

Treatment	Dosage, l/ha	Average larvae number per plant				Bioeffectiveness, % ($M\pm SD$)		
		before treatment	after treatment			day 5	day 10	day 15
			day 5	day 10	day 15			
2020								
BtH_1 800/15 (YPM)	20	8.05	0.38	2.00	3.38	95.3±1.6	74.4±3.1	58.8±4.4
BtH_1 800/15 (MMBt)	20	14.80	2.38	4.98	3.85	83.3±5.0	65.4±8.7	73.7±8.7
Chemical standard								
Borei	0,1	4.70	0.25	0.88	2.05	100	81.4±4.4	56.2±2.9
Control	No tretment	7.10	3.08	2.52	1.30			
LSD_{05}						7.7	16.0	14.9
2021								
$BtH_1$800/15 (YPM)	20	36.30	8.96	14.2	8.97	75.3±6.3	60.7±7.7	47.7±3.6
BtH_1 800/15 (MMBt)	20	35.20	11.4	16.2	20.3	67.7±2.1	53.7±5.5	42.5±5.0
BtH_{10} 25 (MMBt)	20	30.45	16.8	18.0	18.5	47.9±3.1	40.8±3.9	39.4±4.9
Chemical standard								
Borei	0,1	35.10	3.75	6.0	11.3	89.3±5.6	82.8±6.1	69.4±4.9
Control	No tretment	25.90	29.4	19.5	10.1			
LSD_{05}						12.0	8.9	13.6

N o t e. The use of drugs produced in the MMBt medium did not lead to clogging of the nozzles, unlike the drugs obtained in the YPM.

In 2020, the effectiveness of the BtH1 800/15-based drug produced in the

YPM was high and reached 95.3% on day 5, being practically not inferior to the chemical standard (100%). The effectiveness of the BtH1 800/15-based drug produced in MMBt was slightly lower (83.3%) but the protective effect lasted longer. On day 15, the effectiveness was 73.7% vs. that recorded for the drug produced in the YPM and for the chemical drug Borei, 58.8 and 56.2%, respectively. In 2021, the effectiveness of the drugs was lower compared to 2020 and on day 5 after treatment with $BtH_1$800/15 it differed slightly for MMBt and YPM media (67.7 and 75.3%, respectively). When using the drug BtH10 25 produced in MMBt, the effectiveness was inferior to BtH1 800/15 and on day 5 reachrd 47.9%. In all variants, the effectiveness decreased on day 15 (Table 2).

3. Bioeffectiveness of liquid preparations based on *Bacillus thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) and *B. thuringiensis* var. *darmstadiensis* 25 (BtH_{10} 25) cultures in different nutrient media against 28-spotted potato ladybird (*Henoseplachna vigintioctomaculata* Motsch, 1857) on potato (*Solanum tuberosum* L.) cv. Yantar ($N = 4$, $n = 10$; Primorsky Krai, Ussuriysk District, 2020-2021)

Treatment	Dosage, l/ha	Average larvae number per plant				Bioeffectiveness, % (M±SD)		
		before treatment	after treatment					
			day 5	day 10	day 15	day 5	day 10	day 15
			2020					
BtH_1 800/15 (YPM)	15	2.7	1.2	0.6	0.2	60.5±7.0	79.5±5.0	86.4±4.9
BtH_1 800/15 (MMBt)	20	3.2	0.9	0.6	0.2	70.5±5.4	84.3±7.1	90.3±3.8
BtH_1 800/15 (MMBt)	15	3.0	1.2	0.5	0.4	63.9±6.7	78.5±4.1	76.2±5.6
Control	No tretment	3.9	4.3	2.8	2.1			
LSD_{05}						9.4	15.5	13.2
			2021					
BtH_1 800/15 (YPM)	15	4.3	1.8	1.0	1.0	76.8±6.4	79.2±4.1	81.0±5.2
BtH_1 800/15 (MMBt)	15	5.7	2.3	1.2	1.2	78.5±5.7	80.4±3.6	82.7±6.9
BtH_{10} 25 (YPM)	15	6.5	3.5	1.5	0.7	72.1±4.9	76.8±5.9	89.6±6.7
BtH_{10}25 (MMBt)	15	9.3	3.0	1.5	1.4	83.0±4.0	86.2±6.0	86.0±5.7
Control	No tretment	3.9	7.9	5.2	4.9			
LSD_{05}						7.6	9.7	13.0

The high effectiveness of liquid preparations produced in different media was also shown when treating potato plantings against the 28-spotted potato ladybug in the Primorsky Territory. In 2020, with the BtH1 800/15 application rate of 15 l/ha the effectiveness for YPS and MMBt media on day 5 was 60.5 and 63.9%, respectively. For MMBt, an increase in the application rate to 20 l/ha increased the efficiency to 70.5%. Similar data we obtained in 2021 (Table 3).

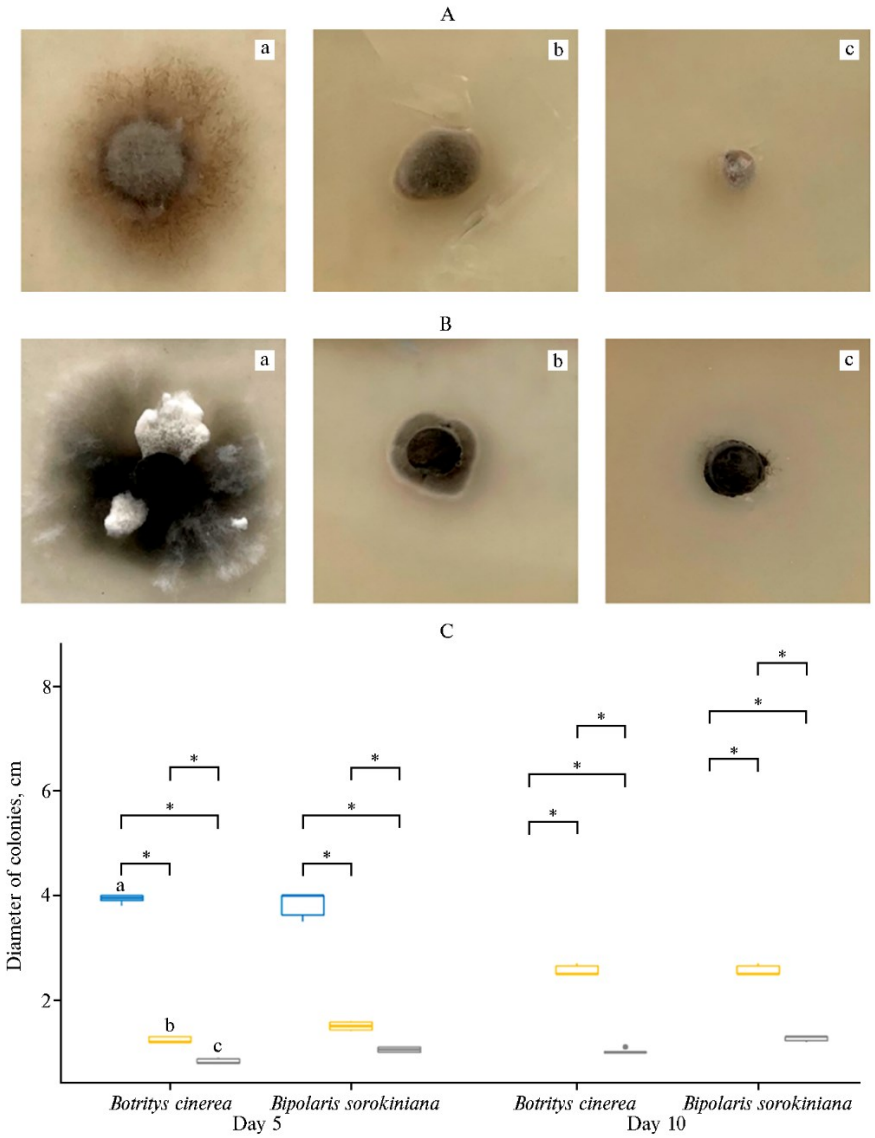
In 2021, along with the drug BtH1800/15, the effectiveness of BtH1025 was studied. On day 5 after treatment, for the preparation in the MMBt medium, the effectiveness was slightly higher than for YPM, up to 83.0% compared to 72.1%. On day 15, the activity of the drugs increased.

Thus, field trials on potatoes against the Colorado beetle and 28-spotted ladybird showed the advantage of MMBt for production of the biologicals.

On day 5 in in vitro tests with BtH10 25, the inhibitory activity of the MMBt-based liquid preparation was 12% higher than that of the YPM-based, for *B. sorokiniana* 72.3 and 60.8%, respectively, for *B. cinerea* 78.9 and 67.4% (Fig.). On day 10, this trend continued. The growth inhibition of *B. sorokiniana* decreased to 57.3%, *B. cinerea* to 44.3% for YPM preparation while for MMBt, the inhibitory activity was not reduced. In the control, on day 5, *B. sorokiniana* and *B. cinerea* showed approximately the same growth rates but differences occurred on day 10.

It should be noted that the diameter of the *B. sorokiniana* and *B. cinerea* colonies on days 5 and 10 differed ($p < 0.0001$) both in the YPM- and MMBt-

based liquid preparations vs. control and betwee each other (see Fig., B).



Growth (day 5) of *Botritys cinerea* Pers. C-5 (A) **and *Bipolaris sorokiniana* (Sacc.) Shoemaker C-20** (B) **and the diameter of colonies (days 5 and 10)** (B) **on potato agar after treatment with a *Bacillus thuringiensis* var. *darmstadiensis* 25 (BtH10 25)-based preparation:** a — control (without treatment), b — culture in the YPS medium, c — culture in the MMBt medium (see the description in the Materials and methods section).

* The differences between the treatments are statistically significant at $p < 0.0001$.

The MMBt medium had a significant advantage over the yeast-polysaccharide media widely used for the production of liquid forms of drugs. Since *Bacillus thuringiensis* is one of the most widely used bacteria in biotechnology [35, 36], optimization of media for their growth is of significant interest. Thus, complex substrates such as molasses, corn extract, corn flour [37)], soy flour, etc. [38] are used as organic ingredients. Note, an important factor in the production of biologicals is the lower cost of culture media, and soybean flour is one of the cheapest components [39]. However, its use often leads to foaming of the culture during preparation production, requires filter replacement, ultimately increasing the product price.

A number of studies have also shown the importance of balancing carbon and nitrogen sources in media to achieve optimal sporulation efficiency in Bt cultures. It was found that the higher concentration of glucose in the medium, the higher is optical density of the culture, while increasing the yeast extract concentration suppresses sporulation [40]. Other nitrogen sources can have either stimulatory or inhibitory effects, depending on the culture growth stage [41]. Moreover, a recent study that provided appearantly the most detailed comparative up-to-date analysis of the influence of nitrogen and carbon balance in the culture medium on Bt sporulation showed that the highest sporulation occurs when the carbon to nitrogen ratio in the medium is 5:1 [42]. It is noteworthy that when bacteria of the genus *Bacillus* are used for the production of enzymes (the vegetative culture stage), increasing the nitrogen concentration, on the contrary, can have a beneficial effect [43]. Thus, the selection of media components to optimize bacterial growth significantly depends not only on the systematic position of the microorganisms, but also on the stage of the life cycle (vegetative or spore). Yeast autolysates traditionally used in Bt culture media delay sporulation. In addition, complex organic components reduce the processability of the resulting media.

So, the modified semi-synthetic medium (MMBt) we developed, which consists of casein hydrolyzate and a standard set of salts supplemented with 1% glucose and 2 g/l Na citrate as carbon sources, eliminates shortcomings discussed hereinabove. MMBt is suitable for the production of *Bacillus thuringiensis*-based liquid preparations and has advantages over yeast-polysaccharide media (YPS). Thus, the use of the MMBt medium can ensure the production of *B. thuringiensis* biologicals that are quite effective and more convenient for use. In MMBt medium, the growth rate accelerates and less time is required for the formation of spores and protein crystalline endotoxin. E.g., in the MMBt medium, the formation ended after 48 hours vs. 72 hours in the YPS medium that reduces energy costs. In field trials, there was no clogging of the equipment nozzles for MMBt-based preparations while the YPS-based preparations require washing of the spraying equipment due to contamination with organic particles. Field trials confirm that *B. thuringiensis* var. *thuringiensis* 800/15 and *B. thuringiensis* var. *darmstadiensis* 25 preparations in MMBt are not inferior to YPS preparations in effectiveness and have a longer insecticidal effect against the Colorado potato beetle (the Tambov Povince) and the 28-spotted potato ladybug (the Far East). The use of the developed medium contributed to enhancing the activity of the drug based on *B. thuringiensis* var. *darmstadiensis* 25 against plant pathogenic fungi. In general, the MMBt is a promising and more technologically advanced alternative to yeast-polysaccharide media, avoiding the use of additional organic components which in some cases can be non-standard and difficult to acquire.

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THE EFFECT OF ENDOPHYTIC BACTERIA *Bacillus thuringiensis* W65 AND *B. amyloliquefaciens* P20 ON THE YIELD AND THE INCIDENCE OF POTATO RHIZOCTONIOSIS AND LATE BLIGHT

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Abstract

Chemical fungicides are chemicals used to combat late blight and potato rhizoctoniosis. However, due to repeated treatments, the resistance of plant pathogens to fungicides increases. Biofungicides serve as an alternative to chemical fungicides. The use of strains of endophytic bacteria of the genus *Bacillus* is promising for the development of novel biofungicides. Endophytes, being inside plants, have an advantage in interactions with the plant compared to bacteria occupying other ecological niches. In this work, for the first time, the effectiveness of experimental samples of preparations based on strains of endophytic bacteria of the genus *Bacillus* was established when growing potato (*Solanum tuberosum* L.) varieties differing in resistance to late blight in the conditions of the North-West of the Russian Federation. It is known that the effectiveness of the use of endophytes with biocontrol activity differed in the field when growing potatoes, while the varietal responsiveness of potatoes to biocontrol agents has not been sufficiently studied. The aim of the research was to study the effect of experimental samples of preparations of endophytic bacteria *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 on the yield and infection of potato plants with rhizoctoniosis and late blight. Strains of endophytic bacteria *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 isolated from potatoes had antagonistic activity to phytopathogens-pathogens of late blight *Phytophthora infestans* (Mont. de Bary) and rhizoctoniosis *Rhizoctonia solani* (Kuhn.) when growing on agar media. Small-scale field experiments (2020-2021) were conducted at the experimental field of the Leningrad Research Agriculture Institute Belogorka. The experiment scheme included the following options: clean control — no treatments; chemical control — treatment with chemical fungicides: CELEST® Top, SC (Syngenta, Russia), Mankoceb, WP (AgroRus and Co., Russia), Rapid Duo, WP (AgroRus and Co., Russia), Infinito, SC (Bayer Crop Science, Germany), Buzzer, SC (CROPEX, Russia) and desiccant Golden Ring (Agro Expert group, Russia); biological control — BisolbiSan (BISOLBI INTER, Russia), the biofungicide based on rhizospheric bacteria *Bacillus subtilis* Ch-13; experimental sample of the preparation *B. thuringiensis* W65, experimental sample of the preparation *B. amyloliquefaciens* P2. Two potato varieties, Charoit (resistant to late blight) and Gusar (susceptible to late blight), were used. In the

experiments, the dynamics of plant growth and development, yield and infection of potato plants with rhizoctoniosis and late blight were evaluated. Statistical treatment of the obtained results (calculations of averages and their standard errors, ANOVA analysis of variance, Duncan's test, was carried out using the Statistica 10 program («StatSoft, Inc.», USA). When inoculated with experimental samples of *B. amyloliquefaciens* P20 and *B. thuringiensis* W65, the duration of flowering of potato plants increased by 8-13 days compared to the control. The potato tuber harvest also increased by 7.9-14.6 % ($p < 0.05$). The largest increase in yield was registered on the Gusar variety in 2020. It was found that responses of potato varieties to inoculation with experimental samples of *B. amyloliquefaciens* P20 and *B. thuringiensis* W65 preparations differed. The yield of potato tubers of the Charoit variety mainly increased due to an increase in the average weight of one tuber while the yield of the Gusar variety increased due to an increase in the number of tubers per plant. When inoculated with experimental samples of *B. amyloliquefaciens* P20 and *B. thuringiensis* W65, the crop structure changed, the yield of the large tuber fraction increased by 22.5-30.6 % ($p < 0.05$) in Charoit variety. The use of experimental samples of *B. amyloliquefaciens* P20 and *B. thuringiensis* W65 did not have a significant effect on the development of rhizoctoniosis in small-scale experiments. The *B. amyloliquefaciens* P20-based preparation showed 42.8 % biological efficacy in reducing the development of late blight on the potato variety Charoit. Preparation of endophytic bacteria based on *B. amyloliquefaciens* P20 can be recommended for further testing in commercials field trials when growing potatoes in an integrated protection system together with chemical fungicides and inducers of systemic plant resistance.

Keywords: endophytic bacteria, *Bacillus thuringiensis* W65, Bacillus amyloliquefaciens P20, biofungicides, tuber harvest, potatoes, rhizoctoniosis, late blight

Late blight and rhizoctonia are the most common and aggressive mycoses among diseases of potato (*Solanum tuberosum* L.), which can lead to loss of 30-50% yield and impares the realization of the potential productivity of the crop [1]. Oomycete Phytophthora infestans (Mont.) de Bary, the causative agent of late blight with asexual reproduction, spreads due to the formation of sporangia which germinate and form motile zoospores at low air temperatures from +4 to +15 °C, then encyst and their growth tubes penetrate into the plant tissue. At elevated temperatures (20-25 °C), zoospores are not formed. The sexual process is possible only if there are two types of mating in the population. As a result of mating, oospores appear, which, after overwintering, germinate into growth tubes. Sporangia are formed at the end of the growth tubes [2]. The main source of infection is diseased tubers and contaminated plant debris [3]. During infection, the pathogen synthesizes protein effector molecules (apoplastic and cytoplasmic) which affect the structure and function of the plant cell [4-7]. EPIC1 is one of the best characterized apoplastic effectors that targets host defense-associated proteases [7-9]. Oomycete cytoplasmic effectors include the RXLR class, containing the conserved Arg-any amino acid-Leu-Arg (RXLR) peptide motif that is required for delivery of these proteins to plant cells [10]. Plant genetic resistance to *P. infestans* is regulated through recognition of specific RXLR effectors by host NB-LRR resistance proteins within plant cells [10].

Over the past decades, there has been an increase in the aggressiveness of the late blight pathogen *P. infestans* [11, 12]. It is associated with recombination of virulence genes during sexual reproduction and mating leading to the emergence of highly aggressive races of the pathogen [13, 14]. The aggressiveness of *P. infestans* may be due to the predominance of the aggressive clonal line 13_A2 in the population [12].

The gembiotrophic fungus *Rhizoctonia solani* Кьhn, a teleomorph, or sexual stage, of *Thanatephorus cucumeris* (A.B. Frank) Donk is the causative agent of potato rhizoctonia blight. The fungus attacks tubers, stems, stolons and roots of adult plants. The damage is visiblr due to the formation of brown spots and ulcerations on sprouts, stolons and roots of potato plants. Black sclerotia appeare on the tubers. The teleomorph appears as a dirty white felt coating on the lower part of the stems at high humidity and an optimal temperature of 15-21 °C. Sclerotia can remain dormant for many years in soil and dead plant debris [15, 16]. Effectors

described for *R. solani* induce necrosis in many plants species [17, 18]. The effector RsRplA is also known which acts as an active protease inhibitor and suppresses the induction of plant hypersensitivity reactions [19].

Currently, the chemical method of protecting potatoes from mycoses is the most effective [20, 21]. Resistance of phytopathogens to fungicides increases due to repeated treatments [22-24]. The development of resistance of pathogens to fungicides is also influenced by the reproduction of the pathogen, the degree of protection by the fungicide, the mechanism of action on the pathogen, and the dynamics of the pathogen population [25]. After the widespread introduction into practice of selective systemic drugs, the frequency of detection of resistant races of pathogens affecting various crops, including potatoes, has increased [26-29]. Resistance to the highly effective systemic fungicide mefenoxam (the R-enantiomer of metaloxyl) in *P. infestans* was observed when previously susceptible isolates were exposed to sublethal doses (5 mg/ml) of the fungicide [27]. Mefenoxam-resistant *P. infestans* isolates showed slow growth compared to susceptible isolates [30].

ABC transporters and detoxifying enzymes (cytochrome P450) are involved in the formation of *P. infestans* resistance to mefenoxam/metalaxyl [31, 32]. Changes occur in the plasmalemma of *P. infestans* that prevent the poison from entering the cells [33]. Thus, when metaloxyl was used in minimal concentrations (0.1 and 1 μg/ml), the growth inhibition of *P. infestan*s from the Priluki population in Belarus exceeded 50.0%. With an increase in the concentration to 10 μg/ml the sensitivity of the pathogen increased to 75.2%, and when using a high concentration (1000 μg/ml), mycelial growth was completely suppressed [34].

As an alternative to chemical fungicides, the widespread introduction of new biological fungicides has been proposed [23, 35]. With the combined use of chemical fungicides and biofungicides, pathogen resistance is reduced and plant immunity is stimulated [23, 36]. In addition, to protect against mycoses in agricultural practice, it is necessary to introduce new potato varieties with resistance to pathogens [14, 37].

Bacteria from the genera *Pseudomonas*, *Bacillus*, *Lysobacter*, *Enterobacter* and *Paenibacillus* are the most effective in suppressing the development of mycoses [38]. The antagonistic activity of bacteria of the genus *Bacillus* against mycoses of various crops has been studied [39-42]. Bacilli are capable of producing metabolites with fungicidal activity [43-45]. Thus, the combination of fengycin B and surfactin, produced by *B. pumilus*, was more effective in protecting potatoes against late blight than each metabolite applied separately [46].

Recombinant endophytic *B. subtilis* 26DCryChS with the *Btcry1Ia* gene encoding Cry1Ia had complex fungicidal and insecticidal effects [47, 48].

The special literature discusses the prospects of using endophytic bacteria of the species B. thuringiensis, which have a complex effect, for plant protection [49-51]. Chitinase-producing *B. thuringiensis* strains can effectively inhibit the pathogenic fungi *Fusarium oxysporum*, *F. graminearum*, *Pyricularia grisea*, and *Physalospora piricola* [49-51]. Chitinases can also enhance the insecticidal activity of *B. thuringiensis* [52].

Endophytic bacteria are promising objects in the biosecurity because they are located inside the host plant and directly interact with it [53-55]. Endophytes are able to reduce the number of phytopathogens due to competition for the ecological niche and the synthesis of biologically active substances (BAS) [56]. In pot and field trials, the effect of endophyte bacteria on the late blight [57-59] and rhizoctonia [60, 61] infection of potato was studied. The effectiveness of these bacteria has been shown to vary under field conditions, with synthetic chemical

fungicides being more effective [59].

The antagonistic activity of four *B. subtilis* strains MTCC-2422 (T-3), KU936344 (T-4), KU936345 (T-5) and KU936341 (T-6) against late blight was studied in field experiments when growing potatoes of the Kafri Jyoti variety. The fungicide mancozeb M 45 (CURZATE®) was a positive control [59]. Treatment with bacteria significantly reduced the incidence of late blight compared to control, but their effectiveness was significantly lower than that of a chemical fungicide. The intensity of disease development was 65% with mancozeb and more than 74% with bacteria [59]. The reduction in the incidence of potato rhizoctoniosis when using the culture filtrate of *B. subtilis* HussainT-AMU was 71% in pot tests and 50% in field conditions, which indicates the high biocontrol activity of this strain against *R. solani* [60]. Treatment of potato tubers with phytosporin (an experimental *Bacillus subtilis* 26-based drug; BashInkom, Russia) in the Kamchatka Territory did not effectively reduce the rhizoctonia disease, the degree of the disease development before harvest decreased by 7.5%, the prevalence of the disease by 8.6%, the yield increased by 4.7 t/ha [61]. However, when tubers were co-treated with the fungicide TMTD (JSC Firm August, Russia) at 1.7 l/t and sprayed with sporobacterin, the development and prevalence of the disease decreased by 11.6 and 48.5%, respectively [61].

Treatment of potato plants with fluopimomide at a minimum dose of 85 g/ha in combination with *B. velezensis* SDTB038 significantly reduced the incidence of late blight and increased yield. Its average efficiency was 69% for 2 years, being comparable to the effect of the maximum dose (170 g/ha) of the fungicide (68.6% average efficiency for 2 years). The *B. velezensis* strain SDTB038 allows fungicide concentrations to be reduced in the field [57]. Currently, the study of new strains of endophyte bacteria to protect potatoes in field conditions is relevant.

In this work, we for the first time established the effectiveness of using experimental samples of preparations based on strains of endophytic bacteria *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 in the conditions of the northwest of the Russian Federation when growing potato varieties that differ in resistance to late blight.

The purpose of the work was to evaluate the effect of experimental samples of preparations based on endophytic bacteria *Bacillus thuringiensis* W65 and *B. amyloliquefaciens* P20 on potato yields and the susceptibility of plants to rhizoctonia and late blight.

Materials and methods. Endophytic *Bacillus* strains were isolated from the internal tissues of potato (*Solanum tuberosum* L.) variety Sudarynya resistant to potato cancer. The bacteria were identified as *B. thuringiensis* (strain W65) and *B. amyloliquefaciens* (strain P20) by the16S rRNA gene sequencing. The nucleotide sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov) under the numbers OP537151 and OP537150, respectively. Strains *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 were also deposited in the Network Bioresource Collection for Genetic Agriculture Technologies (the All-Russian Research Institute of Agriculture).

For endophyte isolation, potato tubers were washed with tap water, sterilized for 5 min in 70% ethanol and 10 min in 15% H_2O_2. After sterilization, they were successively washed in sterile water five times for 2 min. The last water (40 microliters) was surface sown on potato dextrose agar (PDA, Difco, USA), incubated for 2 days at 28 °C, and in the absence of bacterial growth, sterile tubers were used to isolate endophytes. PDA contained (per liter) 4 g of freeze-dried potato decoction obtained from 200 g of potatoes, 20 g of dextrose, 20 g agar, pH 5.6.

Under sterile conditions, the tubers were cut with a scalpel, the internal tissues were cut out and placed in a mortar, 5 ml of distilled water was added, and the sample was ground with pestle until a homogeneous state. Then the tuber tissue homogenates were diluted to 10^{-5}. The lasr dilution was plated on Petri dishes with PDA agar in 3-fold repetition. Petri dishes were placed in a thermostat for 5 days at 28 °C, and indicidual colonies of bacteria were seeded into test tubes with PDA.

Experimental samples of endophytic bacterial preparations based on strains *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 (500×10^6 CFU/ml) were produced using a universal RALF bioreactor (Bioengineering, Switzerland). As a standard, we used the biofungicide BisolbiSan, L (liquid) (Bisolbi-Inter LLC, Russia), registered in the Russian Federation to combat rhizoctonia, late blight and Alternaria potato (State catalog of pesticides and agrochemicals approved for use in the Russian Federation, 2022). Biofungicide BisolbiSan, L is produced on the basis of a strain of rhizosphere bacteria *B. subtilis* Ch-13 (500×10^6 CFU/ml)

A nutrient medium of the following composition was used (in g/dm^3 of distilled water): molasses 25, corn extract 12.5, $MgSO_4\cdot7H_2O$ 0.2, $CaCl_2$ 1.0, $MnSO_4$ 0.01.

The counts of bacteria in experimental samples was determined by the limiting dilution method [62] followed by culture on bovine meat fermented (BMF) agar (BMF base 15.0 g, sodium chloride 9.0 g, microbiological agar 13.5 g, NICF, Russia). The fungicidal activity was assessed by the well method on potato dextrose agar PDA [63]. Suspensions of conidia of the studied strains of phytopathogenic fungi (5 ml, 1×10^6 conidia/ml) were added to 250 ml of warm (45 °C) potato dextrose agar. The medium was mixed and poured into Petri dishes. After hardening, wells 8 mm in diameter and a depth of the entire thickness of the agar were made in each dish using a cork drill (4 pieces per dish). The tested bacterial strains were grown stationary in potato dextrose broth PDB (Difco, USA) for 5 days at 28 °C. Then, 100 μl of the bacterial suspension was added to the prepared wells in triplicate. The antifungal activity was assessed by the zone of growth inhibition around the wells after 3-5 days of incubation of inoculated Petri dishes at 28 °C.

To study the growth-stimulating activity, sterile seeds of watercress (*Lepidium sativum* L., 1753) Dukat variety were soaked in the suspensions of bacterial cells with a titer of 5×10^5 CFU/ml for 30 min. Then 30 seeds were placed in sterile Petri dishes on damp filter paper. In the control treatment, the seeds were soaked in a sterile saline solution. The seedlings were grown for 5 days at 28 °C. The experiments were arranged in triplicate.

In field small-plot experiments, new varieties of table potatoes Charoit and Gusar approved for the North-West Russia, differing in resistance to late blight, were grown.

Charoit (originated by the North-Western Research and Production Association for Breeding and Plant Growing Belogorka, Selection Firm Liga LLC, Branch of the Russian Agricultural Center in the Novgorod Province) is an early ripening variety, moderately resistant to late blight, common scab and rhizoctonia. Seed productivity is 20-30 t/ha, commercial prosuctivity 40-60 t/ha. The period from full germination to harvest is 50-60 days.

Gusar (originated by the LLC Selection Firm Liga) is a mid-season variety, moderately susceptible to late blight, moderately resistant to common scab. Productivity for seeds is 25-30 t/ha, commercial productivity is 40-60 t/ha. The growing season from complete germination to harvesting is 75-80 days.

Small-plot experiments were performed at the Leningrad Research Institute of Agriculture Belogorka (Belogorka village, Leningrad Province, Gatchina District). The soil is soddy-podzolic light loamy, medium cultivated, agrochemical parameters of the arable layer: mobile phosphorus P_2O_5 20.2 mg/100 g, exchangeable K_2O 7.7 mg/100 g of soil, organic matter 2.6%, pHsol. 5.1. The granulometric composition of the soil was optimal for cultivating potato plants. The predecessor in field crop rotation was winter rye.

Mineral fertilizers azofoska (NPK 16:16:16) were applied before cutting ridges at the rate of 500 kg/ha (80 kg/ha a.i.). Potatoes were planted on May 8, 2020 and 2021 with two inter-row treatments, the pre-emergence (June 12, 2020 and June 10, 2021) and post-emergence (June 28, 2020 and June 25, 2021) hilling. Before harvesting, chemical desiccation was carried out with Golden Ring (2.0 l/ha, Agro Expert Group, Russia). Spraying of vegetative plants was carried out using a battery-powered backpack sprayer Solo (SOLO Kleinmotoren GmbH, Germany), the flow rate of the working fluid was 500 l/ha.

The test design was randomized with 3-fold repetition. The area of each registration plot was 11.2 m^2, 2.8 m width (4 ridges), 3 m length. The yield was harvested on September 15 in 2020 and September 10 in 2021. The development of diseases was determined in 2021. When assessing the incidence of rhizoctoniosis, the count was carried out on stems and stolons on 24 bushes in each experimental treatment, when assessing the spread of late blight on 45 bushes in each experimental treatment. The degree of development of rhizoctoniosis and late blight was determined according to the guidelines for registration testing of fungicides [64].

The development of late blight was assessed by an 8-point scale with the following gradations: 0 — no signs of damage; 1 — less than 2.5% of the leaf surface affected; 2 — 2.5-5% of leaves affected; 3 — 6-10% of leaves affected; 4 — 11-15% of leaves affected; 5 — almost every leaf affected, 16-25% leaf drying; 6 — 26-50% drying of leaves, beginning of damage to stems; 7 — 51-75% drying of leaves, damage to stems progresses; 8 — the plant died.

The development of rhizoctonia on stolons was assessed on a 3-point scale: 0 — no signs of damage; 1 — up to $^1/_3$ of the stolons are affected; 2 — $^1/_3$-$^2/_3$ stolons are affected; 3 — more than $^2/_3$ of the stolons are affected. On sprouts and stems, a 4-point scale was used to assess the development of rhizoctonia: 0 — no signs of damage; 1 — spots (ulcers) on sprouts or stems are single, superficial, distributed no more than $^1/_4$ of the length of the sprout and the underground part of the stem; 2 — ulcers cover the entire circumference, up to half the sprout and the underground part of the stem; 3 — deep ulcers, covering the entire circumference and more than half of the sprout and the underground part of the stem, the stems have partially withered, the leaves have curled and turned yellow; 4 — complete rotting of the sprout, the lower part of the stem and roots, death of the plant.

Biological effectiveness was calculated by the formula:

$$BE = (C - b)/C \times 100\%,$$

where BE is the biological effectiveness of the drug, C is the intensity of disease in the control (point), b is the intensity of disease in the test (point).

Statistical processing of the obtained results was carried out using the Statistica 10 program (StatSoft, Inc., USA). Arithmetic means (M) and standard errors of the means (±SEM) were calculated, as well as the least significant difference (a value indicating the limit of random deviations in the experiment at a significance level of 95%). To assess the statistical significance of differences between the average experimental variants, Duncan's test was used.

Results. Before small-plot field tests, we assessed the growth-stimulating

and fungicidal activity of experimental samples of endophytic bacterial strains *B. thuringiensis* W65 and *B. amyloliquefaciens* P20. As shown by the lab tests, the strains had both growth-stimulating (increase in the root length of the test pant vs. control by 12.3 and 18.9%, respectively) ($p < 0.05$) and fungicidal activity (Table 1, Fig. 1).

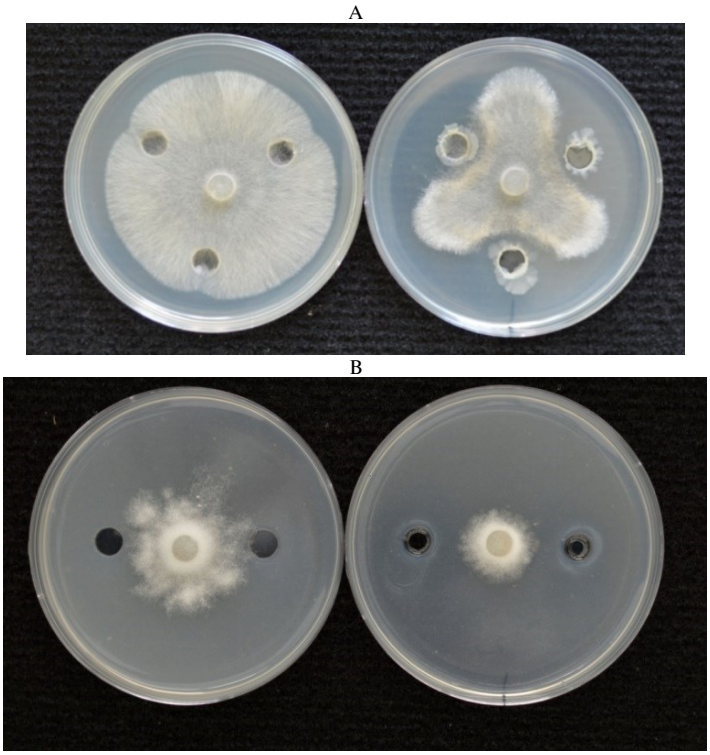

Fig. 1. Fungicidal activity of the *Bacillu amyloliquefaciens* P20 against the micromycetes *Rhizoctonia solani* Kühn (A) **and *Phytophtora infestans* (Mont.) de Bary** (B)**:** on the left — control (sterile water), on the right — bacterial suspension of B. amyloliquefaciens P20.

1. Growth-stimulating and fungicidal activity of experimental preparations of the genus *Bacillus* strains of endophytic bacteria isolated from the tissues of potatoes (*Solanum tuberosum* L.) variety Sudarynya (*M*±SEM)

Treatment	Root length increase, % from cotrol (n = 100)	Diameter of fungal growth inhibition zones, mm (n = 3)			
		Phytophtora infestans (Mont.) de Bary	*Rhizoctonia solani* Kühn	*Fusarium solani* (Mart.) Sacc.	*Fusarium coeruleum* (Lib. ex Sacc.)
B. amyloliquefaciens P20	18.9±1.3	17.5±1.0	25.1±1.9	19.9±1.5	21.5±1.7
B. thuringiensis W65	12.3±0.9	13.5±0.3	18.7±0.5	8.7±0.7	13.1±1.1

N o t e. Growth-stimulating activity was assessed using watercress (*Lepidium sativum* L., 1753) cv. Dukat.

Thus, samples of endophytic bacteria *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 were selected for field small-plot experiments.

Main meteorological indicators of the growing season 2020 and 2021. according to the United Hydrometeorological Station Belogorka are presented in Figure 2. The scheme of field small-plot experiments with two potato varieties is presented in Table 2.

The 2021 season was characterized by severe soil drought because of which the seedlings were very uneven with sections without growth and those stunted in growth. From the beginning of June to the end of the first ten days of July, no precipitation occurred. Under these conditions, the Gusar variety showed resistance to drought, the yield in the control was 27% higher compared to 2020

(Table 3). The yield of the Charoit decreased by 4.7%. In 2021, due to dry conditions during the potato flowering period, flowering of both varieties was poor with massive fall of buds in the Gusar variety. Precipitation in August 2021 led to an increase in the aboveground mass of plants and increased branching in both potato varieties. The trend of increasing flowering duration with the use of microbiological preparations, noted in 2020, continued in 2021.

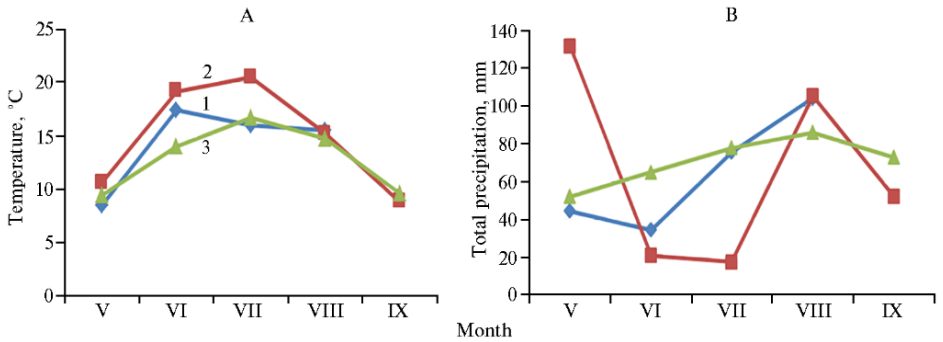

Fig. 2. Average air temperature (A) **and total precipitation** (B) **by month in the years of observation:** 1 — 2020, 2 — 2021, 3 — average long-term data for 1990-2020 (the United Hydrometeorological Station OGMS Belogorka, village Belogorka, Leningrad Perovince, Gatchina District).

2. Scheme of application of chemical and microbiological plant protection products in small-plot field trials on potato (*Solanum tuberosum* L.) varieties Charoit and Gusar (experimental field, the Leningrad Research Institute of Agriculture Belogorka, village Belogorka. Leningrad Province, Gatchina District, 2020-2021)

Treatment	Drugs	Rate	Date
Pure control	Treatment of tubers and spraying with water		
Chemical control	CELEST® Top, SC (Syngenta, Russia)	0.4 l/t	May 28
	Buzzer, SC (CROPEX LLC, Russia)	0.3 l/ha	July 05
	Infinito, SC (Bayer Crop Science, Germany)	1.2 l/ha	July 13
	Rapid Duo, WP (AgroRus and Co., Russia)	2.0 kg/ra	July 22
	Infinito, SC (Bayer Crop Science, Germany)	1.4 l/ha	August 02
	Mankoceb, WP (AgroRus and Co., Russia)	1.2 kg/ra	August 10
	Golden Ring, WS (Agro Expert Group, Russia) + Buzzer, SC (CROPEX LLC, Russia)	2.0 + 0.3 l/ha	August 19
Biological standard	BisolbiSan, L (Bisolbi-Inter LLC, Russia)	4.0 l/t 10 l/ha	May 28 July 01. July 05. 13 July. 22 July. 02 August. 10 August
	Golden Ring, WS (Agro Expert Group. Russia) + Buzzer, SC (CROPEX LLC, Russia)	2.0 + 0.3 l/ha	August 19
B. thuringiensis W65	An experimental drug	–	–
Bacillus sp. X20	An experimental drug	–	–

N o t e. Dashes mean that the application scheme is similar to that for the biological standard.

3. Plant biometric parameters of potato (*Solanum tuberosum* L.) varieties in small-plot field trials with chemical fungicides and experimental preparations of endophytic *Bacillus* bacteria isolated from potato cv. Sudarynya (n = 45. M±SEM; experimental field, the Leningrad Research Institute of Agriculture Belogorka, village Belogorka. Leningrad Province, Gatchina District, 2020-2021)

Treatment	Plant height, cm		Average tuber weight, g		Tuber number per plant		Productivity, g per plant	
	2020	2021	2020	2021	2020	2021	2020	2021
	C h a r o i t (resistant variety)							
Pure control	51.2±4.8^{a}	50.1±4.6^{a}	79.8±5.4^{ab}	70.0±4.7^{b}	8.1±0.4^{b}	8.8±0.4^{b}	647±28^{b}	617±32^{c}
Chemical control	54.3±4.6^{a}	52.4±4.8^{a}	78.6±5.2^{b}	83.7±6.2^{a}	9.0±0.5^{a}	8.8±0.4^{b}	708±36^{ab}	732±51^{a}
BisolbiSan L	56.1±7.0^{a}	53.6±4.4^{a}	85.5±5.8^{a}	79.5±5.4^{a}	9.2±0.7^{a}	9.2±0.4^{ab}	787±54^{a}	727±48^{a}
B. amyloliquefaciens P20	58.4±6.6^{a}	53.7±4.6^{a}	79.1±5.0^{a}	75.6±5.0^{ab}	8.6±0.4^{ab}	9.0±0.3^{ab}	625±30^{c}	681±34^{b}
B. thuringiensis W65	55.2±5.2^{a}	51.1±4.0^{a}	85.5±5.4^{a}	70.3±4.0^{b}	7.9±0.3^{b}	9.8±0.6^{a}	736±50^{a}	686±36^{b}
LSD_{05}	7.3	4.8	3.9	4.3	0.8	0.6	32.3	40.6

Continued Table 3

Gusar (susceptible variety)								
Pure control	52.3±4.2[a]	44.5±4.0[b]	42.3±3.4[a]	45.4±3.8[ab]	12.0±0.7[b]	14.2±0.9[b]	508±31[b]	645±32[b]
Chemical control	53.3±4.0[a]	50.9±4.6[a]	40.6±3.6[a]	44.5±3.6[b]	12.2±0.7[b]	15.5±0.8[a]	496±28[b]	689±30[ab]
BisolbiSan L	56.3±6.8[a]	49.9±4.4[a]	40.3±4.2[a]	49.6±4.0[a]	13.0±0.8[a]	14.3±1.0[b]	524±32[b]	709±34[a]
B. amyloliquefaciens P20	56.0±6.2[a]	48.2±4.2[a]	40.0±2.8[a]	45.0±3.8[ab]	14.1±0.9[a]	16.1±1.1[a]	564±30[ab]	702±40[a]
B. thuringiensis W65	55.4±4.6[a]	47.1±4.0[a]	39.5±3.0[b]	43.2±2.8[b]	14.7±0.9[a]	15.6±0.6[b]	581±25[a]	695±36[a]
LSD_{05}	6.4	4.2	2.4	2.8	0.6	0.4	25.4	34.2

[a. b. c] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different according to the Duncan's test at $p < 0.05$.

The results of 2-year field small-plot experiments showed that there was no significant increase in plant height upon inoculations with experimental preparations (see Table 3). These data are consistent with the results of other studies [65].

On average, with a drug based on *B. amyloliquefaciens* P20, potato yields in the 2020 growing season decreased by 3% compared to the control, while in 2021 it increased by 10.3% for the late blight-resistant variety Charoit. The yield of the susceptible variety Gusar increased with the use of a *B. amyloliquefaciens* P20 preparation by 11 and 8.8% in 2020 and 2021, respectively. With a *B. thuringiensis* W65 preparation, the potato yield of both studied varieties increased significantly ($p < 0.05$) from 7.7 to 14.4%. The greatest increase in yield with the *B. thuringiensis* W65-based drug was in 2020 and amounted to 13.7% for the Charoit variety and 14.4% for the Gusar variety.

The increase in potato tuber yield of the Charoit variety treated with microbiological preparations was mainly due to an increase in the average tuber weight, of the Gusar variety due to an increase in the number of tubers per plant.

4. Fractions of tubers of potato (*Solanum tuberosum* L.) varieties in small-plot field trials with chemical fungicides and experimental preparations of endophytic *Bacillus* bacteria isolated from potato cv. Sudarynya ($n = 30$. $M \pm$SEM; experimental field, the Leningrad Research Institute of Agriculture Belogorka, village Belogorka. Leningrad Province, Gatchina District, 2020-2021)

Treatment	Tuber yield by fractions, %							
	< 35 mm		35-45 mm		46-55 mm		> 55 mm	
	2020	2021	2020	2021	2020	2021	2020	2021
Charoit (resistant variety)								
Pure control	22.2±1.1[b]	20.5±0.8[b]	37.0±1.4[a]	38.6±1.4[ab]	34.6±1.6[b]	38.6±1.2[a]	6.2±0.4[c]	2.3±0.1[c]
Chemical control	21.6±0.8[ab]	14.8±0.4[d]	35.5±1.2[ab]	39.8±1.3[a]	36.7±1.8[a]	37.5±1.6[ab]	6.7±0.3[c]	8.0±0.3[b]
BisolbiSan L	19.4±0.6[c]	17.4±0.4[c]	32.6±0.8[c]	34.8±1.8[b]	38.0±2.7[a]	39.1±1.6[a]	10.0±0.6[a]	8.7±0.2[a]
B. amyloliquefaciens P20	24.1±1.0[a]	23.5±0.9[a]	32.9±0.6[c]	34.7±0.8[b]	35.4±1.4[ab]	36.7±1.8[b]	7.6±0.5[b]	5.1±0.1[b]
B. thuringiensis W65	19.8±0.4[c]	20.0±0.4[b]	34.9±0.5[b]	41.1±1.6[a]	37.2±2.0[a]	33.3±1.4[c]	8.1±0.6[b]	5.6±0.1[b]
LSD_{05}	1.2	0.9	1.1	1.4	1.9	1.8	0.8	0.3
Gusar (susceptible variety)								
Pure control	41.6±2.7[b]	29.6±0.8[b]	43.3±2.2[a]	45.8±2.7[ab]	15.0±0.6[a]	22.6±1.2[b]	0.08±0.00[d]	0
Chemical control	46.4±2.8[a]	36.8±1.8[a]	38.5±1.4[ab]	47.2±3.1[a]	15.0±0.8[a]	15.5±0.6[c]	0.13±0.01[c]	0
BisolbiSan L	44.5±2.4[ab]	27.3±1.1[c]	38.5±1.6[ab]	46.8±3.0[ab]	15.0±0.4[a]	25.2±1.4[a]	0.13±0.01[c]	0.7±0.0
B. amyloliquefaciens P20	47.3±2.4[a]	38.5±2.0[a]	39.8±1.4[b]	44.8±2.4[b]	11.1±0.4[c]	16.7±0.8[c]	1.80±0.12[a]	0
B. thuringiensis W65	46.7±3.0[a]	34.8±0.9[b]	38.1±1.5[b]	50.3±3.2[a]	13.6±0.7[b]	14.9±0.4[d]	1.60±0.10[b]	0
LSD_{05}	2.7	1.3	1.5	3.1	0.7	1.3	0.02	0

[a. b. c. d] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different according to the Duncan's test at $p < 0.05$.

Treatments with chemical fungicides did not have a statistically significant effect on the average tuber weight in both studied varieties, with the exception of the Charoit in 2021. Our results are consistent with the data of other researchers. It is known that endophytic strains can stimulate plant growth due to phytohormones they produced, increasing the availability of nutrients and stress resistance [53, 66]. Bacteria of the genus *Bacillus*, producing various metabolites, exhibit plant protection in arid conditions [67]. The effectiveness of the *B. velezensis* AFB2-2 against potato late blight under greenhouse conditions was 85.7% due to the

biosynthesis of bacillomycin D, iturin and surfactin [58]. Another strain, *B. velezensis* SDTB038 provided effective control of potato late blight in greenhouses and fields and promoted the growth of potato plants [57].

When we used experimental *B. amyloliquefaciens* P20 and *B. thuringiensis* W65 preparations, the yield of the tuber fraction larger than 55 mm in size increased in the Charoit potato variety by 22.5-30.6% ($p < 0.05$) (Table 4). The use of the biofungicide BisolbiSan had the same effect on the tuber yield structure of this variety. However, no such changes were noted in the Gusar variety (Table 4).

The maximum yield (t/ha) of potato variety Charoit was harvested in 2020 with the biological standard BisolbiSan (Table 5). In 2020, using experimental samples of preparations *B. amyloliquefaciens* P20 and *B. thuringiensis* W65, significant differences ($p < 0.05$) occurred for the yield of the Gusar variety compared to the chemical control, however, these differences were not revealed in 2021. It should be noted that the increase in yield upon treatment with the experimental samples was comparable or higher than for chemical fungicides (see Table 5).

5. Productivity of potato (*Solanum tuberosum* L.) varieties in small-plot field trials with chemical fungicides and experimental preparations of endophytic *Bacillus* bacteria isolated from potato cv. Sudarynya ($n = 45$, $M \pm$SEM; experimental field, the Leningrad Research Institute of Agriculture Belogorka, village Belogorka. Leningrad Province, Gatchina District, 2020-2021)

Treatment	2020			2021		
	yield, t/ha	Δ, %		yield, t/ha	Δ, %	
		to control	to chemical control		to control	to chemical control
Charoit (resistant variety)						
Pure control	39.3±1.1[b]	–	–	35.2±1.7[c]	–	–
Chemical control	43.0±4.1[b]	9.4	–	41.7±1.6[a]	18.4	–
BisolbiSan L	47.8±2.0[a]	21.6	11.2	41.4±1.4[a]	17.6	–
B. amyloliquefaciens P20	38.0±1.0[bc]	–	–	38.8±1.5[bc]	10.2	–
B. thuringiensis W65	44.7±2.2[ab]	13.7	4.0	39.1±1.2[b]	11.1	–
LSD_{05}	1.9			1.8		
Gusar (susceptible variety)						
Pure control	30.8±1.5[b]	–	–	36.7±0.3[b]	–	–
Chemical control	30.2±3.0[b]	–		39.2±1.2[ab]	6.8	–
BisolbiSan L	31.9±1.5[ab]	3.5	5.6	40.4±1.7[a]	10.1	3.1
B. amyloliquefaciens P20	34.3±2.1[a]	11.4	13.6	40.0±1.4[a]	9.0	2.1
B. thuringiensis W65	35.3±1.5[a]	14.6	16.9	39.6±1.5[a]	7.9	1.0
LSD_{05}	1.5			1.7		

Note. Dashes mean that the application scheme is similar to that for the biological standard.
[a. b. c] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different according to the Duncan's test at $p < 0.05$.

6. Incidence (I) and severity (S) of rhizoctonia and late blight infections of potato (*Solanum tuberosum* L.) plants in small-plot field trials with chemical fungicides and experimental preparations of endophytic *Bacillus* bacteria isolated from potato cv. Sudarynya (budding stage, the scale of the All-Russian Research Institute of Plant Protection; $M \pm$SEM; experimental field, the Leningrad Research Institute of Agriculture Belogorka, village Belogorka. Leningrad Province, Gatchina District, 2021)

Treatment	Rhizoctonia					Late blight		
	stems		stolones		BE, %	I, %	S, points	BE, %
	I, %	S, points	I. %	S, points				
Charoit (resistant variety)								
Pure control	100	1.0	100	2.6±0.3[a]		100	2.1±0.30[a]	
Chemical control	100	1.0	100	2.5±0.3[a]	3.8	6.7	0.1±0.01[c]	95.2
BisolbiSan L	100	1.0	100	2.5±0.3[a]	3.8	88.9	0.9±0.1[b]	57.2
B. amyloliquefaciens P20	100	1.0	100	2.3±0.3[a]	11.5	90.5	1.2±0.07[b]	42.9
B. thuringiensis W65	100	1.0	100	2.6±0.4[a]	–	100	2.1±0.2[a]	–
LSD_{05}				0.6			0.8	
Gusar (susceptible variety)								
Pure control	100	1.0	100	2.9±0.1[a]		100	7.2±0.2[a]	
Chemical control	100	1.0	100	2.4±0.2[b]	17.2	28.9	0.7±0.04[c]	90.3

Continued Table 6

BisolbiSan L	100	1.0	100	2.6±0.2[ab]	10.3	100	6.9±0.2[a]	4.2
B. amyloliquefaciens P20	100	1.0	100	2.5±0.1[ab]	13.7	98.0	6.2±0.1[b]	13.8
B. thuringiensis W65	100	1.0	100	2.9±0.3[a]	–	100	7.1±0.5[a]	1.4
LSD_{05}				0.5			0.8	

Note. BE — biological effectiveness of the drug. The number of plants when assessing the incidence of rhizoctonia in each variant was 24, for the incidence of late blight 45. Dashes mean the absence of a statistically significant increase compared to the control.

[a. b. c] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different according to the Duncan test at $p < 0.05$

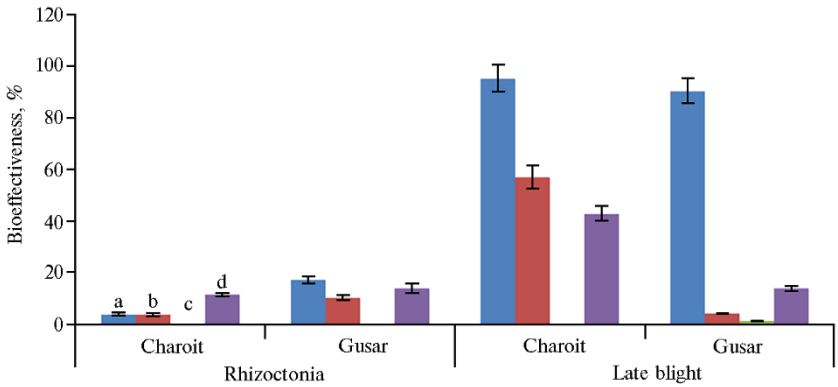

Fig. 3. Biological effectiveness of chemical fungicides and experimental samples of preparations based on strains of endophytic *Bacillus* bacteria isolated from the Sudarynya variety against rhizoctonia and late blight for two potato (*Solanum tuberosum* L.) varieties in small-plot field trials: a — chemical control, b — BisolbiSan, c — *B. thuringiensis* W65, d — *B. amyloliquefaciens* P20 ($n = 24$. M±SEM; the Leningrad Research Institute of Agriculture Belogorka, village Belogorka, Leningrad Province, Gatchina District, 2021).

The weather conditions in 2020-2021 were not favourable for the development of rhizoctoniosis. In all treatments the number of affected plants (stems and stolons) during the budding phase for both potato varieties was 100%, however, the lesion was very weak, 1 point on the rhizoctonia development scale (Table 6). Despite the fungicidal activity of the studied preparations based on *B. thuringiensis* W65 and *B. amyloliquefaciens* P20 identified in lab tetst they did not have an effective influence on the development of rhizoctonia in field conditions (Table 6). However, for the Charoit variety, a drug based on the *B. amyloliquefaciens* P20 was more effective against rhizoctonia than chemicals and for the Gusar variety, the effect was comparable (see Table 6, Fig. 3).

Thus, the weak development of rhizoctonia and late blight in the field conditions of 2021 did not allow us to fully evaluate the fungicidal effects. Obviously, the fungicidal effect of *B. thuringiens*is W65 and *B. amyloliquefaciens* P20 preparations must be assessed in model experiments upon artificial infestation under phytotron conditions.

To date, not a single potato variety with immunity to rhizoctonia blight has been found in the Russian Federation [24]. Analysis of special publications showed that the *B. subtilis* SR22 strain exhibited high antagonistic activity against *Rhizoctonia solani* and reduced the development of the disease by 53.8% on tomato plants. Treatment with this strain increased the total phenolic content (by 76.8%) and the activity of the antioxidant enzymes peroxidase (by 56%) and polyphenoloxidase (by 29.2%) in tomato roots [40]. *Bacillus subtilis* HussainT-AMU isolated from potato tubers produced surfactin and reduced the development of potato rhizoctonia blight by 50% in field conditions [60].

An analysis of the incidence of late blight in potato plants showed that chemical fungicides minimized the development and prevalence of the disease.

whereas preparations based on strains *B. amyloliquefacien*s P20 and *B. thuringiensis* W65 had little effect on its spread (Table 6). Under the influence of weather conditions in the second half of August causing soil drought, protection with microbiological preparations was not effective enough. The prevalence of the disease when using the BisolbiSan standard and the *B. amyloliquefaciens* P20 preparation was 89 and 90%, respectively, on the Charoit variety. However, the biologicalscals reduced the late blight development on the Charoit variety by 57.1 for BisolbiSan and 42.9% for *B. amyloliquefaciens* P20 (see Fig. 3, 4). Our data confirm the results of other researchers. The use of a microbiological preparation based on *Bacillus velezensis* SDTB038 made it possible to reduce the incidence of late blight by 40.79 and 37.67% in 2018-2019 [57]. As some researchers believe, progress in the use of endophytes against late blight can be achieved with an integrated protection scheme for joint use of biologicals with small doses of fungicides and inducers of systemic plant resistance [57, 68-70].

Fig. 5. Development of late blight on the potato (*Solanum tuberosum* L.) variety Charoite on the date of phytopathological registration: left — pure control, right — treatment with a *Bacillus amyloliquefaciens* P20 preparation (the Leningrad Research Institute of Agriculture Belogorka, village Belogorka, Leningrad Province, Gatchina District, August 29, 2021).

So, in small-plot field experiments upon inoculation of Charoit and Gusar potato plants with experimental preparations of endophytic bacteria *Bacillus amyloliquefaciens* P20 and *B. thuringiensis* W65, the flowering stage in both varieties was by 8-13 days longer compared to the control and tuber yield was higher by 7.9-14.6%. The yield structure changed. In the Charoit variety, the yield of the maximum-sized fraction increased by 22.5-30.6%. The experimental preparations do not have a significant effect on the development of rhizoctoniasis, and the *B. amyloliquefaciens* P20 (42.8%) reduced the late blight famage to the Charoit variety. The drug based on the *B. amyloliquefaciens* P20 strain can be recommended for further in commercial testing as a biofungicide and the drug based on the *B. thuringiensis* W65 strain as a growth stimulant.

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EFFICACY OF *Stagonospora cirsii* S-47 AGAINST PERENNIAL SOWTHISTLE IN POTATO CROPS

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Abstract

Potato (*Solanum tuberosum* L.) is a crop that needs biological control of perennial weeds (for example, perennial sowthistle *Sonchus arvensis* L.) due to the insufficient assortment of post-emergent chemical herbicides. The fungus *Stagonospora cirsii* J.J. Davis from the VIZR culture collection (All-Russian Institute of Plant Protection), being a producer of herbicidal metabolites, is able to infect *Sonchus arvensis* plants. In the present work, the possibility of using strain *Stagonospora cirsii* S-47 to control perennial sowthistle in small-scale field experiments was shown for the first time. The aim of the study was to evaluate the effectiveness of the use of *Stagonospora cirsii* S-47 in the form of chopped mycelium against perennial sowthistle on potato plantings in small-scale field trials. The trials were conducted during the growing seasons of 2020 and 2021 at the experimental field of the All-Russian Institute of Plant Protection (VIZR, Leningrad Province). Experiments were conducted on plantings of potatoes (*Solanum tuberosum* L.) of Nevsky variety belonging to the medium-early group. Soil of the experimental site is sod-podzolic, loamy, with a humus content in the arable layer of 3-4 %, pH 6.3. The soil was ploughed in the autumn, and in the spring, the site was disked, cultivated, and furrows were cut. The planting rate of tubers was 25 per ha. Fertilizers were not applied. To exclude the influence of non-target objects on the results of the experiments, the treatment of the experimental plots with the herbicide Gezagard (2.0 l/ha) (OOO Syngenta, Russia) was carried out before the emergence of potato plants. The starter inoculate of *Stagonospora cirsii* S-47 was obtained by culturing the fungus for 3 days in liquid sucrose-soybean meal nutrient medium. The biomass was grown in a glass fermenter with a working volume of 5 l (Applikon Biotechnology, the Netherlans). The fermentation medium (4.8 l) was inoculated with 200 ml of the starter culture. After 6 days, the raw biomass was separated from the culture liquid by centrifugation (4000 rpm, SL40, Thermo FS, USA) and weighed. A 0.01 % solution of Tween 80 was added to the raw mycelium to a concentration of 50 g/l, and the mycelium was chopped with a blender (MaxoMixx, Bosch, Germany) for 1 min. Potato plantings were treated using a Mesto RESISTENT 3610 manual knapsack sprayer (MESTO Spritzenfabrik Ernst Stockburger GmbH, Germany) in accordance with the experimental scheme. The herbicide Agritox (1.2 l/ha; Nufarm GmbH & Co. KG, Australia) containing 500 g/l MCPA (2-methyl-4-chlorophenoxyacetic acid) in the form of a mixture of dimethylamine, potassium and sodium salts was used as a standard. We used the treatments: 1 — *S. cirsii* S-47 (50 kg/ha; working fluid consumption was 1000 l/ha), 2 — *S. cirsii* S-47 (100 kg/ha; 2000 l/ha), 3 — *S. cirsii* S-47 + Agritox (50 kg/ha + 0.6 l/ha; 1000 l/ha), 4 — Agritox (0.6 l/ha; 300 l/ha), 5 — Agritox (1.2 l/ha; 300 l/ha), 6 — untreated control. During the treatments, the height of potato plants was 10-15 cm, and perennial sowthistle plants were in the stages from rosette to stalking, not exceeding 10 cm in height. The counts were performed on day 14 and day 28 after treatment by quantitative weight method. Biological efficacy (BE) was calculated vs. untreated control. Potato tubers were harvested manually from each plot to quantify the yield. In the absence of extreme weather conditions, the application of 50 kg/ha of *S. cirsii* mycelium significantly (by 53.9-59.2 %) reduced the weight of perennial sowthistle plants. However, the fungus did not completely eliminate the weed and was less effective than the herbicide Agritox at a dose of 0.6 l/ha. A twofold increase in the rate of application of *S. cirsii* led to an increase in its effect on the number of perennial sowthistle by 13 % on average. The use of *S. cirsii* in combination

with Agritox (0.6 l/ha) improved treatment efficiency by an average of 15 % compared to the use of the herbicide alone. This made it possible to reduce the amount of the applied chemical by half without reducing the effectiveness of perennial sowthistle suppression. In 2020, the use of microbiological and chemical products contributed to an increase in crop yield by 4.7-10.1 %. The statistically significant ($p < 0.05$) increase in crop yield was with an individual application of 100 kg/ha of *S. cirsii* S-47 mycelium and 1.2 l/ha of herbicide Agritox. In 2021, the crop yield from the treated plots increased by 6.8-8.3 %, however there were no statistically significant differences between the treatments and the untreated control. To ensure maximum effect from the mycoherbicide, it should not be used in dry conditions (with a lack of moisture and high temperatures).

Keywords: mycoherbicide, *Stagonospora cirsii*, potato, *Sonchus arvensis*, MCPA, 2-methyl-4-chlorphenoxyacetic acid

Advances in pest biocontrol techniques to improve plant protection are of particular importance in recent decades. Despite the obvious successes in the protection of greenhouse crops by entomophages and the fairly widespread practical use of insecticidal and fungicidal microbial preparations, it should be recognized that there is some lag in the use of methods of biological weed control [1]. It is necessary to test new developments taking into account the needs of the market and the current range of herbicides. In our opinion, potato (*Solanum tuberosum* L.) is one of the most promising crops for the practical implementation of biological and biorational herbicides. It is widely cultivated and in demand among the population of the Russian Federation, and a significant amount of commercial chemicals for protecting this crop is available [2].

The modern herbicides for protecting potatoes from weeds are divided into groups depending on the harmful objects that they can combat. Narrowly specific anti-cereal herbicides based on clethodim, quizalofop-P-tefuryl, fluazifop-P-butyl and chisalofop-P-ethyl can effectively control annual and perennial cereal weeds in potato plantings [3, 4]. The largest group includes drugs to combat annual dicotyledonous and annual cereal weeds, based on metribuzin, prometrin, prosulfocarb, flurochloridone, clomazone and diquat [5-10]. The drugs based on rimsulfuron have the widest range of action. They can affect annual and perennial cereals and some dicotyledonous weed species [11, 12].

Analysis of modern means for protecting potato plants from weeds allows us to conclude that a vacant area for the introduction of biological products is the control of perennial weeds, such as *Sonchus arvensis* L. and *Cirsium arvense* (L.) Scop. [2].

Typically, glyphosate-based herbicides are used to control such species [13], but these drugs are only allowed in the post-harvest period (late summer or autumn) in fields intended for planting potatoes, or after planting, 2-5 days before the appearance of crop seedlings. That is, this is a preventive measure which does not fully correspond to modern ideas about the ecologically friendly plant protection. Restrictions on the glyphosate also must be accounted [14].

In this regard, it seems relevant to develop biologicals controlling perennial dicotyledonous weeds, in particular field sow thistle, in potato plantings.

To control weeds, their pathogens or various natural phytotoxins can be used [15, 16]. For example, the fungus *Stagonospora cirsii* J.J. Davis and its phytotoxins are promising for suppressing root sucker weeds such as thistles [17, 18]. However, field trials of their effectiveness have not yet been conducted.

In laboratory experiments, it was shown that crushed deep mycelium of *S. cirsii* C-163 infects the leaves of thistle more effectively than conidia [19]. A liquid nutrient medium was selected using soybean flour as a source of nitrogen and the duration of cultivation of this fungus was selected to significantly increase the pathogenicity of the mycelium [20, 21].

It has been established that *S. cirsii* C-163 and S-47 serve as producers of

the phytotoxic ten-membered lactones stagonolide A and herbarumin I in technologically significant quantities. They are believed to be responsible for the herbicidal activity of *S. cirsii* [22, 23]. A likely mechanism of action for stagonolide A is inhibition of photosynthesis in sensitive plants [24], while herbarumin I is thought to inhibit cAMP phosphodiesterase [25].

This work demonstrates for the first time the possibility of using the *Stagonospora cirsii* S-47 to control field sow thistle in a small-plot experiment.

The purpose of this study was to evaluate the effectiveness of using *Stagonospora cirsii* S-47 in the form of crushed mycelium against field thistle plants in potato plantings.

Materials and methods. Small-plot tests were performed during the growing seasons of 2020 and 2021 (the experimental field of the All-Russian Research Institute of Plant Protection — ARRIPP, Leningrad Province; 59.74′N, 30.42′E) on potato (*Solanum tuberosum* L.) mid-early variety Nevsky with a marketable yield of 380-500 c/ha. The soil of the site was typical for the North-Western region (soddy-podzolic, loamy, the arable layer is 3-4% humus, pH 6.3). Tillage was plowing in the fall and disking and cultivated in the spring, and furrows were cut. The tuber planting rate was 25 c/ha. No fertilizer was applied.

To exclude the influence of non-target objects, before the potato plants emerged, test plots were treated with the herbicide Gesagard, SC (suspension concentrate, 2.0 l/ha) (Syngenta LLC, Russia) against annual dicotyledonous and cereal weeds.

The strain used was *S. cirsii* S-47 from the collection of the Laboratory of Phytotoxicology and Biotechnology ARRIPP. The strain was stored in tubes on potato-glucose agar slants at 4 °C. For deep culture, a liquid sucrose-soy nutrient medium (SS) was used (sucrose 60 g/l, soy flour 15 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, pH 6.0). The inoculum was a 3-day culture of the fungus in SS medium (500 ml conical flasks with 100 ml of medium, an orbital shaker at 180 rpm, 24 °C). To grow *S. cirsii* S-47 biomass, a 5-l glass fermenter (Applikon Biotechnology, Holland) with an ez-Control process control system and BioXpert software was used. Fermentation medium (4.8 l) was inoculated with 200 ml of the culture in SS medium. The fermentation parameters were as follows: 24 °C, air supply speed 5 l/min, stirring speed 200 rpm for 2 days and 400 rpm until the fermentation is completed. An antifoam agent (refined sunflower oil, 1% of the medium volume) was added to the medium before inoculation. After 6 days of culture, the raw biomass was separated from the culture liquid by centrifugation at 4000 rpm (a centrifuge SL40, Thermo FS, USA) and weighed. The yield of raw biomass was approximately 200 g/l. After 0.01% Tween 80 solution was added to a concentration of 50 g/l, the mycelium was crushed using a blender (MaxoMixx, Bosch, Germany) for 1 minute. Before the experiment, the crushed biomaterial was stored without loss of viability for 5 days at 5 °C.

The potato plantings were treated using a RESISTENT 3610 manual backpack sprayer (MESTO Spritzenfabrik Ernst Stock-burger GmbH, Germany) in accordance with the experimental design. To completely cover the plants with the preparation and provide sufficient moisture to infect field thistle, an increased rate of liquid was used, the 2000 l/ha (or 100 kg of raw mycelium/ha). When used together with a herbicide, the application rate of the drug was reduced to 1000 l/ha (50 kg of mycelium/ha).

The herbicide Agritox, WSC (water-soluble concentrate, 1.2 l/ha; Nufarm GmbH & Co KG, Australia) containing 500 g/l MCPA (2-methyl-4-chlorophenoxyacetic acid) as a mixture of dimethylamine, potassium and sodium salts. The choice of the standard was due to the fact that this active substance is used in the fight against harmful dicotyledonous plants (including perennial root sucker weeds

including field sow thistle) in haylands and pastures, and it has already been used as a standard in experiments with mycoherbicides [26, 27]. An important feature of the herbicide Agritox, WSC is that in potato plantings, complete destruction of sow thistle from this herbicide is, as a rule, not occurred due to the lower application rate with regard to the permitted regulations. This made it possible not to create too harsh conditions for evaluating the biological product, since the use of pathogens against weeds in real field conditions is, as a rule, significantly inferior in effectiveness to the use of the chemical method. This deficiency can be compensated by co-application of biologicals and chemicals. Guided by this approach, we added the experiment scheme with the use of 50 kg/ha of *S. cirsii* in a tank mixture with 0.6 l/ha of the herbicide Agritox, WSC.

The experimental design included following treatments 1) *S. cirsii* S-47 (50 kg/ha, or 1000 l/ha), 2) *S. cirsii* S-47 (100 kg/ha; 2000 l/ha), 3) *S. cirsii* S-47 + Agritox, WSC (50 kg/ha + 0.6 l/ha; 1000 l/ha), 4) Agritox, WSC (0.6 l/ha; 300 l/ha), 5) Agritox, WSC (1.2 l/ha; 300 l/ha), and 6) control (without treatment).

Fourteen days after treatment, the plant pathogen was reisolated into a pure culture to make sure that the symptoms of the lesion were caused by *S. cirsii* and not a natural infection. The treated potato plants were 10-15 cm in height. Field sow thistle plants did not exceeding 10 cm in height and were in the stage from rosette to stemming. Counts were performed by quantitative-weight method 14 and 28 days after treatment, on four 0.25 m^2 sections for each test plot as per the Guidelines for registration testing of herbicides in agriculture [28] and Methodological recommendations for conducting registration tests of herbicides [29].

Biological efficiency (BE) was calculated by the formula

$$BE = (K - O)/K \times 100\%,$$

where K is the number (wet weight) of thistle plants in the control, nos/m^2 (g/m^2); O is the number (wet weight) of thistle plants in the treatment option, nos/m^2 (g/m^2).

The potato harvest was recorded manually from each test plot.

Statistical processing was carried out using Microsoft Excel. Based on the data obtained, the sample mean (M) and standard deviation of the mean (±SEM) were calculated. Yield data was processed by one-way analysis of variance (*F* test) with the calculation of LSD_{05}.

Results. Meteorological conditions in both years of the experiments were generally hotter and drier compared to the long-term average (Table 1). However, in 2020, air temperature during the growing season was only 7% higher than the long-term average, and air humidity was 8% lower. The parameters in 2021 differed more from the long-term average values, temperature was 21% higher, air humidity was 16% lower, and precipitation was 23% lower. All this had a direct impact on the results of the experiments: the effect of *S. cirsii* S-47 on field thistle plants in the first year was significantly stronger than in the second.

1. Meteorological parameters during assessment of the *Stagonospora cirsii* S-47 mycelium effectiveness against sow thistle (*Sonchus arvensis* L.) plants on potato (*Solanum tuberosum* L.) variety Nevsky (Leningrad Province)

Parameter	Month, decade											
	May			June J			July			August		
	I	II	III	I	II	III	I	II	III	I	II	III
Air temperature, °C:												
average long-term	8.5	11.1	12.3	14.3	15.7	16.6	17.3	17.8	17.9	17.2	16.0	14.4
2020	9.1	6.5	11.8	16.1	19.8	20.1	17.1	17.4	16.6	18.2	16.0	16.1
2021	5.7	17.6	11.4	18.0	19.8	24.2	23.4	24.2	19.6	17.3	18.0	13.9
Precipitation, mm:												
average long-term	10.3	12.2	14.7	13.8	17.0	24.5	22.1	21.2	22.7	24.2	20.4	24.8
2020	7.0	27.6	8.8	13.6	8.5	10.0	17.6	5.3	43.9	15.5	0.3	78.7

Continued Table 1

2021	45.0	40.9	38.1	0.0	5.8	19.6	7.9	10.1	25.9	87.4	51.6	27.2
Air humidity, %:												
average long-term	67	73	71	66	68	71	72	74	76	77	79	82
2020	58	68	52	68	62	56	70	67	75	73	67	77
2021	73	60	69	51	55	61	52	54	59	74	71	80

In both years, the predecessor for potato plantings was a fallow plot, which provides significant infestation with field thistle, in the absence of treatment, 24 plants/m^2 in 2020 and 32 plants/m^2 in 2021.

Four days after treatment with the drugs, the effect of *S. cirsii* S-47 began to be apparent. At the initial stage, round spots of yellow and brown color appeared on the leaves of weeds. Over time, the number of spots increased, and they merged, covering the edge of the leaf blade, which led to the death of leaves (Fig. 1, A). As a result of this effect of *S. cirsii* S-47, the decrease in the weight of sow thistle plants vs. control was more pronounced than the decrease in the number of weeds confirmed by the results of surveys carried out later, 14 and 28 days after the treatment.

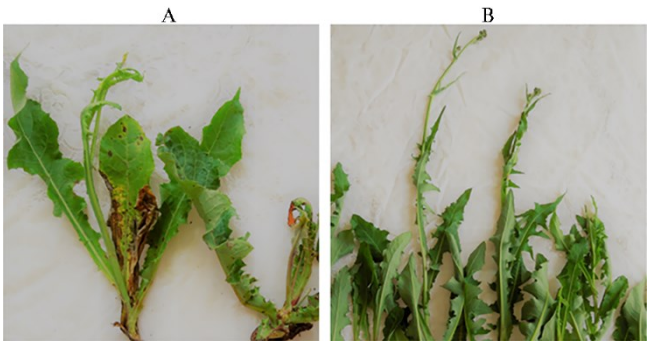

Fig. 1. Field sow thistle (*Sonchus arvensis* L.) plants in potato (*Solanum tuberosum* L.) Nevsky variety plantings 14 days after protective treatment: A — *Stagonospora cirsii* (50 kg/ha) + Agritox, WSC (0.6 l/ha); B — control (without treatment) (Leningrad Province, July 14, 2020; a small-plot test).

Visually distinguishable symptoms of the action of a chemical preparation based on MCPA on field thistle plants were detected later, on the days 7-10 after treatment. They consisted of damage to the growing points and twisting of the upper parts of the stems. Thistle plants damaged by MCPA were noticeably retarded in growth compared to control plants. Among the latter, by the first mass count, specimens were found that had reached the budding and flowering stages (see Fig. 1, B).

Phytoexamination of the selected leaves showed that *Stagonospora cirsii* was reisolated from the affected leaves, but not from the control leaves.

2. Bioeffectiveness (BE) **of crushed *Stagonospora cirsii* S-47 mycelium against field sow thistle (*Sonchus arvensis* L.) plants on potato (*Solanum tuberosum* L.) Nevsky variety** (M±SEM, $N = 4$; Leningrad Province, 2020-2021; small-plot tests)

Treatmetnt	Days after treatmetnt	Field thistle number				Field thistle plant weight			
		number per m^2		BE, %		g/m^2		E,%	
		2020	2021	2020	2021	2020	2021	2020	2021
1	14	14±3.0	24±1.9	39.1	20.0	173.2±64.0	265.0±104.3	59.2	43.5
	28	15±3.9[a]	25±1.2	37.5	21.9	244.2±58.6	453.2±142.1	53.9	44.0
2	14	12±1.9	19±2.9	47.8	36.7	158.4±34.0	105.4±21.0	62.6	77.5
	28	11±2.2	21±3.5	54.2	34.4	179.9±34.5	243.0±51.6	66.0	70.0
3	14	11±1.2	22±3.0	52.2	26.7	134.1±31.2	247.9±70.5	68.4	47.1
	28	11±3.9	18±4.0	54.2	43.8	128.7±29.1	242.4±88.5	75.7	70.1
4	14	12±2.7	23±2.2	47.8	23.3	199.7±58.7	229.7±64.1	52.9	51.0
	28	11±2.2	22±3.0	54.2	31.3	229.9±20.4	326.0±63.4	56.6	59.8
5	14	11±2.2	15±3.5	52.2	50.0	150.6±16.7	110.3±35.2	64.5	76.5
	28	10±3.0	16±3.8	58.3	50.0	192.4±53.4	203.3±75.8	63.7	74.9
6	14	23±4.8	30±3.0			424.0±20.4	469.0±61.4		
(control)	28	24±7.3	32±1.9			529.8±184.4	810.0±56.1		

N o t e. For a description of the experimental options, see the Materials and methods section. Plants were counted on 0.25 m^2 test plot sections. In all treatments, differences with the control (except for the value marked with the letter [a]) were statistically significant ($p < 0.05$), there were no significant differences between treatments

The results of the surveys we carried out in both years of research demonstrated a decrease ($p < 0.05$) in the number and weight of field thistle plants in all test treatments compared to the untreated control (Table 2). The only exception was the number of sow thistle plants 28 days after treatment with 50 kg/ha of *S. cirsii* S-47 in 2020.

In the growing season of 2020, the use of 50 kg/ha of *S. cirsii* S-47 provided a decrease in the weight of thistle plants by 53.9-59.2% and in the number by 37.5-39.1% (see Table 2). The effect of 50 kg/ha of *S. cirsii* S-47 on the biomass of weeds was comparable to that of 0.6 l/ha herbicide Agritox, WSC but was inferior to the standard in the number of field sow thistle plants. A twofold increase in the rate of application of the mycoherbicide led to an increase in its BE in terms of the number of the weed plants by an average of 13%, and in terms of biomass by 8%, being comparable to the effect of Agritox, WSC at 1.2 l/ha.

The use of 50 kg/ha of *S. cirsii* S-47 mycelium mixed with 0.6 l/ha of Agritox, WSC had a better effect vs. the pure mycoherbicide, on average by 15% (in terms of both number and weight of the weed). In this treatment, the reduction in the number of field sow thistle plants 28 days after treatment was 54.2%. i.e., as upon a separate use of 100 kg/ha *S. cirsii* S-47 and 0.6 l/ha Agritox, WSC, while the reduction in the biomass of the weed was 75.7%, being higher than that for pure Agritox, WSC at a dosage of 0.6 l/ha.

In the extremely hot and dry conditions of the 2021 growing season, the mycoherbicide had a weaker effect on field sow thistle plants (see Table 2). At a rate of 50 kg/ha, the pure mycoherbicide reduced the weed number by 20.0-21.9%, weight by less than 44%, which differed little from the BE of Agritox, WSC used at a rate of 0.6 l/ ha. Increasing the application rate of *S. cirsii* S-47 to 100 kg/ha significantly increased its effectiveness, in terms of the number of weeds up to 36.7-37.5%, in terms of their weight up to 70.0-77.5 %. Agritox, WSC has the same BE at an application rate of 1.2 l/ha.

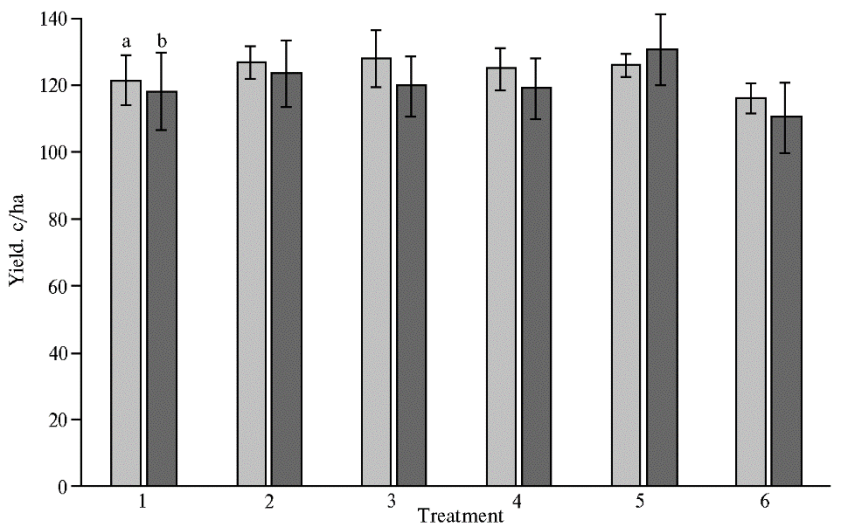

Fig. 2. Tuber yield of potato (*Solanum tuberosum* L.) variety Nevsky after protective treatments against field sow thistle (*Sonchus arvensis* L.) in 2020 (a) **and 2021** (b)**:** 1 — treatment with *Stagonospora cirsii* S-47 (50 kg/ha; consumption rate 1000 l/ha), 2 — *S. cirsii* S-47 (100 kg/ha; 2000 l/ha), 3 — *S. cirsii* S-47 + Agritox,WSC (50 kg/ha + 0.6 l/ha; 1000 l/ha), 4 — Agritox, WSC (0.6 l/ha; 300 l/ha), 5 — Agritox, WSC (1.2 l/ha; 300 l/ha), 6 — control (without treatment). LSD_{05} for 2020 was 16.0 c/ha, for 2021 26.4 c/ha ($M \pm SEM$, $N = 4$, plants were counted on 0.25 m^2 test plot sections; Leningrad Province, 2020-2021; small-plot tests).

A tank mixture of 50 kg/ha *S. cirsii* S-47 mycelium with 0.6 l/ha Agritox,

WSC acted gradually. During the first survey, 2 weeks after treatment, the reduction in the number and weight of the weeds statistically corresponded to the BE for separate use of *S. cirsii* S-47 mycelium (50 kg/ha) and Agritox, WSC (0.6 l/ha). Four weeks after treatment, the effectiveness of the tank mixture increased to BE from the separate use of 100 kg/ha *S. cirsii* S-47 and 1.2 l/ha of Agritox, WSC.

Despite the suppression of field sow thistle plants and the treatments against other types of annual dicotyledonous and cereal weeds, potato plants were not able to fully realize their yield potential, primarily due to the significant overgrowing of the test plots with annual weeds by the end of the growing season. Nevertheless, in 2020, in a control free from treatments, the potato yield was 116.0 c/ha (Fig. 2). Microbiological and chemical preparations contributed to an increase in crop yield by 4.7-10.1%. Statistically significant ($p < 0.05$) was the increase in yield for 100 kg/ha of *S. cirsii* S-47 mycelium and 1.2 l/ha of Agritox, WSC used separately. In 2021, the potato yield in the control was 110.5 c/ha. Upon treatments increased by 6.8-8.3%, but there were no statistically significant differences between the treatments and the control.

The results we obtained suggest that *S. cirsii* S-47 is of particular interest for the development of a mycoherbicide against field sow thistle in potato plantings. Like other research [30-33], our data indicate the preference for using a biological control agent in combination with chemical treatment. In our experiments, the combined use of microbiological and chemical preparations halved the rate of Agritox, WSC, containing 500 g/l MCPA, without losing its effectiveness. This is especially important for acidic soils common in the north-west of the Russian Federation. It has been established that the degradation of MCPA in acidic soils is difficult [34], which aggravates the adverse effects of this active substance on the environment, and in the future can lead to inhibition of plant development, increased soil toxicity and pollution of surface and ground waters [35, 36].

An important result of our research was also the decrease in the effectiveness of *Stagonospora cirsii* that we identified under dry and hot weather conditions. Previously, similar trends were noted abroad for *Phoma herbarum* Westend, *Sclerotinia minor* Jagger, *Phytophthora palmivora* Butler and *Colletotrichum gloeosporioides* Penzig [37-39].

Thus, under non-extreme weather conditions, the application of 50 kg/ha of *Stagonospora cirsii* S-47 mycelium significantly (by 53.9-59.2%) reduces the biomass of field sow thistle in the Nevsky potato varie plantings. However, the fungus, as a rule, did not ensure complete death of the weed plants, which was inferior to the 0.6 l/ha herbicide Agritox, WSC. A twofold increase in the application rate of *S. cirsii* S-47 leads to approximately 13% increase in its effect on the number of field sow thistle plants. *S. cirsii* S-47 combined with 0.6 l/ha Agritox, WSC provide a higher efficiency in both the number and weight of field sow thistle plants, by an average of 15% compared to the herbicide without *S. cirsii* S-47. This reduces the amount of the applied drug by 2 times without a decrease in suppressing field sow thistle plants. The increase in yield of the Nevsky variety after these protective measures ranged from 4.7 to 10.1%. The effectiveness of *S. cirsii* S-47 highly depends on weather conditions, especially during treatment. To ensure maximum effect of the mycoherbicide, it should not be used in dry conditions (lack of moisture and high temperatures).

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RELATIONSHIP OF THE ROSE VARIETIES INFESTATION LEVEL BY SPIDER MITE WITH THE BUSH STRUCTURAL ELEMENTS UNDER THE *Phytoseiulus persimilis* APPLICATION IN GREENHOUSES

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Abstract

Varieties of roses grown for cutting differ in the degree of costs for protection against pests, primarily from the two-spotted spider mite *Tetranychus urticae* Koch. To control this pest, from 6-8 to 25 or more treatments with acaricides are required. The predatory mite phytoseiulus *Phytoseiulus persimilis* A.-H. can be used as an alternative or addition to chemical treatments. Here, we report on a long-term monitoring of the spider mite abundance in commercial rose greenhouses. The observation allows us, for the first time, to assess a relationship between two *Rosa hybrida* variety-specific morphometric parameters, the area of a compound leaf segment and the total leaf area per bush, and an abundance of the spider mite in a triotrophic system, i.e., rose plant—spider mite—predatory mite. From this data, we obtained the equations to predict the development of the pest and determined the predatory mite number effective on a certain variety. This work aimed i) to assess the two-spotted spider mite infestation in a set of rose varieties, ii) to establish the relationship of the spider mite infestation level with the bush structure elements, and iii) to choose mathematical models for prediction of the pest infestation levels and the number of the predatory mite phytoseiulus necessary to use for the control of the pest. Observations on the two-spotted spider mite development were carried out in a block glass greenhouse of ZAO Agroleader (Vyborgsky District, Leningrad Province) on rose plants (*Rosa* sp., hybrid tea group) of 18 varieties. The area of the greenhouse was 45,000 m^2. A scoring system was used to assess the infestation levels of roses by spider mites. The greenhouse was divided into plots. Each plot was a 3.95 m long (8.02 m^2 in area) segment of a double row of rose bushes. The survey consisted of a visual inspection of plants and assignment of the infestation level score from 1 to 5. Surveys were carried out twice a month, the total number of counts per year was at least 24. The dynamics of rose plant infestation by spider mites was assessed over 8 years (2011-2018). Since 2011, on particular varieties, and since 2012, on the entire area of the rose greenhouse, the predatory mite *Ph. persimilis*, introduced continuously or into the infestation foci, was used to control the two-spotted spider mite. Continuous application from 3 to 10 individuals/m^2 over the entire area of the greenhouse was carried out 1-1.5 times a month; from 10 to 60 individuals per bush were introduced into foci weekly until new significant foci of the pest continued to appear. Acaricides were used only in cases where the *T. urticae* infestation level exceeded 2.5 points. Seven days after the first treatment the second treatment was carried out. We determined the number of stems in the upper part of the bush (crown) and on the whole bush, the productive stem length, the number of lobes of the complicated leaf, the number of leaves on the entire stem and on 10 centimeters of the stem, the number of leaves in the bush crown and on the entire bush, the surface areas of the lobule and the entire leaf, the area of the leaves surface in the crown and in the entire bush. Correlation analysis was used to assess the relationship between the occupancy of individual varieties of roses and the structural elements of their bushes, and regression analysis was used to describe it mathematically (rectilinear regression equations). To establish the relationship between the parameters of individual elements of the structure of rose

bushes and the infestation level of spider mites, a two-factor ANOVA was used. When comparing the parameters of regression models built from sample data, the least squares method was used. In the most contrasting varieties Brazil and Aqua, the average long-term level of infection differed by 17.8 times. The remaining varieties could be divided into 6-8 groups, of which the most contrasting ones differed by 5.0 times. The rose varieties differed significantly in the average values of individual elements of the bushes structure. These were the number of stems per crown and per entire bush; the number of lobules of the compound leaf; the number of leaves per entire stem and per 10 cm of the stem; the number of leaves per crown and per entire bush; the productive stem length; the areas of the leaf lobule and the entire leaf; the leaf surface per bush and per its crown. Of the 12 indicators of the rose bush structure, a significant relationship with the infestation level of varieties by spider mites in the presence of phytoseiulus was found only for four indicators. These were the number of lobules in a compound leaf ($r = 0.49 \pm 0.218$, $0.95 < P < 0.99$), the area of the leaf lobule ($r = -0.52 \pm 0.214$, $0.95 < P < 0.99$), the leaf area of the bush crown ($r = -0.70 \pm 0.179$, $P > 0.998$), the leaf area of the entire bush ($r = -0.65 \pm 0.189$, $P > 0.995$). A very close relationship was found between the pest infestation of rose varieties and the multiplication of the leaf lobule area by the area of leaves per entire bush ($r = -0.89 \pm 0.134$, $P > 0.99999$) or by the leaf area per crown ($r = -0.94 \pm 0.096$, $P > 0.999999$). Rectilinear regression equations were chosen for predicting the level of rose variety average infestation by *T. urticae*. It was $y_p = 2.57 - 0.073xz$ (with an error of 0.102 ± 0.0154 points) for the first year of phytoseiulus application and $y_p = 2.89 - 0.127xz$ (with an error of 0.081 ± 0.0156 points) for continuous use of phytoseiulus. For predicting the required releases of predatory mites, it was $y_{ph} = 345 - 11.3xz$ (an error of 22.0 ± 5.52 individuals per 1 m^2 per year) for the first year and $y_{ph} = 278 - 11.1xz$ (the error of which is 9.8 ± 1.36 individuals per 1 m^2 per year) for continuous use. In the equations, y_p is the level of a particular rose variety average infestation by the two-spotted spider mite, points; y_{ph} is the number of *Ph. persimilis* required for releases in order to protect this variety from spider mites during a year, individuals per m^2; x is the average area of a leaf segment (lobule) of a given rose variety, cm^2; z is the average area of a bush crown leaves of a given rose variety, m^2. These equations are recommended for use in the biological control of two-spotted spider mite on roses using *Ph. persimilis*.

Keywords: *Rosa hybrida*, rose varieties, bush, structure elements, commercial greenhouses, *Tetranychus urticae*, pest infestation level, *Phytoseiulus persimilis*, correlation analysis, regression analysis, forecasting models, rectilinear regression equations

The cut rose cultvaion is characterized by a significant, ever-increasing number of varieties which differ not only in the decorative properties of the flower, but also in the structural elements and architecture of the bush [1]. Varieties differ in the degree of costs for protection from pests, primarily from the common spider mite *Tetranychus urticae* Koch., to combat which some varieties require 25 or more acaricide treatments, while others require only 6-8 [2-4].

Colonization by phytophages can be influenced by plant height [5], leaf surface area [6, 7], structural complexity [8] and their relationship (leaf contact) [9], number of leaves on the plant [1, 10], leaf area and thickness, their morphological structure (pubescence, density of trichomes, their types) [11-14]. Elements of plant structure determine the presence of shelters for phytophages, distribution [16] and abundance of phytophages [17], and also indirectly influence natural enemies due to the spatial distribution of prey [18, 19]. In addition, plant structure affects the choice of the host plant by natural enemies [20], their movement and survival [18, 21], other features of the behavior of predators and parasites [22], for example, the predatory activity of acarifages, their reproductive behavior, dispersal, and search ability [23-25].

Rose varietal properties affecting the phytophage *T. urticae* were assessed mainly in turms of biochemical traits (terpene content, tannins, essential oils) and leaf morphological traits (trichomes, glands, leaf thickness) [26, 27]. Interaction between the plant, *T. urticae* and its predator *Phytoseiulus persimilis* A.-H. on rose varieties with different resistance to the phytophage has not been enough studied [28]. In addition, little is known about the influence of plant architectural features on their infestation by spider mites and the effectiveness of *Phytoseiulus* [29], although this is of scientific interest and necessary for successful, cost-effective crop protection from the pest. Identification of the elements of the rose bush structure that determine the development of the phytophage and its predator will make it

possible to predict the protective measures on cultivated and new varieties.

Previously, we established significant differences in varieties of roses grown for cutting in the degree of their infestation by spider mites, both under conditions of acaricide use and when using phytoseiulus [2, 3].

In this work, the elements of the rose bush structure that determine the development of the phytophage *T. urticae* and its predator *Ph. persimilis* have been identified for the first time, and equations were suggested to predict protective measures on different varieties of *Rosa hybrida* grown in greenhouses for cutting.

The goal of the work was a long-term assessment of the infestation of various rose varieties by the common spider mite, establishing its connection with the parameters of individual elements of the bush structure and to develop math models to predict the infestation rate for this pest and the number of the predatory phytoseiulus mite to combat it.

Materials and methods. Observations on the development of the common spider mite were carried out in a 45,000 m^2 block glass greenhouse (Agroleader LLC, Leningrad Province, Vyborg District) on rose (*Rosa hybrida*) varieties Aqua, Avalanche, Peach Avalanche, Wow, Dark Wow, Grand Prix, Miss Piggy , Penny Lane, Jumilia, Taleya, Myrna, Brazil, Heaven, Dolomiti, Hot Shot, Red Naomi, Deep Water, Fiesta.

Roses were grown using low-volume hydroponics with Grodan mineral wool (Grodan B.V., the Netherlands) as a substrate. The microclimate in the greenhouses was regulated, drip irrigation, artificial lighting (4500 lux), and curtains were used. During lighting (from 4.00 to 0.00), the temperature was maintained at least +20 °C and relative air humidity 60-65%, without lighting, the parameter were at least +16 °C and 70-75%, respectively (an automatic mode).

A scoring system was used to assess the infestation of roses with spider mites. The entire greenhouse was divided into sections. Each plot was a section of a double row of rose bushes. The length of the segment was 3.95 m, its area was 8.02 m^2. The number of bushes per site was on average 60 at the rate of 7-8 bushes/m^2. The number of plots varied among different varieties, since the area under them was not the same. The minimum area and number of plots (76 in total) were for the Fiesta variety, the maximum (988 plots) for the Grand Prix variety. In a survey, the plants were visually inspected to assign an infestation score for each area on the following scale: 1 — spider mites are found in a part of the bush formed by bending shoots down to increase photosynthesis; 2 — the spider mite is found in the crown (the productive part of the bush, consisting of marketable shoots, peduncles, and shoots for bending), moves to the middle and upper tiers of productive stems but does not yet reach the bud (dozens of individuals on infested leaves); 3 — appearance of the first mites and cobwebs on the buds (hundreds of individuals per site plant); 4 — cobwebs have appeared on more than 50% of the leaves, “caps” of cobwebs appear on the buds (thousands of individuals per site plant); 5 — the entire plant in a web, accumulations of phytophage on the buds and tips of leaves, cessation of shoot growth and their deformation, drying out and falling of leaves (this situation is not allowed in greenhouses) [2-4]. After each survey, the average pest infestation score was determined for each variety on the recording date. Surveys were carried out 2 times a month, the total number of surveys per year was at least 24. The dynamics of the spider mite population was assessed over 8 years (2011-2018). For each variety, the total number of surveys varied, since during the study period some varieties were removed from production and new ones were introduced. The average long-term population was also estimated for each variety. As a result, the minimum number of surveys (48 in total)

was carried out on the Brazil variety, and the maximum (195 surveys) on the Deep Water variety. The average number of surveys for all varieties over 8 years was 137.5±11.21. Based on the survey results, a decision was made to carry out protective measures.

Since 2011 on some varieties, and since 2012 on the entire area of the enterprise, the predatory phytoseiulus mite *Phytoseiulus persimilis* introduced by continuous and local (to the pest foci) methods was used to combat the common spider mite. A continuous application of 3 to 10 mites/m^2 over the entire area of the greenhouses was carried out 1-1.5 times a month, in the foci from 10 to 60 mites per bush weekly [30] until new significant foci of the pest continued to appear. With such an introduction, as a rule, the required predator-to-prey ratio of 1:10-1:20 is created [30, 31]. If the presence of acarifage was detected in the foci (1-5 mites/leaf), its additional application was canceled. Acaricides were used only in cases where the *T. urticae* infestation of roses exceeded 2.5 points. The treatments were paired, i.e., the second treatment was 7 days after the first treatment.

To study the influence of elements of the bush structure on the development of the common spider mite and the effectiveness of phytoseiulus, the morphometric parameters assessed in all 18 varieties of roses were as follows. The productive stem number in the crown and in the bending part was counted in 30 randomly selected bushes of each variety, the sum of the crown stems and the bending stems gave the total number of stems per bush, then the average number of stems per bush was calculated for each variety. The length of productive stems was measured. The number of segments of a compound leaf and leaves on the entire stem were assessed by counting on 30 randomly selected stems of each variety (the quotient of dividing the number of leaves on the entire stem by its length multiplied by 10 gave the number of leaves per 10 cm of stem). The number of leaves in the crown of the bush and on the entire bush was calculated by multiplying the average number of leaves per entire stem by the average number of stems in the crown and in the entire bush. The area of a lobule (simple leaf) was calculated by the formula for an ellipse using the length and width of 30 compound leaves of each variety (the number of measurements varied from 140 to 173, since the number of leaf lobules, deviating from the standard five, varied among varieties). The leaf surface area per bush or per its crown was calculated by multiplying the average area of a compound leaf by the number of leaves per entire bush or its crown.

Statistical data processing was carried out using the SPSS program (https://www.ibm.com/products/spss-statistics) and Microsoft Excel. For each variety, the mean (*M*) colonization score for the entire observation period, the mean values of the bush structure parameters and the standard errors of the means (±SEM) were calculated. The significance of differences was assessed by Student's *t*-test. Correlation analysis was used to evaluate the relationship between the infestation of the rose variety and its bush structural elements, and regression analysis (linear regression equations) was used to describe it mathematically. To reveal the relationship between the element of bush structure of the variety and the degree of its infestation, two-factor analysis of variance was used [32]. When comparing parameters of regression models built from sample data, the least squares method was applied [33]. The errors of the regression equations were calculated by averaging the deviations of the actual values of the average long-term degrees of infestation by spider mites for all studied varieties or the nimber of phytoseiulus released from those expected according to the estimated mathematical model.

Results. Increasing the number of varieties and combining the results over

several years made it possible to identify a wider composition of groups compared to previous studies [2, 3], differing in traits that affect the development of the common spider mite when phytoseiulus is released (Fig. 1). Thus, two varieties with the smallest (Aqua, No. 1) and the highest (Brazil, No. 18) pest infestation highly significantly ($p < 0.001$) differed from the others and from each other (by 17.8 times), and should be considered the most contrasting.

The second most infestead (Grand Prix, No. 17) and penultimate (Deep Water, No. 2) varieties also differed statistically significantly ($p < 0.001$) from all the others and 5-fold between themselves, therefore, they should be considered representatives of two more groups. The fifth group with a relatively low mite population density was represented by the varieties Jumilia, Peach Avalanch, Avalanche, Taleya (Nos. 3-6) (see Fig. 1), which did not differ significantly in this trait (with sample sizes from 72 to 168). The varieties Taleya, Wow, Fiesta, Dark Wow, Hot Shot, Myrna, Red Naomi (Nos. 6-12) which showed moderaate colonization (0.61-1.19 points), represented several groups, since each of them did not differ significantly from the previous one, but differed (mainly at $p < 0.05$) from the variety with a number two units less. Finally, one or two more groups were represented by the varieties Heaven, Dolomiti, Miss Piggy, Penny Lane (Nos. 13-16), among which only the varieties Heaven (No. 13) and Pany Lane (No. 16) differed significantly ($p < 0.05$) in the rate of spider mite infestation in the presence of phytoseiulus.

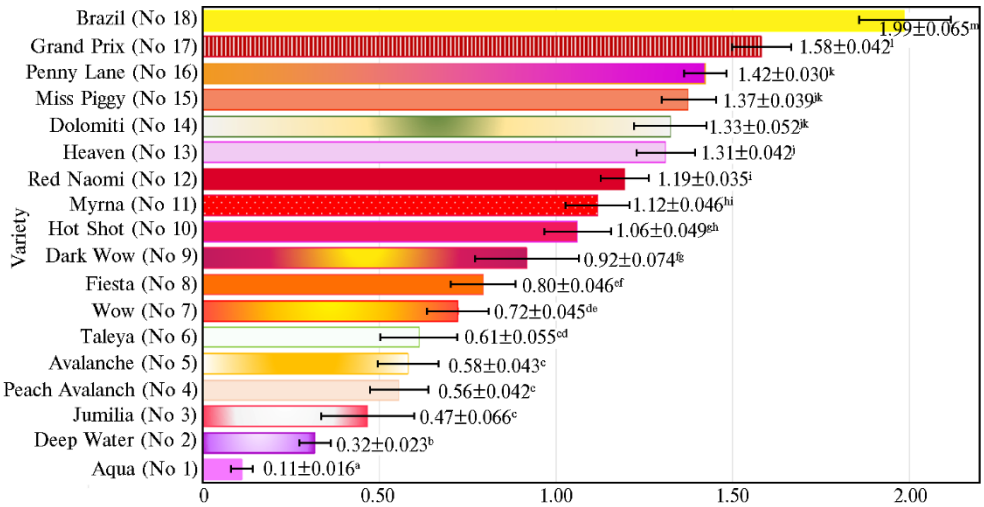

Fig. 1. Average long-term infestation (points) **of different rose (*Rosa* sp.) varieties of the hybrid tea group by the common spider mite *Tetranychus urticae* Koch. during releases of the predatory mite *Phytoseiulus persimilis* A.-H. (***M***±SEM**, experience in greenhouses, OOO Agroleader, Leningrad Province, 2011-2018). Error bars indicate confidence intervals for a probability of 0.95; the same letters indicate values that do not differ significantly ($p > 0.05$) according to Student's *t*-test.

Analysis of the elements of the structure of rose bushes showed significant intervarietal variability [29]. Between the most contrasting varieties, the differences were highly significant ($p < 0.001$) for all studied parameters. However, in absolute value they turned out to be not as high as in *T. urticae* colonization. Thus, the fewest stems in the crown of the bush and on the entire bush was in Brazil variety (3.6±0.14 and 6.2±0.21), and the largest values were characteristic of Peach Avalanch (9.5±0.43 and 13.9±0.62). The longest stems were found in the Grand Prix variety (74.1±1.49 cm), the shortest in Heaven (60.4±0.98 cm). Aqua had the fewest segments in a complex leaf (4.7±0.14), and in Heaven the leaf segment number is the largest (5.7±0.17).

1. Leaf surface parameters in 18 rose (*Rosa* sp.) varieties of the hybrid tea group (*M*±SEM, greenhouse tests, OOO Agroleader, Leningrad Province, 2011-2018)

Variety	Leaf area			
	whole leaf, cm^2 (n = 30)	leaf segments, cm^2 (n from 140 to 173)	bush crown, m^2 (n = 30)	whole bush, m^2 (n = 30)
Aqua	115.7 ± 3.81^{efg}	24.8 ± 1.00^{kl}	0.79 ± 0.050^{qr}	$1.16\pm0.077^{\alpha-\delta}$
Deep Water	129.4 ± 4.05^{bc}	25.4 ± 0.98^{kl}	0.79 ± 0.040^{qr}	$1.16\pm0.057^{\alpha\beta\gamma}$
Jumilia	172.5 ± 7.60^{a}	35.2 ± 1.35^{i}	$0.61\pm0.091^{s-v}$	$0.91\pm0.066^{\zeta\eta}$
Peach Avalanch	110.9 ± 4.13^{fgh}	20.5 ± 0.57^{no}	0.94 ± 0.040^{q}	$1.37\pm0.097^{\alpha}$
Avalanche	112.8 ± 4.43^{fgh}	20.9 ± 0.68^{no}	0.95 ± 0.066^{q}	$1.40\pm0.125^{\alpha\beta}$
Taleya	128.2 ± 4.18^{bcd}	24.3 ± 0.70^{l}	0.81 ± 0.042^{qr}	$1.32\pm0.065^{\alpha\beta}$
Wow	141.9 ± 7.80^{bc}	29.0 ± 0.95^{j}	0.56 ± 0.040^{uv}	$0.98\pm0.067^{\delta-\eta}$
Fiesta	133.4 ± 6.86^{bc}	27.6 ± 1.00^{jk}	0.64 ± 0.040^{su}	$1.14\pm0.072^{\beta-\varepsilon}$
Dark Wow	140.7 ± 7.60^{bc}	28.7 ± 0.85^{j}	0.60 ± 0.041^{tuv}	$0.99\pm0.069^{\gamma\eta}$
Hot Shot	127.9 ± 4.57^{bcd}	25.4 ± 0.9^{kl}	0.66 ± 0.034^{stu}	$1.00\pm0.052^{\delta\varepsilon\zeta}$
Myrna	136.7 ± 3.90^{b}	25.2 ± 0.80^{kl}	0.62 ± 0.034^{stu}	$1.00\pm0.055^{\delta-\eta}$
Red Naomi	125.7 ± 3.78^{cde}	23.2 ± 0.76^{lm}	0.59 ± 0.039^{uv}	$0.96\pm0.062^{\varepsilon\zeta\eta}$
Dolomiti	114.7 ± 4.08^{efg}	21.2 ± 0.60^{no}	0.66 ± 0.032^{stu}	$1.07\pm0.050^{\gamma\delta\varepsilon}$
Heaven	$116.0\pm5.06^{d-g}$	20.2 ± 0.76^{nop}	0.71 ± 0.049^{rst}	$1.11\pm0.076^{\gamma\delta\varepsilon}$
Miss Piggy	104.8 ± 5.81^{gh}	18.2 ± 0.60^{p}	0.74 ± 0.056^{rs}	$1.13\pm0.079^{\beta-\varepsilon}$
Pany Lane	$123.6\pm5.29^{b-f}$	22.2 ± 0.68^{mn}	0.53 ± 0.031^{v}	$0.86\pm0.051^{\eta}$
Grand Prix	112.2 ± 4.30^{fgh}	20.7 ± 0.68^{no}	0.51 ± 0.035^{v}	$0.81\pm0.057^{\eta}$
Brazil	101.1 ± 4.89^{h}	20.2 ± 0.70^{o}	0.37 ± 0.024^{w}	$0.64\pm0.040^{\theta}$

N o t e. The same letters indicate indicators that do not have statistically significant differences ($p > 0.05$) according to Student's *t*-test.

2. Pair correlation coefficients ($r_n\pm S_r$) of infestation by the common spider mite *Tetranychus urticae* Koch. 18 varieties of roses (*Rosa* sp.) of the hybrid tea group with some parameters of the bushe structure, linear regression equations and the sum of squared deviations of the actual colonization from that expected by the regression equations (greenhouse tests, OOO Agroleader, Leningrad Province, 2011-2018)

Parameter	$r_n\pm S_r$	Probability of difference r_n from zero	Regression equation (y = a + bx or y = a + bxz)	Sum of squared deviations
Stems in crown (x)	–0.36±0.233	0.8 < P < 0.9	y = 1.53 – 0.100x	3.60
Stems in a bush (x)	–0.30±0.238	0.5 < P < 0.8	y = 1.50 – 0.061x	3.74
Stem length (x)	0.27±0.241	0.5 < P < 0.8	y = –0.97+0.031x	3.81
Number of segments of a compound leaf (x)	0.49±0.218	0.95 < P < 0.99	y = –3.09+0.779x	3.07
Number of leaves:				
on the entire stem (x)	–0.03±0.250	P < 0.2	y = 1.25 – 0.024x	3.88
per 10 cm stem (x)	–0.19±0.246	0.5 < P < 0.8	y = 1.97 – 0.695x	4.05
in the crown of the bush (x)	–0.42±0.227	0.9 < P < 0.95	y = 1.73 – 0.014x	3.14
all over the bush (x)	–0.35±0.234	0.8 < P < 0.9	y = 1.70 – 0.008x	3.59
Square:				
leaf segments (x)	–0.52±0.214	0.95 < P < 0.99	y = 2.44 – 0.060x	2.94
entire sheet (x)	–0.44±0.225	0.9 < P < 0.95	y = 2.55 – 0.013x	3.23
bush crowns (x)	–0.70±0.179	P > 0.998	y = 2.54 – 2.326x	2.12
whole bush (x)	–0.65±0.189	P > 0.995	y = 2.67 – 1.604x	2.31
bush crowns (x) and leaf segments (z)	–0.95±0.081	P > 0.999999	y = 2.92 – 0.120xz	0.406
whole bush (x) and leaf segment (z)	–0.89±0.116	P > 0.99999	y = 2.92 – 0.077xz	0.790

The largest number of leaves on the entire stem was in the Fiesta variety (12.5±0.32), per 10 cm of the stem in the Deep Water variety (1.74±0.025), in the bush crown in Avalanche (84.8±7 .55), on the entire bush in Peach Avalanch (125±7.4). One variety, the Jumilia had the fewest leaves for all four indicators (7.8±0.27; 1.22±0.036; 35.5±2.16 and 53±3.5, respectively). On the contrary, the Jumilia variety showed the largest area of the lobule and the entire leaf (Table 1). The smallest leaf segment area was in the Miss Piggy variety, and the Brazil variety had the smallest leaf area. The largest leaf area of the crown and of the entire bush was found in the Avalanche variety, and the smallest in Brazil. The remaining varieties occupied an intermediate position in a number of indicators. They were statistically significantly different or not different from each other.

A more definitive pattern of the relationship between the structural

elements of rose bushes and their infestation by spider mites was provided by the results of correlation and regression analyzes (Table 2). It was not possible to identify a reliable connection between 8 of the 12 studied morphometric features of the structure of rose bushes and their colonization by *T. urticae*, since the probability of the correlation coefficient differing from zero was less than 0.95, and for the number of leaves on the entire stem it approached zero. Moreover, the sum of squared deviations of the expected colonization according to the calculated regression equations for 18 varieties was significantly greater than 3.

There was a connection (with a probability of a correlation coefficient different from zero > 0.95, but < 0.99) of the *T. urticae* population of rose varieties with the area of a leaf lobule (average negative) and the number of lobes of a compound leaf (average positive). The negative relationship between the infestation of roses by spider mites and the leaf surface area of the crown and the entire bush turned out to be higher, with a probability of > 0.99 but < 0.999, and the sum of squared deviations was more than 2. However, the predictive accuracy of the expected spider mite infestation of a variety when using such simple models is low. The average error in the degree of infestation will range from 0.27 points when using the total area of the leaf surface of the bush as a predictor, to 0.33 points when using the area of the leaf lobule. In this regard, we tried to find a model that takes into account both the factors that determine the climate in the bush zone (the area of the leaf surface of the crown and the entire bush) and the factor that influences the microclimate in the laminar layer of the leaf (the area of the leaf lobule), by multiplying them. The correlation coefficients of such indicators with the population of the studied rose varieties by *T. urticae* sharply increased during the release of phytoseiulus, and the sum of squared deviations decreased (see Table 2). Two-factor analysis of variance did not show the interaction of the area of a leaf lobule and the area of the leaf surface of the crown or the entire bush in their influence on the infestation of rose varieties by spider mites in greenhouses, which indicates the possibility of their use in the model as independent predictors.

Using the average number of segments of a complex rose leaf as a second argument turned out to be ineffective. The correlation coefficients of the products of the average number of leaf segments on the area of the leaf surface of the crown or the entire bush with spider mite infestation were only 0.52±0.214 (0.99 > P > 0.95) and 0.46±0.223 (0.95 > P > 0.90), and their predictive error is 0.34±0.055 and 0.33±0.063 points. In addition, a significant interaction was revealed between the average number of segments and the area of the leaf surface of the crown and the entire bush in their influence on the infestation of rose varieties by spider mites, which makes it difficult to use these indicators in a linear regression model.

3. Pair correlation coefficients ($r_n \pm S_r$) of infestation by the common spider mite *Tetranychus urticae* Koch. in 14 varieties of roses (*Rosa* sp.) of the hybrid tea group with some bush structure parameters, linear regression equations and the sum of squared deviations of the actual colonization from that expected by the regression equations (greenhouse tests, OOO Agroleader, Leningrad Province, 2011-2018)

Parameter	$r_{n14} \pm S_r$	Probability of difference r_n from zero	Regression equation (y = a + bx or y = a + bxz)	Mean deviation	
				14 сортов	4 сорта
Leaf segments (x)	–0.58±0.235	0.98 > P > 0.95	y = 2.70 – 0.069x	0.28±0.074	0.43±0.131
Total sheet (x)	–0.47±0.254	0.95 > P > 0.90	y = 2.73 – 0.014x	0.31±0.076	0.43±0.132
Bush Crowns (x)	–0.74±0.193	0.998 > P > 0.995	y = 2.74 – 2.649x	0.14±0.040	0.23±0.069
Whole bush (x)	–0.68±0.211	0.995 > P > 0.990	y = 2.77 – 1.705x	0.30±0.067	0.21±0.090
Bush crowns (x) and leaf segments (z)	–0.94±0.096	> 0.999999	y = 2.88 – 0.118xz	0.12±0.029	0.12±0.037
Whole bush (x) and leaf segments (z)	–0.89±0.134	> 0.99999	y = 2.85 – 0.071xz	0.14±0.043	0.21±0.059

N o t e. 14 varieties served as the basis for calculating correlation coefficients and regression equations, 4 varieties were selected for verification using a table of random numbers..

To verify the proposed model for predicting the infestation of rose varieties by spider mites under releasing phytoseiulus, four varieties were selected from a table of random numbers, the Deep Water, Avalanche, Dark Wow and Miss Piggy. The remaining 14 varieties were used to calculate correlation coefficients and regression equations, based on which the expected population of the four excluded varieties was predicted (Table 3). The average deviations of their expected occupancy rates were practically no different from those for the 14 varieties included in the calculations.

An equation based on the leaf areas of the bush crown multiplied by the leaf lobule areas showed the best predictive properties. Moreover, the average error was only 0.12 points. It should be noted that the identification of these elements of the bush structure as the best predictors of the colonization of rose varieties by *T. urticae* influenced by the predatory mite *Phytoseiulus* is not accidental. Both factors determine the climate, which influences the development of both phytophages and acarifages, i.e., humidity and temperature in the bush zone depend on the total area of the leaf surface, and humidity in the laminar layer of the leaf up to 3-5 mm thick depends on the area of the leaf lobule [34, 35]. Humidity is of particular importance for the effective use of phytoseiulus. It affects its survival, especially at the embryonic and larval stages of development, and the reproductive behavior of females [36-38].

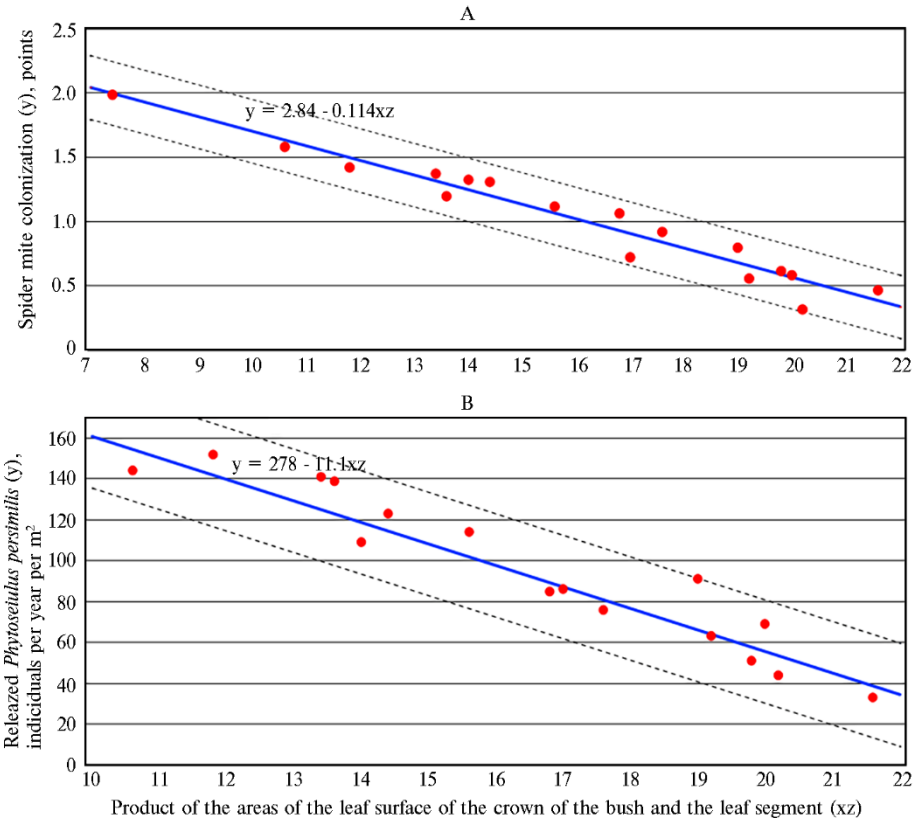

Fig. 2. Dependence of the colonization of roses (*Rosa* sp.) of the hybrid tea group by the common spider mite *Tetranychus urticae* Koch. (A) **and the required number of the predatory mite *Phytoseiulus persimilis* A.-H.** (B) **from the bush crown leaf surface area × a leaf segment area in a certain rose variety:** red dots — actual colonuzation, blue line — expected colonuzation, dotted lines — the boundary of the regression zone ((greenhouse tests, OOO Agroleader, Leningrad Province, 2011-2018).

Further improvement of the model was carried out by clarifying the coefficients in the equations intended to predict colonization of rose varieties in the

first year of phytoseiulus application and with its stable use, since they differed significantly [4], and to reduce the forecast error by excluding the Aqua variety, the actual colonization of which was outside the 95% regression confidence zone. For the first year of the phytoseiulus use, the equation is $y_c = 2.57 - 0.073xz$ with the error of 0.102 ± 0.0154 points, and with stable use $y_c = 2.89 - 0.127xz$, the error of 0.081 ± 0.0156 points (Fig. 2, A). In the recommended equitions, y_c is the average infestation of a rose variety by common spider mites, points; x is the average area of a leaf segment, cm^2; z is the average area of the leaf surface of the bush crown, m^2. These equations can be used when selecting new varieties for commercial cultivation in greenhouses, taking into account the protection against the common spider mite. The data obtained made it possible to draw up predictive equations for calculating the number of phytoseiulus required during its use both in the first years and over a fairly long period of time. This is important for planning the mass breeding or purchasing of predatory mites.

After comparing several versions of the equations, to improve the accuracy of prediction, we excluded the varieties Brazil and Aqua, which were the most contrasting in terms of the required volume of acarifage, which fell outside the 95% confidence zone of the regression [39]. To predict the required volumes of phytoseiulus releases on most varieties of roses in the first year, the recommended equation is $y_p = 345 - 11.3xz$, the error of which is 22.0 ± 5.52 individuals per m^2 per year, and with stable use $y_p = 278 - 11{,}1xz$, the error of 9.8 ± 1.36 individuals per m^2 per year (see Fig. 2, B). In these equations, y_p is the number of phytoseiulus required for releases to protect a particular rose variety from spider mites throughout the year, individuals/m^2; x is the average area of a leaf segment for the variety, cm^2; z is the average leaf surface area of the bush crown in the variety, m^2.

Of the 18 rose varieties studied, protection against spider mites by using phytoseiulus turned out to be most effective in the Aqua variety. In the first year, only 9 releases of the predator were required with a total number of 48 individuals/m^2. In subsequent years, the number of required releases decreased to 4-5, and with 11-19 individuals/m^2 (years 2-4), and then to 6-8 individuals/m^2 per year [4]. For 7 years, the Aqua variety has never required the use of acaricides. On the Brazil variety, on the contrary, protection with phytoseiulus turned out to be extremely ineffective. Acarifage releases had to be carried out 2 times a month. The total number of released predatory mites per 1 m^2 in the first year was 418 individuals, in the second 390 individuals. In this case, 7 and 6 double treatments with acaricides were additionally carried out, respectively.

Subsequently, the farm had to abandon the cultivation of the Brazil variety. The transition to using only acaricides to control *T. urticae* on this variety required 13-15 double treatments per year, which, given the presence of only two drugs approved in the Russian Federation for use on roses in greenhouses [39], extremely limited the possibility of their alternation and led to the rapid emergence of a stable population of the phytophage. Currently, this list has been expanded to five drugs [40], but mainly due to chemical acaricides, the constant use of which in greenhouses is undesirable. Improving biological control of spider mites using the predatory mite *Ph. persimilis* remains extremely relevant. Our research allows us to select rose varieties more suitable for biological protection, as well as plan the use of predatory mites. The fitted models, based on the use of leaf lobule area and bush crown leaf area as predictors, are easy to use and seem to have satisfactory accuracy. Thus, when predicting the infestation of a variety by spider mites and the required number of phytoseiulus with its constant use, the coefficients of determination r^2 are 95.6 and 90.9%, respectively.

Identification of morphological, biochemical and other factors [26-28] influencing the reproduction of *T. urticae* and *Ph. persimilis* in the triotroph system

(plant variety, phytophage, entomophage) is of particular scientific interest. There has been no significant work on roses in this area of research. There are observations by Australian scientists made on roses that were grown in open ground, where it was noted that in densely growing varieties, the abundance of contacting leaves contributed to the rapid movement of *Ph. persimilis* along the plant. Long-term interactions between the common spider mite and the predator resulted in satisfactory control of the pest compared to varieties where the crown was not in contact [41, 42].

In Iran, laboratory studies were carried out on 10 varieties of roses to assess the main vital parameters of the development of the common spider mite. Some varieties differed significantly in their effects on survival, developmental duration of immature ticks, and fertility of adult ticks, as well as on the rate of population growth, reproductive capacity, and average generation time [27]. The relationship with morphological or biochemical characteristics of the varieties was not assessed.

In Mexico, some of the 13 cultivars showed significant differences in their ability to favor *T. urticae* development, as well as a negative correlation with essential oil content and a positive correlation with terpene content, nitrogen content, and leaf thickness [26]. There, in Mexico, 1 week after the pest uniformly colonized two varieties of roses, contrasting in conditions for the development of *T. urticae*, it was noted that the density of damage caused by *T. urticae* and the chlorophyll content in rose leaves did not differ. After *Ph. persimilis* release, the density of *T. urticae* changed on both varieties. Differences between varieties in the percentage of damage and *Ph. persimilis* density was not recorded. One of the varieties had the lowest chlorophyll content, but *Ph. persimilis* was more effective [28].

Our studies revealed a significant negative relationship between the development of the common spider mite in the presence of phytoseiulus, that is, in the triotroph system, and changes in the area of a compound leaf lobule and the area of the leaf surface of the bush crown and the entire bush in different varieties. This relationship is reflected in the required predator application on varieties with different parameters of these structure elements.

Thus, our surveys on 18 rose varieties grown in greenhouses for cutting under the use of the predatory mite *Phytoseiulus persimilis* A.-H. for biocontrol of the common spider mite *Tetranychus urticae* Koch. for 8 years, showed significant diversity in the colonization by the phytophage. The two most contrasting varieties in terms of average long-term colonization, Brazil and Aqua, differed by an average of 17.8 times. Other varieries could be divided into several (six to eight) groups, of which the most contrasting differed 5-fold. Rose varieties differed significantly in the following elements of the bush structure: the number of stems in the crown and in the entire bush, the number of segments of a compound leaf, the number of leaves per entire stem and per 10 cm of the stem, the number leaves in the crown and per the entire bush, the length of the productive stem, the area of the segment and the entire leaf, the leaf area per bush and its crown. Of the 12 assessed indicators of bush structure, a significant relationship with the rose variety infestation by spider mites controlled by phytoseiulus was noted only for the number of lobes in a compound leaf ($r = 0.49 \pm 0.218$; $0.95 < P < 0.99$), for leaf segment area ($r = -0.52 \pm 0.214$; $0.95 < P < 0.99$), for the crown leaf area ($r = -0.70 \pm 0.179$; $P > 0.998$) and for the leaf area per bush ($r = -0.65 \pm 0.189$; $P > 0.995$). A very close relationship was found between the infestation of rose varieties by the pest and the product of the leaf lobe area by the area of the leaves per bush ($r = -0.89 \pm 0.134$; $P > 0.99999$) or by the crown leaf area ($r = 0.94 \pm 0.096$; $P > 0.999999$). Straightforward regression equations were selected to predict the average infestation of the variety by the spider mite *T. urticae* in the first year of

the use of phytoseiulus $y_c = 2.57 - 0.073xz$ (the error of 0.102±0.0154 points), for stable use of phytoseiulus $y_c = 2.89 - 0.127xz$ (the error of 0.081±0.0156 points). The equiations for forecasting the required annual number of the predator and frequency of its application are $y_p = 345 - 11.3xz$ (the error of 22.0±5.52 individuals per 1 m^2 per year) and with stable use $y_p = 278 - 11.1xz$ (the error of 9.8±1.36 individuals per 1 m^2 per year). The y_c is the average colonization of the variety with spider mites, individuals/m^2, y_p is the number of phytoseiulus required to protect the variety from spider mites throughout the year, individuals/m^2, x is the average leaf segment area, cm^2, z is the average crown leaf area, m^2. These equations are recommended for planning biological protection of rose plantings from common spider mites using *Ph. persimilis.*

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SPECTRAL VEGETATION INDEXES AS INDICATORS OF LEAF PIGMENT CONTENT IN APPLE (*Malus domestica* Borkh.)

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Abstract

Methods of operational remote (satellite and unmanned) agricultural monitoring are currently based on the use of spectral vegetation indices as some integral indicators of plant condition. Since the first of them (Normalized Difference Vegetation Index — NDVI) appeared in the early 1970's, rich experience has been accumulated in their use to detect various properties of agricultural plants and agrophytocenoses as a whole. About a hundred different indices have been proposed to detect different properties, e.g., moisture, leaf structure, architecture of plants in crops, the content of various substances, including pigments regulating photosynthesis and plant productivity. In many cases, the proposed indices function reliably for specific plants or for the vegetation as a whole. For fruit crops and, in particular, for apple-tree, there are practically no such indices. In this paper, it is shown for the first time that the spectral vegetation indices proposed for the detection of pigments in agricultural plants need to be refined when they are used for similar detection of pigments in the leaves of an apple tree of a particular variety. Our goal was to analyze the relationship between the spectral vegetation indices calculated for the leaves of the Imrus apple tree (*Malus domestica* Borkh.) with the leaf content of chlorophyll and carotenoids. We evaluated the applicability of several dozen vegetation indices proposed for determining the content of chlorophylls and carotenoids in the leaves of various plants to the non-contact determination of these pigments in the leaves of the Imrus apple tree. On October 19, 2021, leaves were collected at noon randomly from 2-5-year old branches of the middle part of the crown of model Imrus trees grown from 2011 at the test plot (Stupino District, Moscow Province, Russia). In total, 26 mixed leaf samples were collected for pigment content analysis. The content of chlorophylls a + b was determined in the laboratory by the Wintermans-De Mots method, carotenoids by the von Wetshtein method. For the same leaves, spectral reflectance was measured under field conditions using a SR-6500 field spectroradiometer (Spectral Evolution, USA), which operates in the 350-2500 nm range with a resolution of 1 nm. Spectral reflectivity curves were plotted in 5 replicates for the upper surface of the leaves, averaged for each leaf, and then for each of the 26 mixed groups of leaves. Based on the averaged spectral reflectance curves, the most common spectral vegetation indices were calculated, followed by an analysis of the relationship between the values of the spectral vegetation indices and the content of pigments in the leaves. It has been established that the previously proposed numerous vegetation indices cannot be used for non-contact detection of the content of chlorophyll and carotenoids in the leaves of the Imrus apple tree. There is practically no connection between the index value and pigment content. It is also not possible to group the analyzed leaves according to the content of pigments based on the construction of a dendrogram of the similarity between the spectral reflectance curves of leaves in the range of 350-2500 nm. Based on the correction of the indices that showed the most accurate dependence, new vegetation indices were proposed for non-contact detection of the content of carotenoids and chlorophyll in apple leaves, which make it possible to obtain regression models with R^2 above 0.65. Before widespread use, they must be tested

for leaves of apple trees of other varieties, as well as for leaves at different stages of development.

Keywords: spectral reflectance, *Malus domestica*, apple leaves, chlorophyll content, carotenoids content, vegetation indexes

Remote sensing data (mainly satellite data) is now widely used as the main source of information for quickly and cost-effectively obtaining information about the condition of agricultural plants over large areas. According to scientific publications, satellite agricultural monitoring allows for assessment of sown areas [1, 2], operational monitoring of crop state [3-5], yields [6-8], and monitoring of agronomically important soil properties [9].

Methods of satellite agricultural monitoring during their development since the mid-1960s have evolved from visual analysis of paper photographs to interactive interpretation on a computer monitor [10] and further to the construction of fully automated analysis algorithms [11] due to the transition from analogue images to satellite data as a set of digital (pixel) scenes. As a result, it became possible to perform automated computer pixel-by-pixel analysis of satellite data using a combination of several imaging channels in the form of derivative images obtained by arithmetic operations on individual channels. This significantly expanded the list of potential satellite predictors of the vegetation or soil properties as objects of remote monitoring. It turned out that in many cases the use of derivatives of satellite images rather than original satellite images is more effective for detecting and monitoring the properties of soils and vegetation.

In 1972, the first spectral vegetation index NDVI (normalized difference vegetation index) [12] was proposed for remote monitoring of vegetation, which was calculated as $NDVI = (R - NIR)/(R + NIR)$, where R is the image brightness in the red shooting channel, NIR is the image brightness in the near-infrared shooting channel.

Numerous studies using the example of different plant associations have shown that this index well reflects the state of vegetation and correlates with many of its properties (leaf color, aboveground phytomass, leaf surface, etc.) [13]. Until now, NDVI is widely used in agricultural remote monitoring systems around the world [2, 4, 14].

However, a search was carried out for other spectral vegetation indices that would be more sensitive to the specific properties of vegetation and soils. Currently, there are more than a hundred of them proposed and the number is constantly growing [15]. Of significant practical interest are vegetation indices developed for non-contact (remote) detection of the amount of pigments in plant leaves (mainly chlorophyll and carotenoids), since the efficiency and productivity of photosynthesis depends on their content [16, 17].

As a rule, authors test and validate their models and their proposed indices using the example of specific plants (in agriculture, these are mainly annual plants) [18], and the convenience of their use for other plants remains unexplored.

The possibilities of using vegetation indices to monitor perennial fruit plantations have been least studied. Moreover, many publications focus on the development of new methods for extracting information about the content of pigments in plants. Thus, for apple leaves in China [19, 20], approaches based on machine learning methods and neural networks have been proposed. C. Li et al. [21] assessed the capabilities of remote (satellite) detection of chlorophyll content for individual apple trees. However, there are few such publications and non-contact methods for assessing the content of pigments in apple leaves are still not sufficiently developed.

The presented article shows for the first time that spectral vegetation indices proposed for detecting pigments in agricultural plants need to be clarified

when used for similar detection of pigments in the leaves of a particular apple tree variety.

Our goal was to analyze the relationship between the spectral vegetation indices calculated for the leaves of the Imrus apple tree with the content of chlorophyll and carotenoids in them.

Materials and methods. An analysis of the leaf spectral reflectance of apple tree (Malus domestica Borkh.) Imrus variety planted in 2011 was carried out on the territory of the experimental garden of the Federal Scientific Center for Horticulture (Mikhnevo village, Moscow Province, Stupinsky District) on October 19, 2021. At this time, the leaves of the trees are in different states (from completely green to already yellowed or reddened), which ensured the most complete coverage of possible options for pigment content. Imrus is a winter scab-immune (*Vf*) variety (Antonovka vulgaris × OR18T13) bred at the All-Russian Research Institute for Breeding Fruit Crops (Oryol Province)

Mixed leaf samples were taken from two adjacent rows in plots with 15-20 trees in each row, which were located opposite each other. Leaves were selected randomly at midday and from branches 2-5 years old in the middle part of the crown. A total of 26 samples, each from 30-40 trees, were mixed to analyze the pigment content. Chlorophylls a + b were measured in lab test by the Wintermans-De Mots method [22], carotenoids by the von Wettstein method [23].

Spectral reflectance was assessed using a field spectroradiometer SR-6500 (Spectral Evolution, USA) that operates in the range of 350-2500 nm with a resolution of 1 nm. Spectral reflectance curves were obtained in 5 replicates for the upper leaf surfaces and averaged for each leaf and then for each of the 26 mixed leaf groups.

Based on the averaged spectral reflectance curves, the most common spectral vegetation indices were calculated. After this, an analysis was carried out of the relationship between the values of spectral vegetation indices and the content of pigments in the leaves.

At the first stage, a simple correlation analysis was carried out. Then clustering of the spectral reflection curves was performed and an analysis of the grouping of the chlorophyll and carotenoids contents in different groups of curves, identified by the similarity dendrogram of the reflection curves, was carried out. At the last stage, an attempt was made to correct the most suitable indices in order to adapt them to determine the pigment content in apple leaves based on linear regression analysis.

Statistical processing of data. i.e., the calculation of average values, confidence intervals, assessment of the statistical significance of differences ($t_{0.05}$), preliminary processing of spectral reflection curves (their smoothing and removal of outliers) was carried out using the stats and prospectr packages in the R environment (https://www .r-project.org/). The similarity dendrogram was constructed using the Statistica 6.0 package (StatSoft, Inc., USA). Regression analysis and calculation of p-value using the *F*-test were performed in Microsoft Excel.

Results. Table 1 presents formulas for calculation of vegetation indices for non-contact determination of pigment contents in leaves.

Regression analysis between the content of pigments in leaves and the value of various vegetation indices showed an almost complete absence of reliable regression dependencies. For carotenoid content, the highest R^2 value was found for the ARI index (ARI = 0.36), for chlorophyll content for the G index (G = 0.36). All other R^2 values turned out to be lower than 0.2 (Table 2). Only two models were statistically significant (at p = 0.01).

By the dendrogram of similarity of spectral reflection curves of apple tree

leaves in the analysis of 26 mixed samples, all curves were quite reliably divided into two large groups and one curve (19av) was not included in any of these groups (Fig. 1).

1. Spectral vegetation indices for non-contact determination of the chlorophyll and carotenoids contents in plant leaves

Formula for calculation	Pigment	Reference
$ARI = 1/R_{550} - 1/R_{700}$	Carotenoids	[24]
$CRI = 1/R_{510} - 1/R_{550}$	Carotenoids	[24]
$CRI2 = 1/R_{510} - 1/R_{700}$	Carotenoids	[24]
$PSSRc = R_{800}/R_{500}$	Carotenoids	[25]
$SIPI = (R_{445} - R_{800})/(R_{670} - R_{800})$	Carotenoids	[26]
$CSI1 = R_{695}/R_{420}$	Chlorophyll	[27]
$CSI2 = R_{695}/R_{760}$	Chlorophyll	[27]
$G = R_{554}/R_{677}$	Chlorophyll	[28]
$GM1 = R_{750}/R_{550}$	Chlorophyll	[29]
$GM2 = R_{750}/R_{700}$	Chlorophyll	[29]
$gNDVI = (R_{750} - R_{550})/(R_{750} + R_{550})$	Chlorophyll	[30]
$MCARI = [(R_{700} - R_{670}) - 0{,}2\cdot(R_{700} - R_{550})]\cdot(R_{700}/R_{670})$	Chlorophyll	[31]
$NPQI = (R_{415} - R_{435})/(R_{415} + R_{435})$	Chlorophyll	[32]
$PRI = (R_{528} - R_{567})/(R_{528} + R_{567})$	Chlorophyll	[33]
$SR705 = SR_{705} = R_{750}/R_{705}$	Chlorophyll	[34]
$TCARI = 3\cdot[(R_{700} - R_{670}) - 0{,}2\cdot(R_{700} - R_{550})]\cdot(R_{700}/R_{670})/$ $/(1 + 0{,}16)\cdot(R_{800} - R_{670})/(R_{800} + R_{670} + 0{,}16)$	Chlorophyll	[35]
$TVI = 0{,}5\cdot[120\cdot(R_{750} - R_{550}) - 200\cdot(R_{670} - R_{550})]$	Chlorophyll	[36]
$VOG1 = R_{740}/R_{720}$	Chlorophyll	[37]
$VOG2 = (R_{734} - R_{747})/(R_{715} - R_{720})$	Chlorophyll	[37]
$ZTM = R_{750}/R_{710}$	Chlorophyll	[38]
$SR\ (Chl\ a) = R_{675}/R_{700}$	Chlorophyll	[30]
$SR\ (Chl\ b) = R_{675}/R_{650}\cdot R_{700}$	Chlorophyll	[30]
$SR\ (Chl\ b2) = R_{672}/R_{708}$	Chlorophyll	[30]
$SR\ (Chl\ tot) = R_{760}/R_{500}$	Chlorophyll	[30]
$PSSRa = R_{800}/R_{675}$	Chlorophyll	[25]
$PSSRb = R_{800}/R_{650}$	Chlorophyll	[25]
$LCI = (R_{850} - R_{710})/(R_{850} + R_{680})$	Chlorophyll	[39]

N o t e. R_{xxx} in formulas means reflection at the specified wavelength (xxx, nm).

2. The effectiveness of vegetation indices for regression modeling of pigment content in the leaves of the apple tree (*Malus domestica* Borkh.) variety Imrus (Mikhnevo village, Moscow Province, Stupinsky District, 2021)

Index	Linear Regression R^2	p-value	Pigment
ARI	0.36	8.84184E–05	Carotenoids
CRI	0.08	0.11982	Carotenoids
CRI2	0.03	0.41109	Carotenoids
PSSRc	0.12	0.66258	Carotenoids
SIPI	0.17	0.11075	Carotenoids
CSI1	0.02	0.10390	Chlorophyll
CSI2	0.04	0.06195	Chlorophyll
G	0.36	8.89972E–06	Chlorophyll
GM1	0.11	0.46123	Chlorophyll
GM2	0.07	0.13564	Chlorophyll
gNDVI	0.03	0.48826	Chlorophyll
MCARI	0.16	0.37879	Chlorophyll
NPQI	0.19	0.37090	Chlorophyll
PRI	0.08	0.65917	Chlorophyll
SR705	0.03	0.24438	Chlorophyll
TCARI	0.18	0.37874	Chlorophyll
TVI	0.03	0.33811	Chlorophyll
VOG1	0.02	0.11980	Chlorophyll
VOG2	0.04	0.78741	Chlorophyll
ZTM	0.09	0.14587	Chlorophyll
SR (Chl a)	0.11	0.02127	Chlorophyll
SR (Chl b)	0.17	0.08067	Chlorophyll
SR (Chl b2)	0.08	0.13811	Chlorophyll
SR (Chl tot)	0.12	0.02885	Chlorophyll
PSSRa	0.02	0.16915	Chlorophyll
PSSRb	0.06	0.14184	Chlorophyll
LCI	0.16	0.01012	Chlorophyll

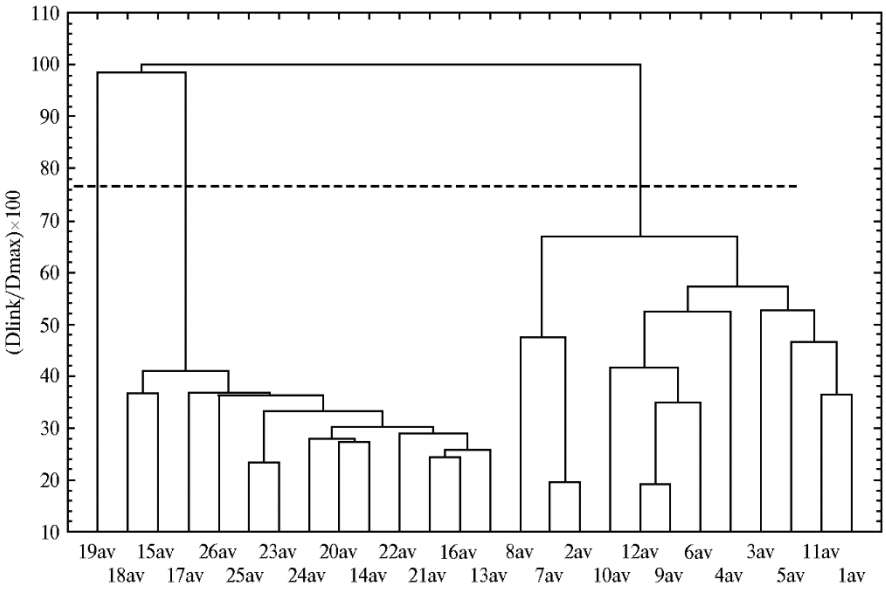

Fig. 1. Dendrogram of similarity of spectral reflection curves of apple tree leaves (*Malus domestica* Borkh.) variety Imrus: 1av-26av — each of 26 mixed leaf samples (Mikhnevo village, Moscow Province, Stupinsky District, 2021).

An attempt to establish connections between the content of pigments in leaves with the indicated groups was also not successful. In particular, the content of carotenoids in one of the groups was 0.57±0.06 mg/g, in the other 0.56±0.06 mg/g, and the content of chlorophylls a + b was 2.27±0.27 and 2.17±0.26 mg/g (at $p = 0.05$), respectively.

Thus, the spectral vegetation indices proposed by other researchers for the non-contact detection of pigments in plant leaves, in our case, did not provide satisfactory results. This is most likely due to the fact that most of the indices (see Table 1) were proposed and tested for vegetation at the level of phytocenosis, rather than individual leaves, without division into species (24, 26, 32) or for specific agricultural plants (28, 30, 31). The structure of the leaves of an apple tree has its own specifics and differs significantly from that of other plants, which determines the characteristics of light reflection.

Having analyzed the relationship between the previously proposed vegetation indices and the content of pigments in apple leaves, we tried to select more reliable indices. Since the general patterns of constructing indices should be preserved, the indices that showed the best results in regression analysis were selected as the base ones, and then we refined them for apple tree leaves by changing the wavelengths involved in the calculation.

The G index was chosen to detect chlorophyll content [28]. When specifying the wavelength for which the reflection value is taken when calculating using the formula, the quality of the regression model (as per R^2) increased almost 2 times. As a result, a new vegetation index was obtained for non-contact detection of chlorophyll content in apple tree leaves:

$$G\_apple = R_{580}/R_{685}.$$

The regression dependence ($R^2 = 0.66$) with this index is presented in Figure 2, A, the parameters of the regression model are in Table 3.

For carotenoids, we used the ARI vegetation index [24] as the base one:

$$ARI\_apple = (1/R_{560}) - (1/R_{690}).$$

The R^2 value of the regression model with this vegetation index reached 0.65 (see Fig. 2, B), table 3 shows the parameters of the regression model.

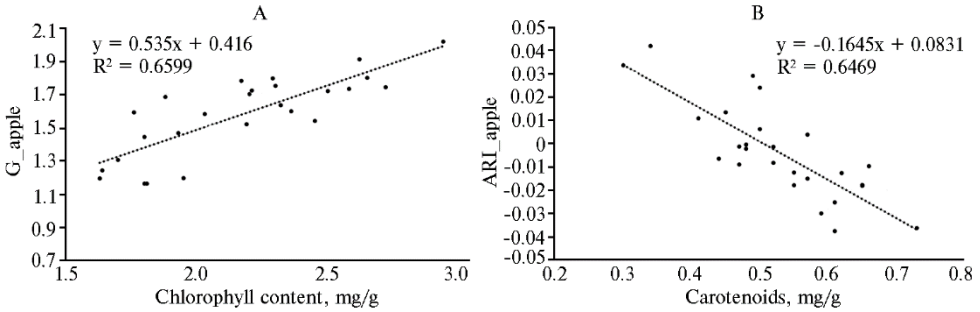

Fig. 2. Regression dependence of the spectral vegetation indices ARI_apple (A) **and G_apple** (B) **on the content of chlorophylls a + b** (A) **and carotenoids** (B) **in the leaves of the apple tree (*Malus domestica* Borkh.) variety Imrus** (Mikhnevo village, Moscow Province, Stupinsky District, 2021).

3. Parameters of regression models characterizing the dependence of the spectral vegetation indices on the content of pigments in the leaves of the apple tree (*Malus domestica* Borkh.) variety Imrus (Mikhnevo village, Moscow Province, Stupinsky District, 2021)

Index ARI_apple—chlorophylls a + b (Fig. 2, A)					
Regression statistics					
Plural R	0.804310212				
R-square	0.646914918				
Normalized R-squared	0.632203039				
Standard error	0.060379302				
Observations	26				
Analysis of variance					
	df	*SS*	*MS*	*F*	*F* significance
Regression	1	0.160308005	0.160308005	43.97228542	7.3744E-07
Remainder	24	0.087495841	0.003645660		
Total	25	0.247803846			
	coefficient	standard error	*t*-statistics	p-value	
Y-intersection	0.51319766	0.012052075	42.5816848	4.00502E-24	
Variable X1	–3.933057307	0.593117523	–6.631160187	7.3744E-07	
Index G_apple—carotenoid (Fig. 2, B)					
Regression statistics					
Plural R	0.812355896				
R-square	0.659922102				
Normalized R-squared	0.645752189				
Standard error	0.217909933				
Observations	26				
Analysis of variance					
	df	*SS*	*MS*	*F*	*F* significance
Regression	1	2.211462417	2.211462417	46.57206635	4.65864E-07
Remainder	24	1.139633737	0.047484739		
Total	25	3.351096154			
	coefficient	standard error	*t*-statistics	p-value	
Y-intersection	0.225012363	0.288248014	0.780620689	0.442657485	
Variable X1	1.23344918	0.18074176	6.824372964	4.65864E-07	

Thereof, our findings show that simple vegetation indices can be applicable to non-contactly determine the pigment content in apple leaves, but they must be adjusted for a specific variety. The quality of the results is quite comparable to that obtained using machine learning methods [19] or methods based on the use of neural networks [20]. Moreover, unlike complex methods, the approaches we propose are easier to use. Our studies confirm the results of C. Li et al. [21], although they were obtained for individual trees.

Therefore, the previously proposed numerous vegetation indices cannot be used for non-contact detection of the chlorophyll and carotenoid contents in the leaves of the Imrus apple tree. There is practically no connection between the index value and pigment content. It is also not possible to group the analyzed leaves according to pigment content based on constructing a dendrogram of similarity between the leaf spectral reflectance curves in the range of 350-2500 nm.

By the correction of the indices that showed the most accurate dependence, we proposed new vegetation indices for non-contact detection of the content of carotenoids and chlorophyll in apple leaves. Based these indices, we suggest regression models with R^2 above 0.65. Before widespread use, such models need to be tested for leaves of other apple varieties, as well as for leaves at different stages of development.

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INVESTIGATION OF THE SUGAR BEET (*Beta vulgaris* L. ssp. *vulgaris*) MICROSATELLITE LOCI STRUCTURE TO DEVELOP A TECHNOLOGY FOR GENETIC ANALYSIS OF SUGAR BEET LINES AND HYBRIDS

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Abstract

The quality control in the course of maintenance and reproduction of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) hybrid parent lines upon seed production is highly important. The method of microsatellite analysis seems to be very perspective tool to provide genotyping during breeding and seed production. Different research groups reported about microsatellite loci in the sugar beet genome. However, the implementation of this technique into the breeding process requires the development of robust and high-throughput technology of analysis. To develop a technology for obtaining stable DNA profiles, a more detailed study of the sugar beet genome microsatellite loci is required using a large set of verified breeding material. The sequencing a number of sugar beet genome regions containing microsatellite loci to clarify the nature of polymorphism as well as ability for providing the stable DNA profiles has been made in this study. Together with breeders (Pervomayskaya Selection and Experimental Station, Krasnodar Krai), a collection of 146 sugar beet plant samples was selected, including 28 male-sterile (MS) lines, 28 O-type lines, 82 pollinator lines, 6 hybrids of Russian selection (Azimut, Corvette, Pervomaisky, Rubin, Fregate, Uspekh) as well as Dobrava and Dorothea hybrids. Five plants of each sample were analyzed for 12 microsatellite loci, FDSB 502, FBSB 1001, FDSB 1033, Unigene 27833, Unigene 26753, Unigene 16898, Unigene 17623B, Unigene 15915, Unigene 17923, SB 04, SB 09, and SB 15. Allelic variants of each locus were amplified, cloned into the pAL2-T plasmid vector and sequenced. The results of sequencing the microsatellite loci FDSB 1001, FDSB 1033, Unigene 16898, Unigene 17623B, Unigene 26753, Unigene 17923, Unigene 27833, and SB 04 revealed that their length polymorphism is solely due to the different number of tandem repeats in the amplified DNA fragment. The locus Unigene 15915 was excluded from further work because of insertions and deletions in the flanking regions of microsatellite repeats $(AC)_n$ in its allelic variants. The polymorphism of allelic variants of the microsatellite loci SB 09, SB 15, and FDSB 502 is due to the complex (composite) repeats. Nevertheless, the SB 09 and SB 15 loci were approved for further study, since they produced stable DNA profiles. The allelic variants of the locus FDSB 502 contained the $(TC)_n(GAT)_n(AAG)_n$ sequence, which in some cases may complicate the analysis. To use this locus for the genetic analysis of sugar beet lines and hybrids, we propose the primers flanking only variable microsatellite repeatsб the $(GAT)_n$ and $(AAG)_n$ separately. The results we report here are prospective to develop a technology for the genetic analysis of sugar beet lines and hybrids as a reliable tool for both breeding and seed production.

Keywords: *Beta vulgaris*, sugar beet, fingerprinting, microsatellite analysis, DNA-profile

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is an important industrial crop, accounting for approximately 40% of global sugar production. It is also used as a

high-energy animal feed (beet molasses and beet pulp) and grown for biofuel production [1, 2].

In the recent past, the main indicators of the effectiveness of the breeding process were non-flowering and sugar yield per unit amount of raw materials and sowing area. Currently, economic priorities are increasing technological suitability of raw materials, seeds with high sowing and physical properties, tolerance to herbicides, resistance to diseases, pests, environmental factors, and, most importantly, the profitability of seed production and the cultivation of commercial crops [3-5].

In this regard, modern sugar beet hybrids are created on the basis of dioecious forms with cytoplasmic male sterility (CMS) and the so-called fixers of the CMS trait (O-type lines) and are multicomponent [6]. Therefore, commercial seed production of sugar beets is very complex and requires compliance with a number of conditions. Firstly, it is necessary to strictly monitor the high-quality maintenance and reproduction of all components, which, at a minimum, include components of the maternal form, the MS line (a line with cytoplasmic male sterility) and the O-type fixative line, as well as the component of the paternal form, the line pollinator.

The creation of a commercial sugar beet hybrid is multi-step and involvs crossing specific parental lines to produce simple intermediate hybrids. The selection of each component for hybridization is carried out based on the specific combinative ability which is determined experimentally in test crosses. Thus, to consistently produce a commercial hybrid, it is necessary to control all components used to generate the final hybrid, as well as intermediate hybrids.

Genetic analysis can be used to evaluate the quality of breeding material at various stages of creating a sugar beet hybrid. Such an analysis is necessary for the genetic identification of lines that are components of the hybrid, as well as for assessing their homogeneity.

A number of molecular genetic methods can be used to analyze plant genomes: RFLP (restriction fragment length polymorphism) [7], AFLP (amplified fragment length polymorphism) [8], RAPD (random amplified polymorphic DNA) [9], SCAR (sequence characterized by an amplified region) [10], SNP (single nucleotide polymorphism) [11], DArT (diversity array technology) [12], SSR (simple sequence repeat). or microsatellite analysis [13]. Among them, the most commonly used methods for identifying plant genotypes are SNP and SSR. Note, the method of studying single nucleotide polymorphisms (SNP) was used to analyze the genome of both sugar beet [14-16] and a number of other crops, e.g., cocoa [17], cucumber [18], cauliflower [19], honeysuckle [20]. However, the use of this method for reliable identification of genotypes requires the development and subsequent recording of a large number of markers (from hundreds to several thousand) and expensive equipment for obtaining and processing the results.

For the purposes we mentioned, the most promising is the analysis of microsatellite loci polymorphisms in the sugar beet genome. The microsatellite markers give a stably reproducible DNA profile (primers are complementary to conservative regions of the genome). In addition, these markers are codominant, allowing their use to track the inheritance of the genomes of parental lines in intermediate and final hybrids.

Despite a number of works have been published on the microsatellite analysis method in sugar beet breeding programs both abroad [21-26] and in Russia [27-29], this technology is not convenient. To create a technology that allows unique and stable DNA profiles to be generated, an in-deep study of genomic microsatellite profiles on a large sample of verified sugar beet breeding material is

required.

In the presented study, we for the first time carried out a detailed analysis of the primary structure of a number of microsatellite loci in the sugar beet genome to determine the nature of the polymorphism of these regions and their suitability for obtaining stable DNA profiles.

The goal of our work was to study the structure of microsatellite loci of the sugar beet genome for subsequent use in creating a technology for genetic analysis of lines and hybrids.

Materials and methods. The study was performed on 146 samples of sugar beet (*Beta vulgaris* L.) plants, including 28 MS lines, 28 O-type lines, 82 pollinator lines, 6 hybrids of domestic selection (Azimut, Korvet, Pervomaisky, Rubin, Fregat, Uspekh), hybrids Dobrava and Dorotea (provided by the Pervomaisk Selection and Experimental Station of Sugar Beet, Gulkevichi, Krasnodar Territory). For reliable results, five different plants of each sample were used.

Genomic DNA was isolated from green leaves by CTAB extraction with additional purification with chloroform [30]. DNA in the resulting preparations was detected by electrophoresis in a 1% agarose gel, followed by staining with ethidium bromide. The quality and quantity of isolated DNA were determined on a SPECTROstar Nano plate spectrophotometer (BMG LABTECH GmbH, Germany).

Amplification of target DNA fragments was carried out with locus-specific primers FDSB 502 [21], FDSB 1001, FDSB 1033, 521.6 [24], SB 04, SB 09, SB 15 [25], Unigene 15915, Unigene 16898, Unigene 17623B, Unigene 17923, Unigene 26753, Unigene 27833 [23], labeled with fluorescent dyes FAM, R6G, TAMRA and ROX. PCR was run in a 25 μl reaction mixture containing 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$ (AppliChem, USA); 2.5 mM $MgCl_2$ (AppliChem, USA); 5 units/μl of Taq-DNA polymerase (DNA-Technology LLC, Russia), 25 mM dNTP (Medigen LLC, Russia), 10 pmol of each primer (NPK Syntol, Russia) and 2 μl of DNA solution. The PCR protocol was 95 °C for 5 min; 30 cycles: 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min (a CFX-96 thermal cycler, Bio-Rad, USA).

PCR products were detected by high-resolution capillary electrophoresis under denaturing conditions (an ABI PRISM 3130XL genetic analyzer, Applied Biosystems, USA). To determine the size of PCR fragments using the DNA Fragment Analysis software (IAP RAS, Russia), 1 μl of the PCR product was mixed with 0.5 μl of the molecular weight marker GeneScan™ 600 LIZ (Applied Biosystems, USA) and 8 μl of Super DI formamide (MCLab, USA) and denatured for 5 min at 95 °C.

Preparation of samples for sequencing included amplification of each allelic variant with unlabeled primers and subsequent purification of the resulting amplified DNA fragment using the Cleanup Mini kit (JSC Evrogen, Russia). Purified PCR products were cloned into the plasmid vector pAL2-T (JSC Evrogen, Russia), which was delivered into *Escherichia coli* XL1-Blue strain by electroporation. Clones after blue-white selection were tested for the presence of the insert using PCR. Plasmid DNA was isolated by a standard method [31]. Plasmid inserts were sequenced by the Sanger method with a standard pair of primers, the M13F 5′-GTTGTAAAACGACGGCCAGTG-3′, M13R 5′-AGCGGATAACAATTTCACACAGGA-3′ (Synthol Research and Production Company, Russia). To ensure the reliability of sequencing results for each allelic variant, two DNA samples were taken from unrelated samples and two clones were selected from a Petri dish.

Nucleotide sequence analysis was performed using Chromas 2.6.6 (Tech-

nelysium Pty, Ltd., Australia) and Clustal Omega (EMBL's European Bioinformatics Institute, EMBL-EBI, UK) software.

The design of new primers for the FDSB502 microsatellite locus, flanking only tandem repeat regions, was carried out with Primer3Plus software, EMBL (https://www.primer3plus.com), the absence of secondary structures in the sequence was checked with Oligo Calc software: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html).

Results. The polymorphism of microsatellite loci in the sugar beet genome was studied using plant material from the collection of the Pervomaisk Sugar Beet Breeding and Experimental Station (Krasnodar Territory, Gulkevichi), used in breeding in 2018-2022.

For reliable discrimination and identification of plants, the selection of the most informative microsatellite loci is of decisive importance. For this purpose, based on an analysis of literature data, 40 microsatellite loci were initially selected [27]. Selection was carried out by the following criteria: the number of alleles in the locus is at least three; the location of loci on different chromosomes, which should ensure independent inheritance of DNA markers; DNA fragment size from 100 bp up to 400 bp for reliable determination of PCR fragment lengths.

The polymorphism of the selected loci was studied experimentally on a set of 129 sugar beet samples. Loci that were monomorphic, difficult to amplify, or that gave ambiguous and unstable results were excluded from the study. As a result, 13 microsatellite loci remained, the 521.6, FDSB 502, FBSB 1001, FDSB 1033, Unigene 27833, Unigene 26753, Unigene 16898, Unigene 17623B, Unigene 15915, Unigene 17923, SB 04, SB 09, SB 15 which were highly polymorphic (from 3 to 11 detected alleles for each locus). Using them, unique DNA profiles were generated for each sample of sugar beet [27].

A study of a larger set of 146 sugar beet samples confirmed the suitability of these loci for genetic analysis. The exception was the 521.6 locus [24] the amplification of which in some cases gave nonspecific DNA fragments in addition to the target product (Fig. 1). Therefore, locus 521.6 was excluded from tests.

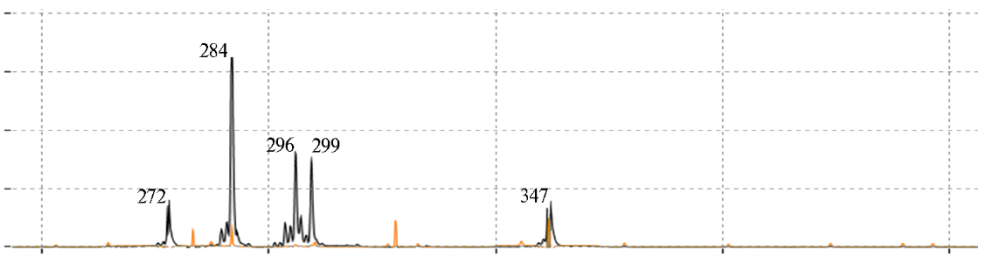

Fig. 1. Electropherogram of PCR products of the microsatellite locus 521.6, labeled with the fluorescent dye TAMRA, in the sugar beet line Op 66279 7/10 from the working collection of the Pervomaisk Research and Development Station of Sugar Beet (Gulkevichi, Krasnodar Territory, 2018-2022). During amplification, nonspecific DNA fragments (272 bp, 296 bp, 299 bp, 347 bp) appeared in addition to the target 284 bp PCR product.

As a result of genetic analysis of 146 sugar beet lines, 35 lines with a high homogeneity were selected. Homogeneous lines (all plants had an identical DNA profile for microsatellite loci) were involved in the breeding as components for the creation of new hybrids. Lines with incomplete homogeneity (less than 80%) were subjected to further self-pollination, followed by annual control of uniformity using microsatellite analysis.

All allelic variants of the studied microsatellite loci that we identified are submitted in Table 1.

1. **Polymorphism of microsatellite loci of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) identified in a set of 146 samples from the working collection of the Pervomaisk Breeding and Experimental Station of Sugar Beet** (Gulkevichi, Krasnodar Territory, 2018-2022)

Locus	Alleles, bp	References
FDSB 502	265, 271, 273, 276, 279, 283, 286, 293, 314	[21]
FDSB 1001	315, 323, 325, 333, 347, 351	[24]
FDSB 1033	167,177, 193, 195, 197, 199, 221, 229	[24]
SB 04	180, 186, 189, 192, 195, 198, 201	[25]
SB 09	130, 133, 136	[25]
SB 15	146, 150, 154, 160, 166, 170, 174	[25]
Unigene 15915	299, 305, 314, 321, 339, 342, 345, 349, 383	[23]
Unigene 16898	276, 279, 285, 291	[23]
Unigene 17623B	147, 153, 156, 159, 162, 165, 168, 171, 174, 177, 180	[23]
Unigene 17923	193, 195, 197, 199, 201, 203, 205, 209, 215, 219, 225	[23]
Unigene 26753	282, 285, 288, 291, 294, 297, 303	[23]
Unigene 27833	190, 199, 205, 208, 211, 214, 217	[23]

N o t e. The size of PCR products was determined by high-resolution capillary electrophoresis under denaturing conditions on an ABI PRISM 3130XL genetic analyzer (Applied Biosystems, USA). The GeneScan™ 600 LIZ molecular weight marker (Applied Biosystems, USA) was used as a size standard.

To obtain reliable results of genetic analysis, the length polymorphism of microsatellite loci must be caused only by the microsatellite repeats without additional insertions or deletions outside the repeat region in the amplified fragment. Therefore, at the next stage, we assessed the primary structure of the 12 microsatellite loci used in the analysis.

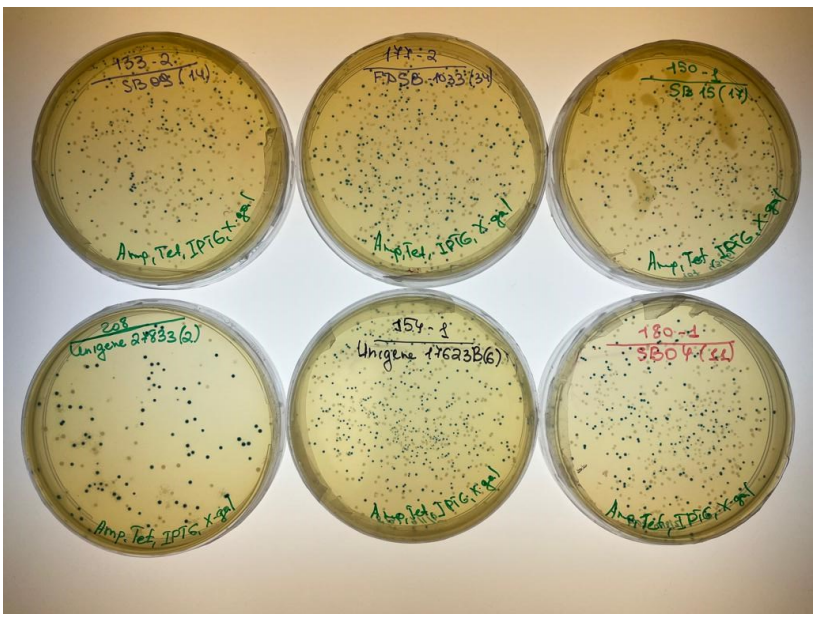

Fig. 2. Petri dishes with *Escherichia coli* XL1-Blue transformants carrying the pAL2-T plasmid vector with inserts of microsatellite loci SB 09, FDSB 1033, SB 15, Unigene 27833, Unigene 17623B and SB 04 target fragments (white clones). DNA fragments of micro-satellite loci were obtained from the analysis of the working collection of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) of the Pervomaisk Sugar Beet Breeding and Experimental Station (Gulkevichi, Krasnodar Territory, 2018-2022).

Allelic variants of each of the 12 loci were individually amplified and cloned into the pAL2-T plasmid vector (JSC Evrogen, Russia). The resulting *E. coli* transformants with inserted target DNA fragments (Fig. 2, white colonies) were selected and their plasmid DNA was sequenced for each allelic variant of the corresponding locus.

The results of the analysis of nucleotide sequences of all microsatellite loci

studied in our work are schematically shown in Figure 3.

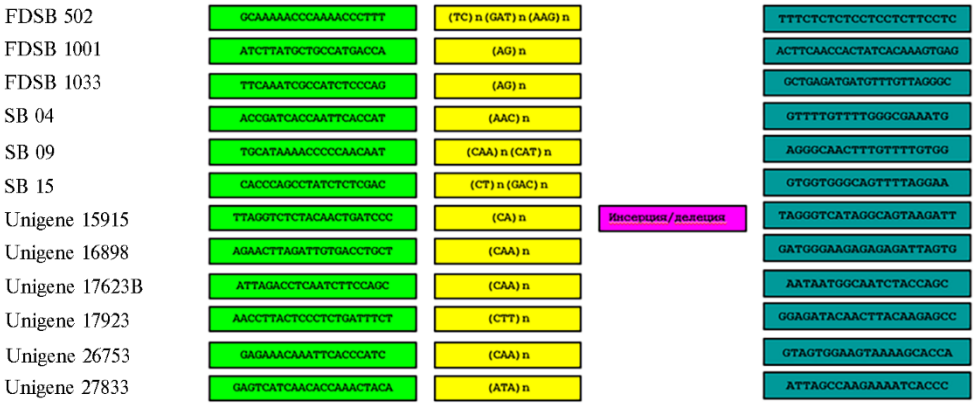

Fig. 3. The structure of microsatellite loci in the genome of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) samples from the working collection of the Pervomaisk Sugar Beet Breeding and Experimental Station (Gulkevichi, Krasnodar Territory, 2018-2022). Sequences marked in light green and dark green are primer regions, in yellow are tandem repeat regions, and in pink are insertions/deletions.

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Unigene 26753_288-1     GAGAAACAAATTCACCCATCAACATCTTCAACAACAACAACAACAACAACGACAACAACC   60
Unigene 26753_288-2     GAGAAACAAATTCACCCATCAACATCTTCAACAACAACAACAACAACAACAACAACAACC   60
Unigene 26753_294-3     GAGAAACAAATTCACCCATCAACATCTTCAACAACAACAACCACAACAACAACAACAACA   60
Unigene 26753_294-4     GAGAAACAAATTCACCCATCAACATCTTCAACAACAACAACCACAACAACAACAACAACA   60
Unigene 26753_303-5     GAGAAACAAATTCACCCATCAACATCTTCAACAACAACAACAACAACAACCCACAACG     60
Unigene 26753_303-6     GAGAAACAAATTCACCCATCAACATCTTCAACAACAACAACAACAACGACAACCACAACA   60

Unigene 26753_288-1     ACAACAA---------------AGGAGCAAGTTCTTACCAATGCTATGCTCCTCATCAAG  105
Unigene 26753_288-2     ACAACAA---------------AGGAGCAAGTTCTTACCAATGCTATGCTCCTCATCAAG  105
Unigene 26753_294-3     ACAACAACAACAA---------AAGAGCAAGTCCTTACCAAAGCTATGCTCCTCATCAMC  111
Unigene 26753_294-4     ACAACAACAACAA---------AAGAGCAAGTTCTTACCAATGCTATGCTCCTCATCAAC  111
Unigene 26753_303-5     ACAACAACAACAACAACAACAAAAGAGCAAGTTCTTACCAATGCTATGCTCCTCATCAAC  120
Unigene 26753_303-6     ACAACAACAACAACAACAACAAAAGAGCAAGTTCTTACCAATGCTATGCTCCTCATCAAC  120

Unigene 26753_288-1     CAAAGAAGTGACCAAGCTTTCGCTTTCGCATCGGCGTAGCTCCTCCGCCTGCTCCACCAC  165
Unigene 26753_288-2     CAAAGAAGTGATCAAGCTTTCGCTTTCGCATCGGCGTAGCTCCTCCGCCTGCTCCACCAC  165
Unigene 26753_294-3     CAAGGAAGTGATCAAGCTTTCGCTTTCGCATCAGCGTAGCTCCTCCACACATGTTCCACCAC  171
Unigene 26753_294-4     CAAAGAAGTGATCAAGCTTTCGCTTTCGCATCGGCGTAGCTCCTCCACATGTTCCACCAC  171
Unigene 26753_303-5     CAAAGAAGTGATCAAGCTTTCGCTTTCGCATCGGCGTAGCTCCTCCACCTGTTCCACCAC  180
Unigene 26753_303-6     CAAAGAAGTGATCAAGCTTTCGCTTTCGCATCGGCGTAGCTCCTCCACCTGTTCCACCAG  180

Unigene 26753_288-1     CTCCTCCACCGTCTCCGAGGACCCTTTATCACCAAAAGTTGGGTGTATGGGCCAAGTTAA  225
Unigene 26753_288-2     CTCCTCCACCGTCTCCGAGGACCCTTTATCACCAAAAGTTGGGTGTATGGGCCAAGTTAA  225
Unigene 26753_294-3     CTCCTCCACCGTCTCCGAGGACCCTTTATCACCAAAAGTTGGGTGTATGGGCCAAGTTAA  231
Unigene 26753_294-4     CTCCTCCACCGTCTCCGAGGACCCTTTATCACCAAAAGTTGCGTGTATGGGCCAAGTTAA  231
Unigene 26753_303-5     CTCCTCCACCGTCTCCGAGGACCCTTTACCACCAAAAGTTGGGTGTATGGGCCAAGTTAA  240
Unigene 26753_303-6     CTCCTCCACCGTCCCCGAGGACCCTTTATCACCAAAAGTTGGGTGTATGGGCCAAGTTAA  240

Unigene 26753_288-1     GAGAAACAACAAAGTTGTTGGTTTTCCAAGTAAGAGTTTTGTTGGTGCTTTTACTTCCAC  285
Unigene 26753_288-2     GAGAAACAACAAAGTTGTTGGTTTTCCAAGTAAGAGTTTTGTTGGTGCTTTTACTTCCAC  285
Unigene 26753_294-3     GAGAAACAACAAAGTTGTTGGTTTTCCCAGTAAGAGTTTCGTTGGTGCTTTTACTTCCAC  291
Unigene 26753_294-4     GAGAAACAACAAAGTTGTTGGTTTTCCAAGTAAGAGTTTTGTTGGTGCTTTTACTTCCAC  291
Unigene 26753_303-5     GAGAAACAACAAAGTTGTTGGTTTTCCAAGTAAGAGTTTTGTTGGTGCTTTTACTTCCAC  300
Unigene 26753_303-6     GAGAAACAACAAAGTTGATGGTTTTCCAAGTAAGAGTTTTGTTGGTGCTTTTACTTCCAC  300

Unigene 26753_288-1     TAC         288
Unigene 26753_288-2     TAC         288
Unigene 26753_294-3     TAC         294
Unigene 26753_294-4     TAC         294
Unigene 26753_303-5     TAC         303
Unigene 26753_303-6     TAC         303
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Fog. 4. Comparison of nucleotide sequences of the Unigene 26753 microsatellite locus allelic variants identified in the genome of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) samples from the working collection of the Pervomaisk Sugar Beet Breeding and Experimental Station (Gulkevichi, Krasnodar Territory, 2018-2022). The primer regions are highlighted in light green and dark green; the tandem repeat region is highlighted in yellow. Sample 1 (allele 288 bp) is line Ot 7994, sample 2 (allele 288 bp) is line Op 19962, sample 3 (allele 294 bp) is line Ot 12122, sample 4 (allele 294 bp) is line MS 11348, sample 5 (allele 303 bp) is line Op 10632, sample 6 (allele 303 bp) is line Op SP-1P2.

Figure 4, as an example of the analysis of a typical microsatellite locus the fragment length polymorphism of which is due only to a different number of repeats (in this case, CAA), shows the alignment of nucleotide sequences of the

most common alleles of the Unigene 26753 microsatellite locus.

The results of sequencing allelic variants of the microsatellite loci FDSB 1001, FDSB 1033, Unigene 16898, Unigene 17623B, Uni-gene 17923, Unigene 27833, SB 04 also confirmed that their polymorphism is caused solely by the number of microsatellite repeats in the amplified DNA fragment.

Analysis of the nucleotide sequences of allelic variants of the microsatellite locus Unigene 15915 showed that the length polymorphism of the amplified fragments is caused not only by a different number of tandem repeats $(CA)_n$, but also by additional insertions and deletions in the DNA regions flanking the repeats (see Fig. 3). This complicates the interpretation of the results of microsatellite analysis; thereof, the indicated Unigene 15915 locus was excluded from further work.

The results of sequencing allelic variants of microsatellite loci SB 09, SB 15 and FDSB 502 showed that these loci contain complex (compound) repeats (see Fig. 3). However, amplification of microsatellite loci SB 09 and SB 15 resulted in stable and reproducible DNA profiles, so these two loci were involved in further tests.

The polymorphism of the FDSB 502 locus is due to quantitative changes in the complex (composite) tandem repeat $(TC)_n(GAT)_n(AAG)_n$ (see Fig. 3). Note, in some cases, the analysis of polymorphism of a locus containing hree types of tandem repeats in the amplified DNA fragment is diffecult. We have previously shown that to obtain stable DNA profiles, it is advisable to simultaneously amplify no more than two polymorphic regions in one locus [32-34].

This locus can be used for genetic analysis of sugar beet lines and hybrids by amplifying each tandem repeat region separately. In this case, the likelihood of obtaining a reliably interpretable DNA profile is much higher. Therefore, we selected primers flanking different groups of tandem repeats in the FDSB 502 locus.

2. Novel primers for amplification of variable regions of the FDSB 502 locus in the sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) genome

Locus	Microsatellite repeats	Primer pair
FDSB 502-2	$(GAT)_n$	502-2F: 5′-ACAATGGCGAATCGCTTTTGGGG-3′
		502-2R: 5′-CGTACTCATCTTCATCGTCTTCTTC-3′
FDSB 502-3	$(AAG)_n$	502-3F: 5′-GAAGAAGACGATGAAGATGAGTACG-3′
		502-3R: 5′-GAATCAACCTTGCCGACATATCC-3′

3. Microsatellite loci that are promising for creating genotyping technology for sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) lines and hybrids

Locus	Microsatellite repeats	Detected alleles	
		size rank, bp	number
Unigene 16898	$(CAA)_n$	276-291	4
Unigene 17623B	$(CAA)_n$	147-179	11
Unigene 17923	$(CTT)_n$	193-225	11
Unigene 26753	$(CAA)_n$	282-303	7
Unigene 27833	$(ATA)_n$	190-217	7
FDSB 1033	$(AG)_n$	165-229	8
FDSB 1001	$(AG)_n$	315-351	6
SB 04	$(AAC)_n$	180-201	7
SB 09	$(CAA)_n(CAT)_n$	130-136	3
SB 15	$(CT)_n(GAC)_n$	146-174	7
FDSB 502-2	$(GAT)_n$	112-154	5
FDSB 502-3	$(AAG)_n$	223-241	4

N o t e. Obtaining stable and unambiguously interpreted DNA profiles when using microsatellite loci in this study is shown on 146 phenotypically characterized samples from the working collection of the Pervomaisk Breeding and Experimental Station of Sugar Beet (Gulkevichi, Krasnodar Territory, 2018-2022).

In a set of 146 sugar beet samples, for polymorphisms caused by different numbers of tandem repeats of each type, it was shown that the DNA region with the microsatellite repeat $(TC)_n$ is conservative (the number of TC repeats in all samples is the same and equal to 10) while the regions $(GAT)_n$ and $(AAG)_n$ are variable. Therefore, for genetic analysis of sugar beet lines and hybrids, only the

primers that flank the microsatellite repeats $(GAT)_n$ and $(AAG)_n$ in the FDSB 502 locus (Table 2) seem to be promising.

Therefore, 12 microsatellite loci provide stable and unambiguously interpreted DNA profiles and are promising for genotyping sugar beet lines and hybrids (Table 3).

So, 146 samples from the working collection of the Pervomaisk Selection and Experimental Station of Sugar Beet were analyzed for 12 microsatellite loci (FDSB 502-2, FDSB 502-3, FBSB 1001, FDSB 1033, Unigene 27833, Unigene 26753, Unigene 16898, Unigene 17623B, Unigene 17923, SB 04, SB 09 and SB 15). The tested samples were 28 MS lines, 28 O-type lines, 82 pollinator lines, hybrids of domestic selection Azimut, Korvet, Pervomaisky, Rubin, Fregat, Uspekh, hybrids Dobrava and Doroteya. The revealed allele length polymorphisms are 265-314 bp for FDSB 502, 315-351 bp for FDSB 1001, 167-229 bp for FDSB 1033, 180-201 bp for SB 04, 130-136 bp for SB 09, 146-174 bp for SB 15, 299-383 bp for Unigene 15915, 276-291 bp for Unigene 16898, 147-180 bp for Unigene 17623B, 193-225 bp for Unigene 17923, 282-303 bp for Unigene 26753, and 190-217 bp for Unigene 27833. Sequencing of allelic variants of microsatellite loci FDSB 1001, FDSB 1033, Unigene 16898, Unigene 17623B, Unigene 26753, Unigene 17923, Unigene 27833, and SB 04 confirmed that their polymorphisms are due to the number of tandem repeats in the amplified DNA fragment. In addition to $(CA)_n$ repeats, the Unigene 15915 locus contains insertions and deletions, so we do not recommend this locus for genotyping. The polymorphisms of the SB 09, SB 15 and FDSB 502 loci are caused by complex repeats. However, the SB 09 and SB 15 loci provide stable DNA profiles. The $(TC)_n(GAT)_n(AAG)_n$ polymorphism was detected in the FDSB 502 locus, which may distort the genotyping results. To use this locus in genotyping sugar beet lines and hybrids, we propose primers flanking only the variable microsatellite repeats $(GAT)_n$ and $(AAG)_n$. The results obtained will help create a reliable laboratory tool for sugar beet breeding and commercial seed production.

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CERTIFICATION OF RUSSIAN RED CLOVER (*Trifolium pratense* L.) VARIETIES BASED ON SSR AND SRAP MARKERS

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Abstract

Molecular-genetic certification is a powerful strategies and efficient addition to the traditional methods of variety testing and agricultural crops identification. Russia, as well as a world in a whole, introduces the current DNA technologies in the breeding programs, in a variety registration process and in a system of seed production. However, the traditional approaches, based on observation and recording the morphological characters, are the prevalent now for the forage crops. It influences negatively on efficiency of selection, increases the terms and coasts of the new varieties development, registration and breeders rights protection. In this paper, the results of creation a system for identification and genetic certification of Russian red clover cultivars on the base of SSR and SRAP-markers are submitted for the first time. The seeds of 15 domestic varieties from gene pool collection of Federal Williams Research Center of Forage Production and Agroecology and 6 accessions of foreign breeding from Vavilov All-Russian Institute of Plant Genetic Resources were used for investigations. The genome DNA was extracted from 7-day seedlings' tissue. Bulk DNA samples were formed from 30 individual genotypes per each variety. We used basic SDS-method in own modifications. Quantity and quality of extracted DNA was analyzed by agarose gel electrophoresis and measurement of concentration and purity. The final concentration of DNA samples was 30 ng/μl. PCR amplification was performed using 35 SSR from the Red Clover Marker Database (http://marker.kazusa.or.jp/Red_clover), and 40 SRAP markers. A total of 476 PCR products were generated with SSR markers for 12 red clover varieties. A set of eight microsatellite loci was selected for identification the tested samples. With application of 40 SRAP markers, we selected 18 informative combinations for analysis of the red clover collection of 16 varieties. Total 812 PCR products were revealed and 85 (10.5 %) among them were determined as polymorphic. The set of 7 informative markers were identified for samples differentiation on the base of SRAP analysis. Unique varieties-specific DNA fragments were sequenced (Evrogen Lab company, Russia) for validation the results of analysis. Nucleotide sequences, identifying Russian red clover varieties Trifon, Mars, Topas, Atlant, Tetraploidniy VIK, Meteor, VIK 77, were included in the GenBank NCBI (https://www.ncbi.nlm.nih.gov/). The data of DNA fingerprinting we used for development the molecular-genetic formulas representing microsatellite loci allele composition and polymorphism in exon and intron regions of genome. As a result of this study, 10 etalon genetic certificates were designed for Russian red clover varieties.

Keywords: forage crops, genetic diversity, SSR markers, SRAP markers, DNA polymorphism, genetic certification

Among genomic biotechnologies in agriculture of the Russian Federation, special attention is focused on DNA identification and genetic certification of breeding achievements. Widespread use of these approaches will increase the efficiency of registration of new varieties and protect the copyright of breeders, and

will help in the fight against counterfeit in the seed market. Currently, the assessment of varieties for compliance with the the DUS (distinctiveness, uniformity and stability) test is based on the description of morphological characteristics. However, this is a labor-intensive and time-consuming protocol that requires appropriate professional scale and, in some cases, special conditions for testing (for example, vernalization of barley and wheat) [1]. The task is also complicated by the limited number of evaluation descriptors, which are also subject to the influence of environmental conditions. These are, in particular, traits of disease resistance that must be assessed when certifying some vegetable varieties (e.g., tomatoes) or flowering time for many outbreeding species (e.g., ryegrass) [2-4]. For a number of crops, identifying the characteristics that determine the originality of a variety is complicated by a high degree of intrapopulation variation or, on the contrary, interspecific morphological similarity (twin species) [5, 6). As the number of varieties increases, the genetic base shrinks because breeding tends to focus on a few of the most important agronomic traits, making it difficult to distinguish differences on a morphological basis [7]. Moreover, in perennial species, the manifestation of a number of traits requires a long time and an appropriate stage of development.

The efficiency of assessment can be significantly increased by integrating methods based on the use of molecular DNA markers into the variety testing system. An almost unlimited number of such markers and a high degree of detectable polymorphism (regardless of the plant part being studied and environmental conditions) allow differentiation of even difficult-to-distinguish varieties. DNA markers can reveal hidden variability which improves the accuracy of results and the resolution of analysis [8-10].

Genetic identification is based on determining the combination of alleles of a particular gene that are characteristic of the organism being studied. If you have informative and convenient methods, the results can be easily documented and a molecular genetic passport of the variety can be developed indicating the length of DNA fragments in specific chromosome regions. The passport allows you to determine the uniqueness of the sample and the level of genetic variability of the species, assess the degree of relationship with known varieties and compliance with the standard, and analyze the uniformity of seeds.

The methods underlying the creation of genetic passports are successfully used not only in variety testing, but also in breeding. The ability to select valuable genotypes at the initial stage of plant development reduces the duration of testing and increases the efficiency of the breeding process, especially in cases where selection by phenotype turns out to be lengthy and not reliable enough [11]. Analysis of DNA polymorphism provides breeders with information that can be used to control the results of hybridization, when selecting parental forms for crosses, and to identify the sources of genes that affect economically valuable traits.

Molecular markers used to assess genetic variability and identify lines, varieties and forms must meet certain requirements, i.e., to be highly polymorphic, reproducible, and evenly distributed throughout the genome, covering its different regions. Most often for this purpose, RFLP- (restriction fragment length polymorphism), RAPD- (random amplified polymorphic DNA), AFLP- (amplified fragment length polymorphism), SSR- (simple sequence repeats) markers, which are anonymous DNA fragments reflecting polymorphism in randomly selected regions of the genome [14, 15].

However, the results of polymorphism assessment do not always correlate with known morphological characters. For the purposes of applied genetics and breeding, analysis of functional molecular markers that identify changes in transcribed coding DNA sequences, i.e., the genes is more rational [16-18]. The main

resource for the development of such markers are EST (Expressed Sequence Tags) libraries which contain sequenced DNA fragments 500-700 bp long. For many crops, databases have now been created with information on EST-SSR markers developed based on complementary DNA (cDNA) [19, 20]. SSR markers provide the detection of polymorphisms of simple repeated DNA sequences (1-10 bp) which vary in length in some plant accessions [21]. The identification is also based on the variability of these genome regions, suggesting that genotypes of the same variety (if it is sufficiently aligned) should contain alleles of the same size.

In recent years, to assess the genetic polymorphism of varieties and species, the system of SRAP markers (sequence related amplified polymorphism) designed to amplify DNA fragments in the intron-exon regions of a gene has been successfully used [22]. Their advantages are ease of use and statistical processing, high information content and stability of results, much higher than with RAPD marking. With the SRAP method, it is easy to detect polymorphism even in closely related breeding material, so it is successfully used to distinguish varieties of different crops [23-25].

In Russia, as throughout the world, the DNA analysis is involved in breeding programs, registration of new varieties and commercial distribution of seeds. Genetic passports have been developed for a number of agricultural crops, mainly those for food purposes (rice, wheat, beets, potatoes, etc.) [26-29]. Forage grasses remain less studied, with phenotypic assessment predominant. This significantly reduces the efficiency of the selection of parental and breeding materials, increases the time for the creation of new varieties and the costs of their testing and registration. The solution will be a methodology for analyzing the genetic variability of varieties and forms at the DNA level, which should be high-performance, relatively inexpensive and independent of morphological parameters.

Of the forage perennial grasses in the regions of the Non-Chernozem Zone of Russia, meadow clover is the most common. It is used not only as a valuable high-protein feed for animals, but also as an excellent precursor for other crops and plays an important role in the biologization of agriculture [30]. To date, through the efforts of Russian breeders, more than 100 varieties of this species have been created. The use of modern methods of molecular analysis expands the possibilities of their reliable identification and legal protection, and also helps to accelerate the process of creating new forms that are high-yielding and disease-resistant.

In the presented work, we for the first time assessed the genetic polymorphism of a collection of Russian and foreign varieties of red clover using SSR and SRAP markers, identified variety-specific DNA fragments for differentiating the studied material, the uniqueness of which was confirmed by sequencing. Based on the results obtained, reference genetic passports have been compiled for a number of Russian varieties.

Our goal was to develop a system for determining varietal identity and reference genetic passports based on SSR and SRAP markers for Russian meadow clover varieties.

Materials and methods. The research was carried out in 2019-2021 at the Williams Federal Scientific Center for Forage Production and Agroecology. The material seeds of 15 Russian varieties of meadow clover (*Trifolium pratense* L.), from the Center for Shared Use Biological Collections of Forage Plants and 6 accessions of foreign origin from the Collection of Genetic Resources VIR ((FSC Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg) were used.

Genomic DNA was isolated from a bulk sample of the 7-day-old seed-

lings, 30 genotypes from each variety. The common SDS method with modifications was used [31]. DNA preparations were evaluated using electrophoresis in agarose gel and spectrometry (a UV-vis Nabi spectrophotometer, MicroDigital Co., Ltd., Korea) to measure the concentration and purity. The final concentration of samples was adjusted to 30 ng/μl before use in PCR. In genotyping, we used 35 microsatellite markers developed by S. Sato et al. [32] for the genome structure of red clover and placed in the Red Clover Marker Database (http://marker.kazusa.or.jp/Red_clover) and 40 combinations of primers for SRAP markers [22, 33].

A reaction mixture for SSR analysis of 15 μl per sample contained 3 μl of 10× PCR buffer (Taq Turbo Buffer); 0.2 μl of polymerase (Tag DNA Polymerase, 5 units/μl); 0.1 μl of each primer (100 μM); 1 μl 50× dNTP (dNTP mix 10 mM each) and 1 μl DNA sample (30 ng/μl) (all reagents from Evrogen Lab LLC, Russia). Amplification (a T100 thermal cycler, Bio-Rad, USA) by a modified Touchdown PCR program includes an initial denaturation for 3 min at 94 °C followed by 3 stages of sequential reduction in the primer annealing temperature by 2 °C every 3 cycles, the 30 s at 94 °C, 30 s at 68 °C (3 cycles), 30 s at 94 °C, 30 s at 66 °C (3 cycles), and 30 s at 94 °C, 30 s at 64 °C (3 cycles). A chain elongation was run for 30 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C (3 cycles); 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C (3 cycles); 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C (3 cycles). Then, the hybridization temperature reduced to optimal value of 55 °C was maintained for the remaining 30 reaction cycles: 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C. The final stage of chain elongation took 10 min at 72 °C [32, 34]. The reproducibility of the results was checked by repeating the experiments three times, including using bulk DNA preparation from different seedling samples as a matrix.

The resulting PCR products were preliminarily analyzed after electrophoresis in a 1.6% agarose gel (LE2, Lonza, USA) and detection using a GelDoc™ XR+ device (Bio-Rad, USA). The size of the fragments was determined with the molecular marker 100 bp GeneRuler DNA Ladder (Thermo Fisher Scientific, USA) in the ImageLab program (Bio-Rad Lab., Inc., USA). The results of the analysis were summarized in a general table and the presence of variety-specific alleles for each marker was revealed.

To validate alleles unique to the variety, the PCR products were cloned in the pAL2-T vector (ZAO Evrogen, Russia) and sequenced (an ABI PRISM 3130XL genetic analyzer, Applied Biosystems, Inc., USA) using a Big Dye terminator v.3.1 cycle sequencing kit (Applied Biosystems, Inc., USA). Sequencing data were analyzed in the Ugene program [35] and then aligned using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). EST/genomic sequences from the Red Clover Marker Database (http://marker.kazusa.or.jp/Red_clover) were used as a reference.

To increase the accuracy of determining the length of all fragments identified in the DNA profile of candidate varieties for certification, control markers were developed. Based on the electrophoresis in 10% acrylamide gel (BIO-RAD Tetra Cell chamber, USA), the allele sizes were determined vs. the control marker.

Components of the reaction mixtures for most of the SRAP markers corresponded to that proposed by H.B. Rhouma et al. [23], however, for successful amplification of some primer combinations, the reagent content and PCR program had to be optimized. The modified mixture, 20 μl per sample, was 3 μl 10× PCR buffer (Taq Turbo Buffer); 0.5 μl 50× dNTP; 0.1 μl 100 μM primer; 0.4 μl polymerase (Tag DNA Polymerase, 5 units/μl); 1.0 μl DNA sample (30 ng/μl).

Amplification (a T-100 Thermal Cycler, Bio-Rad, USA) program was as follows: 4 min at 94 °C (initial denaturation); 1 min at 94 °C, 1 min at 35 °C,

1 min at 72 °C (10 cycles); 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C (30 cycles); 5 min at 72 °C (final elongation). The size of amplified DNA fragments was measured in horizontal electrophoresis vs. a 1 kb molecular marker (Evrogen Lab LLC, Russia). Target (variety-specific) PCR products were excised from agarose gel and purified on columns with a Cleanup Standard kit (Evrogen Lab LLC, Russia), and then the purified amplicons were cloned in the pAL2-T vector (ZAO Evrogen, Russia) as per the manufacturer's instructions and sequenced. The results of sequence analysis performed in the Ugene and BLAST programs [35] were used to select SRAP markers identifying individual varieties.

Genetic relationships between the studied samples were visualized by Principal Coordinate Analysis (PCoA) and the GenAlEx software package (version 6.2) [36].

Results. The red clover varieties used in this study are listed in Table 1.

1. List of red clover (*Trifolium pratense* L.) varieties involved in the study and the labeling system used

Variety	Originator (country of origin/catalog number in the VIR collection)	Year of entry into the State Register	Marking system
Rannii 2	Williams FRC VIK	1995	SSR, SRAP
Trifon	Rudnitsky FASC North-East	2014	SSR, SRAP
Pamyayi Lisitsyna	Federal Scientific Center for Leguminous and Cereal Crops; Williams FRC VIK; Siberian FSC of Agrobiotechnologies RAS	2005	SSR, SRAP
Pelikan	Federal Scientific Center for Bast Crops	1992	SSR, SRAP
Trio	Williams FRC VIK	1995	SSR, SRAP
Veteran	Williams FRC VIK	2011	SSR, SRAP
Tetraploid VIK	Williams FRC VIK	1973	SSR, SRAP
Mars	Williams FRC VIK	1993	SSR
VIK 771	Williams FRC VIK	2006	SSR
Mateor	Siberian FSC of Agrobiotechnologies RAS; Williams FRC VIK	2007	SSR
Topaz	Williams FRC VIK OOO Agrokompleks-N	2000	SSR
Atlant	FRC Tyumen Scientific Center RAS; Siberian FSC of Agrobiotechnologies RAS	2007	SSR
Altyn	Williams FRC VIK; ONO Morshansk breeding station	1999	SRAP
VIK 84	Williams FRC VIK	1991	SRAP
Vorontzhskii	Williams FRC VIK	2015	SRAP
Marathon	K-48013 (France)	–	SRAP
Freedom	K-51532 (USA)	–	SRAP
Ganymed	K-53648 (Czech Republic)	–	SRAP
Metis	K-53792 (Denmark)	–	SRAP
Nemaro	K-50958 (Germany)	–	SRAP
Norlac	K-51526 (Canada)	–	SRAP

N o t e. Dashes mean that the variety is not included in the State Register of the Russian Federation.

To assess the genetic polymorphism of red clover varieties, a representative sample of genotypes is required (at least 30-50 per sample) in order to accumulate the maximum number of markers characteristic of cross-pollinated populations with a high level of heterogeneity. In such cases, the use of individual genotypes for analysis of the total DNA sample (bulk strategy) can significantly reduce the costs of labor, time and financial resources [37, 38]. Previously, our modifications of the SDS method for DNA extraction [39] ensured good quality of preparations suitable for use in PCR with different types of markers from a common sample formed from highly watered tissue of 30 seedlings of each variety [31].

The main our criteria for selecting microsatellite loci were the number of detected alleles, coverage of all linkage groups by location on chromosomes, and the short length of the resulting PCR fragments (100-300 bp). All SSR markers were di- and tri-nucleotide repeats. To determine the degree of homogeneity, random samples of seedlings of the varieties Ranniy 2, Mars, Tetraploid VIK, Trifon (10 genotypes of each) were analyzed for microsatellite loci RCS1307,

RCS5600 and RCS5208. A fairly high genetic evenness of the material was revealed, which is not typical for cross-pollinated crops with a high level of gametophytic self-incompatibility. Other studies have also reported a significant degree of DNA polymorphism identified within red clover populations with markers of different types (RAPD, AFLP, SSR), from 67.5 to 83.6% [17, 38, 40]. Apparently, our results were influenced by the limited genetic material used in the breeding schemes (the samples included in the analysis had a common originator, the Williams Federal Scientific Center WIK).

Figure 1 shows the general scheme of assessing intervarietal genetic polymorphism to develop genetic passports of red clover.

To study intervarietal genetic variability using 35 pairs of SSR primers and subsequent certification, 476 amplification products were generated for 12 varieties of meadow clover, with an average of 13.6 alleles per locus. These data are comparable to those of other researchers. I. Radinovic et al. [41] in a study of 46 red clover genotypes of different ecological and geographical origins revealed an average of 13.36 alleles per locus for 14 SSR markers. Researchers in Brazil [42] genotyped 57 red clover accessions from North America and Europe and determined that there was an average of 9 alleles for each of the 7 SSR loci tested.

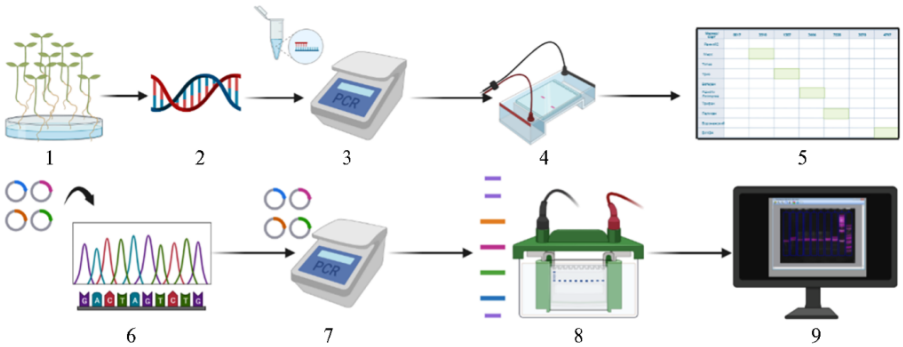

Fig. 1. Scheme of red clover (*Trifolium pratense* L.) DNA fingerprinting for genetic certification: 1-9 — procedures using SSR markers, 1-6 — procedures using SRAP markers; 1 — formation of a total sample of seedlings of 30 genotypes of each variety, 2 — DNA extraction, 3 — amplification in a thermal cycler (Bio-Rad, USA), 4 — preliminary electrophoresis in agarose gel, 5 — selection of variety-specific markers, 6 — Sanger sequencing and analysis of results, 7 — PCR with plasmid DNA, 8 — development of a specific (control) marker and vertical PAGE electrophoresis, 9 — determination of product sizes in comparison with a control marker to compile a molecular genetic formula of the variety (scheme created in the Biorender program (https://app.biorender.com/).

In our work, the size of the amplicons varied from 91 bp (with primer pair RCS5305) to 359 bp (RCS1535). We identified a set of 8 informative microsatellite loci that generated reproducible polymorphic products and were suitable for distinguishing varieties in the sample. Thus, with primers to the RCS1307 marker, unique alleles were discovered that distinguished the varieties Mars, Topaz and Trifon, and the RCS0017 locus distinguished Meteor and Topaz.

In general, based on SSR analysis, a low level of both intravarietal and intervarietal DNA polymorphism was revealed, which is likely due to the origin and selection history of the material. The average value of genetic variability between varieties was 5.3%. This turned out to be significantly lower than the indicators known from the works of other researchers [43, 44].

The use of additional marker systems based on sequence polymorphism in various structural elements of the genome can significantly increase the resolution of the analysis. Using a set of 40 combinations of SRAP primers, we genotyped an expanded collection of red clover samples composed of the Russian varieties that could not be certified using a panel of SSR markers and the foreign

ones involved to compare genetic polymorphism.

The suggested PCR program [23] was ineffective for the SRAP marker combinations F10-R9, Me4-R9, ME1-EM1. By optimizing the components of the reaction mixture, the temperature, time parameters and using 18 combinations of primers, we obtained distinct and reproducible products (812 in total). The largest number of amplified fragments was detected with F13-R9 (62 amplicons), while with ME1-EM2 this figure was minimal (22 amplicons) with an average value of 45.1 for each combination. A total of 85 amplicons turned out to be polymorphic, which amounted to 10.5% of the total value. DNA fragments unique to individual varieties (up to 10) were found using F9-R9, F10-R8 and F10-R9 and only one variety-specific fragment was revealed for F9-R14 and for Me4-EM1.

Based on this information, we assessed the genetic relationships between the studied samples and visualized variations in their distribution on the coordinate plane using PCoA analysis (Fig. 2).

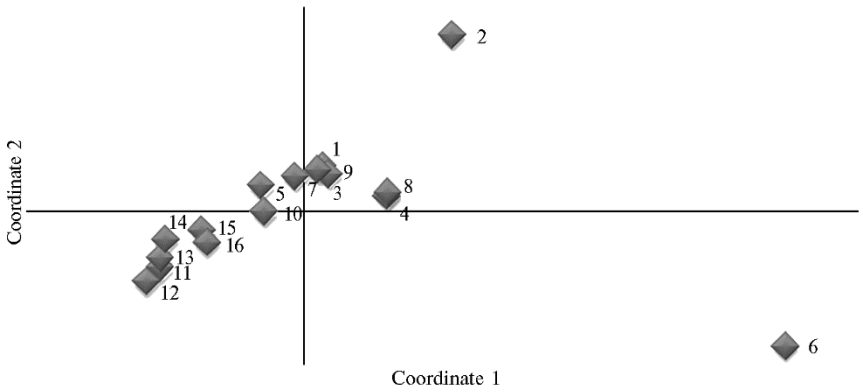

Рис. 2. Clustering of Russian and foreign varieties of meadow clover (Trifolium pratense L.) using the principal coordinate method (PCoA analysis) according to SRAP analysis: 1 — Ranny 2, 2 — Trifon, 3 — Pamyati Lisitsyn, 4 — Pelican, 5 — Trio, 6 — Veteran, 7 — Altyn, 8 — VIK 84, 9 — Tetraploid VIK, 10 — Voronezhsky, 11 — Norlac, 12 — Freedom, 13 — Ganymed, 14 — Metis, 15 — Nemaro, 16 — Marathon.

The clustering showed two relatively compact groups. One group is the varieties of foreign origin, and the other group is ultra-early ripening diploid varieties Trio, Altyn, Voronezhsky, Ranniy 2, tetraploids Pamyati Lisitsyna and Tetraploid VIK originated from Williams Federal Scientific Center VIK. The samples in the first group overlapped significantly, indicating high genetic similarity between them, possibly due to intense gene flow. The most genetically close were Norlac (Canada) and Freedom (USA), as well as the European varieties Ganymed and Metis. At a small genetic distance from them are varieties from Western Europe Nemaro (Germany) and Marathon (France).

The revealed similarity between the varieties of the second cluster was probably a consequence of their genetic relatedness and common breeding history. The tetraploid varieties VIK 84 and Veteran (the Williams Federal Scientific Center VIK) were located on the coordinate plane separately from the other varieties of their group. The Veteran variety stood out among other samples due to its high photosynthetic and symbiotic activity and longevity in agrophytocenoses. The VIK 84 variety has an increased resistance to damage by clover cancer, anthracnose and fusarium, high winter hardiness and drought resistance. It was clustered together with the Pelican variety (Penza Research Institute of Agriculture), bred by the method of a complex hybrid population from several varieties of early ripening clover, including that selected at the Williams Federal Scientific Center VIK. The diploid single-cut variety Trifon (Rudnitsky Research Institute of

North-East Agriculture) generated through the multiple recurrent selection based on the variety Krano (Denmark) is at a considerable distance from the others. The meadow clover variety Trifon is winter-hardy and resistant to sclerotinia and fusarium, since biotypic selection was carried out under an artificial increased load to these pathogens [45]. A high genetic similarity was evidenced by the close location of the Voronezhsky and Trio samples on the graph. The Voronezh variety was created within an ecological selection program at the Voronezh Experimental Station by selection of the best varieties of the Williams All-Russian Research Institute of Feeds under different conditions. The genotyping results suggest that plants of the Trio variety also participated in the formation of the new variety. Currently, the Voronezh variety is considered a source of high winter hardiness, drought resistance and seed productivity. Due to its good adaptive potential, it is widely in demand in the seed market.

To validate the analysis results, Sanger sequencing of the identified unique amplification fragments was performed (21 using SSR markers, 5 using SRAP). Analysis of the obtained data in the BLAST search engine revealed a high degree of sequence homology in comparison with nucleotide sequences from the reference database (Table 2).

2. Alignment of red clover nucleotide sequences obtained by SSR analysis vs. the reference from the database Red Clover Kazusa (http://marker.kazusa.or.jp/Red_clover)

Variety	Primer	Clone number/product length, bp.	Match with reference	
			identity	indels
Mars	RCS1307	1.3/155	149/155 (96 %)	1/155 (0 %)
	RCS3666	1.4/216	210/216 (97 %)	4/216 (1 %)
		1.5/226	28/28 (100 %)[a]	0/28 (0 %)[a]
			179/182 (98 %)[a]	0/182 (0 %)[a]
Topas	RCS1307	2.2/151	140/161 (87 %)	16/161 (9 %)
	RCS0017	9.1/150	100/101 (99 %)[a]	0/101 (0 %)[a]
			24/24 (100 %)[a]	0/24 (0 %)[a]
		9.2/144	100/101 (99 %)[a]	0/101 (0 %)[a]
			44/44(100 %)[a]	0/44 (0 %)[a]
Trifon	RCS1307	3.3/152	149/155 (96 %)	3/155 (1 %)
		3.5/157	152/157 (97 %)	2/157 (1 %)
Pamyati Lisitsyna	RCS1307	4.5/169	154/169 (91 %)	14/169 (8 %)
Atlant	RCS1307	5.5/141	136/155 (88 %)	15/155 (9 %)
		5.6/137	135/155 (87 %)	19/155(12 %)
	RCS2199	7.4/198	190/198 (96 %)	8/198 (4 %)
		7.6/200	190/200 (95 %)	10/200 (5 %)
	RCS4797	8.2/175	36/36 (100 %)[a]	0/36 (0 %)[a]
			141/143 (99 %)[a]	1/143 (0 %)[a]
		8.3/158	116/116 (100 %)[a]	0/116 (0 %)[a]
			44/44 (100 %)[a]	0/44 (0 %)[a]
Tetraploid VIK	RCS1307	6.2/158	152/157 (97 %)	2/157 (1 %)
		6.6/155	151/155 (97 %)	1/155 (0 %)
	RCS5781	9.4/205	187/208 (90 %)	12/208 (5 %)
		9.6/201	184/203 (91 %)	6/203 (2 %)
	RCS4532	10.2/235	234/237 (99 %)	2/237 (0 %)
Meteor	RCS0017	8.4/166	164/166 (99 %)	2/166 (1 %)
		8.6/172	164/172 (95 %)	8/172 (4 %)
	RCS3070	4.1/333	136/137 (99 %)[a]	1/137 (0 %)[a]
			123/125 (98 %)[a]	0/125 (0 %)[a]
		4.2/323	136/137 (99 %)[a]	1/137 (0 %)[a]
			125/125 (100 %)[a]	0/125 (0 %)[a]
	RCS3510	5.5/254	252/271 (93 %)	17/271 (6 %)
		5.6/257	255/271 (94 %)	14/271 (5 %)
Trio	RCS0017	10.5/164	164/164 (100 %)	0/164 (0 %)
Veteran	RCS0017	11.2/164	149/164 (91 %)	0/164 (0 %)
	RCS4797	6.2/180	180/190 (95 %)	10/190 (5 %)
VIK 77	RCS3510	2.1/249	243/271 (90 %)	22/271 (8 %)
	RCS7228	3.5/182	181/185 (98 %)	3/185 (1 %)
		3.6/181	180/185 (97 %)	4/185 (2 %)

N o t e. [a] — nucleotide sequences that have a large insertion or deletion compared to the database reference.

Figure 3 shows, as an example, the results of alignment of the nucleotide

sequence of a PCR fragment obtained with a combination of SRAP markers ME1-EM1.

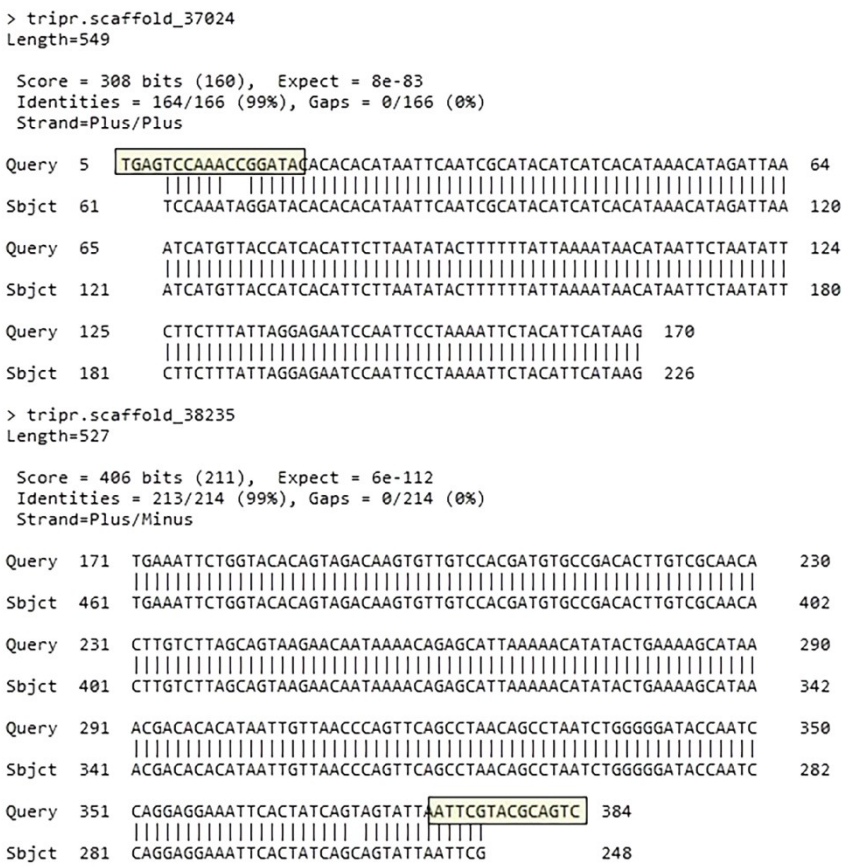

FIg. 3. Sequence alignment of a unique PCR fragment for red clover (*Trifolium pratense* L.) variety Trio amplified with the SRAP combination ME1-EM1 (query) vs. the *T. pratense* genome (sbjct). Primer sequences are marked.

Annotated nucleotide sequences identifying the varieties Trifon, Mars, Topaz, Atlant, Tetraploid VIK, Meteor, VIK 77 are included in the international database of genetic resources GenBank NCBI (https://www.ncbi.nlm.nih.gov/) under individual numbers MW520170; OP493602 and OP493613; OP493603 and OP493612; OP493606 and OP493607; OP493608 and OP493609; OP493610 and OP493611; OP546652 and OP546653. This data can be used for basic and applied research on red clover, including development of novel technologies. To increase the accuracy of determining the sizes of alleles that are not unique to the variety in the sample under study, but were identified as part of the DNA profile (8 loci), we created specific control markers which are PCR products obtained using plasmids.

Commercial standard markers were not suitable, since the large range in the size of divisions of the marker ladder did not allow the establishment of insignificant differences of several nucleotides between amplicons. In our study, we compared the size of alleles in the DNA profiles of the studied samples with the control marker after electrophoresis in 10% PAGE (Fig. 4).

For the studied collection, identification DNA markers were determined: eight pairs of SSR loci and seven SRAP combinations, which revealed the uniqueness of the allelic composition of at least two Russian varieties (Table 3).

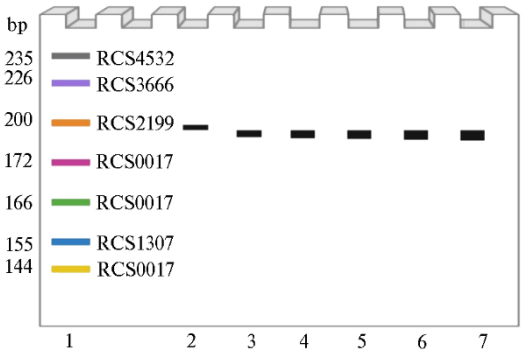

Fig. 4. Electrophoresis with a control marker to verify the allele sizes in DNA profiles of red clover (*Trifolium pratense* L.) varieties certified based on SSR markers: 1 — control marker composed of amplicons produced from clones 9.2 (144 bp), 1.3 (155 bp), 8.4 (166 bp), 8.6 (172 bp), 7.6 (200 bp), 1.5 (226 bp), 10.2 (235 bp), 2 — amplicon from clone 7.4 of the certified Atlant variety (SSR marker RCS 2199, 198 bp), 3-7 — PCR products with genomic DNA of the varieties Mars, VIK 77, Meteor, Topaz, Tetraploid VIK (SSR marker RCS 2199).

3. SSR- and SRAP markers for DNA identification of red clover (*Trifolium pratense* L.) Russian varieties

Marker (reference)	Nucleotide sequence 5′-3′	PCR fragment size, bp	Marker code
	SSR markers		
RCS3666 (32)	CATGGCTGCCTGAGGTTAAT/ TCTGTTTCTTGTCTCGGCCT	216-230	A
RCS3510 (32)	TTCACAAGTTTTTCGGGTGA/ GCCAAAGGGAAGGTTCAATC	249-257	B
RCS7228 (32)	TCAACAATGTGGCTTCTCCT/ AAGGTTCCCAACCCAATTTC	179-187	C
RCS4797 (32)	GCCCGTCTACCTTTTGTTCA/ GCGCCATAAGCAACTGTGTA	155-180	D
RCS2199 (32)	AAAAAGAAAGCGTTAAAGGGG/ GCATTGCCTTTTGCTTCTTC	178-200	E
RCS5781 (32)	GATCGATCCGAAAACCAAAA/ TGCCATCGAGAGAGAAGGTT	165-210	F
RCS1307 (32)	CCCTTCTAGCCTAGCAACCA/ GCGGAAAAGATTCAGCCTAA	137-158	G
RCS0017 (32)	GCGGAAAAGATTCAGCCTAA/ GGACTTCTCTGATATTGAACTGAATG	144-177	H
	SRAP markers		
F10-R9 (23)	GTAGCACAAGCCGGAAG/ GACTGCGTACGAATTTCA	116-1442	A
Me4-R9 (22)	CGAATCTTAGCCGGAAT/ GACTGCGTACGAATTTCA	165-519	B
ME1-EM1 (22)	TGAGTCCAAACCGGATA/ GACTGCGTACGAATTAAT	125-700	C
F13-Em2 (23)	CGAATCTTAGCCGGCAC/ GACTGCGTACGAATTCGG	234-763	D
F13-R9 (23)	CGAATCTTAGCCGGCAC/ GACTGCGTACGAATTTCA	115-1315	E
F11-R7 (23)	CGAATCTTAGCCGGATA/ GACTGCGTACGAATTGAG	232-1762	F
Me4-EM5 (22, 23)	CGAATCTTAGCCGGAAT/ GACTGCGTACGAATTAAC	139-748	G

The information obtained by sequencing DNA fragments unique to the variety and verifying the sizes of the remaining alleles in its DNA profile (compared to the control marker) was used to compile an individual molecular genetic formula. Capital letters of the Latin alphabet in the formulas denote the studied loci (marker code, see Table 3), and the lower digital index indicates the size of the identified alleles in nucleotide pairs (Table 4).

Molecular formulas served as the basis for creating genetic passports of red clover using two marking systems (six with SSR loci, four with SRAP markers). Additionally, the passport included information on the origin of the variety, regions of cultivation, main morphobiological characteristics and economically valuable

properties (Fig. 5).

4. Molecular genetic formulas of the studied red clover (*Trifolium pratense* L.) Russian varieties based on SSR and SRAP markers

Variety	Formula
	SSR marking
MArs	**A216/226**B252/252C180/180D155/169E189/194F192/208**G155/155**H165/177
VIK 77	A219/219**B249/249C182/182**D165/174E180/180F193/207G156/156H144/144
Meteor	A219/219B**254/257**C187/187D170/179E178/189F165/209G142/142**H166/172**
Topas	A220/230B252/252C179/179 D157/164E181/190F194/209**G151/151**H144/**150**
Atlant	A221/221B252/252C179/184**D158/175E198/200**F195/210G137/141H145/145
Tetraploid VIK	A218/230B252/252C179/179D170/180E181/193**F201/205**G155/**158**H144/144
	SRAP marking
VIK 84	A331B286**C446**,356D700,447,290,234E1070,565,180,115F1055,685,534,313,232G748,638,523,359
Trio	A400,305B286**C393**D700,234E1070, 300,115F685,313,232G609,359,139
Voronezhslky	A900,800,585,400,317,168**B129**D700,270,234E1070,300,115F800,685,534,313,232G748,638,204,139
Pelikan	**A338**,175B286C356,234D700,234E1070,115F800,685,313,232G748,638,359

N o t e. Letters of the Latin alphabet are marker code, digital index means size of amplification fragments (bp); Unique products confirmed by sequencing are highlighted in bold.

Fig. 5. Molecular genetic passports of red clover (*Trifolium pratense* L.) variety Mars based on the SSR marker system (left) **and variety Trio based on the SRAP marker system** (right)**.**

Thus, here we assessed the genetic polymorphism of meadow clover varieties for SSR and SRAP markers. The genotyping technologies have been optimized with regard of the properties of the crop studied, including modified DNA extraction from seedlings and amplification conditions for SRAP markers. For each marking system, unique DNA profiles and markers for genetic identification were obtained. The sizes of variety-specific DNA fragments identifying seven commercially important Russian varieties of red clover were confirmed by sequencing. Annotated nucleotide sequences are deposited in the GeneBank NCBI. Molecular genetic formulas of varieties have been compiled that reflect the allelic composition of microsatellite loci and polymorphism of intron-exon regions of the genome. These data formed the basis for the development of 10 reference genetic passports (six for SSR loci, four for SRAP markers) for Russian red clover varieties. Using a reference passport, you can identify a variety, analyze its homogeneity and genetic purity, and establish the compliance of an anonymous sample with a known standard. The proposed method for certification of red clover went through a triple control system, including 3-fold repetition of experiments, cloning and sequencing of the main fragments, and the use of a control marker to increase the accuracy of determining the lengths of amplified products. These techniques provide accurate allelic profiles of varieties, reduce the likelihood of genotyping errors, serve as confirmation of the reliability of the results obtained, and can be used in the

development of genetic passports of various forage crops.

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COMPARATIVE CHARACTERIZATION AND ADAPTIVE MECHANISMS OF SALT TOLERANCE OF DIFFERENT WHEAT GENOTYPES

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Abstract

The study of adaptive mechanisms of salt tolerance for the identification and selection of resistant wheat genotypes remains an urgent task since the area of lands with high salinity is constantly increasing worldwide. This work was focused on the processes of accumulation and excretion of toxic ions from the roots and leaves of different wheat genotypes and the effect of these ions on the state of plant tissues. It was shown that in the studied varieties of durum wheat, with an increase in salinity, the size of the root system decreased and, as a result, the absorption of toxic Na^+ ions decreased. In soft wheat, ionic conductivity increased and the excretion of Na^+ ions increased too. We compared the manifestations of salt stress caused by high concentrations of NaCl, and the mechanisms of salt tolerance and adaptation to its toxic effect in varieties of different types of wheat in the conditions of the Orenburg region in the field. The initial assessment of salt tolerance of wheat varieties was carried out according to the degree of growth inhibition by sodium chloride. Based on the initial assessment, wheat varieties differing in salt tolerance were selected, i.e., two durum wheat (*Triticum durum* Desf.) varieties Zolotaya and Orenburgskaya 10 and two soft wheat (*Triticum aestivum* L.) varieties Ulyanovskaia 105 and Orenburgskaya 22. The final assessment of the salt tolerance of the selected varieties was carried out in 2022, growing plants in small-scale (1.8 m^2 plots) field trials in 3 repetitions of each variant. To create salt stress, sodium chloride was applied for root watering after emergence and before the tillering phase in the form of a solution (200 mM NaCl). Plants grown without the addition of salts served as control. The adaptive mechanisms of resistance of different wheat genotypes to sodium chloride were studied using biochemical, molecular genetic and light-optical methods of analysis. The defense mechanisms of plants against the action of salt stress include blocking and excretion of Na^+ and Cl^- from the cell cytoplasm, activation of the antioxidant defense system, and an increase in plant tolerance due to various mechanisms of regulation of gene activity. Our results show that the Ulyanovska 105 and Orenburgskaya 22 wheat cultivars retain the selectivity of K+ ions with respect to Na^+ ions in the roots and maintain a higher K^+/Na^+ ratio (4.12 and 4.18, respectively) under stress compared to with varieties Zolotaya and Orenburgskaya 10 (1.26 and 3.75, respectively). The regulation of ion flows is provided by ion transporters. Increased activity of the genes of two classes of *HKT* transporters in the Orenburgskaya 22 variety contributes to a greater excretion of Cl^- and Na^+, and, conversely, in the Zolotaya variety, accumulation of toxic ions occurred, which leads to a decrease in the content of chlorophylls a and b (Chl a and Chl b). The lower Chl a/Chl b ratio in Orenburgskaya 22 than in Zolotaya 22 (2.03 vs. 2.43) indicates a high content of Chl b. This expands the light absorption spectrum, which contributes to an increase in photosynthesis. The difference in the accumulation of reactive oxygen species (ROS) indicates the initiation of different mechanisms of antioxidant defense in different wheat genotypes. Accumulation of ROS products in the roots of Ulyanovskaia 105 and Orenburgskaya 22 cultivars during salinization occurs more intensively in the zones of the cap and

meristem, which is 1.3 times lower compared to the Zolotaya and Orenburgskaya 10 cultivars. In these wheat varieties, ROS products accumulate in all the studied zones, however, in the area of the cap is less than in the other zones. NaCl-sensitive wheat genotypes showed damage in root cells, while damage was minimal in resistant wheat genotypes. Salt stress in sensitive wheat varieties can lead to programmed cell death. The degradation of nucleic acids is a marker of the plant death. According to the degree of DNA degradation, the Zolotaya variety turned out to be the most unstable. The integrated approach used by us in this study made it possible to identify various mechanisms of resistance to salt stress in wheat genotypes. The obtained results can be in demand by breeders and specialists of the agrarian complex.

Keywords: soft wheat, durum wheat, salt stress, ion transporters, chlorophyll, reactive oxygen species, nuclease activity, DNA degradation, programmed cell death

Plants are very plastic and quickly adapt to changing unfavorable environmental conditions. Depending on the mechanisms and speed of adaptation, plants can be divided into tolerant and sensitive to abiotic stress. Sodium chloride salinity is one of the most common and studied abiotic factors [1, 2]. Soil salinization is largely associated with human economic activity and the agricultural practices used (irrigation, use of fertilizers). This is becoming one of the major threats to sustainable agriculture, causing a decrease in plant productivity due to disturbances at the physiological, biochemical and molecular levels. The areas of saline agricultural land tend to constantly increase as a result of secondary salinization. The total area of saline soils in Russia is 66.4 million hectares, or 3.9% of the total land fund [3]. The Orenburg region belongs to an area with a high percentage of saline soils (5.3% of the area of agricultural land). Currently, soil salinity is considered to be the main limiting factor that negatively affects the growth and development of wheat, the most important agricultural crop [4]. High concentrations of Na^+ ions are toxic to cellular metabolism and can inhibit the activity of many important enzymes, cell division and reproduction, cause membrane disorganization and osmotic imbalance, which can ultimately lead to growth inhibition and even plant death [1, 2, 5].

Understanding the mechanisms of responses to abiotic stress is important to ensure stable yields. Plants have developed the ability to withstand the effects of stressors at the cellular and tissue levels. Plant resistance to salinity is due to the action of specific and (or) nonspecific mechanisms that support stable metabolism, growth and development in ontogenesis and are associated with sensitivity to one or more types of stress factors, including the osmotic and oxidative stress effects of sodium chloride [6]. These mechanisms include changes in stomatal conductance, hormonal balance, antioxidant defense system, osmotic regulation and removal of toxins, Na^+ and Cl^- from vacuoles, blocking Na^+ transport into the cell, and exclusion of Na^+ from the transpiration stream [7, 8].

The complex control and response to abiotic and biotic stresses is achieved through the coordinated action of multiple genes that are directly or indirectly involved in defense responses in plants [9]. An increase in the activity of certain genes encoding osmolytes, ion channel components, receptors, and some other regulatory signaling factors or enzymes can increase plant tolerance to salt action [10, 11]. Normally, in soil, the water potential in root cells is lower than in the external environment, and the influx of water occurs with the participation of water channel proteins, the so-called aquaporins [12]. In a saline environment, the difference in water potential between soil and root cells is much less than under normal conditions, or even greater, resulting in decreased water uptake or loss of water [13]. As a result, growth inhibition occurs, which ultimately leads to severe damage to plant tissue.

At the cellular level, salt tolerance mechanisms are aimed at reducing the accumulation of Na^+ in the cytoplasm by limiting the penetration of Na^+ into cells, ensuring active transport of Na^+ from cells and movement into cellular compartments, the vacuoles [14]. K^+ ions are preferred for uptake by roots from

the soil, and most plants exhibit a high degree of discrimination between K+ and Na^+. High-affinity K^+ transporters (HKTs) have been reported to be active at the level of the plasma membrane and function as Na^+/K^+ symporters as well as selective Na^+ uniporters [15]. HKTs may have two main functions, i.e., the extraction of Na^+ from soil solution to reduce K^+ demand, and the reduction of Na^+ accumulation in the leaf by removing Na^+ from the xylem and moving Na^+ into the phloem.

For cells, toxicity caused by a high content of Na^+ ions is the predominant ion toxicity; it leads to inhibition of various processes, such as K^+ uptake [9, 10], inactivation of vital enzymes [15], and inhibition of photosynthesis [16]. In the cell, photosynthetic processes are most sensitive to the toxic effects of Na^+, and their disruption is directly related to a reduction in carbon fixation and plant biomass production [16].

As a result of the influence of various unfavorable biotic and abiotic factors (hypoxia, drought, salinity, physical damage at the wound site and infection by pathogens), the content of reactive oxygen species (ROS) in plant tissues increases, but their excess can also lead to damage by oxidative stress [17, 18]. The production and accumulation of ROS occur in the plant during redox metabolism [19, 20]. The plant cell is protected from the toxic effects of ROS by the antioxidant system which controls the ROS concentration [21]. ROS can also act as important signaling molecules that regulate plant defense mechanisms, growth, development, and response to external stress [22].

Many studies have been devoted to the study of chlorophylls, in particular to determining their quantity and ratio, due to the important role of this pigment in plant physiology. Chlorophylls are involved in the absorption and transmission of light energy and the transfer of electrons during photosynthesis. Chlorophyll content can change in response to biotic and abiotic stresses, such as pathogen infection [23] or light stress [24, 25]. Thus, chlorophyll quantification provides important information about the influence of the environment on plant growth [26].

The Orenburg region is located mainly in two soil zones (chernozem and chestnut soils), gray forest soils make up 0.4%. The areas subject to salinity in the region amount, according to various estimates, from 13.9 to 15.0% of the soil fund. In total, solonetz soils here occupy 1971.8 thousand hectares, including 541.5 thousand hectares of arable land (27.5%) [27). Under these conditions, the basis for ensuring stable wheat yields remains the assessment of the ability of varieties to adapt to salt stress conditions, the study of its manifestations in plants and the identification of mechanisms that can effectively resist it.

In this work, the main attention was paid to the accumulation and removal of toxic ions from the roots and leaves of different wheat genotypes and the influence of these ions on plant tissues. We showed that in the studied varieties of durum wheat, with increasing salinity, the size of the root system decreased, and as a result, the absorption of toxic Na^+ ions decreased. In soft wheat, ionic conductivity and the excretion of Na^+ ions increased. It was also noted that in durum wheat varieties, the aboveground part of the plants is more adapted to salinity than the underground part.

The purpose of the work is to compare the manifestations of salt stress caused by high concentrations of NaCl, and the mechanisms of salt tolerance and adaptation to its toxic effects in different wheat varieties under the conditions of the Orenburg region.

Materials and methods. The varieties of soft wheat (*Triticum aestivum* L.) Ulyanovskaya 105 and Orenburgskaya 22 and durum wheat (*Triticum durum* Desf.) Zolotaya and Orenburgskaya 10 were obtained from the collection of the

Federal Scientific Center for Biological Systems and Agrotechnologies RAS (Orenburg, Russia).

Plot tests were carried out in the central zone of the Orenburg Province (experimental field of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS, 1.8 m^2 plots, 3 plots per treatment). The soil of the plots is southern carbonate chernozem, loamy solonetz. The humus horizon is 45-55 cm, the pH of the soil solution is close to neutral (6.8-7.0). Humus content in the arable layer is 3.5-4.2%, total nitrogen from 0.2 to 0.6%, available phosphorus 1.5-2.5 mg/100 g of soil, exchangeable potassium 30-40 mg/100 g soil. The crop predecessor was fallowing soil. The varieties were sowed on May 3, 2022 using a CH-16 seeder (Russia) at a viable seed rate of 4.5 million/ha. The effect of salt exposure on wheat was studied by root watering of seedlings with a solution of sodium chloride (200 mM, 100 ml of solution per seedling) from the stage of full germination to the tillering (3 times within 14 days). Control wheat samples were watered. During this period, there was an excess of the average daily air temperature with insufficient precipitation (33% of the norm). The content of productive moisture in the experimental plot at the beginning of the growing season was sufficient and remained without significant changes until the tillering stage. Thirty plants in 3 replicates were collected at tillering stage for morphometric, biochemical, and cytological analyses.

The proportion of dead cells in shoots was determined during the tillering stage by intravital staining with a 0.5% aqueous solution of trypan blue for 5 min. The samples were washed 3 times with running water, and the staining was visualized by light microscopy (Olympus BX51 microscope, Olympus Corporation, Japan; ×10 objective; images were made with a Color View II digital camera, Germany).

For intravital visualization of ROS in root cells at the tillering stage, an aqueous solution of Carboxy-H2DFFDA (Thermo Fisher Scientific, USA) was used according to protocol [28]. The preparations were analyzed (an Olympus BX51 fluorescence microscope, ×10 objective, λ = 490 nm). Fluorescence intensity was measured using a Color View II digital camera (Cell program, Soft Imaging System, Germany). The images were analyzed and processed in the ImageJ program (https://imagej.softonic.ru).

Chlorophyll was extracted from crushed aboveground biomass (shoot samples, 500 mg each) with 80% acetone for 30 min. The optical density of the solution was measured (a SmartSpec Plus spectrophotometer, Bio-Rad, USA). The content of chlorophylls was quantified using formulas [29]:

$$\text{Chl a, mg/ml} = 11.63 \times A665 - 2.39 \times A649;$$

$$\text{Chl b, mg/ml} = 20.61 \times A_{649} - 5.18 \times A_{665}.$$

Total protein from shoots and roots (1 g of test material each) was extracted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.8 M sucrose and 0.35 M NaCl (for 1 h at 25 °C). The amount of protein was determined using the Bradford method. The OD_{595} of the extract was measured and the concentration was calculated by a calibration curve constructed with bovine serum albumin (Serva, USA).

Nuclease activity was determined spectrophotometrically (SmartSpec Plus spectrophotometer, Bio-Rad, USA) at λ = 260 nm, using a solution of thymic DNA (Reakhim, Russia) (A_{260} = 1.0 in 0.5 M acetate) as a substrate buffer, pH 5.0). The reaction was carried out at 37 °C for 2 hours. A unit of activity was taken to be the amount of enzyme that caused an increase in the optical density of the solution by 0.01 units [30].

DNA was isolated from plant roots at the tillering stage (2 g each) according to the protocol of OOO Syntol (Russia) and visualized using electrophoresis in a 1.2% agarose gel.

RNA was isolated separately from shoots and roots of wheat seedlings at the tillering stage (100 mg each) using the RNA-Extran reagent kit as per the manufacturer's protocol (OOO Syntol, Russia). The RNA concentration in the resulting preparations was measured (an IMPLEN nanophotometer, IMPLEN, USA).

cDNA was obtained by standard methods using a set of reagents for reverse transcription according to the protocol (OOO Synthol, Russia). Information on the primary structure of the *HKT* genes was taken from the NCBI database (http://www.ncbi.nlm.nih.gov). Primers for the transcripts of these genes were selected with the NCBI Primer-BLAST online service (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized at OOO Synthol LLC (Russia), the TaHKT1;4 is 5′-ATT CAG GCA ACA CCT AAT CAT GC-3′ and 5′-GCA TCA CAA GAA TGA GGA TGA GC-3′; TaHKT2;1 is 5′-TAT GTG ATG AGT CGC AGC TTG AA -3′ and 5′-GCA ACA AGA GGC CTG AAT TCT TT-3′.

Real-time PCR (RT-PCR) was performed in a CFX 96 Real-Time System thermal cycler (Bio-Rad, USA). The RT-PCR mode was the same for all samples: 95 °C, 5 min for polymerase activation, then 45 cycles 94 °C, 30 s, 58 °C, 30 s, 72 °C, 30 s. RT-PCR was performed in 3 replicates for each sample and in 3 analytical replicates. The level of relative gene expression was calculated using a calibration curve constructed with PCR products that were obtained with primers to the *GaPDh* gene encoding the glyceraldehyde-3-phosphate dehydrogenase protein, taken as a reference (5′-GCC CCA GAG GAG TGT TCA AA-3′ and 5′-AAA ATG TGA GCC GCT AAG CC-3′).

The efficiency of RT-PCR was calculated by the formula:

$$E, \% = (10^{-1/s} - 1) - 100,$$

where s is the slope of the dependence of the decimal logarithm of Ct values on cDNA concentration. The efficiency of RT-PCR with primers for the studied genes was 95-96%.

To analyze the ion content in the leaves and roots of wheat at the tillering stage, cell walls were destroyed (100-300 mg in 25 ml of deionized water) (ultrasonic disintegrator, NPP Sapphire, Russia; 35 kHz for 30 min at 40 °C). The resulting suspension was filtered through a 0.45 μm Millipore membrane (Millipore, USA). Samples were analyzed using an ITAN ionometer (NPP Tomanalit, Russia). The ion concentrations (mg/l) in the samples were determined by a calibration curve. The electrolyte concentrations in the samples was measured by the electrical conductivity of the solution (an Expert-002 conductometer, Econix LLC, Russia) [31].

The mean values of the parameters (M) and their standard deviations (±SD) were calculated. Statistical processing was carried out using Statistica 6.0 (StatSoft, Inc., USA) and STATAN (Statanly Technologies, Russia) programs. The significance of differences was determined by Student's t-test at $p < 0.05$.

Results. As the NaCl content in the soil increased, the total biomass of 14-day plants of Orenburgskaya 22 and Ulyanovskaya 105 varieties decreased (up to 20%), while in the Zolotaya variety it increased (up to 30%) (Table 1; data for 30 plants are shown in 3 replicates in each variant at the tillering stage).

Sodium chloride had different effects on root length and shoot height in different wheat genotypes. The varieties of soft wheat in terms of shoot height showed greater tolerance to salinity, while the varieties of durum wheat turned out to be more sensitive to sodium chloride, especially the Zolotaya variety (a decrease in shoot height by 25% compared to varieties of soft wheat, for which this value was 15%). The root system of the Zolotaya variety also turned out to be the most sensitive (reduction in root length by 30%) compared to other studied varieties of both durum and soft wheat, in which we recorded a decrease in root length by 15-20% (see Table 1).

1. Morphometric parameters of plant at the tillering stage (14 days) **of the studied wheat varieties under chloride salinity** (200 mM NaCl) (N = 3, n = 30, M±SD; experimental field of the FSC RAS, Orenburg Province, 2022)

Variety	Option	Total raw biomass, g	Stem height, cm	Root length, cm
Soft wheat (*Triticum aestivum* L.)				
Ul'yanovskaya 105	Control	6,61±0,33[a]	69,6±3,48[a]	15,6±0,78[a]
	NaCl	5,58±0,28[b]	57,6±2,88[d]	13,4±0,67[c]
Orenburgskaya 22	Control	5,42±0,27[c]	61,5±3,07[c]	14,5±0,72[b]
	NaCl	4,54±0,23[e]	51,4±2,57[f]	11,1±0,55[d]
Hard wheat (*Triticum durum* Desf.)				
Zolotaya	Control	4,89±0,24[d]	64,8±3,24[b]	13,1±0,65[c]
	NaCl	3,67±0,18[f]	49,1±2,45[g]	9,2±0,46[f]
Orenburgskaya 10	Control	5,50±0,27[b]	61,5±3,07[c]	13,2±0,66[c]
	NaCl	4,45±0,22[e]	54,2±2,71[e]	10,6±0,53[e]

[a-g] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different by Student's t-test at $p < 0.05$ (letter designations are assigned in descending order of the M value)

K^+ ions are necessary for the regulation of water-salt balance in plants [32]. In all wheat varieties presented in Table 2, the K^+ content in leaf tissues was more than 2 times higher than this indicator in roots. In the studied varieties, the content of K^+ ions in the leaf increased under salt stress. The exception was the variety Ulyanovskaya 105 in which we noted a decrease in the amount of K^+ in the leaf. In the root, under salt stress, an increase in the content of K^+ ions was observed only in the varieties Orenburgskaya 22 and Orenburgskaya 10.

Our data demonstrate a limited supply of Na^+ to wheat roots, followed by transport of the ion to the shoots and its removal through the leaves to maintain acceptable Na^+ levels. These results are consistent with data previously obtained by other investigators [33]. Because the roots are in direct contact with the soil and absorb nutrients, higher accumulation of Na^+ occurred in the roots compared to the control.

A comparison of the Na^+ distribution between leaf and root tissues under salt stress revealed the accumulation of Na^+ in higher concentrations in the leaf (except for the Zolotaya variety) (see Table 2) which indicates that the leaf serves as the main Na^+ accumulator. Under salt stress, a significant amount of Na^+ was transported from the leaf to the root. In the salinity-sensitive variety Zolotaya, there was probably no outflow of excess Na^+ from the leaves and no restriction on the entry of Na^+ into the root.

Our results show that common wheat genotypes retained selectivity for K^+ over Na^+ and maintained a higher K^+/Na^+ ratio under salt stress, while durum wheat genotypes did not exhibit this ability, especially the salinity-sensitive variety Zolotaya. With an increase in the concentration of Cl^- in the soil solution, the accumulation of Cl^- in the roots of durum wheat varieties occurred 2 times more intensely; smaller amounts were noted in soft wheat, especially in the Orenburgskaya 22 variety. The Zolotaya variety accumulated most Cl^- ions in the leaves. According to our data, the Zolotaya variety should be considered sensitive to the action of Cl^- ions. We believe that this variety has impaired mechanisms for the removal of ions from the xylem into root vacuoles [33], as well as the outflow of excess Na^+ and Cl^- ions from the leaves [33].

The electrical conductivity value is proportional to the concentration of electrolytes and characterizes the accumulation of the sum of ions ($K^+/Na^+/Cl^-$) in plant tissues. With high electrical conductivity, a generally larger number of ions accumulate in tissues. For example, in the Ulyanovskaya 105 variety, the electrical conductivity of leaf tissues was the highest and was determined by the highest concentration of K+ ions. The same was noted in the roots. In the Zolotaya variety, under salt stress, electrical conductivity in the leaves and roots increased, but this was due to the accumulation of Cl- ions in these organs.

2. Ion concentration and electrical conductivity in leaves and roots of the studied wheat varieties at the tillering stage (14 days) **under chloride salinity** (200 mM NaCl) ($N = 3$, $n = 5$, M±SD; experimental field of the FSC RAS, Orenburg Province, 2022)

Variety, plan organ	K^+, mg/g sample	Na^+, mg/g sample	K^+/Na^+	Cl^-, mg/g sample	Electrical conductivity, μSm
Soft wheat (*Triticum aestivum* L.)					
Ul'yanovskaya 105:					
leaf					
control	15.37±0.77[a]	1.65±0.08[e]	9.31[b]	8.38±0.42[f]	813.38±40.67[b]
NaCl	11.85±0.59[d]	2.03±0.10[c]	5.84[f]	11.96±0.60[c]	660.50±33.02[d]
root					
control	6.17±0.31[f]	0.93±0.05[i]	6.63[d]	5.08±0.25[j]	398.73±19.94[h]
NaCl	5.36±0.27[g]	1.30±0.06[f]	4.12[k]	9.24±0.46[e]	384.75±19.20[h]
Orenburgskaya 22:					
leaf					
control	12.03±0.60[c]	1.26±0.06[g]	9.55[a]	9.11±0.45[e]	802.80±40.14[b]
NaCl	14.30±0.71[b]	1.98±0.10[c]	7.22[c]	13.35±0.67[b]	797.44±39.87[c]
root					
control	5.38±0.27[g]	1.03±0.05[h]	5.22[h]	5.57±0.28[i]	316.56±15.83[i]
NaCl	6.64±0.33[e]	1.59±0.08[e]	4.18[l]	7.92±0.40[g]	300.35±15.02[i]
Hard wheat (*Triticum durum* Desf.)					
Zolotaya:					
leaf					
control	9.86±0.49[e]	1.36±0.07[f]	7.25[c]	9.18±0.46[e]	562.34±28.12[e]
NaCl	12.08±0.60[c]	1.91±0.09[c]	6.32[e]	19.63±0.98[a]	949.33±47.47[a]
root					
control	4.82±0.24[h]	1.20±0.06[g]	4.02·	5.06±0.25[j]	386.05±19.30[h]
NaCl	4.64±0.23[i]	3.67±0.18[a]	1.26[o]	10.28±0.51[d]	468.56±23.43[f]
Orenburgskaya 10:					
leaf					
control	11.37±0.57[d]	2.27±0.11[b]	5.01[i]	8.50±0.42[f]	765.93±38.30[c]
NaCl	12.48±0.62[c]	2.22±0.11[b]	5.62[g]	13.92±0.70[b]	825.31±41.26[b]
root					
control	4.81±0.24[h]	1.01±0.05[h]	4.76[j]	3.71±0.18[k]	308.55±15.43[i]
NaCl	6.53±0.33[e]	1.74±0.09[d]	3.75[n]	7.44±0.37[h]	420.20±21.01[g]

[a–o] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different by Student's t-test at $p < 0.05$ (letter designations are assigned in descending order of the M value).

In general, K^+ is preferred for root uptake from the soil, and most plants exhibit a high degree of K^+/Na^+ discrimination for their uptake. The genes of the *HKT* family (K^+ and Na^+ ion transporters in plants) are divided into two subfamilies. The *HKT1* subfamily is found in all higher plants. The genes of this subfamily encode selective ion transporters; the genes of the *HKT2* subfamily are transporters of both ions (K^+ and Na^+) [11, 14].

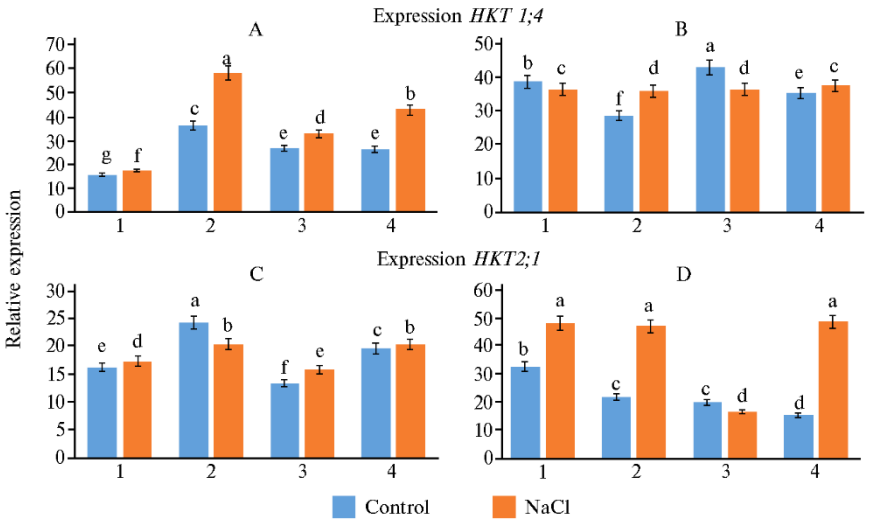

Fig. 1. Expression of the K^+ and Na^+ ion transporter genes *HKT 1;4* and *HKT2;1* in leaves (A and C) **and roots** (B and D) **of the studied varieties of common wheat *Triticum aestivum* L.** (1 — Ulyanovskaya 105, 2 — Orenburgskaya 22) **and durum wheat *Triticum durum* Desf.** (3 — Zolotaya, 4 — Orenburgskaya 10) **at the tillering stage** (14 days) **with chloride salinity** (200 мМ NaCl) (N = 3, n = 5, M±SD; experimental field of the FSC RAS, Orenburg Province, 2022).
[a-g] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different by Student's t-test at $p < 0.05$ (letter designations are assigned in descending order of the M value).

An increase in the expression of the selective K^+ ion transporter gene *HKT1;4* should be accompanied by a decrease in K^+ accumulation. The lowest level of *HKT1;4* expression was observed in the Ulyanovskaya 105 variety in both leaves and roots (Fig. 1). As can be seen from the data presented in Table 2, the Ulyanovskaya 105 variety has the highest K^+ content in both organs. In the Orenburgskaya 22 variety, the K^+ concentration is not the lowest, but the expression level of *HKT1;4* was the highest (see Fig. 1, Table 2). This fact is probably due to the fact that ion exchange in all organs (not only in leaves and roots) in the Orenburgskaya 22 variety is more intense than in other wheat varieties. Class II transporters, as we have already noted, do not have selectivity and are capable of transporting both K^+ and Na^+. The highest *HKT2;1* expression occurred in the Orenburgskaya 22 variety in the leaf, the lowest in the Orenburgskaya 10 variety. These data on the expression of the *HKT2;1* genes are consistent with the accumulation of Na^+ ions in the leaf that we observed.

Probably, the observed level of *HKT1;4* expression is due to the need for intensive transport of K^+ ions (for example, to other plant organs). High activity of the *HKT1;4* expression in the Ulyanovskaya 105 variety leads to the removal of K^+ ions from the roots. The expression level of *HKT1;4* in the Zolotaya variety is also one of the highest, which is consistent with a decrease in the content of K^+ ions in the roots of this variety compared to that in the Orenburgskaya 10 and Orenburgskaya 22 varieties. In the Orenburgskaya 22 variety, salt stress leads to a significant increase in expression of the *HKT1;4* in leaves, but the K^+ ions

concentration also increases. It is possible that class I transporters exhibit selectivity towards K^+ ions only under normal conditions. Under salt stress and an excess of Na^+ ions, transporters of this class also begin to actively move toxic Na^+ ions. It is interesting to note that under salt stress in all studied wheat varieties, the level of the *HKT2;3* expression in the roots is almost the same. Thus, in the Orenburgskaya variety, 22 class II ion transporters are the most active, and this wheat variety is subject to the least toxicity by Na^+ ions, they are more actively removed from the xylem. On the contrary, the greatest accumulation of Na^+ ions in the leaf occurred in the Orenburgskaya 10 variety.

Green plants contain two main types of chlorophylls, Chl a and Chl b which are noncovalently associated with membrane proteins [34]. Chl a is part of the reaction center of the antenna array which contains the main proteins that bind Chl a to carotenoids [34]. Salt stress, resulting from excess Na^+ and Cl^- ions, leads to a decrease in the content of chlorophylls and carotenoids, leaf necrosis and a decrease in metabolic functions in the cell, including photosynthesis [26, 35]. Analysis of Chl a and Chl b content allows us to quantify damage to the photosynthetic apparatus caused by abiotic stress [35].

3. Content of chlorophylls a and b in leaves of the studied wheat varieties at the tillering stage (14 days) **with chloride salinity** (200 мМ NaCl) (N = 3, n = 5, M±SD; experimental field of the FSC RAS, Orenburg Province, 2022).

Variety	Option	Content		Chl a/Chl b
		Chl a	Chl b	
Soft wheat (*Triticum aestivum* L.)				
Ulyanovskaya 105	Control	11.82±0.59[d]	6.87±0.34[c]	1.72[c]
	NaCl	6.37±0.32[g]	4.45±0.22[d]	1.43[d]
Orenburgskaya 22	Control	17.37±0.87[a]	10.37±0.52[a]	1.67[c]
	NaCl	15.33±0.77[b]	7.54±0.38[b]	2.03[b]
Твердая пшеница (*Triticum durum* Desf.)				
Zolotaya	Control	7.38±0?37[f]	2,89±0,14[e]	2,55[a]
	NaCl	3.52±0.18[h]	1,45±0,07[f]	2,43[a]
Orenburgskaya 10	Control	12.67±0.63[c]	10,01±0,50[a]	1,26[e]
	NaCl	7.96±0.40[e]	6,76±0,34[c]	1,18[e]

[a-h] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different by Student's t-test at $p < 0.05$ (letter designations are assigned in descending order of the M value).

We observed a decrease in Chl a content under salt stress in all studied wheat genotypes (Table 3). The most pronounced changes occurred in the Orenburgskaya 22 and Zolotaya varieties. The variety Orenburgskaya 22 was characterized by the largest amount of Chl a, and the variety Zolotaya by the smallest. Under salt stress, the Chl a content in the Orenburgskaya 22 variety decreased 1.1 times, and in the Zolotaya variety 2.1 times. It should be noted that the Chl a/Chl b ratio in the Zolotaya variety was higher than in the Orenburgskaya 22 variety, although the content of Chl a and Chl b for the Orenburgskaya 22 variety turned out to be higher than for the Zolotaya variety. Under salt stress, in the Orenburgskaya 22 variety the Chl a/Chl b ratio increased compared to the control while in the Zolotaya variety it remained virtually unchanged. The fact that the Chl a/Chl b value in the Orenburgskaya 22 variety is lower than in the Zolotaya variety indicates a high content of Chl b which expands the light absorption spectrum, contributing to increased photosynthesis [35].

Limited photosynthesis inhibits plant growth. This leads to an increase in the content of reactive oxygen species (ROS) due to their excess production and functional imbalance of protective mechanisms [19]. Increased ROS accumulation leads to decreased net Na^+ influx into roots, decreased xylem Na^+ load, and K^+ retention in roots, with subsequent increased salinity tolerance [36]. ROS are inevitable by-products of aerobic metabolism and important

signaling molecules involved in the regulation of many physiological processes associated with plant growth and development, but excess ROS causes lipid oxidation, leading to membrane damage, protein degradation, enzyme inactivation, base modification and DNA breaks, leading to mutation and ultimately programmed cell death [37].

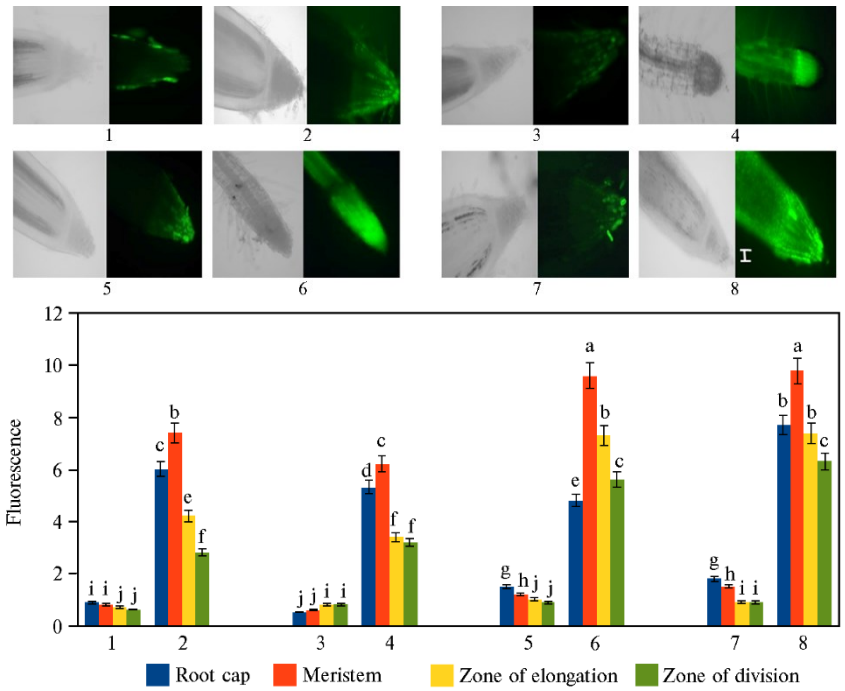

Fig. 2. Distribution of cells containing and not containing reactive oxygen species (ROS$^+$ and ROS$^-$, respectively) **in the root zones of the studied varieties of common wheat *Triticum aestivum* L.** (1, 2 — Ulyanovskaya 105, 3, 4 — Orenburgskaya 22) **and durum wheat *Triticum durum* Desf.** (5, 6 — Zolotaya, 7, 8 — Orenburgskaya 10) **at the tillering stage** (14 days) **with chloride salinity** (200 mM NaCl) (2, 4, 6, 8) compared to the control (1, 3, 5, 7) ($N = 3$, $n = 5$, $M \pm SD$; experimental field of the FSC RAS, Orenburg Province, 2022). Scale bar: 400 μm; fluorescent microscope Olympus BX51 (Olympus Corporation, Japan; for intravital visualization of ROS, an aqueous solution of Carboxy-H2DFFDA, Thermo Fisher Scientific, USA was used).

[a-j] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different by Student's *t*-test at $p < 0.05$ (letter designations are assigned in descending order of the *M* value)

Staining the roots with a fluorescent dye to vizualize ROS showed that under salt stress, ROS were detected in the tissues of many root zones, but the intensity of staining in the cells of different zones varied. We studied the distribution of cells with increased levels of ROS in different root zones (Fig. 2). In the studied varieties of bread wheat, the accumulation of ROS products under salinity was more intense in the zones of the cap and meristem, but was 1.3 times lower than in durum wheat varieties. In durum wheat, ROS products accumulated in all studied zones, but in the root cap their content was lower than in other zones. Moreover, in durum wheat, the increase in ROS content compared to the control occurred to the greatest extent in the cells of the epidermis and cortex and to a lesser extent in the zone of the central cylinder (see Fig. 2). The fact that epidermal and root cortex cells are most transcriptionally active under salinity conditions has also been reported [38]. According to reports, the induction of transcriptional activity in the tissues of the inner layers of the root under the influence of salt stress indicates the spatial regulation of this signaling pathway [38, 39].

We observed the most intense fluorescent staining in the roots of the Zolotaya variety plants. This accumulation of ROS in root cells under the influence of salinity indicates a disruption of ROS homeostasis in these cells and tissues, which can provoke programmed cell death (PCD). Soft wheat varieties turned out to be more resistant to salinity than durum wheat varieties (see Fig. 2).

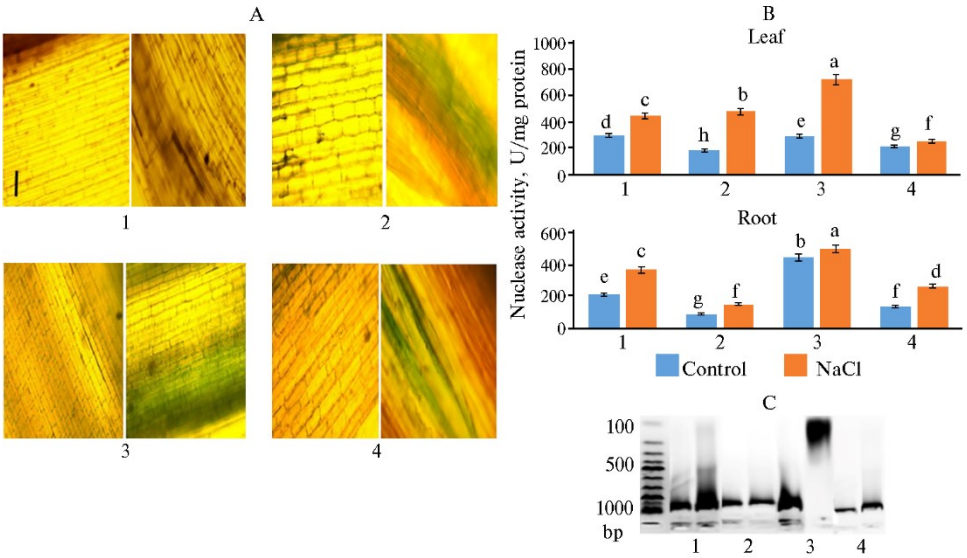

Fig. 3. Viability of coleoptile cells (A, trypan blue staining)**, nuclease activity in leaves and root**s (B, left — control, right — salt stress) **and electrophoretic separation of DNA from root cells in 1.2% agarose gel** (C) **in the studied varieties of soft wheat *Triticum aestivum* L.** (1 — Ulyanovskaya 105, 2 — Orenburgskaya 22) **and durum wheat *Triticum durum* Desf.** (3 — Zolotaya, 4 — Orenburgskaya 10) **at the tillering stage** (14 days) **with chloride salinity** (200 mM NaCl) (N = 3, n = 5, M±SD; xperimental field of the FSC RAS, Orenburg Province, 2022). Scale bar: 400 μm (Olympus BX51 fluorescence microscope, Olympus Corporation, Japan; trypan blue staining). DNA molecular weight marker (100-1000 bp) (OOO Synthol, Russia).
[a-j] Different letters mean that the average values of the indicator for the options in the column are statistically significantly different by Student's t-test at $p < 0.05$ (letter designations are assigned in descending order of the M value).

To assess cell viability under salt stress, we stained seedling coleoptiles with trypan blue (Fig. 3). In the control, there were practically no visible changes in staining, but under salt stress, cell damage turned out to be quite severe (darker staining) and varied depending on the wheat variety. Thus, in the Zolotaya variety, more than 50% of the cell area was damaged due to salinity, while in the Orenburgskaya 10 varieties this figure was no more than 40%, in Orenburgskaya 22 20%, in Ulyanovskaya 105 25%. Thus, under the influence of high concentrations of NaCl, the viability of coleoptile cells in the varieties Orenburgskaya 22 and Ulyanovskaya 105 is higher than in the varieties Zolotaya and Orenburgskaya 10, which are more sensitive to salinity (see Fig. 3).

The degradation of nucleic acids at the final stages of plant ontogenesis (during death) is massive [40, 41]. Figure 3, B shows DNA electrophoresis data from the roots of four wheat varieties grown under different conditions. Control samples of all varieties are characterized by the presence of high molecular weight DNA, while its degradation occurred under salt stress. The Zolotaya variety showed the highest degree of degradation that is consistent with the asseed total nuclease activity (see Fig. 3, B), which in the Zolotaya variety turned out to be the highest in both leaves and roots.

Thus, according to biometric indicators, the varieties of soft wheat turned out to be the most resistant to the action of high concentrations of NaCl in the

soil. The durum wheat variety Zolotaya showed the greatest sensitivity to salt stress. One of the mechanisms for increasing plant tolerance is the removal of toxic ions from the cytoplasm of plant cells. The increased activity of the genes of ion transporters of two classes of NKT in the Orenburgskaya 22 variety contributes to a greater excretion of Cl^- and Na^+ ions and, conversely, in the Zolotaya variety there is an accumulation of toxic ions [42]. It is accompanied by a decrease in the content of chlorophylls a and b and an increase in the content of ROS; as a result, damage to root tissue increases, especially in the Zolotaya and Orenburgskaya 10 varieties. The difference in the accumulation of ROS products indicates the activation of different antioxidant defense mechanisms in different wheat genotypes. The greatest accumulation of ROS products in roots under the influence of NaCl is observed in durum wheat varieties, especially in the Zolotaya variety, which initiates programmed cell death.

So, our results showed that different wheat genotypes have developed different mechanisms of adaptation to salt stress. An increase in soil salinity led to a decrease in the size of the root system in durum wheat varieties, which was accompanied by a decrease in the absorption of toxic Na^+ ions. Common wheat varieties increased ionic conductivity and the excretion of Na+ ions, especially the Orenburgskaya 22 variety. The Ulyanovskaya 105 variety had the highest content of K^+ ions which create a barrier to the penetration of Na^+ ions. The aboveground part of plants in durum wheat varieties is more adapted to salinity than the underground part. This is supported by our data on the content of chlorophylls, in particular on the Chl a/Chl b ratio, indicating an increase in photosynthesis. To deepen our understanding of the mechanisms of adaptation to salinity in different genotypes, we plan to use additional methods for analyzing the processes that shape the response to salt stress depending on the stage of plant development, and to expand the range of studied wheat varieties.

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INFLUENCE OF γ-IRRADIATION AND LEAD ON THE DYNAMICS OF GERMINATION OF SPRING BARLEY SEEDS

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Abstract

Crops are simultaneously affected by factors of different nature; therefore, it is important to study the separate and combined effects of technogenic stressors on plants. During seed germination, there is a transition from heterotrophic to autotrophic type of nutrition, which largely determines the further development of the plant, the size and quality of the crop. The impact of biotic and abiotic factors on seeds can significantly affect the passage of germination phases. In this work, for the first time, the dynamics of development in the first phases of germination of barley variety Nur under the conditions of separate and combined action of gamma radiation and heavy metal $Pb(NO_3)_2$ was studied in detail. The antagonistic effect of preliminary irradiation on the toxic effects of lead salt during germination was revealed. The aim of the work is to evaluate the influence of separate and combined effects of gamma radiation and lead, including possible synergistic and antagonistic effects of the interaction of stressors, on the dynamics of germination of spring barley seeds. The seeds of spring barley (*Hordeum vulgare* L.) of the Nur variety of the first reproduction of 2019 were used. The germination process was assessed visually for 70 hours, with detailed observation every 2 hours from the 18th to the 38th hour and every 4 hours from the 46th to the 70th hour. The seeds were irradiated with a dose of 20 Gy (dose rate 60 Gy/h) at the GUR-120 (^{60}Co) unit (RIRAE, Obninsk). We also used the $Pb(NO_3)_2$ salt at a concentration of 2 mg/ml which inhibited the development of seedlings but did not lead to their death. In the control group, non-irradiated seeds were germinated in 7 ml of distilled water. In experimental group I, seeds irradiated at a dose of 20 Gy were germinated in the same volume of water. In experimental group II, non-irradiated seeds were germinated in water with the addition of $Pb(NO_3)_2$ at a concentration of 2 mg/ml; in experimental group III, the seeds were subjected to a combined action of γ-irradiation and lead. In total, 800 seeds were studied, 200 seeds in each group. Seeds were germinated in a MIR-254 thermostat (Sanyo, Japan) in Petri dishes (20 pieces each), on a double layer of filter paper (Belaya Lenta, Russia), in the dark, at 20±0.5 °C. The germination process was divided into 6 main phases: "point' — pecking, the appearance of the germinal root, roots 1 (K-1), "fork" — differentiation of the germinal root into several roots 1-2 mm long; roots 2 (K-2) — the initial growth of roots, their size is less than the length of the seed; roots 3 (K-3) — mature roots larger than the length of the seed, no sprout; sprout — the appearance of a coleoptile, the seed has several roots and a sprout less than half the length of the seed; seedling — the formation of a full-fledged sprout, having at least two roots larger than the length of the seed and a sprout larger than half the length of the seed.. The nonparametric Mann-Whitney test was used to compare mean values. The coefficient of interaction Kw was used as a quantitative measure of the deviation of the observed effect from the additive effect and classification of the effects of combined action into groups of additivity, synergy, and antagonism. Under γ-irradiation of seeds, statistically significant differences from the control appeared in phases K-1 and K-3. Significant differences were noted in the "sprout" and "seedling" phases by the end of the observations. In general, γ-irradiation at a dose of 20 Gy did not significantly disrupt the passage of microphenological phases in seeds. Treatment with $Pb(NO_3)_2$ at a concentration of 2 mg/ml slowed down seed germination, which manifested itself in a delay in the transition to each subsequent microphenological phase, as well as in a decrease in the proportion of seeds at late stages of development compared to the control. In addition, lead had a negative effect on the development of the root, almost completely excluding the K-3 phase from the development of the seedling. The combined effect of γ-irradiation and lead also led to a slowdown in development, but in

this variant, the proportion of seeds that reached the K-3 phase increased and approached the rate in the control, that is, γ-irradiation at a dose of 20 Gy mitigated the toxic effect of lead. Therefore, a dose of 2 mg/ml $Pb(NO_3)_2$, regardless of the effect of γ-irradiation, has an inhibitory effect on the development of seeds, but does not completely suppress it, only reducing the rate of development..

Keywords: *Hordeum vulgare*, barley, seeds, germination phases, lead, γ-irradiation, combined action of radiation and lead

Technogenic pollution limits the yield and quality of agricultural plant products. The areas of emissions from industrial enterprises reach enormous sizes. In the Russian Federation alone, the area of heavy metal (HM) contamination is more than 3.6 million hectares [1]. Lead is one of the most common agricultural pollutants. It belongs to the first hazard class [1] and is capable of influencing the morphology and physiology of plants. Lead is not an element essential for plants, and its toxic effect is largely associated with various disorders of cell metabolism and inactivation of enzymes [2]. As a result, lead inhibits germination, root elongation, seedling development, chlorophyll production, and inhibits Calvin cycle enzymes, thereby affecting plant development [2, 3].

After the discovery of ionizing radiation (IR), research began on its effect on living organisms. The biological action of IR is based on the direct interaction of radiation quanta with biological macromolecules and the formation of reactive oxygen species in the process of water radiolysis [4]. Already the first experiments showed that with increasing radiation dose, damage to biological structures increases, loss of their functions is observed, inhibition of reproduction and growth and, as a result, death of the organism. However, in the low-dose region, deviations from the monotonic nature of the dose-effect relationship were found, which is associated with a qualitative difference in cell responses to irradiation at high and low doses [5]. Moreover, in the low-dose region, radiation hormesis is observed, when inhibition of physiological processes is replaced by stimulation [6].

In real conditions, plants are affected by combinations of factors that differ in toxicity and mechanisms of action. In this regard, it is important to study the combined action of factors of different nature, which can influence biochemical processes, accelerate or slow down metabolism and, accordingly, affect the rate of plant development [2, 7]. Qualitative differences between the mechanisms of biological action of factors and their targets in the cell can cause fundamentally different plant responses, from antagonism to synergism [8]. In particular, preliminary irradiation of barley (*Hordeum vulgare* L.) seeds, *Arabidopsis thaliana* L. and faba beans (*Vicia faba* L.) increased plant resistance to the toxic effects of lead and cadmium [9-11].

When a seed germinates, a transition occurs from a heterotrophic to an autotrophic type of nutrition, which largely determines the further development of the plant, the size and quality of the harvest. Exposure of seeds to biotic and abiotic factors can significantly influence the progression of germination phases [12, 13]. In the scientific literature, there is practically no data on the detailed dynamics of the germination process, both in the absence of technogenic factors and when factors of different nature act separately or together.

In this work, for the first time, the dynamics of germination in barley variety Nur under the separate and combined action of γ-radiation and $Pb(NO_3)_2$ was studied in detail. Pre-irradiation has been shown to mitigate the toxic effect of lead salt during germination.

The purpose of the work was to assess the influence of each of the studied factors, the γ-radiation and lead salt and their combination, including possible synergistic and antagonistic effects of the interaction of stressors, on the germination of spring barley seeds.

Materials and methods. We used seeds of spring barley (*Hordeum vulgar*e

L.) Nur variety of the first reproduction in 2019. The germination was assessed visually over 70 hours, with detailed observations every 2 hours from the 18th to the 38th hour and every 4 hours from the 46th to the 70th hour.

The seeds were irradiated at 20 Gy (the dose rate 60 Gy/h) using a GUR-120 (^{60}Co) installation (ARRIRAE, Obninsk). In our previous experiments [14], this dose stimulated the development of barley Nur and Grace seedlings. The seeds were placed in paper bags with a surface area of 25 cm^2, which ensures an even distribution of the dose. The radiation dose absorbed by the seeds was assessed with a DKS-101 dosimeter (Politechform-M, Russia). We also used $Pb(NO_3)_2$ salt concentration 2 mg/ml, which inhibited the development of seedlings, but did not lead to their death [15].

The control was non-irradiated seeds germinated in 7 ml of distilled water. The first treatment was seeds irradiated at 20 Gy and germinated in the same volume of water. The second treatment was non-irradiated seeds germinated in water with 2 mg/ml $Pb(NO_3)_2$; the third treatment was the seeds subjected to the combined action of γ-radiation and lead salt. A total of 800 seeds were examined, 200 seeds per treatment. Seeds were germinated in a MIR-254 thermostat (Sanyo, Japan) in Petri dishes (20 seeds per each) on a double layer of filter paper (White Lenta, Russia) in the dark at 20±0.5 °C.

Microphenological stages of seed germination were assessed as described [16]. The germination process was divided into six main stages, "point" means pecking, appearance of the embryonic root; roots 1 (R-1), "fork" means differentiation of the embryonic root into several roots 1-2 mm long; roots 2 (R-2) corresponds to initial growth of roots, their size is less than the length of the seed; roots 3 (R-3) means mature roots larger than the length of the seed, no sprout; sprout means the appearance of a coleoptile, the seed has several roots and a sprout measuring less than half the length of the seed; seedlings correspond to the formation of a complete sprout that has at least two roots larger than the length of the seed and a sprout measuring more than half the length of the seed. The "point" stage determines the beginning of pecking of the rudimentary root, the length of which should not exceed 1 mm. During seed germination, shoot and root growth stages were distinguished [17], and root growth, in turn, was divided into three stages. The scale is consistent with the approaches underlying the GOST on methods for determining germination [18].

The nonparametric Mann-Whitney test was used to compare mean values. As a quantitative measure of the deviation of the observed effect from the additive one and the classification of the combined effect as additivity, synergism, and antagonism, the interaction coefficient K_w [8] was calculated:

$$K_w = \frac{\Delta A(\gamma, HM)}{\Delta A(O, HM) + \Delta A(\gamma, O)},$$

where $\Delta A(X,Y) = A(X,Y) - A(O,O)$ is the increment (the excess of the level induced by stressors over the spontaneous one) at the ionizing radiation dose X and the heavy metal concentration Y.

Since the level of an observed effect is a random variable that has a probability distribution, the value of the interaction coefficient K_w is also a random variable. To classify the response of plants to a combined effect, it is necessary to test the statistical hypothesis that Kw is equal to 1. The effect is recognized as additive if $K_w \sim 1$, as antagonistic if K_w is statistically significantly less than 1, and as synergistic if K_w is statistically significantly greater than 1.

Calculations were carried out in Microsoft Excel 2010 and Statistica v. 8.0 (StatSoft, Inc., USA). The differences were confirmed statistically with a significance level of $p < 0.05$.

Results. In the "point" phase (Fig. 1), in seeds irradiated with a dose of

20 Gy (treatment I), significant differences ($p < 0.05$) vs. control occurred only at the 20th hour. In the R-1 phase, differences vs. controls appeared at the 30th hour of germination (Fig. 2, A), while the peak of development of both groups occurred at the 32nd hour. Upon transition to the R-3 phase (see Fig. 2, B), there was a statistically significant excess vs. control in irradiated seeds from the 58th to the 62nd hour. During sprouting (Fig. 3, A), differences from the control were significant ($p < 0.05$) at the 54th and 66th hour of germination. In general, the development of irradiated seeds repeated the dynamics of control seeds, and the few deviations were not systematic.

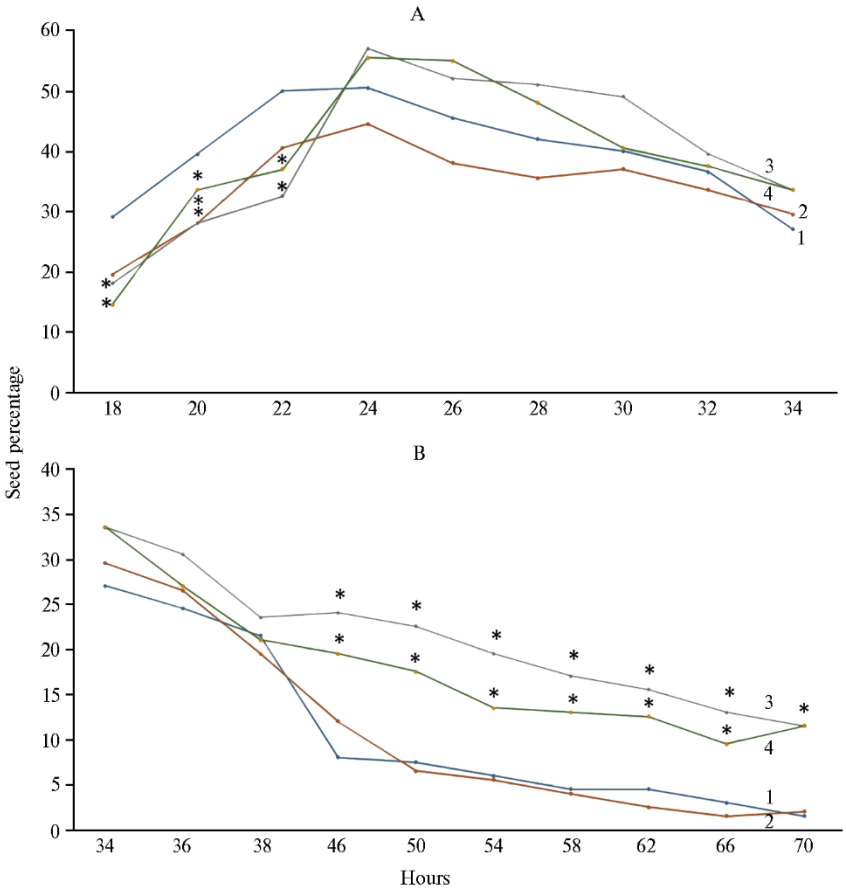

Fig. 1. The proportion of seeds of spring barley (*Hordeum vulgare* L.) variety Nur at the "point" stage from the 18th to 34th hour (A) **and from the 34th to 70th hour of germination** (B)**:** 1 — control (no treatment), 2 — irradiated seeds 20 Gy (treatment I), 3 — seed treatment with $Pb(NO_3)_2$ (treatment II), 4 — seeds exposed to the combined action of γ-radiation and lead (treatment III) (lab tests). A total of 800 seeds were studied, 200 seeds per treatment.
* Differences from control are statistically significant at $p < 0.05$.

When treated with lead (treatment II), a slowdown in the passage of all stages of development was recorded. From the first hours, a slowdown in swelling was observed, as a result of which the proportion of seeds that entered the "point" phase was significantly lower ($p < 0.05$) compared to the control (see Fig. 1, A) up to the 22nd hour. However, by the 24th hour, the proportion of seeds treated with lead in the "point" phase reached and even slightly exceeded the control, which indicated an equalization of growth rates. A statistically significant ($p < 0.05$) increase in the proportion of lead-treated seeds that reached the "point" stage compared to control was recorded starting from the 46th hour until the end of the observations (see Fig. 1, B).

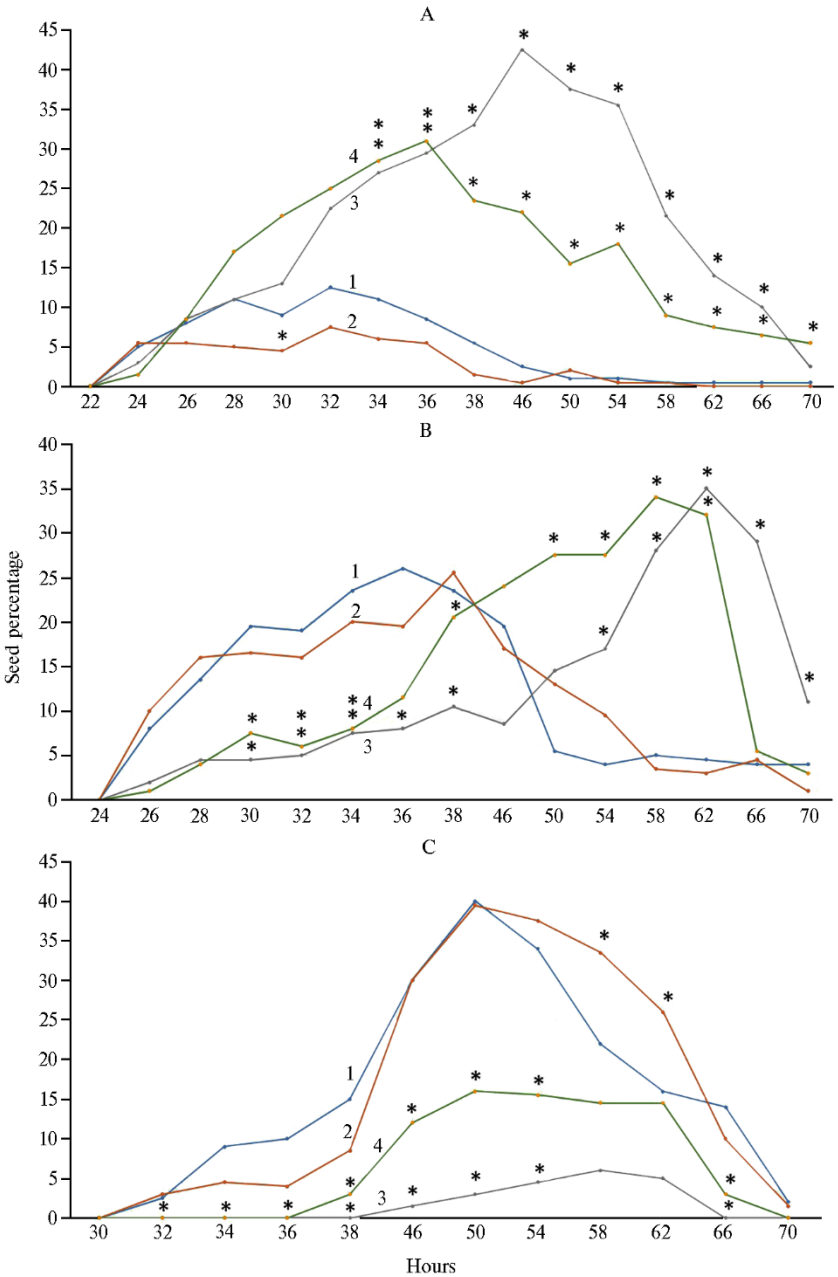

Fig. 2. The proportion of seeds of spring barley (*Hordeum vulgare* L.) variety Nur at stages R-1 (A)**, R-2** (B) **and R-3** (C) **from the 30th to the 70th hour of germination:** 1 — control (no treatment), 2 — irradiated seeds (20 Gy) (treatment I), 3 — seed treatment with $Pb(NO_3)_2$ (treatment II), 4 — seeds exposed to the combined action of γ-radiation and lead (treatment III) (lab tests). A total of 800 seeds were examined, 200 seeds per treatment.

* Differences from control are statistically significant at $p < 0.05$.

At the R-1 stage, significant differences from the control ($p < 0.05$) were observed from the 34th to the 66th hour (see Fig. 2, A). The proportion of seeds that reached the R-1 stage (43%) in treatment II was the greatest at the 46th hour. This value turned out to be higher than in treatment III (31%), in the control (13%) and in treatment I (7.5%), that is, exposure to lead significantly slowed down the development at the R-1 stage. The main proportion of lead-treated seeds reached the R-2 stage (see Fig. 2, B) by the 62nd hour, which is 26 hours later

than in the control group. Significant differences vs. control aooeared from the 30th hour until the end of observations. From the 46th to the 50th hour, there were no significant differences in the proportion of seeds that entered the R-2 stage, since in this interval the number of such seeds in treatment II began to increase and their number sharply decreased in the control.

The proportion of seeds in the R-3 phase (see Fig. 2, B) when exposed to lead decreased significantly compared to the control from the 32nd to the 54th hour. In the sprout phase, a statistically significant excess ($p < 0.05$) over the control was observed from the 66th hour (see Fig. 3, A). Since the proportion of seeds in the seedling stage in treatment II increased much more slowly (see Fig. 3, B), the number of such seeds in the control was statistically significantly greater. In general, lead-treated seeds showed a slow entry into the seedling phase (+1% per 4 hours, starting at 62 hours).

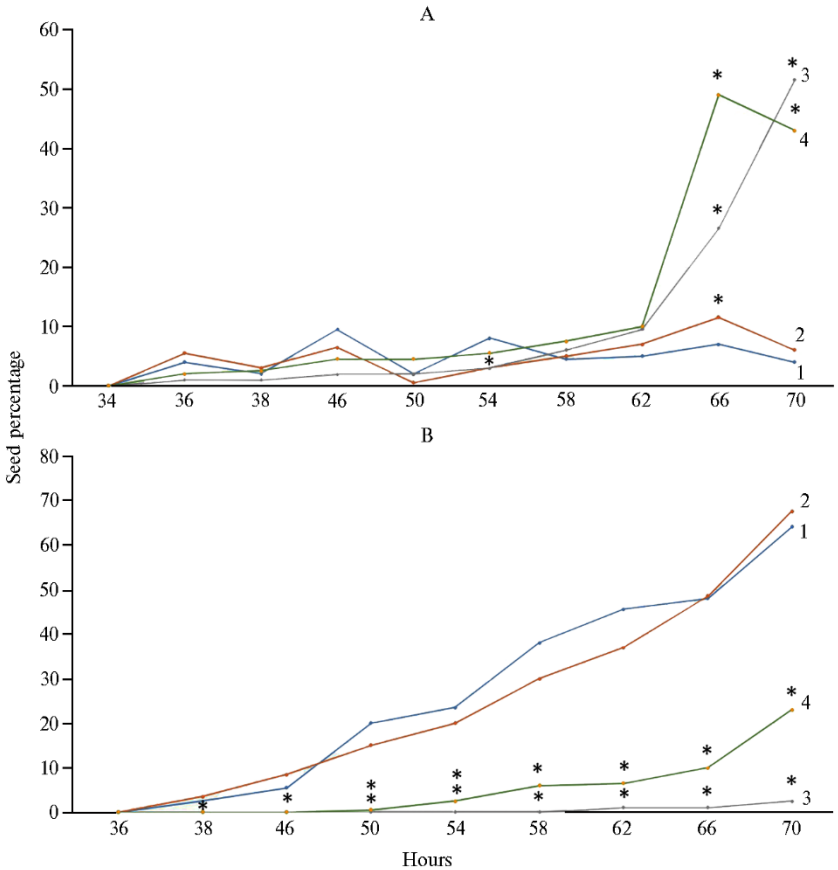

Fig. 3. The proportion of seeds of spring barley (*Hordeum vulgare* L.) variety Nur at the sprout (A) **and seedling** (B) **stages and from the 34th to the 70th hour of germination:** 1 — control (no treatment), 2 — irradiated seeds (20 Gy) (treatment I), 3 — seed treatment with $Pb(NO_3)_2$ (treatment II), 4 — seeds exposed to the combined action of γ-radiation and lead (treatment III) (lab tests). A total of 800 seeds were examined, 200 seeds per treatment.

* Differences from control are statistically significant at $p < 0.05$.

Under the combined influence of γ-radiation and lead (treatmen III) at the "point" stage, the seeds developed similarly to those treated only with lead (see Fig. 1, A, B). The largest proportion of seeds in treatmen III reached the R-1 stage at the 36th hour (31%). This value was less than with lead treatment (43%), but statistically significant ($p < 0.05$) more vs. control (13%) and treatment I (7.5%). At the R-2 stage, seeds exposed to the combined influence of two factors

in the interval from 30 to 38 hours and from 50 to 62 hours demonstrated significantly less ($p < 0.05$) activity compared to the control (see Fig. 2, B).

In phase R-3 in treatment III, a significant ($p < 0.05$) effect was observed, although it was smaller than in the control and under the influence of γ-radiation. The increase occurred at the 50th hour, but the period of significant differences covered from 32nd to 54th hour (see Fig. 2, B). From the 46th to the 62nd hour in treatment III, the proportion of seeds in the R-3 phase exceeded the value in treatment II. Moreover, from the 46th to the 54th hour, the interaction coefficient K_w was statistically significantly ($p < 0.05$) less than 1, which indicates an antagonistic interaction of factors and allows us to conclude that γ-irradiation partially neutralizes the toxic effect of lead.

During the sprouting stage, significant ($p < 0.05$) differences vs. control occurred in treatment III from the 66th hour (see Fig. 3, A). The proportion of seeds that reached the seedling stage in treatment III differed significantly ($p < 0.05$) from the control, starting from the 38th hour until the end of the experiment due to a significant slowdown in development. At the 70th hour, the difference between seeds in variants II and III became statistically significant ($p < 0.05$), that is, with preliminary exposure to IR, the germination rate returns to normal faster than when treated with lead alone. In addition, the K_w values in these groups indicate the antagonistic interaction of factors.

Time during which the maximum number of seeds of spring barley (*Hordeum vulgare* L.) variety Nur reached a certain stage of development depending on the effect of γ-radiation and lead on the seeds (lab tests)

Treament	Stage	Development peak, h	Seed number
Control	«Point»	24	101
	R-1	32	25
	R-2	36	52
	R-3	50	80
	Sprout	46	19
	Seedling	70	128
I, γ-radiation	«Point»	24	89
	R-1	32	15
	R-2	38	51
	R-3	50	79
	Sprout	46	13
	Seedling	70	135
II, $Pb(NO_3)_2$	«Point»	24	114
	R-1	46	85
	R-2	62	70
	R-3	58	12
	Seedling	70	103
	Shoot	70	5
III, γ-radiation + $Pb(NO_3)_2$	«Point»	24	111
	R-1	36	50
	R-2	58	68
	R-3	50	32
	Sprout	66	98
	Seedling	70	46

N o t e. For a detailed description of the options, see the Materials and methods section.

From the presented results it follows that seeds treated with γ-radiation and lead develop unevenly, and the transition to each subsequent stage occurs at different times.For each treatment, the average time was determined during which most of the seeds passed a certain stage of development (Table).

In general, lead-treated seeds lagged behind in development compared to control and γ-irradiated seeds. They reached earlier stages in greater numbers than in the control and treatment I over the same periods. However, the number of seeds treated with $Pb(NO_3)_2$ in later stages of development, on the contrary, was smaller compared to the control and treatment I.

Seed is a special state of a plant in which metabolism is almost completely

suppressed [12] in order to conserve resources for the development of the sprout. The entry of water into the seed initiates the swelling process, and reserve substances are converted into soluble compounds used to nourish the embryo. A cascade of events is launched aimed at transferring the cells of the embryo into an active state. Genome derepression caused by ionizing radiation or heavy metals gives rise to key metabolic processes: the synthesis of nucleic acids and proteins increases, the activity of many enzymes increases, and the content of phytohormones, the growth activators that control plant growth and development, increases [14, 19, 20].

The energy of ionizing radiation absorbed by the seeds is converted mainly into free radicals that exist for a long time in air-dry seeds. When water and oxygen enter the seed, they quickly react to form strong oxidizing agents, the hydroperoxides and hydroxyl radicals [21]. Reactive oxygen species transform the genome of embryonic cells into an active state. Previous studies [14, 19] have shown that irradiation at stimulating doses is sufficient to influence plant regulatory systems and accelerate the development program.

Different doses of irradiation of barley seeds can induce qualitatively different effects, from inhibition (at 50 Gy) to stimulation (at 20 Gy) of plant development [14]. In our experiment, a statistically unreliable stimulation of the rate of irradiated seed development was observed at the R-1 stage, but the seeds soon became equal in rate of development to the control group.

Similar to the effects of ionizing radiation, exposure of seeds to heavy metals can lead, depending on the concentration, to either inhibition or stimulation of growth [20]. The main barrier to HMs is root tissues that can bind cations [22], but this is not the only way a plant can reduce HM uptake. Thus, endodermal cells, which play an important role in the development of lateral roots, are the first to be exposed to HM, which significantly inhibits their functioning and, accordingly, the overall development of the root. For this reason, when there is an excess of heavy metals in the soil, the development of the root system is primarily disrupted [22].

In our study, obvious damage to the root system occurred when assessing the stages of root development. According to the methodology we used, seeds are considered full-developed seedlings when they reach the R-3 stage and form a sprout more than half the length of the seed [16]. We have changed the germination criterion for seeds treated with lead nitrate. The germination phase was considered reached in the case of germination of the initial coleoptile, without entering the R-3 phase. This was associated with the accumulation of more lead in the root compared to other parts of the plant [23]. In the remaining parts of the seedling, at a concentration of 2 mg/ml, the development rate was close to normal. Germination of lead-treated barley seeds delayed coleoptile development by approximately 16 hours, bypassing the R-3 stage, which may pose a risk for future plant development.

Another target of HM is the plasmalemma. Exposure to lead ions changes its permeability and ion balance, and interferes with the functioning of H^+-ATPases [24]. The cause of these disorders is errors in lipid synthesis and their increased oxidation by reactive oxygen species [25]. In addition, lead can affect enzyme metabolism by binding to SH groups. As a result, thylakoids are destroyed, disruptions in the Calvin cycle and water stress occur, cell division is inhibited (impaired cytokinesis is due to a decrease in the rate of microtubule formation) and mitochondria are damaged.

In our opinion, the described features of the action of IR and HM could lead to the results obtained in this work. In wheat seeds treated with $Pb(NO_3)_2$, a decrease in root development was observed [26]. Data presented by A.V. Dikarev

et al. [15] indicate significant sensitivity of barley roots to lead. The length of the roots sharply decreased even at a $Pb(NO_3)_2$ concentration of 1 mg/ml. Treatment with heavy metals resulted in a significant retardation of wheat germination [27]. After adding Cu, Cd, Ni, the length of the sprout decreased by 51, 48 and 33%, respectively, of roots by 91, 63 and 72% [27]. Growth inhibition was associated with significant metal accumulation in wheat seedling tissues. In lentil plants exposed to 0.5 mM (~ 0.56 mg/ml) and 1000 mM (~ 1.12 mg/ml) lead concentrations, germination was inhibited by 2.5 and 10% [28].

Effects of individual stressors and their combination vary markedly [8, 29-31]. The reason may be the increased level of reactive oxygen species (ROS), induced by either one of the stressors or due to their combined effect. The heavy metal lead and γ-radiation, both separately and together, cause a number of specific reactions. For example, by interacting with DNA, each of the stressors can partially suppress repair systems or induce mutations [8]. The formation of mutations is extended over time, therefore, external influences modify the proportion of potential damage recorded in the mutation. Small doses of IR can activate repair and the antioxidant systems [32].

We did not observe an effect of γ-radiation on the rate of barley seed development, while lead significantly slowed down the plant development. The combined action led to statistically confirmed antagonistic effects. Similar results were reported by H.I. Mohamed [33], that is, the combined effect of 25 Gy IR and 300 μM (~ 0.6 mg/ml) lead ions statistically significantly increased the length of the root and sprout of cowpea (*Vigna sinensis* L.). The dose load elevated to 80 Gy statistically significantly slowed down the development of the sprout, and the size of the root increased. The lead concentration of 600 μM (~ 1.2 mg/ml) and 25 Gy irradiation led to a statistically significant increase in all parameters of seedlings. Considering that when the seeds were treated only with lead in two concentrations, there was a statistically significant decrease in all parameters of seedlings while a combined effect was the opposite, it can be assumed that γ-irradiation partially neutralized the toxic effect of lead on the seeds of cowpea (*Vigna sinensis* L.). The reason for this may be an increase in the efficiency of protein and metabolite utilization, modulation of the amount of ROS, and the triggering of repair mechanisms [34]. Similarly, pre-treatment of mountain barley seeds with 50 Gy γ-radiation increased the tolerance to heavy metals in seedlings by reducing the H_2O_2 concentration [10]. The activity of antioxidant enzymes increased, which alleviated oxidative stress caused by heavy metals.

This phenomenon is also confirmed in a study on *Arabidopsis taliana* L. [9]. The authors considered the effect of the combined action of $Pb(NO_3)_2$ and 25-150 Gy γ-radiation [9]. At 50 Gy, a statistically significant stimulation of root development occurred compared to plants exposed to 500 μM (~ 1.02 mg/ml) $Pb(NO_3)_2$. When the dose was increased to 150 Gy, root growth was inhibited.

In our experiment, the dynamics of plant development in the control and with γ-irradiation coincided (see Table 2). In both cases, the peak of the R-3 stage occurred later than the peak of the sprout stage. This may be due to polymorphism in seed germination, as well as the specificity of the development of some seeds, in which the sprout begins to develop simultaneously with the root, without reaching the R-3 phase. In the case of combined action and separate exposure to lead, there was a shift in the peak of R-2 development to a later time relative to the next stage. Due to the specificity of the effect of lead on root development [15], only a small part of the seeds reached the R-3 stage. Most of the seeds have passed this phase and entered the sprout stage. Note that the difference between the time to reach the peak development of stages R-2 and R-3 reached 4 hours with separate lead exposure and 8 hours with combined action, however, in the latter case,

the sprout appeared earlier. Apparently, preliminary γ-irradiation activated the antioxidant and repair systems, which partially neutralized the effect of lead on the development of the sprout. Similar results were obtained in other works [9-11]. The peak of the seedling stage for all plants was at 70 h, since it was before this time that observations were made.

Thus, γ-irradiation of spring barley (*Hordeum vulgare* L.) variety Nur seeds at a dose of 20 Gy did not significantly disrupt the passage of microphenological phases of development. Treatment with $Pb(NO_3)_2$ at a concentration of 2 mg/ml slowed down seed germination, which was manifested in a delay in the transition to each subsequent microphenological phase, as well as a decrease in the proportion of seeds at late stages of development compared to the control. In addition, lead negatively affected root development, almost completely excluding the R-3 stage from the seedling formation. The combined effect of γ-radiation and lead also led to a slowdown in development, but the proportion of seeds that reached the R-3 phase increased and approached the control value, that is, γ-radiation at a dose of 20 Gy mitigated the toxic effect of lead. Therefore, a dose of 2 mg/ml $Pb(NO_3)_2$, regardless of exposure to γ-irradiation, has an inhibitory effect on seed development, but does not suppress it completely, only reducing the rate of development. An hourly assessment of seed germination under separate and combined action of lead and γ-radiation gives a deeper understanding of the mechanisms of plant adaptation to technogenic impacts at the early stages of development. Our findings will be used in research on genetic technologies for obtaining high-yielding barley varieties that are resistant to technogenic factors.

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GROWTH AND VIABILITY OF COLEOPTILES UNDER OXYGEN DEFICIENCY IN *Oryza sativa* L. FROM THE COLLECTION OF THE FEDERAL RICE RESEARCH CENTER

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Abstract

The distinctive ability of rice seedlings lies in the ability to germinate under conditions of oxygen lack. At the germination stage, the first to develop is the coleoptile, a juvenile organ that protects the true leaf in cereals. The mechanisms of regulation of growth and development of this organ have not been studied to a large extent. Special attention may be paid to a coleoptile in seedlings capable of germinating under oxygen conditions. In the presented study, for the first time, the importance of the growth rate and viability of coleoptiles of rice seedlings during flooding in determining survival and development was demonstrated. A total of 36 varieties and forms from the collection of the Federal Research Center for Rice, Krasnodar, were tested. Sprint and Kuban 3 were among the fastest growing varieties. Their coleoptiles reached 19-25 mm length both under normoxia and hypoxia. The slowest growing group included three Philippine varieties harbouring the *SUB1A* allele (HHZ11 Y6-Y2-SUB1, HHZ8 SAL 14 SUB1, HHZ9 DT12 SUB1), Chinese variety Xiannui and domestic varieties Amethyst, Zhemchug, Natasha, Rapan and Yuzhnaya noch. This group of varieties was characterized by inhibition of the growth of coleoptiles by 2.5-3 times under submergence. In main a positive correlation was estimated between the coleoptile length in normoxia and hypoxia ($\rho = 0.70$, $p = 10^{-6}$), i.e. forms that actively grow in an aerobic environment also grow rapidly when flooded. Further detailed analysis of the growth of coleoptiles under hypoxic conditions showed that growth changes correspond to several patterns. The most common reaction to hypoxia in rice coleoptiles of the first group was growth suppression, but with the preservation of a small part of the plants that continue to grow. This group included all Philippine cultivars harbouring the *SUB1A* allele. In the second group, a significant proportion was plants which length under submergence exceeded that at normoxia. Approximately half of the plants of the third group slowed growth arrest, while the other part continued to grow as in normoxia. The fourth group included the variety Sprint, which was the only one to have traits of avoidance strategy (LOES — low oxygen escape syndrome) associated with growth enhancement, although this enhancement was not intensive. The variety Yuzhnaya noch had a unique growth pattern, the coleoptiles of which grew slowly both in normoxia and being submerged. In addition to growth, the viability of coleoptiles was analyzed in the work, which

was assessed using a tetrazolium test. Under hypoxic conditions, the viability of all tested forms significantly decreased. In the fastest growing varieties (Sprint, Kuban 3), the color intensity of tetrazolium salts was higher both in the control (6-fold) and in the experiment (2-fold), compared to slow growing forms (Amethyst, Yuzhnaya noch, Philippine *SUB1A* varieties). In general, growth rate correlates with metabolic rate and submergence tolerance. Obtained results illustrate the tolerance to oxygen deficiency of the genotypes from the collection of the Federal Research Center for Rice, and show that coleoptile elongation can be used as a criterion for assessing the tolerance of rice varieties to the lack of oxygen.

Keywords: *Oryza sativa*, rice, submergence, hypoxia, coleoptile, growth, tolerance

Rice (*Oryza sativa* L.) is the oldest agricultural crop, the cultivation of which began more than 5 thousand years BC. It forms the basis of nutrition for several billion people. In 2022, 525.6 million tons of rice grain was harvested, which is grown on more than 167 million hectares (http://www.fao.org/worldfoodsituation/csdb/ru/). Rice is cultivated quite intensively in Russia. The area under rice as of June 2022 in the Russian Federation amounted to 162.5 thousand hectares (https://sfera.fm/news/zernovye/minselkhoz-otmetil-snizhenie-ploshchadiseva-zerna-v-rf-na-07-mln-ga-god-k-god). In Kuban, the main rice-growing region of the Russian Federation (92.3 thousand hectares), the rice harvest according to Rosstat, in 2022 amounted to 582.6 thousand tons (https://rosstat.gov.ru/compendium/document/13277). Understanding the mechanisms of rice plant adaptation to floods facilitates breeding varieties resistant to long-term flooding in view to reduce the pesticide load on rice paddies and to ecologize commercial rice farming.

A distinguishing feature of rice plants is their ability to germinate under oxygen deficiency (hypoxia) or complete absence (anoxia). Plants growing in wetland environments, including rice, use two strategies to adapt to oxygen deficiency. The first strategy is aimed at actively avoiding oxygen deficiency (low oxygen escape syndrome, LOES), the second is a passive rest strategy (low oxygen quiescence syndrome, LOQS) [1].

The LOES strategy provides rapid elongation of shoots, hyponastic bending and changes in leaf anatomy to improve the diffusion of gases, the formation of aerenchyma, and an increase in the number of superficial adventitious roots [2-6]. The formation of aerenchyma necessary for the intensification of gas exchange between plant organs and tissues is under regulation by phytohormones, primarily ethylene. It is considered as a key regulator of the plant hormonal status under the LOES strategy. Ethylene initiates a decrease in the abscisic acid concentration and promotes an increase in auxin and gibberellins [3, 5, 7, 8]. In rice, the *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*) genes of the *ERF-VII* (ethylene response factor) family regulate the LOES strategy. With an increase in their expression in internodes, both the content of gibberellins and sensitivity to them increases, which stimulates the activity of intercalary meristems [9].

An important adaptation to oxygen deficiency is the formation of a gas film on the hydrophobic surface of leaves and stems of flooded plants. It is known that rice plants retain a surface gas film even during long-term (4-5 days) flooding [10]. The film is responsible for the delivery of oxygen from the above-water surface and is important for underwater photosynthesis, which provides plants with energy [5, 6]. Accelerated growth also occurs due to the metabolization of sugars with the participation of protein kinases. For example, CIPK15 (calcineurin B-like protein interacting protein kinase15) initiates a SnRK1A-dependent cascade of anaerobic starch degradation [11]. In turn, SnRK1A (sucrose-nonfermenting1-related protein kinase 1A), interacting with the transcription factor MYBS1, stimulates the expression of the α-amylase which is especially important for the starch breakdown during seed germination.

Inhibition of growth and metabolism are the main criteria for an alternative

LOQS strategy. This strategy means metabolic adaptations that prevent energy starvation, cytoplasmic acidification, and the toxicity of anaerobic metabolites. The energy generated during starch metabolism, the glycolysis and fermentation is primarily spent on transport processes, protecting cells from reactive oxygen species (ROS), and preventing protein denaturation [5, 11, 12]. In rice, ethylene is the main regulator of LOQS as in the avoidance strategy. The protein encoded by the *SUB1A* gene of the group of ethylene-sensitive transcription factors (ERF-VII) inhibits ethylene synthesis, leading to a decrease for gibberellins. This stimulates the accumulation of brassinosteroids, the growth regulators that promote the breakdown of biologically active gibberellins and the accumulation of the SLENDER RICE1 (SLR1) protein which suppresses the transduction of the gibberellin signal [13]. In addition, SUB1A inhibits sugar metabolism, reducing the expression of amylase and sucrose synthase genes [14]. When implementing the LOQS strategy, plant growth is inhibited during flooding, and the saved resources are used to wait out the period of oxygen deficiency and subsequent regrowth after gas exchange is restored.

The coleoptile is a special juvenile organ of seedlings in cereals. It grows through the soil, keeping the true leaves intact. If light hits the coleoptile, it stops growing and a true leaf grows through it. The coleoptile has a limited period of development, when all its cells undergo an extensional growth stage. It is believed that longer coleoptiles in many cases have the ecological advantage, for example, they provide protection from high temperature and dense environments [15, 16]. The ability to elongate the coleoptile has proven beneficial for crops in unfavorable conditions, as deep seeding is important for growth under more favorable temperature and humidity conditions, reduces the risk of damage from attacks by mice or other animals, and reduces damage from pre-emergence herbicides [17-19].

A significant number of factors have been identified that regulate coleoptile elongation at the transcription and post-translation levels [20, 21]. Extensional growth has been well studied in coleoptiles of cereals, in particular oats, corn and canarygrass. Note that in rice, at the germination stage, the coleoptile is the first to develop from the grain. The coleoptiles of this plant complete their growth on days 6-9. Under flooding, the growth of coleoptiles in rice varieties adhering to the LOES strategy is significantly accelerated, while in LOQS forms, on the contrary, it is inhibited [22]. In this regard, it is advisable to use the length of the coleoptile as a criterion when choosing genotypes that contrast in their ability to grow, and therefore, in resistance.

Currently, the Federal Research Center for Rice (Krasnodar) stores 7.3 thousand rice samples in the Unique Scientific Installation — UNU Collection of Rice Genetic Resources. The rice gene pool in UNU is represented by varieties, accessions, mutants, dihaploids of *O. sativa* of *indica* and *japonica* subspecies and includes 82 varieties from 42 rice-growing countries. The conserved gene pool is extensively studied to identify sources and donors of valuable traits [23, 24]. However, testing collection samples for resistance to various adverse environmental factors (including oxygen deficiency), unfortunately, is far from complete. In our opinion, the ability of rice plants to change their adaptation strategy under flooding conditions during early ontogenesis can largely determine the final crop yield. In addition, it is not clear whether the growth and stability of a juvenile organ can be extrapolated to the stability and development of an adult plant.

The presented work demonstrates for the first time the importance of the growth rate and viability of coleoptiles of rice seedlings under flooding in determining the stability of plants and their development in subsequent ontogenesis.

The purpose of the work was to assess the growth rate and viability of coleoptiles under flooding conditions in rice varieties and accessions from the collection

of the Federal Research Center for Rice.

Materials and methods. Growing rice (*Oryza sativa* L.) and phenotyping plants for economically valuable traits was carried out in 2019-2022 in a collection nursery (irrigation system of the Federal Research Center for Rice, Krasnodar, Belozernoye village).

The seeds of 36 varieties and accessions from the collection of the Federal Research Center for Rice were used. These were 24 rice varieties of domestic breeding (Amethyst, Anait, Veles, Viola, Vita, Gamma, Zhemchug, Krasnoarmeysky 313, Kuban 3, Leader, Natasha, Novator, Olimp, Privolny 4, Rapan, Regul, Sonata, Sprint, Titan, Ussur, Fontan, Khazar, Sharm, Yuzhnaya noch), one variety from Uzbekistan (Devra), three varieties from China (Xiannui, Zhongyon, Zhongyon 207) and eight samples from the International Rice Research Institute (IRRI, Philippines) (AA WAB 56-125, HHZ11 Y6-Y2-SUB1, HHZ8 SAL 14 SUB1, HHZ9 DT12 SUB1, IR 50, IR14 L 110, Kirkpinar, PV-1 IRBLSH). Among the Philippine accessions, there were three ones carrying the *SUB1A* allele characteristic of the most resistant and slow-growing LOQS rice varieties.

As part of international cooperation, rice samples of world selection entered the collection of the research center from various unified variety testing nurseries (IRRI, Philippines) in which various cultivation methods were prcaticized, e.g., with shortened flooding (IRLON), from nurseries of cold-resistant rice (IRCTN) and artificially irrigated rice from temperate latitudes (IRTON), of deep-water, flood-tolerant rice (IRLYN-SUB), of irrigated lowland rice from green technology nursery (GAR-IRLL), and irrigated super rice from green technology nursery (GSR-Rell) (http://www.knowledgebank.irri.org/images/docs/rice-standard-evaluation-system.pdf).

The growth rate of seedlings was determined visually on a 9-point scale, where 1 point is low, 3-5 points is medium, 7-9 points is high. The varieties with a high growth rate (9 points) at the initial stage of germination were genotypes with a seedling length on day 7 of more than 2 cm; 7 points correspond to 1.5-2.0 cm length, 3-5 points to 1.0-1.5 cm, 1 point to less than 1 cm. As a result, rice varieties and forms were grouped for low, medium and high early growth rate. The development of plants under normoxia was assessed for the total height, the length of the panicle, the duration of the development period before flowering and the total growing season. The duration of growing season was assessed from the date of germination to flowering, and then until the grain is completely ripe. Elements of productivity were determined, including 1000-grain weight, and the overall productivity of the panicle. For biometric analysis, test sheaves of 10 plants were selected from the plots.

For a series of lab studies, the seeds were surface sterilized with a 50% sodium hypochlorite solution for 15 min, washed 10 times with sterile water and soaked in hot water (55 °C) for 1 hour. Next, 50 seeds of control samples were placed in an enamel tray on glass bridges, covered with gauze. A 4% Knop nutrient solution [25] was poured into the tray to the level of the glass bridges, covered with glass and germinated under normal air access. For hypoxia, the seeds were placed in 750 ml containers filled with the same solution to the very top (water column height of 12 cm) and hermetically sealed with a lid. In both treatments, plants were grown for 4 days at 29 °C in the dark. On day 4, the oxygen concentration in the hypoxic solution averaged 0.46 mg/l. It was measured using an Expert-009 dissolved oxygen analyzer (Econix-Expert, Russia). Dishes, gauze and solutions for working with plants were previously sterilized.

To measure the length of coleoptiles, the seedlings were placed in Petri dishes, scanned using an HP ScanJet G2710 (Hewlett-Packard, USA) and the

images were digitized in the ImageJ program (version 1.8.0_172) (https://imagej.nih.gov/ij/download.html). All plants from 50 sown seeds were examined. After scanning, 5 coleoptiles from the control and test plants were used to determine viability by the tetrazolium salt reduction test. Coleoptiles were placed in test tubes with 5 ml of 2,3,5-triphenyltetrazolium chloride (8 g/l) in 0.1 M sodium phosphate buffer (pH 6.9). The solution with pieces of plant tissues was vacuum-infiltrated at –70 Pa for 15 min and allowed overnight in a thermostat at 29 °C.

The next day, triphenylformazan (a reduced form of triphenyltetrazolium colored red) was extracted from plant tissues. Coleoptiles, washed 3 times with distilled water, were placed into 2 ml microtubes, added with 1.5 ml of 96% ethyl alcohol and heated for 10 min at 85 °C in a TDB-120 solid-state thermostat (BioSan, Latvia). Optical density was measured at $\lambda = 485$ nm on a SPECTROstar Nano spectrophotometer (BMG LABTECH GmbH, Germany). Viability was expressed in units of absorbtion A_{485} per seedling. Experiments were carried out in three biological replicates.

Statistical analysis was performed in the R language environment [26]. The mean values of the trait (M) and standard errors of the means (±SEM) were calculted. Normality was tested using the Shapiro-Wilk test. Most samples showed a non-normal distribution of lengths, so the nonparametric Mann-Whitney-Wilcoxon test was used for comparison. A t-test was used to compare tetrazolium staining effects. For multiple comparisons, p-values were corrected by FDR (false discovery rate) method. Checking whether the seedling length in hypoxia and normoxia has the same distribution patters was carried out using the Kolmogorov-Smirnov test with dissimilarity (D) and p-value calculations. The kernel density estimate of the random variable was analyzed using the Epanechnikov function. To determine the correlation, the Spearman coefficient (ρ) was calculated.

Results. According to phenotyping data, the development of *O. sativa* plants from the collection of the Federal Research Center for Rice did not depend on the country and nursery of origin (Table 1). Growth rate was assessed at the initial stages of seedling development. It is during this period that plants have to overcome a 10-centimeter layer of water formed as a result of flooding rice paddies after sowing ("shortened flooding").

The varieties Yuzhnaya noch, Natasha, Veles and HHZ8 SAL 14 SUB1, classified as low group with low growth intensity, had limited stem height (see Table 1). The tall group included tall plants of the varieties Devzra, Kirkpinar and Kuban 3. Interestingly, the tall group also included varieties that reached only half the height of the tall ones, the IR 50, Zhongyon and Zhongyon 207. However, we note that this mainly concerned varieties of foreign origin that were selected according to other criteria. In addition, plant tolerance to flooding varies greatly under different temperature conditions [27].

That is, the growth rate and stability of varieties bred in the Philippines or China can change significantly when grown in the conditions of the Krasnodar Territory. In 2022, during the sowing period (first ten days of May), the average daily temperature did not exceed 16-17 °C (https://o-pogode.ru/prognoz-may-2022/krasnodar). Perhaps this was the reason for the lack of a clear correlation between the growth rate at the initial stage and the subsequent development of rice plants (see Table 1).

The slowest growing varieties were three Philippine varieties carrying the *SUB1A* allele, the Chinese variety Xiannui and domestic varieties Amethyst, Zhemchug, Natasha, Rapan and Yuzhnaya noch. The length of their coleoptiles was apprx. 10 mm in the control and apprx. 3 mm in hypoxia that means a 2.5-3.0-fold growth suppression.

1. Characteriszation of *Oryza sativa* L. varieties and accessions of different origins from the UNU Collection of rice genetic resources of the Federal Research Center for Rice (Krasnodar) **when grown in the Krasnodar Territory** ($n = 10$, $N = 3$, $M \pm$SEM, 2019-2022)

Variety, accession	Country of origin	Year of seed reproduction	Growth rate, seed color	Days		Plant height, cm	Panicle length, cm	Spikelet number per panicle	Panicle productivity, g	1000-grain weight, g
				before flowering	vegetation					
AA WAB 56-125	Philippines	2021	B, IRCTN	84	128±3	93.0±3.5	16.9±0.4	104±6	2.2±0.2	30.2±0.2
HHZ11 Y6-Y2- SUB1	Philippines	2018	H, GAR-IRLL, IRLYN-SUB	102	140±2	73.0±2.8	15.4±0.6	56±10	1.0±0.1	21.7±0.3
HHZ8 SAL 14 SUB1	Philippines	2018	H, IRLYN-SUB	113	146±2	71.0±2.6	22.1±1.1	92±8	1.2±0.2	22.7±0.3
HHZ9 DT12 SUB1	Philippines	2018	H, GSR-Rell	104	140±2	70.0±3.5	17.4±0.3	99±7	1.3±0.2	14.0±0.1
IR 50	Philippines	2020	B, IRTON	80	118±3	66.0±2.8	17.8±0.6	82±12	1.3±0.3	18.7±1.2
IR14 L 110	Philippines	2020	C, IRLON	95	134±1	76.0±3.8	19.6±0.7	115±17	1.6±0.3	22.3±0.3
Kirkpinar	Philippines	2019	B, IRLON	84	125±2	113.0±4.1	16.2±0.5	94±6	2.7±0.4	35.2±0.5
Xiannui	China	2021	B, IRCTN	75	112±4	88.0±4.6	21.4±0.5	155±12	2.7±0.4	26.3±0.5
Zhongyon	China	2019	B, IRCTN	70	110±4	68.0±4.5	20.2±1.0	203±21	4.3±0.3	27.9±0.3
Zhongyon 207	China	2020	B, IRCTN	92	130±2	71.0±2.2	19.3±0.5	125±24	2.7±0.5	22.1±0.1
Ametist	Russia	2019	H	86	120±2	94.0±4.3	13.8±0.5	98±7	2.8±0.5	30.6±0.5
Anait	Russia	2021	H	67	102±1	90.0±7.3	18.4±0.6	78±10	2.9±0.5	37.2±0.5
Veles	Russia	2021	H	88	125±3	73.0±3.7	16.2±0.2	189±21	3.8±0.5	26.4±0.2
Viiola	Russia	2021	C, violet-grained	82	118±3	97.0±5.0	14.2±0.4	67±5	1.7±0.3	23.5±0.3
Vita	Russia	2021	B, violet-grained	70	106±4	88.0±3.3	18.3±0.4	121±13	2.6±0.6	22.1±0.3
Gamma	Russia	2019	B	77	115±4	79.0±6.1	16.0±1.0	124±12	3.4±0.6	26.8±0.5
Devzra	Uzbekistan	2020	B	70	105±2	130.0±5.4	20.5±0.7	82±7	2.6±0.4	30.7±0.5
Zhemchug	Russia	2019	B	67	102±2	90.0±4.8	13.9±0.3	88±12	2.0±0.2	24.0±0.2
Krasnoarmeisky 313	Russia	2020	B	61	98±3	105.0±2.2	15.4±0.2	76±10	1.4±0.2	30.2±0.5
Kuban 3	Russia	2021	B	63	100±4	114.0±3.5	16.0±0.4	67±15	1.6±0.2	28.7±0.5
Lider	Russia	2021	B	78	116±5	101.0±5.1	16.3±0.7	127±12	2.9±0.5	26.2±0.3
Natasha	Russia	2019	H	77	118±2	74.0±8.2	18.5±0.5	91±7	1.7±0.2	25.6±0.7

Continued Table 1

Novator	Russia	2021	B	60	98±2	83.0±3.7	15.1±0.2	86±10	2.3±0.3	24.6±0.2
Olimp	Russia	2019	B	85	122±4	89.0±3.4	15.3±0.4	130±15	3.0±0.7	23.5±0.5
PV-1 IRBLSH	Philippines	2020	B, red-grained	100	140±1	101.0±4.5	19.1±0.4	117±8	2.6±0.8	24.2±0.2
Privolny 4	Russia	2021	H	77	115±3	92.0±6.2	15.8±0.6	120±22	3.6±0.6	24.8±0.3
Rapan	Russia	2021	C	78	116±2	85.0±2.4	15.3±0.6	121±10	3.2±0.3	26.3±0.3
Regul	Russia	2021	B	82	118±2	92.0±2.7	16.5±0.5	105±15	2.5±0.3	30.4 ±0.5
Sonata	Russia	2019	H	84	120±2	87.0±2.5	14.3±0.4	97±12	2.5±0.3	26.7±0.2
Sprint	Russia	2021	B	74	106±4	87.0±7.1	18.1±1.1	135±24	3.8±0.5	27.7±0.9
Titan	Russia	2021	B	74	115±5	86.0±4.3	14.6±0.8	104±6	3.1±0.3	35.0±0.4
Ussur	Russia, Primorye	2020	B	63	98±1	86.0±6.0	19.7±0.6	122±10	3.2±0.3	29.2±0.4
Fontan	Russia	2020	B	64	102±3	100.0±5.1	22.1±1.1	102±7	2.2±0.4	26.4±0.5
Khazar	Russia	2021	H	74	110±4	89.0±3.2	17.1±0.6	103±13	2.7±0.4	26.6±0.3
Sharm	Russia	2019	B	65	100±2	84.0±4.1	19.8±0.8	102±22	2.3±0.2	26.9±0.5
Yuzhnaya noch	Russia	2021	H, violet-grained	80	120±2	68.0±3.3	16.5±0.5	96±9	2.3±0.5	21.3±0.3

N o t e. H, C, B — varieties and variety forms with low, medium and high growth rates, respectively, at the early stage of ontogenesis. All varieties are white-grained, with the exception of those for which a different grain color is indicated. IRLON is shortened flooding, IRCTN is cold-resistant nursery, IRTON is artificially irrigated, temperate rice, GARIRLL is irrigated lowland, green technology nursery; GSR-Rell is irrigated, super-rice, green technology nursery; IRLYN-SUB is deep-water rice.

It should be noted that of the five varieties that have anthocyanin-colored grains, i.e., Viola, Vita, PV-1 IRBLSH, Natasha and Yuzhnaya noch, the varieties Natasha and Yuzhnaya noch were turned out to be slow-growing. The slowest growing variety was Yuzhnaya noch, in which coleoptiles grew to 4.0 mm in the control and up to 2.5 mm in the test. Thus, our study for the first time revealed a slowdown in the growth of coleoptiles with a lack of oxygen for the varieties examined. However, the intensity of suppression under flooding was consistent with the native growth rate of rice seedlings in control.

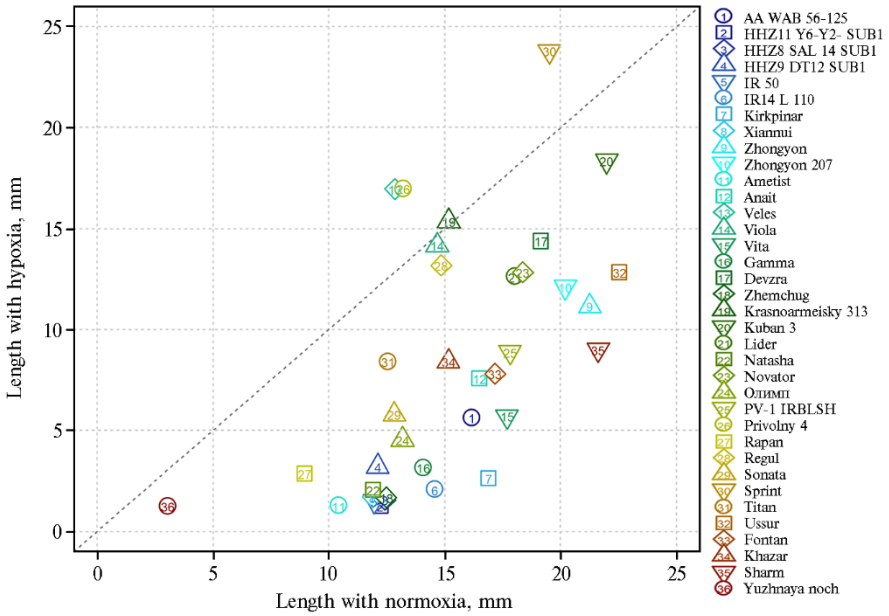

Fig. 1. Sample median of seedling coleoptile lengths in rice (*Oryza sativa* L.) varieties of different origins from the UNU Collection of rice genetic resources (the Federal Research Center for Rice, Krasnodar) **4 days after germination under normoxia and hypoxia** (lab test). The diagonal line corresponds to equal lengths for anoxia and hypoxia.

For most varieties and accessions, showed a decrease in the median (below the diagonal corresponding to equality) of coleoptile length during oxygen starvation (see Fig. 1), only in the varieties Viola and Krasnoarmeysky 313 the differences between normoxia and hypoxia were unreliable (Table 2). Under flooding, plant growth was most suppressed in HHZ11 Y6-Y2-SUB1, HHZ8 SAL 14 SUB1, IR14 L 110, IR 50, Xiannui, Amethyst, Zhemchug, Natasha and Yuzhnaya noch. Three varieties (Sprint, Veles and Privolny 4) showed a statistically significant ($p \le 0.05$) increase in length under hypoxia (see Fig. 1, Table 2). It should also be noted that there is a positive correlation between the length of the coleoptile under normoxia and hypoxia ($\rho = 0.70$; $p = 10^{-6}$), that is, forms that actively grow in an aerobic environment also grew rapidly under flooding.

2. The difference reliability for the length of seedling coleoptiles in rice (*Oryza sativa* L.) varieties of different origins from the UNU Collection of rice genetic resources (the Federal Research Center for Rice, Krasnodar) **under normoxia, hypoxia and by the tetrazolium test** ($n = 5$, $N = 3$, $M \pm$SEM, lab test)

Variety, accession	Test values		Tetrazole test, rel. unts	
	Mann-Whitney-Wilcoxon	FDR	normoxia	hypoxia
AA WAB 56-125	4.32E–20	1.30E–19	0.077±0.007	0.008±0.001
HHZ11 Y6-Y2-SUB1	2.61E–08	3.91E–08	0.013±0.002	0.004±0.001
HHZ8 SAL 14 SUB1	8.05E–11	1.26E–10	0.027±0.005	0.005±0.001
HHZ9 DT12 SUB1	1.66E–13	3.41E–13	0.012±0.001	0.004±0.001

Continued Table 2

IR 50	5.52E–47	1.99E–45	0.050±0.012	0.006±0.001
IR14 L 110	1.15E–20	3.77E–20	0.131±0.017	0.008±0.0001
Kirkpinar	5.90E–31	1.06E–29	0.502±0.014	0.007±0.001
Xiannui	1.25E–17	2.99E–17	0.046±0.007	0.004±0.001
Zhongyon	3.46E–11	5.66E–11	0.269±0.013	0.005±0.001
Zhongyon 207	1.77E–14	3.99E–14	0.087±0.010	0.005±0.001
Ametist	4.94E–29	5.93E–28	0.057±0.009	0.006±0.001
Anait	6.64E–22	2.39E–21	0.083±0.011	0.006±0.001
Veles	2.69E–05	3.34E–05	0.090±0.005	0.008±0.001
Viiola	0.193686*	0.193686*	0.094±0.019	0.010±0.001
Vita	1.56E–18	4.02E–18	0.160±0.019	0.010±0.001
Gamma	5.98E–24	3.07E–23	0.221±0.018	0.010±0.001
Devzra	4.14E–07	5.74E–07	0.550±0.009	0.006±0.001
Zhemchug	5.05E–19	1.40E–18	0.275±0.025	0.005±0.001
Krasnoarmeisky 313	0.134287*	0.138124*	0.148±0.007	0.015±0.002
Kuban 3	0.000117	0.00014	0.172±0.009	0.017±0.001
Lider	4.98E–06	6.64E–06	0.232±0.010	0.010±0.001
Natasha	4.47E–27	3.22E–26	0.050±0.006	0.008±0.001
Novator	8.66E–08	1.25E–07	0.069±0.009	0.010±0.001
Olimp	2.94E–28	2.64E–27	0.201±0.043	0.006±0.001
PV-1 IRBLSH	6.87E–13	1.18E–12	0.028±0.003	0.010±0.001
Privolny 4	0.000828	0.000932	0.334±0.015	0.011±0.001
Rapan	3.53E–13	6.68E–13	0.020±0.004	0.004±0.001
Regul	0.001238	0.00135	0.160±0.006	0.010±0.001
Sonata	2.91E–22	1.31E–21	0.149±0.021	0.008±0.001
Sprint	1.58E–05	2.03E–05	0.129±0.024	0.011±0.001
Titan	0.004084	0.004324	0.092±0.013	0.014±0.001
Ussur	1.71E–13	3.41E–13	0.453±0.014	0.006±0.001
Fontan	2.34E–24	1.40E–23	0.107±0.022	0.010±0.001
Khazar	5.18E–13	9.33E–13	0.268±0.011	0.006±0.001
Sharm	5.55E–22	2.22E–21	0.234±0.005	0.011±0.001
Yuzhnaya noch	0.000305	0.000354	0.061±0.013	0.009±0.002

N o t e. FDR — false discovery rate. For all varieties, the normoxia and hypoxia variants in the tetrazolium test had statistically significant differences (*t*-test, $p \leq 0.05$).

* Differences in conditions of normoxia and hypoxia are unreliable.

An adaptive strategy at the population level may involve changing distributions of trait values in response to changing environmental conditions. Histograms of probability density estimates for the rice seedling coleoptile lengths under various aeration conditions indicate that growth changes corresponded to several patterns.

The most common response (Fig. 2, pattern I) was a shift in the distribution to small length values (an increase in the asymmetry coefficient). Under hypoxia, histograms showed a high peak at small, close to zero values, in combination with an extended "tail" of values on the right. This indicates that the most common response to hypoxia in rice coleoptiles in this group was growth suppression, with a small portion of plants remaining continuing to grow. This strategy is apparently associated with the expectation of more favorable conditions and is close to the LOQS resting strategy. Moreover, it was this group that included all the Philippine varieties with the *SUB1A* allele which are the most prominent representatives of this adaptation strategy.

Pattern II (see Fig. 2) consisted of a decrease in the sharpness (kurtosis) of the probability distribution of length values under hypoxia. A significant proportion of this was made up of plants that were so large that there were none or almost none under normoxia. That is, in response to stress, the population distributed risks evenly across all possible options, which may allow at least some individuals to survive in an uncertain situation.

Pattern III was a combination of the previous two. Distributions during hypoxia became smoothed and shifted to the left. At the same time, most of them were concentrated in the area of small values, as well as in the area of values characteristic of normoxia. Approximately half of the plants slowed down, while

the other part continued to grow as under normoxia. Consequently, at the population level, the proportion of losses that depends on the choice of strategy can be recorded.

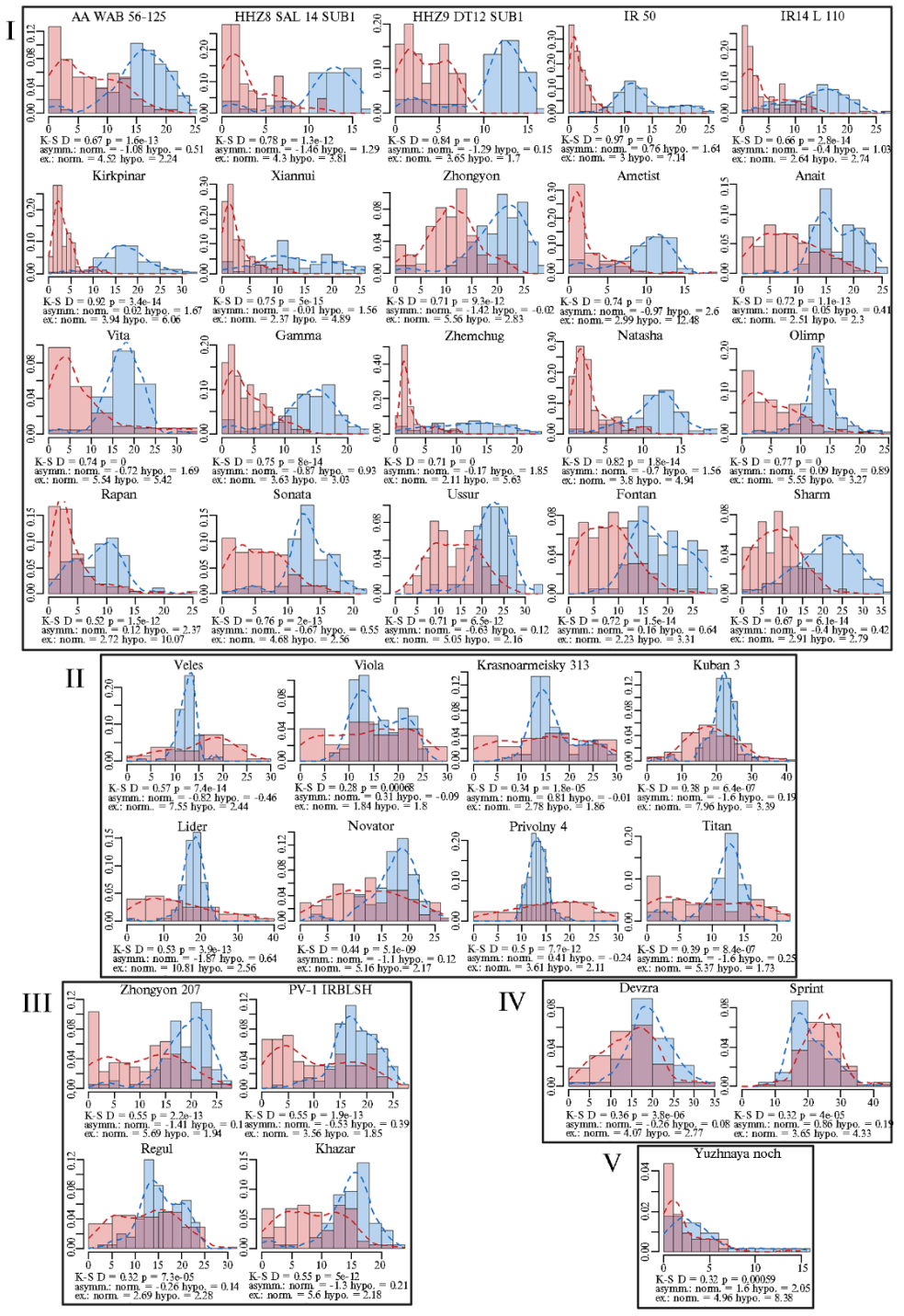

Fig. 2. Histograms of the seedling coleoptile length distribution in rice (*Oryza sativa* L.) varieties of different origins from the UNU Collection of rice genetic resources (the Federal Research Center for Rice, Krasnodar) **4 days after germination under normoxia** (blue) **and hypoxia** (red)**:** I-V — response patterns. Abscissa is length, mm, ordinate is probability density. Dashed lines are kernel density estimate with Epanechnikov function, K-S is Kolmogrov-Smirnov test, asymmetric. is asymmetry coefficient, ex. is kurtosis coefficient, norm. is normoxia, hypo. is hypoxia

Plants of the IV pattern showed the least changes in distribution. In the Devzra variety, there was a slight decrease in the spiciness of the distribution and a shift to the left which is similar to pattern III. In turn, we revealed a unique response in the Sprint variety, i.e., a clear shift to the right in the length distribution. That is, only this variety showed signs of LOES associated with a slight increase in growth. (see Fig. 1, 2). The Yuzhnaya noch also had a unique growth pattern (V pattern), the coleoptiles grew slowly both in the control and under flooding (see Fig. 2).

We can conclude that the growth response of coleoptiles that we identified corresponded to the onset of adaptation processes already at the first stages of ontogenesis during rice seedling development, and the patterns of changes in the growth response reflected the intensity of manifestation of one or another adaptation strategy.

It is well known that coleoptiles increase in length through cellular elongation growth [28]. Such growth, like any other, requires energy expenditure, but it is the cell's energy resources that are limited when there is a lack of oxygen. The diversity of growth response patterns is apparently due to changes occurring in the plant metabolism during hypoxia. Oxygen starvation leads to energy deficiency [3, 6]. Oxidative phosphorylation is inhibited, and the production of reactive oxygen species (ROS) is reduced. The only source of ATP is glycolysis, which turns into fermentation. Cytoplasmic acidosis and accumulation of toxic metabolic products, in particular acetaldehyde and ethanol, are triggered [1, 3, 6]. In addition, hypoxia inhibits the biosynthesis of protein and other polymers [6], induces the generation of reactive oxygen and nitrogen species [6, 29, 30] which cause oxidative damage to lipids and proteins [31, 32]. As discussed earlier, rice seedlings spend their meager energy reserves from starch metabolization depending on their tolerance strategy, plants with an avoidance strategy (LOES) spend on stimulating growth, and those with a growth inhibition strategy (LOQS) on maintaining cellular structures [2-6, 11, 12].

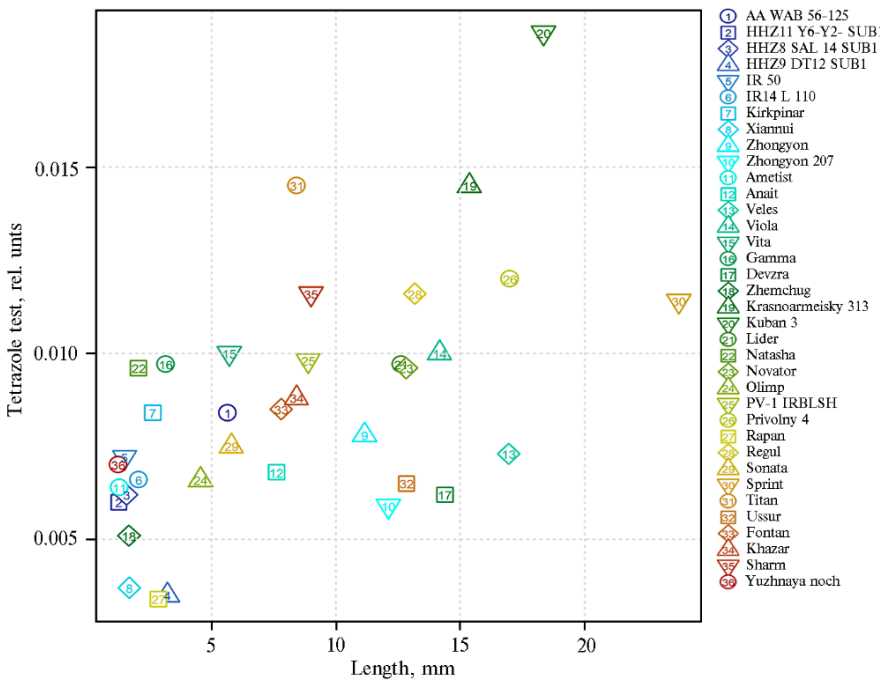

Fig. 3. Relationship between sample median of seedling coleoptile lengths in rice (*Oryza sativa* L.)

varieties of different origins from the UNU Collection of rice genetic resources (the Federal Research Center for Rice, Krasnodar) **4 days after germination under hypoxia with the results of the tetrazolium test.**

Thereof, we next focused on assessing the viability of coleoptiles in the tetrazolium test which assesses the metabolic rate. It is known that the more intense the color that develops in the test with tetrazolium salts, the higher the viability. In all tested forms, viability according to the tetrazolium test was significantly reduced under hypoxic conditions compared to the control (*t*-test, $p \leq 0.05$, see Table 2). However, in slow-growing forms this decrease was not as strong as in fast-growing forms, which may be due to both the metabolic rate and the chosen adaptation strategy. The tetrazolium test characterizes the metabolic activity of tissues. In fast-growing forms, metabolism is intense, and under flooding, its inhibition was significant (by 10-20 times). In slow-growing forms, it is already slowed down, and its decrease during hypoxia was not so apparent, but in all cases statistically significant (by 7 times). Moreover, a positive relationship is shown between the intensity of tetrazolium staining and the length of the coleoptile ($\rho = 0.69$; $p = 10\ 5$), especially under hypoxia (Fig. 3). In the fastest-growing varieties (Sprint, Kuban 3, Krasnoarmeysky 313), the intensity of tetrazolium staining was higher both in the control (by 6 times) and in the test (by 2 times) compared to slow-growing forms (Amethyst, Rapan, Yuzhnaya noch, Xiannui, Philippine *SUB1A* varieties).

Variation in tetrazolium staining intensity has previously been used to test the viability of wheat and rice seedlings that differ in their tolerance to oxygen deficiency [33, 34]. It is shown that the test is applicable to assess the stability of the aboveground part. Rice shoots are less damaged under oxygen starvation.

Thus, the tested rice accessions from the collection of the Federal Research Center for Rice differed significantly in growth rate. In field trials, we identified three groups of varieties with high, medium and low growth rates at the germination stage. It was during this period that the developing seedlings were flooded. However, the growth intensity at the early ontogenesis did not correlate with the height of the adult plant, which can be explained by the lack of standardization of the germination stage (7 days), as well as the imposition of an additional stress factor, e.g., the low temperature (14 °C). The lab analysis of the coleoptile growth on day 4 showed that the fastest growing varieties were Sprint and Kuban 3, the length of their coleoptiles reached 19-25 mm both in the control and under flooding. Note that the domestic variety Kuban 3 reached its maximum height in field studies. Kuban 3 was assigned to pattern II type when analyzing the distribution of coleoptile lengths in seedlings of different varieties. The Sprint variety was assigned to pattern IV. The slowest growing domestic varieties were Amethyst, Zhemchug, Natasha, Rapan and Yuzhnaya noch. In the control, their coleoptile reached 10 mm, and in hypoxia apprx. 3 mm. All these varieties were assigned to pattern I togather with the Philippine varieties carrying the *SUB1A* allele and exhibiting a growth inhibition strategy (LOQS). Thus, our results indicate for the first time the variability of adaptation mechanisms which are reflected in growth rates already at the early stages of ontogenesis. Staining of coleoptile tissues with tetrazolium salts indicates inhibition of metabolism due to lack of oxygen. In fast-growing varieties, e.g., Kuban 3 and Sprint, the staining was 6 times more intense than in the slow-growing Amethyst, Rapan, Southern Night, Xiannui, and Philippine *SUB1A* varieties. Our data allow us to conclude that the growth rate of coleoptiles correlates with the intensity of metabolism and resistance to flooding. Unfortunately, the mechanisms of coleoptile growth under oxygen deficiency have not yet been fully established. Continued research is required to

understand which resistance strategies the tested varieties belong to and what factors determine their possible relationship. However, the presented results have already characterized the resistance to oxygen deficiency of the genotypes stored in the collection of the Federal Research Center for Rice.

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INTRA-CALLUS VARIABILITY FOR RICE BLAST RESISTANCE GENES IN *Oryza sativa* L. INDICATED BY GENETIC ANALYSIS OF ANDROGENIC DOUBLED HAPLOIDS

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Abstract

In vitro culture of cells and tissues of agricultural crops can be conditionally divided into two groups, those to generate a genetically modified initial breeding material and those for mass cloning of existing forms and varieties. Androgenesis in vitro makes it possible to redirect the microspore development from the gametophytic to the sporophytic pathway with the formation of doubled haploids (DHs) in diploid species or the fixation of dihaploids (polyhaploids) in tetraploid species for their wide use in plant breeding. The variability of plants derived from anther or microspore cultures of one donor plant has been studied to a greater extent at the genomic and chromosomal level, since researchers and breeders are primarily interested in spontaneous chromosome duplication and, as a result, completely homozygous fertile offspring. In this work, for the first time, the frequency of intra-callus genetic variability for *Pi* family blast resistance genes (two and three genes) was estimated using rice (*Oryza sativa* L.) doubled haploids (DHs) obtained via androgenesis in vitro of hybrid plants. No significant increase in intra-callus genetic variability was shown with an increase in the number of detected genes. The intra-callus variability frequency in androgenesis in vitro in rice was studied in order to determine the genetic homogeneity degree of doubled haploids (DHs) from one anther. Studies were carried out on doubled haploids obtained in androgenesis in vitro of thirteen F_1 hybrids and one F_2 hybrid of rice *O. sativa*. Molecular genetic analysis of 1271 plants (83 callus lines) was performed to reveal resistance/susceptibility alleles of the genes *Pi-z*, *Pi-b*, *Pi-1*, *Pi-2*, *Pi-ta* for rice blast-resistance to *Pyricularia oryzae* Cav. [*Magnaporthe grisea* (Hebert Barr.)]. In doubled haploids, one to four blast-resistance genes were identified depending on the presence of heterozygotes in the original hybrids. When determining one gene in DHs, the frequency of variable callus lines accounted for 24.0 %. For two genes, polymorphism occurs among 47.7 % of calli. For three genes, 62.5 % of callus lines were polymorphic. No more than four combinations of rice blast resistance gene alleles are present in one callus line. There are no differences in the monomorphic callus lines frequency detected for one, two and three genes (χ^2 = 0.21-0.95, p = 0.33-0.65). With the same combination of two resistance gene alleles, up to 66 plants were formed, and with the same combination of three genes alleles, up to 18 plants were produced per callus line. There was no dependence of polymorphism on the number of doubled haploids in the callus line. The correlation coefficients between the number of DHs and the number of alleles for one, two and three genes in the combination accounted for $r = -0.14$, $r = 0.25$, and $r = -0.35$ ($p < 0.05$). Genetic analysis of rice doubled haploids revealed a low intra-callus genetic variability during in vitro androgenesis due to gametoclonal variability. Thus, the polymorphic callus lines frequency is high, but with a limited set of allele combinations of rice blast resistance genes among DHs. There is true cloning of rice doubled haploids within the callus lineage in androgenesis in vitro. However, due to the DHs polymorphism within one callus, it is expedient to select lines of doubled haploids as breeders usually do. This work is relevant for optimizing the breeding process, including haploid technology.

Keywords: *Oryza sativa*, doubled haploids, intra-callus genetic variability frequency, blast resistance genes

In vitro cultures of cells and tissues of agricultural crops can be divided into two groups according to their intended purpose, i.e., those for obtaining genetically modified initial breeding material and for mass cloning of existing forms and varieties. Anther culture (androgenesis in vitro) allows you to switch the microspore development program from the gametophytic path to the sporophytic one. Spontaneous doubled haploids (DHs) appeared in diploid species or dihaploids (polyhaploids) fixed in teraploid species are widely used in plant breeding [1-3]. Variation among plants regenerated from gametes is commonly called gametoclonal [1, 4]. Strictly speaking, these regenerants are not clones of the donor plant genotype because of the lack of complete genetic identity due to recombinations that occur during the microspore formation. In fact, each microspore is a new genotype, albeit with the same combination of genes. In addition, variability can be induced by the in vitro cultureing itself [4, 5].

The variability generated in the culture of anthers or microspores from one donor plant has been studied to a greater extent at the genomic and chromosomal level, since researchers are primarily interested in spontaneous chromosome duplication and, as a consequence, completely homozygous fertile offspring. The proposed main mechanisms of genomic duplication are endoreduplication, nuclear fusion, endomitosis, and C-mitosis [6, 7]. D.E. Daghma et al. [8] used time-lapse imaging technology and detected only nuclear fusion in barley anther culture. Indirect methods have shown the emergence of regenerants from unreduced 2*n* gametes [9]. The origin of wheat haploids from a single cell has been proven [10]. In direct embryogenesis from microspores, all plants a priori differ genotypically. As a result, among the androgenic regenerants of one donor plant, multiple and non-multiple changes in the number of chromosomes [11-13], molecular genetic variability [14], variability in valuable traits [11, 15] and resistance to pathogens [13, 16, 17] have been identified.

In in vitro anther culture, rice *Oryza sativa* L. undergoes an additional stage of callus formation from which regenerant plants derive [18, 19]. During microsporogenesis, a highly vacuolated microspore of cereals undergoes symmetrical mitotic division with the formation of two equal cells which undergo the stage of callus formation by mitosis in vitro and implement different morphogenesis pathways [20]. In this case, additional variability of cells and regenerants arises, associated with the phenomenon of somaclonal variability [21]. Haploid, diploid, tetraploid, hexaploid and octaploid cells were identified in rice calli. Long passages reduced the number of haploid cells and the cells with high ploidy levels [22]. During prolonged calli culture, the proportion of diploid and high ploidy regenerants increases and deviations from the normal distribution of agronomically important rice traits [23] and from expected segregation for molecular markers [24] occur. Moreover, if the culture of barley microspores produces up to 50 regenerants per anther [25], then the rice callus, if formed, in some cases produces several hundred haploid plants or more than 120 doubled haploids [26]. There are calli (17%) with small numbers (up to 18) of tetraploids or seedless non-haploid regenerants [27]. On the rice variety Cascade, intracallus morphological variability of plants derived from calli with multiple regeneration was investigated ex vitro. The study revealed a significant variability of haploids and monomorphism of doubled haploids for biometric parameters [28]. However, there were several callus lines of the hybrid rice plant F_1, where, among the doubled haploids of one callus line, variability in awning and anthocyanin coloring of the stem was demonstrated, and molecular genetic variability

was detected in the rice genes *Pi-ta* and *Pi-ta2* for resistance to *Pyricularia oryzae* Cav. [*Magnaporthe grisea* (Hebert Barr.)] [29]. Theoretically, a callus line can be formed by one or more microspores. In rice, more than 1000 pollen grains mature in the anther [30]. Studying the frequency of intra-callus variability occurrence provides information on the degree of genetic homogeneity of doubled haploids from the same anther.

The variability of plants obtained via the culture of anthers or microspores from one donor plant has been studied to a greater extent at the genomic and chromosomal level, since researchers are primarily interested in spontaneous chromosome duplication and, as a consequence, completely homozygous fertile offspring. However, to optimize the selection process, including haploid technology (especially with heterotic selection), it is important to assess genetic homogeneity of regenerated plants and to understand the patterns of its change.

In this work, for the first time, we assessed the frequency of intra-callus genetic variability for two and three blast resistance genes of the *Pi* family in rice *O. sativa* doubled haploids derived from hybrid plants of various crossing combination by androgenesis in vitro. It was shown that there was no significant increase in intra-callus genetic variability with an increase in the number of detected genes.

Materials and methods. Doubled haploids of *Oryza sativa* L. subspecies *japonica* Kato generated by in vitro androgenesis were studied for F_1 rice hybrids Lugovoi × Maratelli 5A (L×5A), Rassvet × (Oxy 2x × Dary 23) (P×O×23), Almaz × [(Maratelli 5A × Boyarin) × Maratelli 5A] (4P), 242-01 × Rassvet (242×P), Dolinny × Magnat (D×M, two plants); Dubrava × Viola (Db×V), Dolinny × Maratelli 5A (D×5A, two plants); Almaz × Magnat (A×M), Kaskad × [(Dary 8 × Hayayuki) × Slavutich] (K×3R). Lugovoi × [(Dary 8 × Khayayuki) × Slavutich] (L×3R), Kaskade × (Auguazta × Othello No. 1) (K×2P) and hybrid F_2 Rassvet × Oxy 2x (P×O). The original plants of the hybrids vegetated in containers on the growing site until the panicle collection. Before introducing anthers into in vitro culture, F_1 and F_2 plants heterozygous for rice blast resistance genes of the *Pi* family were selected by molecular labeling.

The anthers were cold treated at 5 °C for 7 days. N6 medium was used to induce callus formation [31], a modified N6-based nutrient medium [32] for regeneration. For rooting, regenerants were planted onto MS medium with half the mineral composition modified by Yu.K. Goncharova [33]. Anthers were cultured in the dark at 25-27 °C, calli and regenerants were grown in a culture room at 25 °C, 5000 lux lighting and 16 h daylight.

2-5 mm callus aggregates (calli) were transplanted from the induction nutrient medium to the regeneration medium with a 7 day interval and assigned a serial number.

R_0 regenerants with a developed root system were planted in vegetation pots and grown in a culture room until seeds formed.

DNA was isolate from fresh plant leaves by salting-out method [34]. The DNA concentration was determined in a volume of 1 μl (a BioSpec-nano spectrophotometer, Shimadzu, Japan).

In PCR, the nucleotide sequence of the forward and reverse primers, the annealing temperature, and the size of the target product of the studied genes were as described [32, 33]. The reaction was run in a 25 μl reaction mixture containing 10× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μl of forward and reverse primers, 1 unit. Taq DNA polymerase (Synthol LLC, Russia) and 70-120 ng of DNA of the studied samples. Temperature response profiles varied among genes.

PCR protocol for the *Pi-1* and *Pi-2* genes was 5 min at 94 °C for initial denaturation; 35 cycles: denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C, elongation for 35 s at 72 °C; 3 min at 72 °C for final elongation. For the *Pi-z*, *Pi-b* and *Pi-ta* genes: initial denaturation for 1 min at 96 °C; 35 cycles including for denaturation 15 s at 94 °C, annealing for 30 s at 60 °C, elongation for 2 min at 72 °C; final elongation for 5 min at 72°C. Amplification was run in 3 repetitions (an MJ Mini thermal cycler, Bio-Rad, USA). Plants of differentiator varieties and known varieties with resistance alleles of the target genes served as control.

Amplification products were separated electrophoretically in a 1.4% agarose gel based on 0.5× TBE buffer (an SE-1 electrophoresis chamber, OOO Helicon Company, Russia; an Elf-4 power source, DNA-Technology LLC, Russia). Bands were pre-stained with 1.0% ethidium bromide for UV-visualization (the Gel Doc XR+ gel documentation system, Bio-Rad, USA).

Characterization of the callus lines of the hybrid was based on the mean (*M*), maximum (max) and minimum (min) number of doubled haploids per callus. The frequency of intra-callus genetic variability was expressed as the proportion of polymorphic calli (%) for one gene in hybrids P×O×23, P×O, K×3P, L×3P (one or two combinations of alleles). In hybrids L×5A, 4P, 242×P, K×2P, D×5A(1), D×5A(2), which were analyzed for two genes, the proportion of calli (%) with one to four allele combinations was determined. In hybrids 242×P, D×M(1), D×M(2), Al×M, one to eight expected combinations of alleles for three genes were identified and the proportion of polymorphic calli (%) was calculated. Differences in the frequency of monomorphic callus lines detected by one, two and three genes were determined by the χ^2 test. The correlation coefficient (r) between the number of doubled haploids and the number of allele combinations per callus was calculated. The obtained data were processed in the Statistica 10 program (StatSoft, Inc., USA).

Results. The compositions of the media used are given in Table 1.

1. Compositions of nutrient media for in vitro androgenesis of rice (*Oryza sativa* L.)

Ingredient	Concentration per 1 l medium		
	induction, N_6	regeneration, $N_{6\text{-}pk}$	rooting, MS
Macrosols, ml	50.0	50.0	25.0
Microsalts, ml	1.0	1.0	1.0
Iron chelate, ml	5.0	5.0	5.0
Thiamine, mg	10.0	1.0	Absent
Pyridoxine, mg	0.5	0.5	Absent
Nicotinic acid, mg	0.5	0.5	Absent
Glycine, mg	2.0	2.0	Absent
2,4-Dichlorophenoxyacetic acid, mg	2.0	Absent	Absent
Naphthylacetic acid, mg	Absent	Absent	0.25
6-Benzylaminopurine, mg	Absent	1.0	Absent
Kinetin, mg	Absent	1.0	Absent
Sucrose, g	30.0	60.0	20.0
Agar-agar, g	8.0	8.0	8.0
pH	5.8	5.8	5.8

Plants of differentiator varieties and known varieties with resistance alleles of target genes were used as controls (Table 2).

Molecular genetic analysis confirmed the heterozygous state for alleles of blast resistance genes (Table 3) in hybrid plants. From the anthers of hybrid plants, 83 callus lines with three or more DHs were formed (the term “callus line” means all callus aggregates formed from one anther). A total of 1314 doubled rice haploids were obtained and 1271 plants were analyzed (96.7% of all DHs). One callus line produced up to 70 doubled haploids (see Table 3).

2. Molecular markers to identify blast resistance genes in androgenic doubled haploids of rice (*Oryza sativa* L.)

Gene	Marker		Primer nucleotide sequence 5′→ 3′	Resistance allele size, bp	Primer annealing temperature, °C	Reference	Control variety
Pi-1	Rm224	F	ATCGATCGATCTTCACGAGG	158	56	[32]	Magnat
		R	TGCTATAAAAGGCATTCGGG				
Pi-2	Rm527	F	GGCTCGATCTAGAAAATCCG	233	56	[32]	Magnat
		R	TTGCACAGGTTGCGATAGAG				
Pi-b	Pi-b	F	CATCAACGAAGTCCAGCTCA	490	60	[32]	Oxy 2x
		R	CCGCGCTATCTTGTACATTC				
		R	CTCAGCATATGTGGCAGCTC				
Pi-ta	Pi-ta	F1	GCCGTGGCTTCTATCTTTACCTG	270	60	[32]	Differentiator CD 8 Pi No 4
		F2	TTGACACTCTCAAAGGACTGGGAT				
		R1	ATCCAAGTGTTAGGGCCAACATTC				
		R2	TCAAGTCAGGTTGAAGATGCATAGA				
Pi-z	Z56592	F	GGACCCGCGTTTTCCACGTGTAA	292	60	[33]	Maratelli 5A
		R	AGGAATCTATTGCTAAGCACGAC				

3. Characterizqtion of androgenic callus lines with doubled haploids (DHs) derived from rice (*Oryza sativa* L.) hybrids with different blast resistance genes

Hybrid	Number of callus lines	Genes	Number of DHs		
			M	min	max
P×O×23	2	*Pi-ta*	10.5	7	14
P×O	20	*Pi-b*	16.5	4	69
K×3P	3	*Pi-ta*	5.3	5	6
Л×3P	4	*Pi-ta*	13.0	3	25
Л×5A	6	*Pi-z, Pi-ta*	8.8	3	22
4P	9	*Pi-z, Pi-ta*	15.8	5	43
242×P	1	*Pi-1, Pi-2*	31.0	31	31
K×2P	21	*Pi-2, Pi-ta*	16.8	4	70
Д×5A(1)	3	*Pi-2, Pi-ta*	14.3	3	26
Д×5A(2)	4	*Pi-2, Pi-ta*	17.5	3	55
242×P	3	*Pi-z, Pi-1, Pi-2*	8.3	3	17
Д×M(1)	1	*Pi-1, Pi-2, Pi-ta*	20.0	20	20
Д×M(2)	1	*Pi-1, Pi-2, Pi-ta*	15.0	15	15
A×M	3	*Pi-z, Pi-1, Pi-2*	5.7	4	8
Дb×B	2	*Pi-z, Pi-1, Pi-2, Pi-ta*	5.0	3	7

N o t e. *M*, min, max — the mean, minimum and maximum number, respectively, of doubled haploids in a callus line.

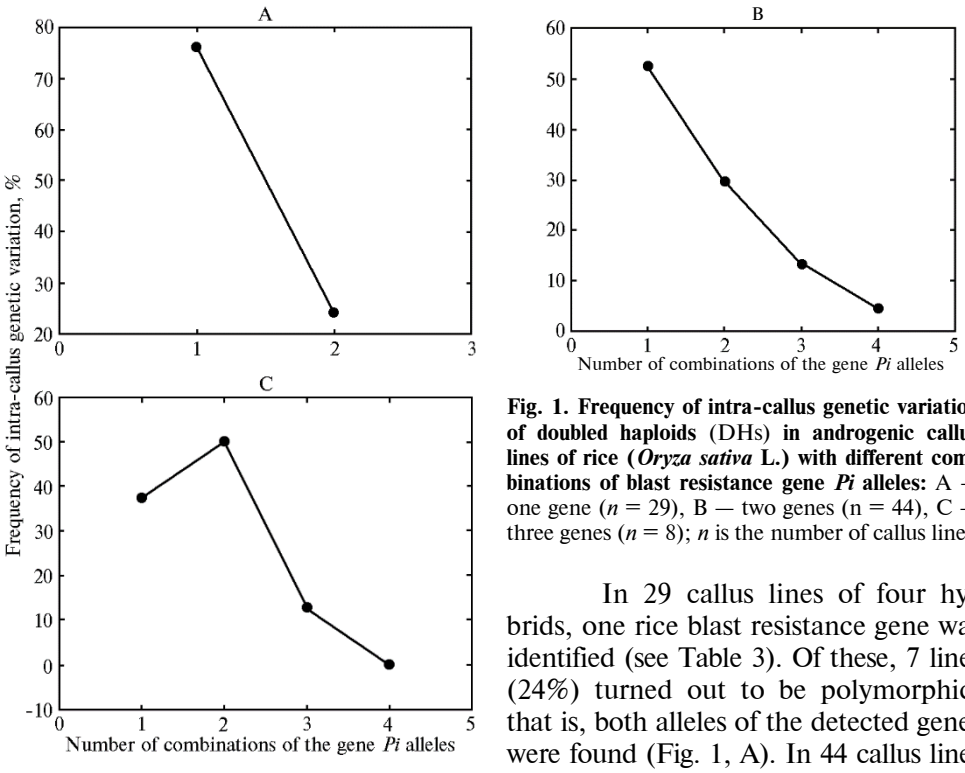

Fig. 1. Frequency of intra-callus genetic variation of doubled haploids (DHs) **in androgenic callus lines of rice (*Oryza sativa* L.) with different combinations of blast resistance gene *Pi* alleles:** A — one gene ($n = 29$), B — two genes (n = 44), C — three genes ($n = 8$); n is the number of callus lines.

In 29 callus lines of four hybrids, one rice blast resistance gene was identified (see Table 3). Of these, 7 lines (24%) turned out to be polymorphic, that is, both alleles of the detected genes were found (Fig. 1, A). In 44 callus lines of six hybrids, two *Pi* genes were detected (see Table 3). Of the four possible combinations of resistance and susceptibility gene alleles, all four were found in only two callus lines (see Fig. 1, B); more than half of the calli were monomorphic. In eight callus lines of four hybrids, alleles for three genes were identified (see Table 3). Theoretically, eight combinations of alleles are possible for three genes. However, we found the largest number of combinations (three) in doubled haploids of one callus line; three out of eight calli were monomorphic (see Fig. 1, B). In the Db×B hybrid, two callus lines with four blast resistance genes were formed (see Table 3). In both lines, doubled haploids had only two combinations of Pi resistance/susceptibility gene alleles out of a possible 15 (Table 4). There were no differences in the frequency of monomorphic

callus lines detected for one, two and three genes ($\chi 2 = 0.21$-0.95, $p = 0.33$-0.65).

4. Examples of intra-callus variability for blast resistance genes in doubled haploids (DHs) derived from androgenic callus lines of rice (*Oryza sativa* L.) hybrids

Hybrid	Callus line	Number of doubled haploids.	Gene combination
By two genes:			
K×2P	95.2	6	*Pi-2* (–), *Pi-ta* (–)
		3	*Pi-2* (–), *Pi-ta* (+)
K×2P	99.2	66	*Pi-2* (+), *Pi-ta* (+)
		4	*Pi-2* (–), *Pi-ta* (–)
D×5A(2)	112.2	55	*Pi-2* (+), *Pi-ta* (+)
By three genes:			
Al×M	564.2	1	*Pi-z* (+), *Pi-1* (+), *Pi-2* (–)
		1	*Pi-z* (–), *Pi-1* (+), *Pi-2* (–)
		3	*Pi-z* (–), *Pi-1* (+), *Pi-2* (+)
242×P	582.2	17	*Pi-z* (–), *Pi-1*(–), *Pi-2* (+)
By four genes:			
Db×B	610.2	1	*Pi-z* (+), *Pi-1* (+), *Pi-2* (–), *Pi-ta* (+)
		6	*Pi-z* (–), *Pi-1* (+), *Pi-2* (+), *Pi-ta* (–)

N o t e. «+» — a resistance allele, «–» — a susceptibility allele.

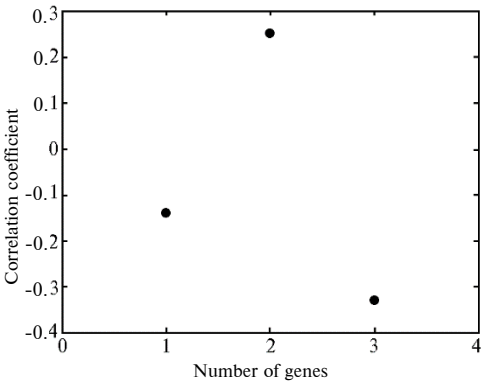

Fig. 2. Correlation coefficients (r) between the number of blast resistance genes and the number of doubled haploids (DHs) in androgenic callus lines of rice (*Oryza sativa* L.) hybrids: 1 — one identified gene ($n = 29$), 2 — two identified genes ($n = 44$), 3 — three identified genes ($n = 8$); n is the number of callus lines. The correlation coefficients are statistically significant at $p < 0.05$.

In the experiment, we used callus lines with multiple regeneration (see Table 3). There was no dependence of polymorphism on the number of doubled haploids in the callus line (Fig. 2). Insignificant negative correlation coefficients indicate that the search for polymorphic callus lines is best carried out among lines with a small number of doubled haploids. Multiple regeneration is more likely to result in the replication of identical DHs genotypes.

Nineteen callus lines formed from two to four callus aggregates with green regenerants. Analysis of the data from callus line 415.2 of the L×5A hybrid showed that the resistance alleles of the two genes *Pi-z* and *Pi-ta* were detected in all doubled haploids of the first callus aggregate, and the second callus aggregate had the resistance allele *Pi-z* and the susceptibility allele *Pi-ta*. This means that callus aggregates are initiated by different immature microspores, indicaying the phenomenon of gametoclonal variability.

Figure 3 illustrates the production of regenerated rice plants from calli based on androgenic doubled haploids.

In haploid breeding, it is important to generate the greatest possible variety of doubled haploids per plant. This can be achived through active callus formation and a large number of induced immature microspores. For species capable of direct embryogenesis, microspore culture provides up to 50 regenerants per anther [25]. Rice is a species that forms callus during androgenesis in vitro [18], and inoculation of microspores on nutrient media is unsuccessful [37, 38]. When ripe, the anther of rice opens on both terminal parts [39]. We have repeatedly observed the beginning of callus formation in vitro at opposite ends of the anther. Subsequently, these callus aggregates grew to a size suitable for transplantation into a regeneration nutrient medium. Visually, they can be mistaken for a single callus aggregate. In general, a limited number of polymorphic callus lines with three or

more gene combinations was noted (see Fig. 1, B, C). Possibly, the induction of callus formation on one microspore inhibits the development of neighboring microspores, but does not interfere with their development at the terminal ends.

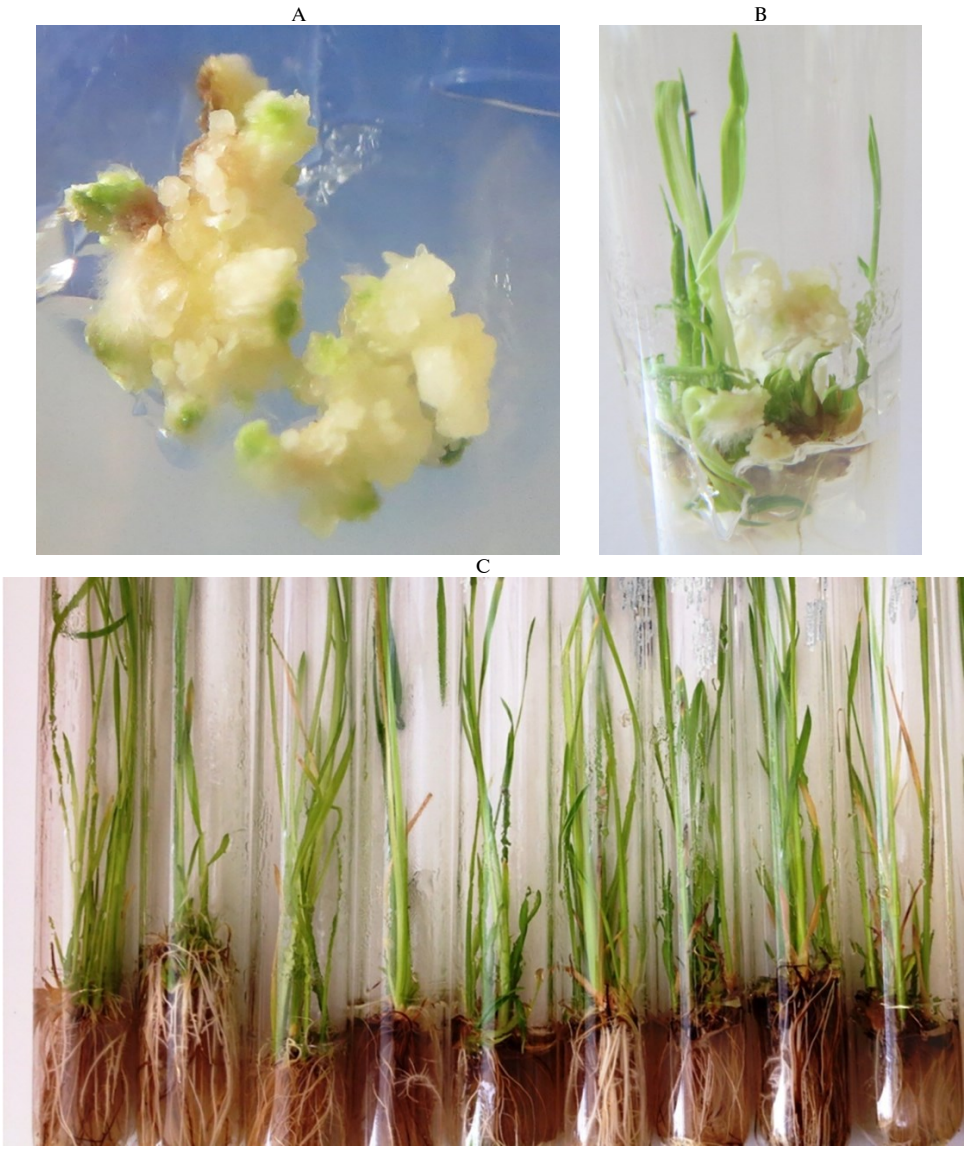

Fig. 3. Plants regenerated from androgenic calli in the studied rice (*Oryza sativa* L.) hybrids: A — androgenic callus at the stage of morphogenesis, B — multiple regeneration on androgenic callus, C — obtained regenerant plants.

The second and subsequent callus aggregates of one anther are often, but not always, identical in the combination of genes of doubled haploids in the first aggregate. For example, callus line 35.1 of hybrid L×3P is formed by two callus aggregates, they contain 25 DHs of the same genotype (resistance allele of the *Pi-ta* gene), and callus line 112.2 of hybrid D×5A(2) is formed by four callus aggregates with 55 genotypically identical doubled haploids (resistance alleles of both genes). This indicates true clonal micropropagation of doubled haploids both for one callus aggregate and for different callus aggregates from the same anther. The number of regenerants of different ploidy per the callus aggregate indirectly supports the cloning. Up to 18 tetraploids are formed per callus, up to 125 doubled

haploids, and up to 349 haploids [26, 27]. The mitotic index of haploid cells exceeds this parameter in doubled haploids and tetraploids [40], which promotes rapid micropropagation of haploids in the callus culture. In addition, the previous process of cell fusion or endomitosis reduces the number of cells involved in the formation of doubled haploids. Cell fusion can occur under certain research protocols at the initial stage during the pretreatment of anthers and microspores [41] and immediately before the formation of regenerants with a high level of ploidy (doubled haploids, triploids, tetraploids, etc.) in the light stage of callus culture [38].

Callus formation in rice during androgenesis in vitro contributes to the production of a large number of doubled haploids, but with a limited number of genotypes. The increase in genetic variability of DHs may be facilitated by somaclonal variability inherent in in vitro rice anther culture [21, 29], as in any culture of plant cells and tissues [5]. This is rather an undesirable phenomenon in in vitro androgenesis, since it is not clear whether somaclonal cell variants arise before cell fusion followed by mitotic division and regeneration or after this event. During regeneration after fusion, the cell that arose as a somaclonal variant multiplied and participated, along with other groups of cells, in the formation of doubled haploids. Hence the mixoploidy of plants occurs in androgenesis in vitro [11, 42]. The appearance of heterozygous doubled haploids is even possible, which contradicts the idea of haploid selection, where the goal is to obtain homozygous seed offspring. Although it is generally accepted that heterozygosity in androgenesis in vitro is a sign of proliferation of somatic tissues of the anther walls [1], the absence of this phenomenon has been proven in rice, that is, callus is always formed from immature microspores [43]. The tendency to somaclonal variability in diploid cells of various crops is genetically determined [44]; obviously, this pattern also applies to haploid cells. And since each microspore of an anther and plant represents a separate genotype, the somaclonal variability may differ in callus lines initiated by different anthers.

Thus, based on analysis of rice doubled haploids (DHs), we estimated the frequency of intra-callus genetic variation in in vitro androgenesis driven by gametoclonal variation. When one gene is detected in DHs, the frequency of occurrence of variable callus lines is 24.0% of all studied lines, for two genes polymorphism occurs among 47.7% of calli, for three genes 62.5% of callus lines are polymorphic. One callus line has no more than four combinations of rice blast resistance gene alleles. Consequently, the frequency of occurrence of polymorphic callus lines is high, but the range of gene allele combinations among DHs is limited. There is true cloning of doubled rice haploids within a callus line in androgenesis in vitro. Nevertheless, due to the polymorphism that occurs in regenerants within the same callus, the selection of lines of doubled haploids, adopted by breeders, is rational.

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TOXIN-PRODUCING SMALL-SPORE *Alternaria* SPECIES FROM OAT GRAIN CONTAMINATED WITH ALTERNARIOL

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Abstract

For many years, the problem of grain infestation with toxin-forming fungi *Alternaria* has been under the close attention (S.M. Tralamazza et al., 2018). Extensive studies have been carried out on wheat (M.T. Amatulli et al., 2013; M.E. Müller, U. Korn, 2013) and barley (V. Sanchis et al., 1993; T.T.T. Nguyen et al., 2018). The grain of oats has been studied much less, and it is still unclear which species of fungi of this genus and to what extent are responsible for the accumulation of the toxin alternariol (AOL). In our country, data have been obtained on the infection with *Alternaria* fungi of oat grains from a number of regions (O.P. Gavrilova et al., 2016; Yu.I. Vargach et al., 2019), as well as grain samples of varieties and lines from the VIR collection detected in field tests (A.S. Orina et al., 2017), however, the frequency of occurrence of the toxin was estimated for several regional lots in total (A.A. Burkin et al., 2015; G.P. Kononenko et al., 2020). In this study, it was established for the first time that the species *A. tenuissima* (Nees et T. Nees:Fries) Wiltshire, which is known as an active AOL producer, and to a lesser extent representatives of *A. arborescens* E.G. Simmons and the '*A. infectoria'* complex can participate in the contamination of oat grains. The aim of the work was to study the species affiliation and toxin-forming ability of *Alternaria* fungi isolated from oat grain with natural AOL contamination. The object of the study was a sample obtained in October 2020 from an agricultural enterprise of the Moscow Province (Odintsovo District) containing AOL in the amount of 630 ppb. Isolation of pure fungal cultures was carried out after surface sterilization of grain and its sowing on Chapek-Dox agar containing bile and antibiotics. Color, structure, and growth rate of colonies were described on yeast extract sucrose agar (YES) and potato-carrot (PCA) on day 8. To assess their toxin formation, oat grain and a panel of four mycological media were used — PCA, malt extract agar (MEA), hay infusion agar (HAY) and an analog of vegetable agar (V-8). After cultivation (7 days, 25 °C, without lighting) and extraction of biomass samples with a mixture of acetonitrile and water in a volume ratio of 84:16, AOL was determined by ELISA test (A.A. Burkin, G.P. Kononenko, 2011) with a detection limit of 0.01 μg/g. In the subepidermal mycobiota of the studied sample, representatives of the genus *Alternaria* were predominant, the degree of infection was 36.0 %, and they were accompanied by fungi *Fusarium* spp. (14.7 %) and *Epicoccum* spp. (2.7 %). After carrying out mycological procedures for the isolation and identification of *Alternaria* cultures, they were assigned to the species *A. tenuissima* (Nees et T. Nees:Fries) Wiltshire (7 strains), *A. arborescens* E.G. Simmons (2 strains) and to the species complex '*A. infectoria*' (4 isolates). On the grain substrate, all strains of *A. tenuissima*, *A. arborescens* and three isolates of '*A. infectoria*' produced AOL in amounts of 370, 5 and 0.8 μg/g. When testing cultures under the same conditions on agar media, the intensity of AOL accumulation in *A. tenuissima* was highest on HAY and MEA (56 and 23 μg/g), in *A. arborescens* and '*A. infectoria*' — on PCA and MEA. Taking this into account, commercial substrate MEA and oat grains are recommended for in vitro evaluation of the biosynthetic potential of fungi and their involvement in contamination of AOL grains in an expanded format. The cultural and morphological features of several cultures of *A. arborescens* and '*A. infectoria*' and their ability to AOL biosynthesis are discussed in a comparative aspect.

Keywords: oat grain, *Alternaria tenuissima*, *A. arborescens*, '*A. infectoria'*, alternariol, ELISA

Recently, the problem of grain infection by toxin-producing fungi of the genus *Alternaria*, mainly wheat [1-3] and barley [4, 5], has been actively discussed in the scientific literature. Oat grain, which in the coarsely ground form is widely used in feeding dairy cows, sheep, pigs, rabbits, poultry and is dietary indispensable for horses [6)], has been studied much less [7]. Among the toxins of *Alternaria* fungi, experts are particularly concerned about alternariol (AOL), a metabolite of the dibenzo-α-pyrone group for which a genotoxic effect has been confirmed [8, 9].

Signs of intoxication in animals when fed with infected AOL containing oats were reported at the end of the last century [10]. However, further studies used either mycological or toxicological analyses. Thus, in a sample of oats from Greece, potential producers of *A. alternata* were identified, but no search for the toxin was carried out [11], and when AOL was detected in grain from Sweden [12], southern Norway [13], Canada [14], Ireland [15], and Slovenia [16], mycological analysis was not performed.

In our country, the species composition of *Alternaria* fungi was studied on grain from a number of regions [17, 18] and in field tests on breeding varieties and lines from the collection of the Vavilov All-Russian Institute of Plant Growing (VIR Collection) [19, 20], but toxin contamination is reported only for several regional samples [21, 22]. Recently, attempts to compare toxin contamination and the DNA amounts of fungi of the sections *Alternaria* and *Infectoriae* were made on several samples from the Ural region [23] and Western Siberia [24]. The *Alternaria* species that are responsible for the accumulation of this toxin in grain remain unclear.

In the present study, it was established for the first time that the species *Alternaria tenuissima* (Nees et T. Nees:Fries) Wiltshire, known as an active producer, and to a lesser extent representatives of *A. arborescen*s E.G. Simmons and the '*A. infectoria*' complex may be involved in the oat grain contamination.

The purpose of the work is to study the species and toxin-forming ability of *Alternaria* fungi isolated from oat grains naturally contaminated with alternariol.

Materials and methods. The mycological study was conducted on grain of oat (*Avena sativa* L.) variety Yakov, obtained in October 2020 from an agricultural enterprise located in the Moscow Province (Odintsovo District). According to enzyme immunoassay measurement, the grain samples were contaminated by AOL (630 μg/kg) and T-2 toxin (5 μg/kg), other mycotoxins (deoxynivalenol, zearalenone, group B fumonisins, aflatoxin B_1, sterigmatocystin, ochratoxin A, citrinin, cyclopiazonic acid, mycophenolic acid, emodin, ergoalkaloids, roridin A, PR-toxin) were absent.

The seeds were superficially disinfected with a 3% formaldehyde solution for 1.5 min, followed by double treatment with an aqueous ammonia solution prepared by adding 4 ml of a 5% ammonia solution to 1 liter of sterile distilled water. The seeds were then placed in Petri dishes on the surface of Czapek-Dox agar containing bile and antibiotics [25]. After 7 days of culture at 25 °C, *Alternaria* were seeded onto Petri dishes and, after confirming their purity, onto agar slant. The isolated cultures were identified to species using manuals [26, 27]; monoconidial strains were obtained as described previously [28]. Description of the color, structure of colonies, and growth rate of cultures was performed on syeast extract sucrose agar (YES) and potato-carrot agar (PCA) on day 8.

To quickly assess the ability of cultures to produce AOL, grain substrate (oat flakes), PCA, malt extract agar (MEA; Liofilchem®, Italy), hay infusion agar (HAY), analogue of agar V-8 from vegetable juice (Southern Juice Company LLC, Krasnodar Province, Belorechensk, Russia) prepared according to the appropriate recipe [29] were used as growth media. The inoculum (10-day cultures on Czapek-

Dox agar) was placed in triplicate into 10-ml vials with a bottom diameter of apprx. 18 mm, each containing 1.5 ml of agar media or 1.0 g of oat flakes with 1.0 ml water added before sterilization. The vials were closed with cotton-gauze caps and wrapped in a layer of laboratory film (Parafilm "M"® PM-996, Pechiney Plastic Packaging, USA). After incubation in the dark for 7 days at 25 °C, each vial was added with 1.5 or 3.0 ml (for grain substrate) mixture of acetonitrile and water (84:16 v/v) and shaken vigorously at the beginning and end of a stationary 14-hour-long extraction. The extracts were analyzed for AOL using a test system for enzyme immunoassay determination of the toxin [30] with its detectable limit of 0.01 μg/g.

The data was processed using descriptive statistics in Microsoft Excel 2013, the results were expressed as arithmetic mean values (*M*) with standard error of mean (±SEM).

Results. Representatives of the genus *Alternaria* predominated in the subepidermal mycobiota of the studied sample. The infection incidence rate was 36.0%, additionally, *Fusarium* spp. (14.7%) and *Epicoccum* spp. (2.7%) were detected. Intensive infection with Alternaria fungi was quite consistent with significant grain contamination with AOL (630 μg/kg), and the detection of *Fusarium* fungi explained the presence of T-2 toxin in grain.

1. Alternariol (AOL) **production by representatives of the genus *Alternaria* isolated from grain of oat (*Avena sativa* L.) variety Jacob grown on a grain substrate** (oat flakes; 7 days, 25 °C, no light) ($n = 3$, M±SEM)

Strain No.	AOL, μg/g substrate
A. tenuissima	
1	140±30
2	290±70
7	370±70
9	460±90
11	115±20
12	1200±70
15	11±1
A. arborescens	
5	4±1
8	6±1
'*A. infectoria*'	
6	–
13	1.5±0.40
14	0.7±0.15
16	0.2±0.04

N o t e. A dash means that AOL was not detected.

After mycological isolation and identification procedures, *Alternaria* cultures were assigned to the species *A. tenuissima* (Nees et T. Nees:Fries) Wiltshire (7 strains), *A. arborescens* E.G. Simmons (2 strains) and to the species complex '*A. infectoria*' (4 isolates). Previously, for grain from five regions of the North-West region, based on the results of morphological identification, the dominance of *A. tenuissima* was shown with a lower occurrence of *A. arborescens* and '*A. infectoria*' [17]. For 5 grain samples from two regions of the Ural Federal District studied by quantitative PCR, a higher infection was reported for the *Alternaria* section fungi (40.8±5.6%) compared to the *Infectoriae* section (2.0±1.1%) [23]. The same relationship was observed for the sample studied. It should be noted that the traditional assignment of fungi to species levels is still considered quite acceptable, despite the increasing use of molecular technologies for DNA detection, e.g., real-time PCR and quantitative digital PCR [31].

In an experiment with short-term culture of fungi on a grain substrate, all cultures except one ('*A. infectoria*' No. 6) produced AOL (Table 1). For *A. tenuissima* strains, the sample average amount was 370 μg/g, indicating a high potential

for toxin production. In *A. arborescens* and '*A. infectoria*' accumulation was significantly less, 5 and 1 μg/g, respectively (see Table 1).

The data obtained for the *Alternaria* isolates from the sample clearly indicated that A. tenuissima was the predominant contributor to the toxin contamination, with the joint participation of *A. arborescens* and '*A. infectoria*'. Of course, this result cannot be extrapolated to the situation as a whole. To establish the composition of the producers responsible for the contamination of oat grain, an extensive survey of the population of fungi associated with this biological object is necessary, organized according to the "one isolate—one sample" principle. When using this strategy for a large set of 58 samples, it was established that *A. alternata*, *A. tenuissima* and *A. arborescens* are involved in the contamination of feed grain products and shown that mycological media may be used for testing fungi. Moreover, from this work, the observations of correspondence between toxin production and color, colony structure, and growth rate of fungi began [28]. This approach was developed in the present study.

Results of testing cultures of A. tenuissima, A. arborescens and 'A. infectoria' for the ability to produce AOL on a panel of four agar media PCA, HAY, MEA, an analogue of V-8, recommended for species identification [26, 27], are submitted in Table 2.

2. Alternariol (AOL) **production by representatives of the genus *Alternaria* isolated from grain of oat (*Avena sativa* L.) variety Jacob grown on mycological agar media** (7 days, 25 °C, no light) (n = 3, M±SEM)

Strain No.	AOL, μg/g substrate			
	PCA	HAY	MEA	analogue V-8
		A. tenuissima		
1	0.06±0.020	2.9±0.50	40±9	0.8±0.05
2	0.9±0.05	77±8	18±2	9±3
7	0.7±0.10	36±8	11±1	5±2
9	0.9±0.30	98±1	21±3	13±3
11	15±5	16±1	38±11	2.0±0.20
12	2.5±0.30	107±27	7±1	16±5
15	–	–	–	–
		A. arborescens		
5	0.4±0.20	0.09±0.020	0.2±0.02	0.1±0.02
8	0.1±0.03	–	0.03±0.010	–
		'*A. infectoria*'		
6	–	–	–	–
13	–	–	–	–
14	1.3±0.90	0.08±0.030	0.1±0.02	0.03±0.006
16	0.08±0.020	–	0.03±0.003	–

Note. A dash means that AOL was not detected. For description of media composition, see Materials and methods section.

On all media, 6 *A. tenuissima* strains produced AOL. The accumulation of the toxin varied from 0.06 to 107 μg/g both between strains and on different media. In one strains (No. 15), the toxin was not detected. The unevenness of AOL biosynthesis in *A. tenuissima* was previously reported [28], and this feature was associated with intraspecific differences that could not be detected by morphological features [32]. In general, the intensity of AOL accumulation in *A. tenuissima* turned out to be greatest on HAY and MEA media (56 and 23 μg/g) and was 1-2 orders of magnitude less than on grain (see Table 1). The same reduction from 300 μg/g (on grain) to 26 μg/g (on MEA) was previously reported for the strain *A. tenuissima* Al 392 isolated from oats [33].

Cultures of *A. arborescens* produced much less AOL than *A. tenuissima*. On HAY and V-8, its amount is detectable only in strain No. 8. On PCA and MEA, the amount of toxin varied from 0.03 to 0.4 μg/g (see Table 2) and, as in *A. tenuissima*, was significantly lower compared to that for grain substrate. Previously, in

three strains of *A. arborescens* from wheat grain and sunflower seeds, a more intense accumulation of AOL on MEA was noted, from 3.3 to 36 μg/g [34], in five crops from grain feed and three collection strains from 2 to 79 μg/kg [28]. Unfortunately, due to the small number of isolates available for study, information on the potential for AOL biosynthesis in this species remains insufficient.

Representatives of the species complex '*A. infectoria*' in terms of toxin production on agar media differed in pairs No. 6, No. 13 and No. 14, No. 16, the first two strains did not produce toxin, while in others it was detected. In both isolates, the ability to produce was detected only on PCA and MEA in comparable quantities, 0.08-1.3 and 0.03-0.1 μg/g, respectively.

Compilation of these data shows that of all mycological media tested, commercial MEA media provided a consistent positive metabolic response for *A. tenuissima*, *A. arborescens* and '*A. infectoria*' and, therefore, can be recommended, along with the grain substrate, for in vitro assessment of their biosynthetic potential in an expanded format.

According to morphological characteristics, all strains of *A. tenuissima* were typical. They had unbranched chains consisting of 5-10 subulate conidia with an elongated neck, dense velvety colonies of dark gray color with a black reverse side, and a moderate growth rate on PCA and YES. No peculiarities in color, colony structure, or growth rate were identified in the only non-producing strain No. 15. Considering the detection of AOL during its culturing on a grain substrate, albeit in the smallest quantity (see Table 1), it is possible to assume its biosynthesis on agar media, but in concentrations below 0.01 μg/kg, that is, beyond the detection limit of the method.

Cultural and morphological traits of *A. arborescens* strains with weak sporulation and isolates of '*A. infectoria*' that did not sporulate in out tests are submitted in Table 3.

3. Cultural and morphological traits of *Alternaria arborescens* strains and the species '*A. infectoria*' complex isolated from grain of oat (*Avena sativa* L.) variety Yakov grown for 8 days on two agar media

Strain No.	Structure, color and diameter (d) of colonies	
	PCA	YES
	A. arborescens	
5	Velvety, dark gray	Dense velvety with distinct concentric circles, gray, the reverse side almost black, d = 47 mm
8	Velvety, light gray, dark gray in the center	Dense velvety, gray, the reverse side almost black, d = 39 mm
	'*A. infectoria*'	
6	Loose fluffy, dark gray	Slightly fluffy, pink-white-gray, the reverse side almost black, d = 55 mm
13	Loose, stringy, light gray	Loose fluffy, white-pink, the reverse side gray, d = 39 mm
14	Felt-like, dark gray	Dense, velvety, white-pink with slightly pronounced concentric circles, the reverse side is brownish, d = 40 mm
16	Felt-like, light gray in the center, gray at the edges	Dense, velvety, white-pink, the reverse side is brownish, d = 50 mm

N o t e. For description of media composition and culture conditions, see Materials and methods section.

A. arborescens colonies on PCA and YES practically did not differ in size and appearance, but in strain No. 5, which, unlike No. 8, produced toxin on all four media (see Table 2), there were clearly visible concentric circles on YES. '*A. infectoria*' colonies were also approximately the same in size (with diameters of 39-55 mm), but differed in pairs in density (No. 6, No. 13 and No. 14, No. 16) (see Table 3) and in the ability to form toxins (see Table 2). Isolate No. 6 which differs sharply from all others in the color of the aerial mycelium and the reverse side on YES, did not produce the toxin both on agar media (see Table 2) and a

grain substrate (see Table 1). Its paired isolate No. 13 which did not produce AOL on agar media but synthesized it on grain, had more signs of cultural similarity to producers No. 14 and No. 16. The maximum accumulation of AOL (1.3 μg/g on PCA) was characteristiv of isolate No. 14 forming dense velvety colony with concentric circles (see Table 3).

This work is the first report on the ability of '*A. infectoria*' isolates from oat grain to produce AOL. The production ability of this complex from wheat grain is known, but there is no detailed description of their macromorphological characteristics. For a strain of Russian origin, 2.01 μg/g AOL production was recorded [35], for 12 strains from Italy, it ranged from 0.3 to 20 μg/g with an average value of 4 μg/g [36], for 98% of isolates in Argentina the value ranged from 1.8 to 433.3 μg/g with an average of 62.2 μg/g [37]. The '*A. infectoria*' morphotypes differing in pigmentation was first reported by B. Kosiak et al. [38]. Recently, an '*A. infectoria*' isolate from sunflower seeds atypical for colony structure and increased growth rate was shown to produce from 2 to 220 μg/g AOL on agar media [28]. Continuing the study of toxin production and culture properties of fungi of this systematically complex group remains relevant for clarifying a number of taxonomic aspects [39, 40].

Thus, this paper described correspondences between macromorphological characteristics and the ability to produce alternariol for a number of *Alternaria arborescens* strains and isolates of the '*A. infectoria*' from Yakov oat variety grain. A comparative assessment of alternariol biosynthesis rate by *A. tenuissima*, *A. arborescens* and '*A. infectoria*' on the panel of agar media recommended for species identification shows that commercial malt agar, along with a solid grain substrate, are suitable for advanced toxicological monitoring. The grain contamination with alternariol is mostly due to *A. tenuissima*, and to a lesser extent to *A. arborescens* and the '*A. infectoria*'. A similar complex methodological scheme, including mycological and toxicological analysis, should be applied for furthet detailed assessment of small-spored Alternaria species populations from oat grain.

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