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THE USE OF *Vrn* GENES FOR CREATION OF TRITICALE FORMS WITH DIFFERENT LENGTH OF VEGETATION PERIOD

(review)

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Abstract

The advantages and disadvantages of triticale culture are briefly reviewed. The control of the length of vegetative period of spring triticale forms and spontaneous spring triticale mutants is viewed more particularly. Triticale (× *Triticosecale* Wittmack) is a new agricultural culture that combines valuable traits of wheat and rye. The advantages of triticale are its ability to grow on poor, acid, waterlogged soils; higher, than in wheat, content of protein in grains; its resistance to many fungus diseases. The disadvantages are undersized grains, its tendency to sprouting, lodging, a partial toxicity of grains due to the presence of alkylresorcinols, and a longer, compared to parental forms, vegetation period. The biggest influence on the length of the vegetation period of cereals have vernalization response genes — *Vrn*. Spring plants have one or more dominant *Vrn* genes, in winter plants all *vrn* genes are recessive. Common wheat carries genes *Vrn-A1*, *Vrn-B1*, *Vrn-D1*, located on the chromosomes 5AL, 5BL, 5DL respectively (A.J. Worland, 1996), *Vrn-D4* gene, located on the centromeric region of chromosome 5D (N. Kippes et al., 2015) and *Vrn-B3* gene on the chromosome 7BS (L. Yan et al., 2006). Rye has *Vrn-R1* gene on the chromosome 5RL (J. Plaschke et al., 1993). In triticale there were detected *Vrn-A1a*, *Vrn-B1a*, *Vrn-B1b* and *Vrn-B1c* alleles (M. Nowak et al., 2014; O.I. Zaitseva et al., 2015). The same alleles were detected previously in common wheat (D.K. Santra et al., 2009; A.B. Shcherban et al., 2012, 2015; J. Milec et al., 2013; I.E. Likhenko et al., 2014). Heading time of plants can be influenced not only by an alteration of nucleotide sequence of *Vrn* genes, but also by a change of the copy number of these genes (A. Diaz et al., 2012). *Vrn* genes can influence heading time in the combination with each other. For example, cultivars with three dominant *Vrn* genes are ripening earlier, than cultivars with one or two dominant *Vrn* genes, but they have the least productivity (A.F. Stelmakh, 1993; M. Iqbal et al., 2007). It was also reported, that introgression of chromosome 2D shortened the period of triticale vegetation (A.A. Shishkina, 2008; L.V. Koren et al., 2010). Triticales have more prolonged vegetation period compared to parental wheat lines, which can be due to the inhibition of the *Vrn* genes by rye genome (L.N. Kaminskaya et al., 2005; I.N. Leonova et al., 2005). The genetic control of growth habit of spontaneous spring mutants is currently unknown. It was determined, that the majority of spring mutants are late ripening, and, after autumn sowing, they survive in different extent, what can mean, that they are facultative (P.I. Stepochkin, 2008; P.I. Stepochkin et al., 2008). In Siberia winter triticales occupy considerable areas, but the breeding of spring triticales hasn't been carried on yet. Spring triticales could increase biodiversity of spring cultures. Thereby creation of spring triticales with different length of vegetative period is of great breeding interest.

Keywords: hexaploid and octaploid triticale, spontaneous spring mutant, wheat, length of vegetative period, *Vrn* genes

Triticale (× *Triticosecale* Wittmack), or a wheat-rye amphiploid (WRA),

is an artificially created culture derived from wheat (*Triticum* spp.) and rye (*Secale* spp.) crossing. Octaploid, hexaploid and tetraploid triticale are distinguished. Octaploid triticale ($2n$ = 56, $A_1A_1B_1B_1$DDRR genome with A_1, B_1 of soft wheat genome, and A, B of durum wheat genome) are obtained by crossing 42-chromosome wheat (mainly a soft wheat *T. aestivum* L.) with rye (mainly *S. cereale* L.) followed by the chromosome set doubling. These WRAs are cytologically unstable and generate aneuploids at a high frequency. Over a number of generations, they lose chromosomes until reaching a stable hexaploid level [1, 2]. Because of a reduced grain number per spike, octaploid (×8) triticale are not used in commercial plant growing, These WRAs should be cytologically monitored, and the typical plants should be selected to maintain the ×8 number of chromosomes [3].

Hexaploid (×6) triticale ($2n$ = 42, AABBRR genome) derive from crossing 28-chromosome wheat (mainly *T. durum* Desf.) with rye and subsequent doubling of the chromosome number. These forms are more cytologically stable than ×8 triticale.

Hexaploid and octaploid triticale are primary forms created by doubling the chromosome number of F_1 hybrids between hexaploid or tetraploid wheat and rye. However, most varieties are secondary hexaploid triticale developed by crossing ×8 triticale with ×6 triticale or ×6 triticale with wheat. In the second generation, due to the peculiarities of the A and B genomes of soft and hard wheat, some genotypes appear with a higher productivity and a significantly lower frequency of meiotic disturbances [4].

Tetraploid (×4) triticale ($2n$ = 28) was first obtained by pollination of wheat-rye F_1 hybrids with rye pollen. Among F_2 hybrids, a plant was found with 28 chromosomes, 14 from wheat and 14 from rye [5]. The yield of ×4 triticale is very low. However, the ×4 triticale plants are more cytologically stable than ×8 and ×6 ones; therefore, the fertility of ×4 triticale may be increased by selection methods [5].

This review focuses on the diversity of genes and alleles encoding development processes in triticale, wheat and rye to involve these gene pool in breeding varieties for regions with different lengths of the growing season.

Triticale possesses a number of advantages. Although wheat was crossed with rye mainly in order to give it winter hardiness of rye, triticale on this basis, as a rule, does not differ from winter wheat. As assumed, this is due to the suppression of the rye chromosome activity by wheat cytoplasm [6]. According to some reports, ×8 triticale is more winter-hardy than ×6 triticale [7], however, a decrease in ploidy from ×8 to ×6 led to an increase in frost resistance [8]. Comparing triticale with the parental wheat lines showed more winter-hardiness due to the presence of rye chromosomes [9]. Molecular markers revealed three loci responsible for winter hardiness on chromosomes 5A, 1B, and 5R of hexaploid triticale [10].

The triticale, like rye, is superior to wheat in the ability to grow on acidic, infertile, flooded soils. Triticale is more resistant to powdery mildew, yellow rust and smut. However, triticale, like rye, is affected by brown rust to which ×8 plants are more susceptible than ×6 plants, and by stem rust, ergot, root rot and snow mold [7].

Another valuable trait of triticale compared to wheat is a higher protein content in grain. However, triticale flour is inferior to wheat flour for bread baking because of low quality of gluten, and, therefore, it is used as a 30-50% mixture with wheat flour. This improves elasticity and increases the bread volume [11]. Bread with triticale flour surpasses wheat bread and rye bread in nutritive

value, and also has a characteristic sweetish taste. Pasta, cookies, biscuits, crackers, diet bread made from triticale flour are gluten-free dietary foods for people suffering from metabolic disorders. A way to improve baking qualities of triticale is the replacement of the rye chromosome 1R with the homeologous chromosome 1D of wheat [12]. Substitution of rye chromosome 1R with 1U *Aegilops umbellulata* chromosome 1U is also of potential interest for improving baking qualities [13].

Grain of triticale is used in brewing and alcohol manufacturing. The yield of alcohol in this case is 3-5 % higher than for other cereals [14]. Triticale is a successful forage and feed crop. Triticale grain is a high-protein feed with better digestibility than that of wheat and barley. Cattle prefer green mass of triticale as compared to wheat and rye due to higher content of sugars and carotenoids. Feeding triticale green mass increases milk yield, milk fat content, and animal weight gain [7].

However, triticale also has some disadvantages. Primary triticale exhibits reduced grain number per spike resulting from disturbances in meiosis, which lead to the emergence of aneuploid plants and, consequently, a decreased productivity [15]. The presence of only one rye chromosome in the wheat genome causes structural changes in the karyotype [16]. The problem of low grain number is solved by selection.

Triticale has a shrunk, poorly filled grain due to violated accumulation of fine-grained starch, as well as premature release of α-amylase which decomposes starch [17]. Grain protein content inversely correlates with grain filling, thence selection for grain filling leads to a decrease in its protein content, and vice versa [7]. The activity of α-amylase determines one more drawback of triticale, i.e. the tendency of grain to germinate prior to harvesting, which reduces grain quality and yield. Presence of chromosome substitution 2R/2D increases the resistance to pre-harvest sprouting [18].

Most triticale varieties are long-stemmed and prone to lodging. Plants more than 91 cm in height have a predisposition to lodging [19]. Plants with 2R/2D substitution are shorter than other plants [20, 21]. The replacement of the 2R rye chromosome with the 2U *Aegilops umbellulata* chromosome led to a decrease in height of triticale hybrids [13].

Another disadvantage of triticale is grain toxicity caused by antimetabolites alkylresorcinols. As per the content of these substances, triticale occupies an intermediate position between wheat and rye. Feed containing more than 50% of triticale grain inhibit animal weight gain, and may cause diseases of liver and gastric mucosa. The amount of alkylresorcinols decreases during grain processing and the preparation of feed mixtures [5].

Triticale has a longer vegetation period than the parent forms. It is known that the growing season of polyploids increases with an increase in ploidy [5]. Moreover, due to the hybrid origin, many biological processes in triticale proceed more slowly than in wheat [7]. The period from heading to flowering in this crop is several days longer than in wheat [5]. The phase of dough-like ripeness in triticale is long and can last up to 3 weeks [5]. Triticale grain ripens 3-20 days later than wheat grain [7, 8]. The ×6 triticale occupies an intermediate position between soft wheat and ×8 triticale as to the period before heading [3, 22], and F_1 hybrids of wheat and rye, on the contrary, develop more rapidly than wheat plants [5]. Winter rye has a higher rate of growth and apex development than wheat, and triticale is closer to rye than to wheat on this trait [23].

Wheat, rye, and triticale forms can be of spring, winter, and alternate type of development [24]. Winter forms need a long (1-3 months) exposure to low

positive temperatures for the transition to generative development (vernalization); spring crops are able to go to earing without it. Alternates can develop both in spring and in winter mode. The time of the onset of generative development and the time of heading are important adaptive traits. The type of development, as well as the duration of the growing season, is controlled by the *Vrn* genes (response to vernalization).

In common wheat, *Vrn-A1*, *Vrn-B1*, *Vrn-D1* genes are located on 5AL, 5BL and 5DL chromosomes, respectively [25, 26)], *Vrn-D4* is in the near-centromere region of the 5D chromosome [27, 28], *Vrn-B3* is on chromosome 7BS (29). The rye gene *Vrn-R1* is located on 5RL chromosome [30]. The spring type of development is controlled by one or several dominant *Vrn* genes, the winter type is controlled by recessive *vrn* genes in all these loci [31]. Alternates can carry dominant genes *Vrn-B1* [32], *Vrn-D1*, *Vrn-D4* [33-36] or a "weak" allele of the dominant *Vrn-A1* gene [37].

On the example of substituted and isogenic wheat lines, it was shown that the dominant *Vrn-A1* gene is epistatic with respect to other *Vrn* genes and determines the absence of a response to vernalization, while plants with the *Vrn-B1*, *Vrn-D1*, *Vrn-D4*, and *Vrn-B3* genes respond to varying degrees to vernalization by acceleration of heading [31, 38, 39]. According to the influence on the heading time, *Vrn* genes can be arranged as following: *Vrn-A1* > *Vrn-D1* > *Vrn-D4* > *Vrn-B1*, where the plants with the dominant *Vrn-A1* gene are the earliest, and with the dominant *Vrn-B1* gene are the latest [40, 41]. Among Chinese wheat varieties, plants with the dominant *Vrn-D1* gene, on the contrary, mature later than plants with *Vrn-B1*, which can be explained by the presence of different *Vrn-D1* alleles [34]. The dominant *Vrn-B3* gene in combination with other *Vrn* genes determines a very early heading [34].

Vrn loci are characterized by multiple allelism resulting from the differences in the structure of regulatory regions (the promoter or the first intron). To detect alleles of *Vrn* genes, primers for these regions have been designed. The *Vrn-A1* gene of common wheat has the alleles *Vrn-A1a* (insertion and duplication in the promoter region), *Vrn-A1b* (deletion of 20 bp in the promoter region) [42] and *Vrn-A1c* (deletion of 5504 bp in the first introne) [43]. Alleles *Vrn-A1d*, *Vrn-A1e* and *Vrn-A1f* were found in tetraploid wheat (deletions of 32, 54 and 50 bp in the promoter region, respectively) [42, 44]. In diploid wheat, there are *Vrn-Am1f*, *Vrn-Am1g*, and *Vrn-Am1h* alleles with deletions and/or insertions in the promoter region and/or in the first intron [45]. Hexaploid wheat *T. compactum* has a new variant of the dominant allele *Vrn-A1a*, characterized by the presence of a 16 bp deletion and four single-nucleotide polymorphisms (SNPs) in a mobile genetic element in the promoter region, as well as a new *Vrn-A1j* allele containing a 54 bp deletion in the promoter region [46]. In tetraploid wheats *T. turgidum* and *T. durum*, the *Vrn-A1i* allele with SNP was found in the sequence of the adenine (A) tract of the VRN-box of the *Vrn-A1* gene, presumably determining a reduced sensitivity to vernalization and a facultative type of development [46]. In wheats ×4 and ×6, five variants of the *Vrn-A1b* allele were identified, which differ in the polymorphism of the A-tract and C-enriched segment in the VRN-box sequence [46]. In *T. dicoccum*, the dominant allele *Vrn-A1k* with a 42 bp deletion was found in the promoter region; this allele is responsible for the decrease in the need for vernalization and the spring type of development [47].

The identified wheat *Vrn-B1* gene alleles are *Vrn-B1a* with 6850 bp deletion in the first intron [43], *Vrn-B1b* which differs from *Vrn-B1a* by an additional 36 bp deletion in the first intron [48], and *Vrn-B1c* which, in addition to the

deletion as in *Vrn-B1a*, carries in the first intron an 820 bp deletion and a 431 bp duplication shifted to the beginning of this deletion [49, 50]. In tetraploid wheats *T. turgidum* and *T. turanicum* Jakubz. the promoter regions of the dominant *Vrn-B1* gene have a 5463 bp and 127 bp deletions, respectively [44, 51]. In wheat ×4 *T. carthlicum*, the *Vrn-B1(ins)* allele was detected, which is characterized by the insertion of a retrotransposon in the promoter region [46]. In *Vrn-B1*, two variants were found, differing from the recessive allele *vrn-B1* in 7 bp, 3 bp, 2 bp deletions and 8 SNPs [46].

The *Vrn-D1a* allele of spring soft wheat has a 4235 bp deletion in the first intron [43]. The *Vrn-D1b* allele of alternates differs from *Vrn-D1a* in a single-nucleotide substitution in the promoter region [36]. In three common wheat varieties from China, the *Vrn-D1c* allele with a 174 bp insertion was detected in the promoter region [52]. *Aegilops tauschii* has *Vrn-D^{t}1* allele with a 5437 bp deletion in the first intron [53], *T. spelta* and *T. compactum* have *Vrn-D1s* with an 844 bp insertion in the first intron [54]. Five haplotypes of the *Vrn-D1* gene, Hap-7Tu and Hap-8T, differing in the length of T-tract at the –428 bp were found in five ×6 wheat samples [55]. The *Vrn-D4* gene appeared due to the insertion of a 290 kbp region of 5AL chromosome carrying the *Vrn-A1* gene into the short arm of the 5D chromosome [56].

The *Vrn-B3a* allele in the substituted Chinese Spring/Hope 7B line has a 5295 bp insertion in the promoter region [29], *Vrn-B3b* allele has an 890 bp insertion in the promoter of recessive gene *vrn-B3*, and *Vrn-B3c* has 20 bp and 4 bp deletions in the promoter of the dominant *Vrn-B3a* gene [57].

Allele-specific markers revealed in triticale the dominant alleles *Vrn-A1a*, *Vrn-B1a*, *Vrn-B1b*, and *Vrn-B1c* [58, 59], previously detected in common wheat [48, 60-63].

Not only a change in the nucleotide sequence of the *Vrn* genes, but also an increase in the number of copies was recently reported to affect earing of wheat. Thus, an increase in the number of copies of the *Vrn-A1* dominant allele to two and three caused a delay in flowering initiation compared to wild-type plants bearing one copy of *Vrn-A1* [64].

Dominant *Vrn* genes can affect the length of growing period in combination with each other. Varieties with two *Vrn* genes enter the earing phase earlier than varieties with one dominant gene, and varieties with three *Vrn* genes are the most early-season but the lowest in productivity [65, 66]. Triticale varieties with earlier earing show higher spike fertility and 1000-grain weight [67].

The dominant *Vrn* genes differ in their effect on plant growing period mainly due to the fact that they determine different durations of the second stage of organogenesis (tillering) [41, 68]. So, it is the smallest in early-ripening genotypes with one dominant *Vrn-A1* gene (*Vrn-A1 vrn-B1 vrn-D1*) and two dominant genes (*Vrn-A1 Vrn-B1 vrn-D1* и *Vrn-A1 vrn-B1 Vrn-D1*) and the largest in the late-ripening line with one dominant *Vrn-B1* gene [68]. Moreover, the shorter the period of vegetative development of plants, the longer the period from heading to ripening, and vice versa [41, 69]. Loci affecting the rate of plant development were found in triticale in different chromosomes [70].

Vrn genes are associated with wheat productivity traits indirectly through regulation of carbohydrate and nitrogen metabolism [71, 72].

L.V. Koren and L.V. Khotyleva note [73] that the ×6 triticale with introgression of 2D soft wheat chromosome ripens most early as compared to all lines they studied. Another study also reports that triticale lines with 2R/2D chromosome substitution have a significantly shorter vegetation period compared to lines with a complete set of chromosomes, and triticale lines with 2B/2D sub-

stitution and T:2RS.2RL-2BL translocation ripen later than forms with substitution 2R/2D [21].

Many researchers observed spontaneous appearance of spring plants among winter wheat, rye and triticale upon spring sowing [74, 75]. Genetic control of the type of development of spontaneous spring mutants is not currently known, but the cause of their occurrence is assumed to be mutations either in the promoter region or the first intron of the *Vrn* genes [42, 43], or an epigenetic change in chromatin state in these regions that does not affect the DNA sequence [76, 77]. In both cases, recessive *vrn* genes become dominant, resulting in induction of generative development [78]. The type of development of spontaneous spring mutants is determined by the heterozygous dominant gene, since the progeny from self-pollination of the mutants segregates into spring and winter forms [8]. In the F_2 from crossing between mutant spring rye plants segregation of spring and winter forms was close to 3: 1, which indicates a monogenic dominant control of springiness [8]. Two spring mutants of winter wheat variety Lutescens 105 differed in vegetation duration by almost a month. A genetic analysis of the F_2 generation of these mutants revealed spring to winter type segregation close to 15: 1. Therefore, the genes that determine the type of development of the studied mutants are in different loci [8].

The earliest spontaneous spring mutants of winter triticale eared in late July to early August, but most of the mutants were late ripening and eared in September [75]. In addition, all spontaneous spring mutants of wheat, rye, and triticale sown in autumn overwinter to varying degrees. Therefore, we can assume that they belong to the alternates [8].

The spring mutants occur more frequent with an increase in the shelf life of seeds, as well as with an increase in temperature in June and the amount of precipitation in July [75]. It is assumed that these stresses activate mobile elements leading to mutations in the *Vrn* genes [75]. The findings [79-81] also confirm the effect of physical and chemical mutagens on the duration of the growing season of spring plants.

The researchers from the Institute of Genetics and Cytology of Belarus reported on production of octaploid and hexaploid triticale lines with dominant *Vrn* genes [22]. The maternal forms were the isogenic lines of common wheat with dominant alleles *Vrn-A1*, *Vrn-B1* and *Vrn-D1*, obtained on the basis of the varieties Triple Dirk, Mironovskaya 808 and Bezostaya 1, and a pollinator was the winter diploid rye variety Voskhod and spring alloplasmic rye [22]. In these lines, the inhibitory effect of the triticale genetic background on the expression of the dominant *Vrn* genes was revealed. The triticale plants eared later than the corresponding wheat lines. Moreover, the dominant *Vrn-A1* gene which determines early earing was suppressed to a greater extent than *Vrn-B1* and *Vrn-D1* [22, 82]. Later, octaploid triticale lines with dominant *Vrn* genes were created at the Siberian Institute for Plant Industry and Breeding, Siberian Branch RAS, by crossing Triple Dirk common wheat isogenic lines with *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-D4* genes with winter diploid rye Korotkostebelnaya 69. A sequential arrangement of dominant *Vrn* genes in these triticale lines, as per their influence on heading time, were the same as in wheat lines with these genes [3].

Thus, triticale is a promising crop with the ability to grow on poor soils and resistance to a number of fungal diseases, with higher protein content in grain than wheat, high alcohol yield and frost resistance. The *Vrn* genes have the greatest influence on the duration of the growing season of wheat, rye, and triticale. These genes to varying degrees affect the earing time and the vernalization effect. Numerous alleles of *Vrn* genes differ in mutations located in the promoter

region and/or the first intron, which increase the diversity of plants by the length of the growing period and response to vernalization. Allele-specific primers revealed in triticale the alleles *Vrn-A1a*, *Vrn-B1a*, *Vrn-B1b* and *Vrn-B1c* previously detected in common wheat. In addition, by varying the number of copies of *Vrn* genes or by combining different dominant *Vrn* genes with each other, the length of the plant growing season can be manipulated. Some winter triticale, wheat, and rye seeds sown in spring produced spontaneous spring plants. The type of development of such spontaneous spring mutants is determined by the heterozygous dominant gene, since the offspring from their self-pollination segregates into spring and winter forms. In Siberia, winter cultivars of triticale are successfully grown. Given an unpredictable climate change and increasing demand for feed grain in animal husbandry, it is of interest to involve different dominant *Vrn* alleles and their combinations in breeding spring forms of triticale, differing in the length of growing season.

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PHENOTYPIC AND GENOTYPIC EVALUATION OF SYNTHETIC HEXAPLOID WHEAT LINES (AABBDD) FOR GRAIN PARAMETERS UNDER THE CONDITIONS OF WESTERN SIBERIA

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Abstract

Wheat is both a vital food crop and an economically important commodity for Russia. Plant breeders are increasingly looking to wild relatives to introduce new genes and alleles to obtain high, stable wheat yields. In this paper, we present the study results for grain morphometric parameters and genotyping with usage of 47 SNP (single nucleotide polymorphisms), including 13 markers assosiated with genes responsible for grain size/weight. For the first time in Western Siberia these data were obtained for synthetic hexaploid wheat genotypes set (the genome AABBDD) in which the genome D was transferred from *Aegilops tauschii*, originated from regions with the highest genetic diversity of this species. The evaluation of 47 synthetic hexaploid wheat lines was performed on the experimental field of Omsk SAU (Omsk city) in 2016-2018. The synthetic lines were created in CIMMYT by crossing durum wheat (*Triticum durum* Desf., AB genome) with different entries of *Aegilops tauschii* Coss. (synonym *Ae. squarrosa*, D genome) from Germaplasm Bank (CIMMYT). Synthetic lines of Kyoto University (Japan) were bred via hybridization of durum wheat variety Langdon (USA) with entries of *Aegilops tauschii* of different ecological origin. After harvesting the structural analysis of yield components, i.e. the number of tillers per plant, number of spikelets per spike, grains per spike, grains per plant, grain weight per spike, grain weight per plant, thousand kernel weight, was carried out. The basic morphometric parameters of grain (area, length, width, perimeter, circularity) were evaluated. Synthetic lines were genotyped with 47 SNP markers using KASP^{TM} technology («LGC Genomics», UK). Synthetic lines were characterized by low variability of grain parameters (Cv = 3.3-6.5 %), higher grain lenght (7.58 mm), area (21.1 mm^2), perimeter (19.7 mm), and thousand kernel weight (34.9-46.7 g) than standard variety Pamyati Azieva. In 2017-2018, the grain yield positively correlated with grain lenght, area, perimeter and negativeley correlated with grain quality indicators (protein and gluten content). An insignificant relationship between the grain lenght and width (r from –0.08 to –0.23) was established. The results indicate that synthetic wheat lines are useful genetic donors for increasing thousand kernel weight due to genes *TaTPP6A*, *TaGW2-6A*, *TaGASR-A1*, *TaGS5-3A*, *TaTGW6*, *TaTGW-7A*, *TaCwi-A1*, *TaGS-D1*, and *TaCKX-D1*. The winter durum wheat varieties, which were used in the creation of synthetic lines, and genes of the A genom mainly contribute to grain size/weight. Synthetic lines from Kyoto University had larger and heavier grains (length 8.22-8.51 mm; thousand kernel weight 43.8-46.4 g) compared to the CIMMYT lines. Hybrid combinations Aisberg/*Ae. squarrosa* (511), Ukr.-Od.1530.94/*Ae. squarrosa* (392), Ukr.-Od.1530.94/*Ae. squarrosa* (1027), and Langdon/*Ae. squarrosa* had the highest thousand kernel weight. Lines No. 8 Ukr.-Od.1530.94/*Ae. squarrosa* (392), No. 37 Ukr.-Od.1530.94/*Ae. squarrosa* (310), No. 44, 46 Ukr.-Od.1530.94/*Ae. squarrosa* (1027), No. 27 Leuc 84693/*Ae. squarrosa* (392), No. 41 Ukr.-Od.1530.94/*Ae. squarrosa* (1027), No. 29

Lang-don/IG 126387 (with exception of *TaGW2*) are recommended as promisis soucers for pyramiding genes *TaCwi-A1*, *TaGASR-A1*, *TaGS5-3A*, *TaGW2*, *TaTGW-7A*, *TaGS-D1*, and *TaCKX-D1*, which are responsible for grain size and thousand kernel weight. The indentified entries in this studies are valuable souces of combination of genes responsible for grain size and thousand kernel weight for breeding under conditions of Western Siberia region.

Keywords: synthetic wheat, grain size, thousand kernel weight, SNP marker, breeding

A thousand kernel weight is one of the most stable trait of wheat productivity the increase of which is topical for breeding [1, 2]. The grain size and shape are closely related to crop yields and milling properties. Grain of modern wheat varieties is wider in width and shorter in length compared to wild relatives which demonstrate greater variability in grain size and shape. This is due to the effect of domestication and breeding of wheat during which the shape of the grain became more rounded resulting in higher flour yield [3]. Larger grains also have larger germination energy, which favorably affects the increase in yield [4, 5].

Bread wheat (*Triticum aestivum* L.) is a natural allohexaploid with subgenomes A, B and D, of which D subgenome was introduced from *Aegilops tauschii* Coss. Probably only some accessions of *Ae. tauschii* participated in crossing with tetraploid wheat (*Triticum turgidum* L., AABB), which led to the limited polymorphism of genome D present in cultivated wheat [6].

Genetic potential of subgenome D in wheat breeding are beeing mobilized via created synthetic hexaploids or by direct hybridization between *Ae. tauschii* and commercial varieties [7]. Many researchers consider synthetic hexaploid wheat a promising source of economically valuable traits, i.e. a high thousand kernel weight, spike productivity and the number of productive plants per unit area, for impoving cultivated varieties [8]. The thousand kernel weight is under polygenic control. QTLs responsible for this trait are mapped on almost all wheat chromosomes. The thousand kernel weight has a high coefficient of heritability and is closely related to grain length, width, area, and perimeter [9-11].

Increasing wheat yield include molecular methods for identifying genes that control the size and weight of a wheat grain. Their search is complicated by the complex structure and large genome of common wheat [12]. Many genes that control the size, shape and weight of the grains are orthologous genes of those already identified in other crops, the rice, barley, and sorghum. A comparative search for such genes in wheat contributes to the expansion of polymorphism in productivity parameters [13]. *TaGW2* [14], *TaCwi-A1* [15], *TaGaSR7-A1* [16], and *TaTGW6* [17] genes that determine the grain size and thousand kernel weight in wheat were originally identified in the rice genome [18].

Ae. tauschii accessions possess significant genetic and phenotypic variability for grain size and weight compared to bread wheat [11, 19]. Synthetic hexaploid wheat with genome D from various *Ae. tauschii* forms also have a broader polymorphism of loci controlling size, shape and weight of grain [18]. In synthetic wheat hexaploids, the QTLs with a pleiotropic effect on the grain length, width, area, and perimeter were mapped on chromosomes 2DL and 7DS [20]. In a synthetic wheat line of the ITMI collection tested under various ecological conditions of Russian regions QTLs for thousand kernel weight were mapped on chromosomes 4A, 1B, 3B, 1D, and 2D [9]. More recently, when examining this collection in Western Siberia, QTL for thousand kernel weight was mapped on chromosome 6A [21].

In recent years, functional markers habe been devepoled for genes that contribute to wheat productivity, *TaSus2-2B*, *TaCwi-A1*, *TaGW2*, *TaGW2-6B*, *TaCKX6-D1*, *TaGS-A1*, *TaSAP1-A1*, *TaGASR7-A1*, *TaGS-D1*, and *TaTGW6* [12, 22]. Searching for sources of genes that control grain size and weight, and

the DNA markers closely linked to these genes, ensured creation of commercial varieties with genetically determined traits to increase wheat productivity in Western Siberia.

This paper is the first report on grain morphometry and analysis of yield structure elements coupled with genotyping by 47 SNP (single nucleotide polymorphisms) of synthetic hexaploid wheat lines in Western Siberia. As a result, we identified valuable genetic sources for increasing grain size and weight in bread wheat varieties.

Our objective was the phenotypic and genotypic assessment of synthetic wheat lines (AABBDD genome) for grain parameters and the selection of the best samples for breeding in Western Siberia.

Materials and methods. A total of 47 synthetic hexaploid wheat lines were tested in 2016-2018 (experimental field of Omsk State Agrarian University, Omsk). CIMMYT synthetic lines were developed by crossing durum wheat varieties from the Institute of Breeding and Genetics (Odessa, Ukraine) and variety Pandur (Romania) (*Triticum durum* Desf., genome AB) with *Aegilops tauschii* Coss. (syn. *Aegilops squarrosa*, genome D) from Germaplasm Bank (CIMMYT). No cytological control after colchicization of F_1 was applied. Fertile plants with good agronomic traits were selected in F_4 hybrid populations, followed by their reproduction till F_7. The synthetic lines of Kyoto University (Japan) were obtained via hybridization of the durum wheat variety Langdon (USA) with *Aegilops tauschii* accessions of various ecological origins.

In 2016, each line was sown in a 1 m row, at 25-30 seeds per row. A mid-early season variety Pamyati Azieva variety and mid-late season Serebryristaya variety were the standards alternately sown after every 5th test plant in the row. In 2017-2018, each line was sown on a 1.4 m^2 plot, at 25 g seeds per plot. The same standard varieties were alternatively sown after every 10th test plant. Row spacing was 15 cm. Field trials utilized a randomized complete block design with four replicates.

Plant productivity elements (the number of productive stems per plant, the number of productive spikelets per spike, grain number per main spike, per spike, and per plant, grain weight per main spike, grain weight per plant, and thousand kernel weight) were estimated after harvesting. The thousand kernel weight was evaluated by counting and weighing grains from a plant, followed by recalculation per 1000 grains.

In 2016, 20 plants of each line and standards were analyzed, in 2017-2018, 10 plants were collected from the middle row at each plot (in four replicates). Grain morphometric parameters (area, length, width, perimeter, circularity) were assessed with Smart Grain v. 1.2 software (http://www.kazusa.or.jp/phenotyping/smart-grain/index.html). A total of 40 grains per line were examined in 2016, 400 grains in 2017-2018. Synthetic lines were genotyped by KASP™ technology (LGC Genomics, UK) with 47 SNP markers, including 13 markers linked to genes that control grain size and weight.

An Oktopure automated system (Biosearch Technologies, UK) and sbeadex™ plant kit (LGC Genomics, UK) were used for DNA extraction. The final DNA concentration in the samples was normalized to 50 ng/l, and 5 μl aliquotes were added into wells of a 96-well microplate (a repliKator automated station, LGC Genomics, UK). KASP™ genotyping mixture (LGC Genomics, UK) containing 5 μl of KASP Master-Mix and 0.14 1 of KASP Assay Mix (allele-specific primers) were added to each well in an automated mode. PCR was carried out for 1 h 20 min in sealed microplates (a Hydrocycler2™ thermocycler,

LGC Genomics, UK), followed by measurement of FAM/HEX fluorescence (a multifunctional Pherastar reader, BMG Labtech, USA).

Statistical processing comprised calculation of mean (*M*), standard error of the mean (±SEM), and variance analysis. Statistical significance of differences was assessed by the least significant difference at 5 % significance level (LSD_{05}) [27] (Microsoft Excel software). Two-way analysis of variance (ANOVA) was performed for 2 years (2017-2018) with STATISTICA v. 6.0 software (StatSoft, Inc., USA). Variability of traits, as depended on the conditions of the growing season and genotype was assessed by Fisher's *F*-test. Principal component analysis (PCA) was performed using Microsoft R Open 3.3.3 software (https://mran.microsoft.com/download).

Results. Table 1 comprises the list of spring synthetic wheat lines used in the work, table 2 describes SNP markers.

1. Origin of tested synthetic hexaploid wheat lines (AABBDD)

Combination	Numer	*Aegilops tauschii*	
		origin	subspecies
CIMMYT synthetic lines			
Aisberg/*Ae. squarrosa* (369)	5	Mazenderan, Iran	*tauschii*
Aisberg/*Ae. squarrosa* (511)	5	Unknown	Unknown
Leuc. 84693/*Ae. squarrosa* (409)	1	Dagestan, Russia	*tauschii*
Ukr.-Od.952.92/*Ae. squarrosa* (1031)	4	Zanjan, Iran	*tauschii*
Ukr.-Od.1530.94/*Ae. squarrosa* (310)	3	Jilan, Iran	*strangulata*
Ukr.-Od.1530.94/*Ae. squarrosa* (392)	2	Shamakhi, Азербайджан	*tauschii*
Ukr.-Od.1530.94/*Ae. squarrosa* (458)	3	Unknown	Unknown
Ukr.-Od.1530.94/*Ae. squarrosa* (629)	3	Mazenderan, Iran	*strangulata*
Ukr.-Od.1530.94/*Ae. squarrosa* (1027)	10	Mazenderan, Iran	*tauschii*
Pandur/*Ae. squarrosa* (223)	1	Jilan, Iran	*tauschii*
Pandur/*Ae. squarrosa* (409)	1	Dagestan, Russia	*tauschii*
Kyoto University synthetic lines			
Langdon/Ku-20-9	1	Babulsar, Iran	*strangulata*
Langdon/Ku-2075	1	Babulsar, Iran	*strangulata*
Langdon/Ku-2088	1	Sari, Iran	*strangulata*
Langdon/Ku-2092	1	Babulsar, Iran	*strangulata*
Langdon/Ku-2093	1	Babulsar - Chalus, Iran	*strangulata*
Langdon/Ku-2097	1	Babulsar, Iran	*typica*
Langdon/Ku-2105	1	Baklava, Iran	*typica*
Langdon/IG 48042	1	Jammu Kashmir, India	Unknown
Langdon/IG 126387	1	Ashgabat, Turkmenistan	Unknown

Note. KU — Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, IG — International Center for Agricultural Research in the Dry Areas (ICARDA).

2. SNP markers associated with grain size/weight genes of synthetic wheat (AABBDD)

Gene	Chromosome	Marker	Reference
TaCwi-A1	2A	Cwi-A1_SNP	[15]
TaSus2-2A	2A	TaSus2_2A_20_SNP[a]	[23]
TaTGW6-A1	3A	TaTGW6_SNP	[22]
TaGS5-3A	3A	GS5_2334_ SNP	[24]
TaTPP-6A	6A	TPP-6A	[25]
TaGW2-6A	6A	TaGW2_ SNP	[14]
TaGASR-A1	7A	TaGASR_IND	[22]
TaTGW-7A	7A	TGW7A_985	[30]
TaSus2-2B	2B	TaSus2_2B _SNP[a]	http://www.cerealsdb.uk.net
TaSus1-7B	7B	TaSus1_7B_2932_IND[a]	[26]
TaCKX-D1	3D	CKX-D1_IND	[22]
TaCwi-5D	5D	CWI5D_SNP[a]	[22]
TaGS-D1	7D	TaGS-D1_SNP	[22]

Note. Letter [a] means absence of the marker amplification in synthetic lines.

In May 2016, air temperatures were elevated with the absence of precipitation, which led to strong early spring drought. In the second part of the growing season, the weather was moderately warm with heavy rainfall (hydrothermal coefficient HTC = 1.8), favorable for high leaf and stem rust infection, which

resulted in producing smaller and shrunker grain. In 2017, an early summer drought typical of the southern forest-steppe of Western Siberia occurred in the first part of the growing season (HTC = 0.5). During the heading—ripening period, moisture conditions were favorable for grain filling and ripening. In 2018, the weather was cool with a large amount of precipitation during formation of generative organs (HTC = 1.2), with a positive effect on formation of large and completed grains.

CIMMYT and Kyoto University synthetic lines with six durum wheat varieties and various *Ae. tauschii* accessions in pedigree were significantly polymorphic in grain size and weight, which indicats unexploited potential for breeding for high productivity. Meteorological conditions during the growing season significantly influenced the formation of morphometric parameters of grains (Table 3).

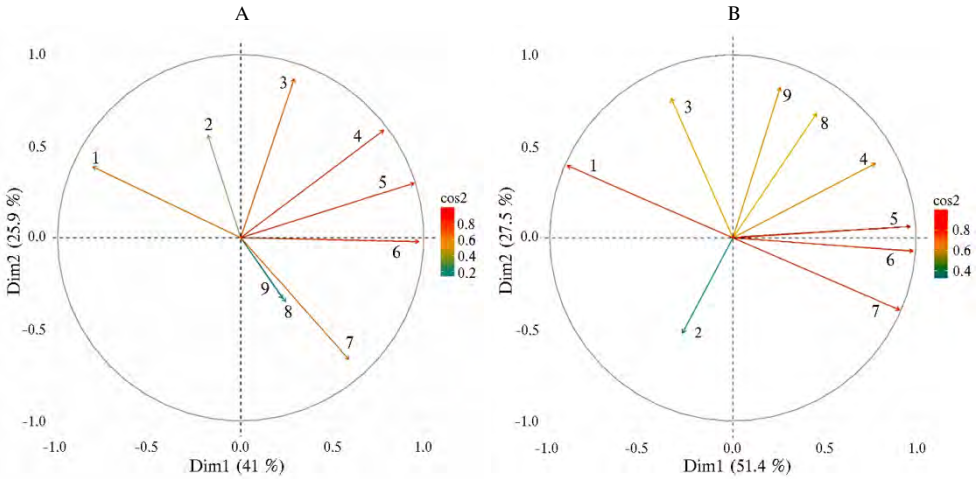
3. Main morphometric parameters of grain amd thousand kernel weight of hexaploid synthetic wheat lines (AABBDD) over years of research (experimental field of Omsk SAU)

Trait	Year	*M*±SEM	Min-max	Pamyati Azieva (standard), *M*±SEM	*Cv*, %	$F_{observed}$	
						genotype	year
Grain length, mm	2016	7.07±0.07	5.66-8.50	6.45±0.04	7.4	4.29**	203.30**
	2017	7.31±0.06	6.48-8.59	6.48±0.03	6.5		
	2018	8.37±0.07	7.43-9.44	7.68±0.02	5.7		
	2016-2018	7.58±0.06	6.74-8.51	6.87±0.03	5.4		
Grain width, mm	2016	2.93±0.03	2.10-3.30	3.29±0.03	8.1	1.47	264.90**
	2017	3.42±0.02	3.10-3.66	3.29±0.02	3.8		
	2018	3.79±0.02	3.48-4.05	3.83±0.01	3.1		
	2016-2018	3.38±0.02	3.04-3.60	3.47±0.02	3.3		
Grain area, mm^2	2016	18.80±0.35	15.90-22.50	15.9±0.17	7.8	2.35**	142.80**
	2017	20.50±0.21	14.20-25.50	15.90±0.14	12.1		
	2018	23.90±0.21	21.00-28.20	22.10±0.14	6.0		
	2016-2018	21.10±0.19	17.90-23.60	18.00±0.15	6.5		
Grain perimeter, mm	2016	18.60±0.15	16.80-21.40	16.60±0.17	3.5	5.87**	53.10**
	2017	19.20±0.15	18.20-20.50	16.80±0.13	5.6		
	2018	20.90±0.13	19.00-22.90	19.50±0.16	4.4		
	2016-2018	19.70±0.14	18.20-21.80	17.70±0.15	4.9		
Grain circularity	2016	0.680±0.004	0.63-0.69	0.730±0.005	2.7	0.27	0.56
	2017	0.690±0.003	0.63-0.75	0.720±0.003	4.1		
	2018	0.690±0.003	0.60-0.75	0.730±0.001	3.8		
	2016-2018	0.690±0.004	0.63-0.75	0.730±0.003	3.8		
Thousand kernel weight, g	2016	34.90±2.10	23.30-44.50	33.20±0.59	14.5	1.68*	146.50**
	2017	45.70±2.37	33.00-57.40	42.10±0.91	11.6		
	2018	46.70±1.66	37.60-53.70	44.70±0.66	8.4		
	2016-2018	42.40±1.39	35.70-51.30	39.40±0.72	7.7		

*, ** Variance is statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

Genotypic differences between synthetic lines on the studied traits were reliable at 5% significance level, with the exception of the width and circularity of the grain. On average, for 2016-2018, synthetic lines had low variability of grain morphometric parameters (*Cv* = 3.3-6.5%) and higher average values of the length, area, perimeter of the grain, and thousand kernel weight than the standard (Pamyat Aziev variety) (see Table 3). The circularity indirectly characterizes the grain shape, i.e. the higher it is, the grain closer approaches in shape to the ball and, accordingly, the higher the flour output [28]. In general, the grain of the synthetic lines had a smaller width and circularity compared to standard Pamyati Azieva.

In 2017-2018, synthetic lines had a grain width comparable to the standard under weak development of rust diseases. The analysis of principal components (PCA) based on the correlation matrix revealed the relationship between the synthetic line yields and grain morphometric parameters (Fig.).



Analysis of principal components (PCA) for 47 synthetic hexaploid wheat lines (AABBDD) shows the relationship of yield with grain morphomentic parameters, protein content and gluten content: A — 2017, B — 2018; 1 — cyrcularity, 2 — yield, g/m^2, 3 — grain wigth, mm, 4 — grain area, mm^2, 5 — grain perimeter, mm, 6 — grain length, mm, 7 — grain lengh to grain width ratio, 8 — gluten content, %, 9 — protein content, %; cos2 — vector of initial variables indicating the significance of the principal component (experimental field of Omsk SAU).

The first main component (Dim1) contributed 41.0% to the total phenotypic variability of the studied parameters in 2017 and 51.4% in 2018. The contribution of the second component (Dim2) was 25.9 and 27.5%, respectively. A two-dimensional graphical representation of the results of PCA analysis revealed a closer relationship between yield and width and shape of the grain (circularity) in 2017 and less close in 2018. This is due to the fact that under favorable moisture conditions in 2018, the number of productive tillers made a greater contribution to the yield.

In both years of research, there was a positive correlation of yield with the grain length, area, perimeter and a negative relationship with grain quality indicators (protein and gluten content). We also established an insignificant relationship between the length and width of the grain (r from –0.08 to –0.23), which is consistent with data from other works [18, 19]. Consequently, the selection of synthetic lines with a wider and rounded grain can reveal high yilding synthetic wheat genotypes for breeding.

Assessment of polymorphism at the loci responsible for grain size and weight with 13 SNP markers revealed the amplification of 9 markers in the genotypes of synthetic forms. The occurrence of the identified loci in the studied synthetic lines was different: 11.1% for *TaTTP6A*, 25.3% for *TaGW2-6A*, 30.6% for *TaGASR-A1*, 30.6% for *TaGS-D1*, 55.6% for *TaGS5-3A*, 66.7% for *TaTGW6*, 72.2% for *TaTGW-7A*, 77.8% for *TaCwi-A1*, and 100% for *TaCKX-D1*. As compared to the standards (Pamyati Azieva and Serebristaya), the synthetic lines have *TaGW2*, *TaTPP6A*, and *TaTGW6* loci. Table 4 presents the best synthetic lines with a combination of 4-7 genes of *TaCwi-A1*, *TaTGW6*, *TaGASR-A1*, *TaGS5-3A*, *TaTPP-6A*, *TaGW2*, *TaTGW-7A*, *TaGS-D1*, and *TaCKX-D1*, selected by the morphomentic parameters and thousand kernel weigh.

Phenotypic estimates of the hexaploid synthetic wheat lines showed that the studied morphometric characteristics of grain in the lines identified over 3 years were higher than the standards, except for the width of the grain. Lines No. 8 Ukr.-Od.1530.94/*Ae. squarrosa* (392), No. 37 Ukr.-Od.1530.94/*Ae. squarrosa* (310), No. 44 and No. 46 Ukr.-Od.1530.94/*Ae. squarrosa* (1027) had higher

thousand kernel weight and two lines exceeded the Serebryristaya standard in the grain width. Japanese breeding lines No. 29 Langdon/IG 126387 и No. 63 Langdon/Ku-2092 also had a higher thousand kernel weight and a longer grain length (see Table 4).

4. Grain morphometric parameters and 1000-grain weight in the best studied lines of hexaploid synthetic wheats (AABBDD) (*M*±SEM, experimental field of Ovsk SAU, 2016-2018)

Line No.	Variety, line	Gene number	Grain				Circularity	Thousand kernel weigh, g
			lenghth, mm	wigth, mm	area, mm^2	perimeter, mm		
8	Ukr.-Od.1530.94/ *Ae. squarrosa* (392)	7	6.94±0.04	3.55±0.03^{b}	20.70±0.33^{ab}	18.40±0.14^{ab}	0.750±0.004^{at}	43,80±1,87^{a}
24	Aisberg/ *Ae. squarrosa* (511)	5	8.00±0.06^{ab}	3.40±0.03	22.50±0.18^{ab}	20.70±0.15^{ab}	0.660±0.003	45,40±2,79^{ab}
27	Leuc 84693/ *Ae. squarrosa* (409)	6	7.87±0.05^{ab}	3.37±0.02	22.00±0.32^{ab}	20.60±0.16^{ab}	0.670±0.003	43,30±3,00^{a}
37	Ukr.-Od.1530.94/ *Ae. squarrosa* (310)	7	7.24±0.07	3.54±0.02^{b}	21.60±0.44^{ab}	18.80±0.12^{ab}	0.730±0.002^{b}	43,50±2,92^{a}
41	Ukr.-Od.1530.94/ *Ae. squarrosa* (1027)	6	7.39±0.06^{ab}	3.43±0.03	20.90±0.41^{ab}	19.80±0.18^{ab}	0.710±0.004	46,80±3,02^{ab}
44	Ukr.-Od.1530.94/ *Ae. squarrosa* (1027)	7	7.52±0.04^{ab}	3.48±0.03	21.90±0.21^{ab}	19.90±0.16^{ab}	0.700±0.002	43,70±1,98^{a}
46	Ukr.-Od.1530.94/ *Ae. squarrosa* (1027)	7	7.64±0.04^{ab}	3.60±0.02	22.70±0.42^{ab}	20.10±0.18^{ab}	0.710±0.003	51,30±2,66^{ab}
29	Langdon/IG 126387	6	8.22±0.06^{ab}	3.04±0.03	21.90±0.29^{ab}	20.70±0.16^{ab}	0.650±0.002	43,80±1,87^{a}
63	Langdon/Ku-2092	4	8.41±0.06 ^{ab}	3.39±0.03	23.30±0.33^{ab}	21.30±0.17^{ab}	0.660±0.004	46,40±3,17^{ab}
65	Langdon/Ku-2105	4	8.51±0.05^{ab}	3.39±0.03	23.60±0.24^{ab}	21.80±0.14^{ab}	0.640±0.004	44,70±3,04^{ab}
Pamyati Azieva (standard)		4	6.87±0.03	3.47±0.02	18.00±0.15	17.70±0.15	0.730±0.003	39.40±0.72
Serebristaya (standard)		4	6.89±0.04	3.33±0.02	17.90±0.27	17.20±0.17	0.720±0.003	40.30±1.31
LSD_{05}			0.47	0.21	2.14	0.73	0.01	3.62

$^{a, b}$ Differences between synthetic lines and standards (Pamyati Azieva and Serebristaya) are statistically signiificant at $p < 0.05$.

We have identified 7 loci (*TaCwi-A1*, *TaGASR-A1*, *TaGS5-3A*, *TaGW2*, *TaTGW-7A*, *TaGS-D1*, *TaCKX-D1*) in lines No. 8 Ukr.-Od.1530.94/*Ae. squarrosa* (392), No. 37 Ukr.-Od.1530.94/*Ae. squarrosa* (310), No. 44 and No. Ukr.-Od.1530.94/*Ae. squarrosa* (1027), and 6 loci we identified in lines No. 27 of Leuc84693/*Ae. squarrosa* (409), No. 41 Ukr.-Od. 1530.94/*Ae. squarrosa* (1027), and No. 29 Langdon/IG 126387 (except *TaGW2*). These lines are recommended for wheat selection as sources of a combination of genes that are responsible for grain size and thousand kernel weight. Several paperes report the possibility of increasing the size and weight of grain in spring bread wheat varieties using sources of similar loci, *TaCwi-A1* (29), *TaGW2* (14), *TaGASR-A1* (22), *TaGS5-3A* [24], TaTGW-7A [30]. However, these loci cannot be deemed crucial until further phenotyping of synthetic lines in specific soil and climatic conditions. Since many of the identified loci that control plant productivity traits are insignificant or minor, it is necessary to identify key loci that determine the formation of a quantitative trait, although the contribution of such loci is certainly determined by the limiting environmental factor [9, 31].

Our paper shows the first rusults of phenotyping performed in Western Siberia for grain morphometric parameters of synthetic hexaploid wheat lines with D genome of *Ae. tauschii* subspecies, originating from regions with the greatest genetic diversity of this species, i.e. from the southwestern coast of the Caspian Sea (*Ae. tauschii* ssp. *tauschii*), and from Azerbaijan and the northern provinces of Iran, Golestan and Mazenderan (*Ae. tauschii* ssp. *strangulata*) [32, 33].

Our findings indicate a high genetic diversity of synthetic wheat lines as genetic sources for increasing size and weight of the grain of bread wheat varieties in Western Siberia. The largest polymorphism of thousand kernel weigh and

the main morphometric characteristics of the grain is noted in hybrids combinations Aisberg/*Ae. squarrosa* (369), Ukr.-Od.1530.94/*Ae. squarrosa* (1027) (Iran, Mazenderan) and Langdon/*Ae. squarrosa* (Iran, Turkmenistan, and India). Aisberg/*Ae. squarrosa* (511), Ukr.-Od.1530.94/*Ae. squarrosa* (392), Ukr.-Od.1530.94/*Ae. squarrosa* (1027) combinations and synthetic lines created on the basis of durum wheat variety Langdon had maximum thousand kernel weight values.

The involvement of Ukr.-Od.1530.94, Aisberg (Ukraine), and Langdon (USA) durum winter wheat varieties in crossing contributed to higher genetic diversity of synthetic lines in terms of grain size and weight, as it is confirmed by larger grain size and higher 1000-grain weight of synthetic lines compared to the standards, together with detection of *TaGW2*, *TaTPP6A*, and *TaTGW6* loci. We have identified *TaCKX-D1* and *TaGS-D1* genes that control these traits [12, 18] in D subgenome introduced into the synthetic lines from *Ae. tauschii*. In the future, we plan to assess polymorphism of genome D in these synthetic lines for kernel size/weight genes with expanded set of SNP markers.

The positive correlation revealed in synthetic lines between the yield and the grain parameters indicates the prospect of selection forms with wider rounded grain and greater endosperm volume, therefore, having a larger weight, which is confirmed by data from other studies [18, 19].

For the success of valuable introgressions from synthetic wheat forms during hybridization with commercial varieties, their pre-selection study is of no small importance. Yan et al. [11] note a negative correlation between the thousand kernel weight and the productive spikes number per plant, and also the grain number per spike. Therefore, valuable introgressions from synthetic hexaploids should be investigated both for the loci that control grain size and for their effect on yield and its components in different genotypes of bread wheat. Kyoto synthetic lines produced a larger and heavier grains compared to the CIMMYT lines, but were less adaptable and productive under conditions of Western Siberia. The lines No. 8 Ukr.-Od. 1530.94/*Ae. squarrosa* (392), No. 37 Ukr.-Od.1530.94/*Ae. squarrosa* (310) and No. 44, No. 46 Ukr.-Od.1530.94/*Ae. squarrosa* (1027) (*TaCwi-A1*, *TaGASR-A1*, *TaGS5-3A*, *TaGW2*, *TaTGW-7A*, *TaGS-D1* and *TaCKX-D1*), No. 27 Leuc 84693/*Ae. squarrosa* (409), No. 41 Ukr.-Od.1530.94/*Ae. squarrosa* (1027), No. 29 Langdon/IG 126387 (except *TaGW2*) can be recommended for pyramiding of the grain size/weight determining genes in wheat breeding.

Thus, grain size phenotyping and genotyping of hexaploid synthetic wheat lines perforemed in Western Siberia, indicates their significance as sources of grain size and thousand kernel weight gene combinations for new commercial varieties ensuring high and stable yield in the region. Assessment of the main grain morphometric parameters, their variability under the influence of the environmental factors, and the relationship of these traits with yield revealed low variability of grain morphometric parameters (Cv = 3.3-6.5%), However, grain average length (7.58 mm), area (21.1 mm^2), perimeter (19.7 mm), and thousand kernel weight (34.9-46.7 g) are higher compared to the standards. In the synthetic lines nine loci determining grain size and thousand kernel weight, the *TaTTP6A*, *TaTGW2-6A*, *TaGASR-A1*, *TaGS5-3A*, *TaTGW6*, *TaTGW-7A*, *TaCwi-A1*, *TaGS-D1*, and *TaCKX-D1* are identified.

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ENLARGEMENT OF GENETIC DIVERSITY OF SPRING BREAD WHEAT RESISTANCE TO LEAF RUST (*Puccinia triticina* Eriks.) IN LOWER VOLGA REGION

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Abstract

Leaf rust (*Puccinia triticina* Eriks.) is the significant disease of winter and spring wheat in Russia. In the Volga region, the epiphytoties of this disease are observed on average once per three to four years. The genetic protection of wheat from leaf rust is a priority. Its successful practical implementation is possible only by the increasing of the genetic diversity of the commercial wheat cultivars, particularly by effective combinations of the known genes for resistance or use in the hybridization donors of new *Lr*-genes, from species of genera *Triticum* and *Aegilops*. On the basis of highly productive and adaptive spring bread wheat cultivars (Prokhorovka, Saratovskaya 29, Saratovskaya 55, Saratovskaya 68, Saratovskaya 70, Saratovskaya 73, Saratovskaya 74, L503, L505, Dobrynya, Favorite, Belyanka, Voevoda of Saratov Breeding Center) and alien species the introgression lines are derived which possess high resistance to leaf rust and are promising as breeding material. It was of interest to study the genetic determination of leaf rust resistance in these new lines and to evaluate their effect on the variability of *P. triticina* population for virulence in Saratov region. A total of 42 introgression lines were investigated. Donors of alien *Lr*-genes were the lines of cultivar Thahcher with *Lr24*, *Lr29*, *Lr36* genes, and cultivars with *Lr37* gene, and also species *Triticum dicoccum*, *T. kiharae*, *T. timopheevii*, *T. durum*, *T. petropavloskyi*, *T. persicum*, *Aegilops tauschii*, *Secale sereale* and *Agropyron elongatum*. Leaf rust resistance genes (*Lr*-genes) were identified by phytopathological tests and DNA markers. The studied lines of spring bread wheat showed high genetic diversity for leaf rust resistance. Among them, we have identified the carriers of known *Lr*-genes which have not yet been used in breeding of spring bread wheat in Russia (L4 with *Lr29*), and also the carriers of presumably new *Lr*-genes transferred from *T. durum* (L8, L39 for *Lr19* + *LrTdur*, L25, L19, L11 for *Lr10* + *Lr19* + *LrTdur*), *T. persicum* (L38 for *Lr19* + *LrT.pers*), *T. timopheevii* (L49 for *Lr10* + *LrT.tim*), *Ae. tauschii* (L6 for *Lr19* + *LrA.tau*), and *T. kiharae* (L33 for *Lr3* + *Lr19* + *LrT.kh*). Lines L10, L13, L46, L24, L48, L5 and L9 have the effective combination of *Lr19* + *Lr26* genes, L2, L28 L29 of *Lr10* + *Lr19* + *Lr26*, L42 of *Lr19* + *Lr37*, L44 of *Lr19* + *Lr26* + *Lr39*, L3 of *Lr19* + *Lr37* + *Lr6Agi*, L4 of *Lr19* + *Lr6Agi*, L7 of *Lr10* + *Lr26* + *Lr6Agi*, L45 of *Lr10* + *Lr19* + *Lr39* + *Lr6Agi*, and L40 of *Lr10* + *Lr39* + *Lr6Agi*. The virulence of the pathogen of the Saratov population was characterized in 2017 and 2018. The samples were collected from susceptible wheat cultivars which grew together with the studied introgression lines. The *Lr9*, *Lr24*, *Lr28*, *Lr29*, *Lr41*, *Lr42*, *Lr45*, *Lr47*, *Lr50*, *Lr51*, *Lr53*, and *Lr6Agi* genes (infection type 0 and 0;) were highly effective. Lines with *Lr28*, *Lr29*, *Lr41, Lr51*, and *Lr6Agi* genes also showed high resistance under field conditions. Thus, all these genes are perspective for breeding in the Volga region to expand genetic diversity of wheat cultivars. The presence of the isolates virulent to Tc*Lr19* lines was moderate, 16 % in 2017 and 20 % in 2018. All isolates virulent to *Lr19* were avirulent to *Lr26*, which confirms the effectiveness of this combination of *Lr*-genes in plant protection from leaf rust. This research resulted in a novel breeding material that combines resistance to leaf rust with adaptability to environmental factors, productivity and grain quality. Its distinctive feature is new donors of resistance involved from related species. Among tested lines there are donors which effectively combine either known *Lr*-genes or known and supposedly

new alien *Lr*-genes. The linkage of *Lr19*, *Lr26*, *Lr34*, *Lr37* genes with effective genes for resistance to other diseases, in particular to stem rust, will determine the resistance of new lines to a complex of diseases.

Keywords: *Puccinia triticina*, virulence, avirulence, *Triticum aestivum*, introgression lines, *Lr*-genes

Brown (leaf) rust (*Puccinia triticina* Erikss.) is a disease of common wheat with significant economic impact in many countries, including the Russian Federation. In the Volga region, the disease occurs almost annually, with epiphytoties observed on average once every three to four years. Crop losses can reach 20-30% (35% under irrigation), while the content of protein and gluten in the grain is significantly lower [1-3]. An analysis of the chronology of epiphytoties suggests that in the Volga region losses from leaf rust have recently become severer than in the first half of the 20th century [4]. Protecting bread wheat from this disease is becoming a priority. Improvement of genetic diversity of locally bred, highly productive and adapted to Volga region spring bread wheat varieties via involvement of *Triticum* or *Aegilops* species as donors or through a combination of known *Lr* genes is deemed most effective.

The first attempts to produce wheat varieties by introgressive hybridization with closely related species were made in Lower Volga Region in the first half of the 20th century by crossing bread wheat (*Triticum aestivum* L.) with *Triticum durum* Desf. [5]. As a result, spring bread wheat varieties Sarroza, Sarrubra, Albosar, Blansar were obtained, of which Sarrubra was regionalized in 1931 and occupied about 1.3 million ha in the early 1940s [6]. Later, species of the genera *Triticum*, the *T. durum*, *T. dicoccum* Schuebl., *T. dicocoides* (Koern. ex Aschers. et Graebn) Schweinf., and *Agropyron*, the *Ag. intermedium* (Host.) Beauv., *Ag. longatum* (Host.) P.B., as well as *Secale cereale* L. were involved to expand the regional genetic diversity of common wheat varieties in the region. The resultant varieties were L503, L505, Dobrynya (with genetic material from *Ag. Elongatum*), Belyanka (*Ag. intermedium*), Favorit, Voevoda (a combination of genetic material from *Ag. intermedium* and hard wheat variety Krasnokutka 10), Lebedushka (genetic material from Ag. *elongatum* and Ag. *intermedium*), Prokhorovka, Yugo-vostochnaya 2 (*Secale cereale*) [6].

Improving plant protection by increasing genetic diversity of highly productive wheat varieties via donors of new *Lr* genes or an effective combination of known *Lr* genes allows the epiphytotic situation with brown rust to be stabilized [2]. The genetic diversity of *Lr* genes among spring common wheat hybrids that are highly resistant to brown rust is an extremely important fundamental and practical issue.

The knowledge on the virulence-based genetic structure of a pathogen population is a background of the advanced breeding for crop resistance. It allows researchers to optimize strategies for using new resistance donors to control the phytosanitary situation [2, 3]. The *P. triticina* virulence in the Volga Region have been studied since 1970 [7]. Long-term observations show that the Lower Volga population of brown rust pathogen is evolutionarily active, and its virulence is increasing [8, 9]. This is primarily caused by the use of new genetically protected wheat varieties, as well as the fact that the territory of the Lower Volga Region is subjected to the inoculum drift from the North Caucasus, from Western Europe and Central Asia [10].

In this paper, we give the first results on the resistance gene diversity of promising spring bread wheat lines in the conditions of Lower Volga Region. Effective combinations of resistance genes, carrier lines of new unidentified *Lr* genes introgressed from durum wheat varieties, as well as the structure of the brown rust pathogen population in the Saratov Region are determined.

Our objective was to identify genetic determinants of brown rust resistance in new introgression lines of spring common wheat, to investigate changes in the composition of the present brown rust pathogen populations and to estimate prospects of using the obtained set of lines carrying *Lr* genes in breeding for brown rust resistance in the Volga Region.

Materials and methods. The promising introgression lines of bread wheat (n = 42) which showed high resistance to brown rust in the Lower Volga region in 2014-2018 we tested. Spring common wheat varieties Saratovskaya 68, Saratovskaya 70, Saratovskaya 73, Saratovskaya 74, Favorit, Dobrynya, Belyanka, Voevoda, L503, L505, Prokhorovka were widely used as recurrent parents. To increase genetic diversity on brown rust resistance, these varieties were crossed with the carriers of alien effective genes (*Lr24*, *Lr29*, *Lr36*, *Lr37*, etc.), with brown rust resistant specimens of *T. durum*, *T. dicoccum* (Schrank) Schuebl., *T. persicum* (Percival) Vavilov., *T. timopheevii* Zhuk., *T. kiharae* Dorof. et Migusch., *Aegilops squarrosa* L. (= *Ae. tauschii* L.) [11, 12] and with the susceptible species *T. petropavloskyi* Udacz et. Migusch.

Brown rust resistance of the introgression lines was assessed in lab tests on seedlings (1st leaf phase) and in field trials (plants in the phase of milk and milk-wax ripeness; Agricultural Research Institute for the South-East Regions, (ARISER), natural infectious). Seedlings were inoculated with four geographically distant populations of *P. triticina* (Saratov, Chelyabinsk, Krasnodar, Dagestan) sampled in 2018, and with three test clones marked by the virulence for *Lr9*, *Lr19* and *Lr26* carriers.

The tested wheat lines were sown in pots with soil. At the 1st leaf (days 10-14), the seedlings were inoculated with an aqueous suspension of pathogen spores (1×10^6/ml) with Tween 80 detergent added. The infected plants were grown in a moist chamber in the dark for 12-14 hours, and then transferred to a climate chamber (Versatille Environmental Test Chamber MLR-352H, SANYO Electric Co., Ltd, Japan) (22 °C, 75% humidity). On day 10 the lesions were recorded as per Mains and Jackson scale [13]: 0 — no symptoms, 0; — necrosis without pustules, 1 — very small pustules surrounded by necrosis, 2 — medium sized pustules surrounded by necrosis or chlorosis, 3 — medium sized pustules without necrosis, 4 — large pustules without necrosis, X — different types of pustules on the same leaf, chloroses and necrosis are present. Plants with a type infection of 0, 0;, 1, and 2 were classified as resistant, 3, 4, X as susceptible.

Molecular markers for identification of 22 *Lr* genes were WR003 (*Lr1*) [14], Xmwg798 (*Lr3*) [15], SCS5 (*Lr9*) [16)], Fi.2245/Lr10-6/r2 (*Lr10*) [17], SCS265 (*Lr19*) [18], STS638 (*Lr20*) [19], Lr21L/R (*Lr21*) (https://maswheat.ucdavis.edu/protocols/Lr21/index.htm), WMS296 (*Lr22a*) [20], Sr24#12, Sr24#50 (*Lr24*) [21], Lr25F20/R19 (*Lr25*) (https://maswheat.ucdavis.edu/protocols/Lr25/index.htm), SCM9 (*Lr26*) [22], SCS421 (*Lr28*) [23], Lr29F24 (*Lr29*) [24], csLV34 (*Lr34*) [25], Sr39=22 (*Lr35*) [26], Ventriup/LN2 (*Lr37*) [27], GDM35 (*Lr41*) [28], marker for *Lr47* [29], WMS382, GDM87 (*Lr50*) (https://maswheat.ucdavis.edu/protocols/Lr50/index.htm), cfd1 (*Lr53*) [30], S13-R16 (*Lr66*) [31], J09/1_pr1,4a (*LrAgi*) [32, 33]. DNA from wheat plants was extracted by the Dorokhov and Kloke method [34].

The virulence of the Saratov population *P. triticina* was analyzed in 2017-2018. The inoculum was collected in the ARISER experimental field. Reproduction of population samples and obtaining monopustular isolates were performed by laboratory cultivation method [35]. Virulence of the pathogen and line resistance to brown rust was studied on the 1st leaf wheat seedlings as per description [36]. In 35 isogenic Thatcher lines and wheat varieties with genes *Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr9*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*,

Lr15, *Lr16*, *Lr17*, *Lr18*, *Lr19*, *Lr20*, *Lr21*, *Lr24*, *Lr26*, *Lr28*, *Lr29*, *Lr30*, *Lr39(=41)*, *Lr42*, *Lr44*, *Lr45*, *Lr47*, *Lr48*, *Lr49*, *Lr51*, *Lr53*, *Lr57* and *Lr6Agi* evaluated the resistance to the combined sample of Saratov pathogen population. The racial composition of the pathogen and the frequency of virulence to 20 differentiator lines were determined using monopuscular isolates. Phenotypes were identified by the North American nomenclature [37], based on the determination of virulence for groups of Tc*Lr* lines. In this paper, the following sequence of Tc*Lr* lines was used (by the set of *Lr* genes): 1 — *Lr1*, *Lr2a*, *Lr2c*, *Lr3a*; 2 — *Lr9*, *Lr16*, *Lr24*, *Lr26*; 3 — *Lr3ka*, *Lr11*, *Lr17*, *Lr30*; 4 — *Lr2b*, *Lr3bg*, *Lr14a*, *Lr14b*; 5 — *Lr15*, *Lr18*, *Lr19*, *Lr20*. The literal code of phenotypes and virulence frequency was received via Virulence Analysis Tool (VAT) software (https://en-lifesci.tau.ac.il/profile/kosman/vat).

Results. Table 1 gives the characterization of the infectious material virulence, and Table 2 comprises the list of markers for identification of pathogen resistance genes.

1. Characterization of *Puccinia triticina* Erikss. virulence to Thatcher lines used in testing resistance of the spring bread wheat introgression lines to the pathogen

Populations and isolates	Origin	Virulence	Avirulence
		to Thatcher lines carrying *Lr* genes	
Test-clone1	Chelyabinsk Province, 2017	*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr30*	*Lr19*, *Lr23*, *Lr24*, *Lr26*, *Lr28*, *Lr29*, *Lr44*
Test-clone2	Tambov Province, 2016	*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr14a*, *Lr14b*, *Lr15*, *Lr17*, *Lr18*, *Lr19*, *Lr20*, *Lr30*, *Lr44*	*Lr9*, *Lr11*, *Lr16*, *Lr23*, *Lr24*, *Lr26*, *Lr28*, *Lr29*
Test-clone 3	Krasnodarskii Krai, 2017	*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr17*, *Lr18*, *Lr20*, *Lr23*, *Lr6*, *Lr30*, *Lr44*	*Lr9*, *Lr16*, *Lr19*, *Lr24*, *Lr28*, *Lr29*
Pop _Sar	Saratov Province, 2018	*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr14b*, *Lr15*, *Lr19*, *Lr20*, *Lr23*, *Lr26, Lr30*	*Lr9*, *Lr24*, *Lr28*, *Lr29*, *Lr44*
Pop_Kr	Krasnodarskii Krai, 2018	*Lr1*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr16*, *Lr17*, *Lr18*, *Lr23*, *Lr26*, *Lr30*, *Lr44*	*Lr9*, *Lr2a*, *Lr15*, *Lr19*, *Lr20*, *Lr24*, *Lr28*, *Lr29*
Pop _Chel	Chelyabinsk Province., 2018 год	*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr9*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr30*	*Lr19*, *Lr23*, *Lr24*, *Lr26*, *Lr28*, *Lr29*, *Lr44*
Pop_Dag	The Republic of Dagestan, 2018	*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr23*, *Lr26*, *Lr30*, *Lr44*	*Lr9*, *Lr19*, *Lr24*, *Lr28*, *Lr29*

2. PCR markers used to determine *Lr genes*

Gene	Marker	Nucleotide sequence 5′→3′	Size, bp	Referencce
Lr1	WR003F	GGGACAGAGACCTTGGTGGA	760	Qiu et al., 2007
	WR003R	GACGATGATGATTTGCTGCTGG		
Lr3	Xmwg798F	GGCTGTCTACATCTTCTGCA	365	Herrera-Foessel et al., 2007
	Xmwg798R	CAAGTGTTGAGAAGGAGAGT		
Lr9	SCS5F	TGCGCCCTTCAAAGGAAG	550	Gupta et al., 2005
	SCS5R	TGCGCCCTTCTGAACTGTAT		
Lr10	Fi.2245	GTGTAATGCATGCAGGTTCC	310	Chelkowski et al., 2008
	Lr10-6/r2	AGGTGTGAGTGAGTTATGTT		
Lr19	SCS265 F	GGCGGATAAGCAGAGCAGAG	512	Gupta et al., 2006
	SCS265 R	GGCGGATAAGTGGGTTATGG		
Lr20	STS638-L	ACAGCGATGAAGCAATGAAA	542	Neu et al., 2002
	STS638-R	GTCCAGTTGGTTGATGGAAT		
Lr21	Lr21L	CGCTTTTACCGAGATTGGTC	669	https://maswheat.ucdavis.edu/
	Lr21R	TCTGGTATCTCACGAAGCCTT		
Lr22a	WMS296F	AATTCAACCTACCAATCTCTG	131	Hiebert et al., 2007
	WMS296R	GCCTAATAAACTGAAAACGAG	121	
Lr24	Sr24 12F	CACCCGTGACATGCTCGTA	550	Mago et al., 2005
	Sr24 12R	AACAGGAAATGAGCAACGATGT		
Lr25	Lr25F20	CCACCCAGAGTATACCAGAG	1800	https://maswheat.ucdavis.edu/
	Lr25R19	CCACCCAGAGCTCATAGAA		
Lr28	SCS421F	ACAAGGTAAGTCTCCAACCA	570	Cherukuri et al., 2005
	SCS421R	AGTCGACCG AGATTTTAACC		

Continued Table 2

Lr29	Lr29F24F	GTGACCTCAGGCAATGCACACAGT	900	Procunier et al., 1995
	Lr29F24R	GTGACCTCAGAACCGATGTCCATC		
	SCM9F	TGACAACCC CCTTTCCCTCGT	207	Weng et al., 2007
	SCM9R	TCATCGACGCTAAGGAGGACCC		
Lr34	csLV34F	GTTGGTTAAGACTGGTGATGG	150	Lagudah et al., 2006
	csLV34F	TGCTTGCTATTGCTGAATAGT		
Lr35	Sr39=22F	AGAGAAGATAAGCAGTAAACATG	800	Mago et al., 2009
	Sr39=22R	TGCTGTCATGAGAGGAACTCTG		
Lr37	Venttriup	AGGGGCTACTGACCAAGGCT	259	Helguera et al., 2003
	LN2	TGCAGCTACAGCAGTATGTACACAAAA		
Lr39=Lr41	GDM 35F	CCTGCTCTGCCCTAGATACG	190	Pestsova et al., 2000
	GDM 35R	ATGTGAATGTGATGCATGCA		
Lr47	PS10F	GCTGATGACCCTGACCGG	282	Helguera et al., 2000
	PS10R	TCTTCATGCCCGGTCGGGT		
Lr50	WMS382-F	GTCAGATAACGCCGTCCAAT	139	https://maswheat.ucdav-is.edu/protocols/Lr50/index.htm
	WMS382-R	CTACGTGCACCACCATTTTG		
	GDM87F	AATAATGTGGCAGACAGTCTTGG	110	
	GDM87R	CCAAGCCCCAATCTCTCTCT		
Lr53	cfd1F	ACCAAAGAACTTGCCTGGTG	225	Dadkhodaie et al., 2010
	cfd1R	AAGCCTGACCTAGCCCAAAT		
Lr66	S13-R16F	GGTGAACGCTAAACCCAGGTAACC	695	Marais et al., 2010
	S13-R16R	CAACCTGGGAAGATGCTGAG		
LrAgi	J09/1	TCTAGTCTGTACATGGGGGC		Schachermayr et al., 1995
	Pr1, 4a	Confidential information		Sibikeev et al., 2018

A characteristic feature of the Saratov breeding school in production of wheat varieties is the continuity and improvement of the local highly adapted gene pool with new genetic material [38]. Spring bread wheat varieties that we used as a recurrent parent belong to the group of highly productive and widely cultivated in the Lower Volga and other Russian regions [39]. These varieties differ significantly in their resistance to brown rust. The group of varieties of the Saratovskaya brand (Saratovskaya 29, Saratovskaya 55, Saratovskaya 68, Saratovskaya 70, Saratovskaya 73, Saratovskaya 74) and the variety Prokhorovka are highly susceptible. PCR analysis showed that most of them have an ineffective *Lr10* gene (with the exception of Saratovskaya 55 and Saratovskaya 70), and Prokhorovka variety additionally carry *Lr26* gene (Table 3).

Lr19 gene protects varieties L503, L505 and Dobrynya. *Lr10* gene is also identified in varieties L503 and L505. Seedlings and adult plants of these varieties showed resistance to pathogen populations, avirulent to lines and cultivars with *Lr19*, and susceptibility to virulent ones (see Table 3). The degree of the damage in the field conditions of the Lower Volga Region varied from 0 to 20%, because of different abundance of isolates that are virulent for plants with the *Lr19* gene, since the area under cultivars carrying this resistance gene has been reducing. In Russia, the first varieties with the *Lr19* gene began to be cultivated since the late 1980s in the Volga Region. When their crop areas in the mid-1990s exceeded 100 thousand ha, the protective effect of *Lr19* was overcome [4]. Currently, virulence to carriers of this gene is recorded both whithin and beyond the regions of cultivation varieties with *Lr19* [40, 41].

Varieties Belyanka, Voevoda, Favorit are the carriers of the Lr6Agi gene which is transferred from the wheatgrass *Elytrigia intermedia* (Host) Nevski and is not identical to the gene included in the gene symbol catalog. These varieties are characterized by high juvenile resistance over a long period of their regionalization [32].

To date, 77 *Lr* genes have been identified worldwide and over 50% of them are alien [42]. Their sources are species *Ae. tauschii* carrying *Lr21*(=*Lr40*), *Lr22a*, *Lr32*, *Lr39*(=*Lr41*), *Ae. umbellulata* (*Lr9*, *Lr76*), *Ae. speltoides* (*Lr28*, *Lr35*, *Lr36*, *Lr47*, *Lr51, Lr66*), *Ae. ventricosa* (*Lr37*), *Ae. kotschyi* (*Lr54*), *Ae. sharonensis* (*Lr56*), *Ae. geneculata* (*Lr57*), *Ae. triuncialis* (*Lr58*), *Ae. peregrina* (*Lr59*), *Ae. neglecta* (*Lr62*), *T. spelta* (*Lr44, Lr71*), *S. cereale* (*Lr25*, *Lr26*, *Lr45*), *T. timo-*

3. Resistance of ARISER spring bread wheat introgression lines to brown (leaf) rust and identified *Lr* genes (experimental field of ARISER, Saratov, 2016-2018)

Line	Pedigree	SH	FD, %/score	Damage to seedlings, score							*Lr* genes
				1	2	3	4	5	6	7	
			Group I								
L3	Milan/Prinia*4//Dobr/3/Fav	*Aegilops ventricosa*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr37* + *Lr6Agi*
L42	Dobr/Trident//Dobr/3/Dobr/4/Dobr	*Aegilops ventricosa*	0/0;	0	0	2–	0	3	0	1-2;	*Lr19* + *Lr37*
L10	L164/Prokh//L164/Dobr *Lr9*	*Ae. umbellulata*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L4	Dobr*2//L2033/Bel/3/Dobr *Lr24*	*Ag. elongatum*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr6Agi*
L52	S70/Tc*Lr29**4//S70	*Ag. elongatum*	0-2/0; and 1	0	0	0	0	0	0	0	*Lr29*
L30	L503 Tc*Lr36*//L503/3/L503	*Ae. speltoides*	0-2/1	0	3	0-1;	0	0	0	0-1	*Lr10* + *Lr19*
			Group II								
L8	L164/Sar. zol//S68	*Triticum durum*	0-5/0 and 3	0	0	0	0	0	0	0	*Lr19* + *LrTdur*
L25	S58*2//Zol. volna/3/S68	*Triticum durum*	0/0;	0	0	0	0	0	0	0	*Lr10* + *Lr19* + *LrTdur*
L39	Dobr/Zol. volna//Dobr/3/Dobr	*Triticum durum*	0/0;	0	0-1	0	0	0	0	0	*Lr19* + *LrTdur*
L43	Dobr*4/Nik	*Triticum durum*	0/0;	0	3	0	0	0	0	0	*Lr19*
L13	Dobr*3//Nik	*Triticum durum*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L19	Dobr*4/Nik/Grekum S2193 L1314/2 16	*Triticum durum*	0-5/0; and 3	0	0	0	0	0	0	0	*Lr10* + *Lr19* + *LrTdur*
L2	L505/L164/4/L503//Trap#1/Bow/3/L503/5/L505/6/Al32	*Triticum durum*	0/0;	0	0	0-1;	0	0	0	0	*Lr10* + *Lr19* + *Lr26*
L11	L505/L164//Prokh	*Triticum durum*	0/0;	0	0-1;	0	0	0	0	0	*Lr10* + *Lr19* + *LrTdur*
L46	Prokh/L164//Prokh/3/L164/4/L164	*Triticum durum*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L38	Dobr/*T. persicum*//Dobr	*T. persicum*	0/0;	0	0-1	0	0	0	0	0	*Lr19* + *LrTpers*
L24	S74/*T. dicoccum* k7507//S73	*T. dicoccum*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L28	S74/*T. dicoccum* k7507//S73/3/S73 L1504/2 16	*T. dicoccum*	0-5/0 and 3	0	0	1-2;	0	0	0	0-1-2;	*Lr10* + *Lr19* + *Lr26*
L29	S74/*T. dicoccum* k7507//S73/3/S73	*T. dicoccum*	0/0;	0	0-1	0	0	0	0	0-1	*Lr10* + *Lr19* + *Lr26*
L47	S74/*T. dicoccum* k7507//S73/3/S73	*T. dicoccum*	0/0;	0	0	3	0	0-2	0	3	*Lr10* + *Lr26*
L48	S74/*T. dicoccum* k7507//S73/3/S73	*T. dicoccum*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L5	S55*5/*T. dicoccoides*// Dobr *Lr9*	*T. dicoccoides* + *Lr9*	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L49	S68/*T. timopheevi**4//Dobr	*T. timopheevii*	0/0;	0	0	0	0	0	0	0	*Lr10* + *Lr28*=*LrTtim?*
			Group III								
L6	Croc/*Ae. squarrosa* (205)//Weaver/3/*3 L505	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0;	0	0	0	0	0	*Lr19* + *LrAtau?*
L7	Bel/3/Croc/*Ae. squarrosa* (205)//Weaver/4/Bel	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr10* + *Lr26* + *Lr6Agi*
L9	Croc/*Ae. squarrosa*//Weaver/3/*3 L505	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26*
L20	Croc/*Ae. squarrosa* (205)//Weaver/3/L505/4/Bel/5/Fav/6/S74	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr10* + *Lr6Agi*
L40	Dobr/3/Croc/*Ae. squarrosa* (205)//Weaver/4/Dobr	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr10* + *Lr39* + *Lr6Agi*
L44	L505/3/Croc/*Ae. squarrosa* (205)//Weaver/4/L505/5/S68	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr19* + *Lr26* + *Lr39*

Continued Table 3

L45	Dobr/3/Croc/*Ae. squarrosa* (205)//Weaver/4/Dobr/5/Dobr	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr10 + Lr19 + Lr39 + Lr6Agi*
L51	Croc/*Ae. squarrosa* (205)//Weaver/3/L505/4/Bel/5/Fav/6/Fav	*Ae. tauschii*(=*Ae. squarrosa*)	0/0;	0	0	0	0	0	0	0	*Lr6Agi*
			Group IV								
L21	Voev/*T. petropavloskyi*//Voev	*T. petropavloskyi*	50/3	2-3	0	3	3–	3	3	3	*Lr10*
L31	Voev/*T. petropavloskyi**3//Voev	*T. petropavloskyi*	0/0;	0	0	0	0	0	0	0	*Lr6Agi*
			Group V								
L17	S70/*T. kiharae*//Dobr/3/Dobr	*T. kiharae*	15/1 и 3	0	3	0	0	0	0	0	*Lr19*
L18	S68/*T. kiharae*//L503	*T. kiharae*	0/0;	0	0-1	0	0	0	0-1;	0	*Lr19 + Lr28=LrTkh?*
L22	S68/*T. kiharae*//S70/3/S68/4/S68	*T. kiharae*	0/0;	0	0-2, 3	0	0	0	0	1-2;	*Lr10 + Lr19*
L32	S68/*T. kiharae*//S70/3/S70/4/S70	*T. kiharae*	0/0;	0	3	0	0	0	0	0	*Lr19*
L33	S68/*T. kiharae*//Dobr/3/Dobr	*T. kiharae*	0/0;	0	0	0	0	0	0	0	*Lr3 + Lr19 + Lr28=LrTkh?*
L53	S68/*T. kiharae*//Dobr/3/Dobr/4/Dobr	*T. kiharae*	0/0;	0	3	0	0	0	0;	0	*Lr3 + Lr19*
L56	Viktoria 95/No. 1	*T. miguschovae*	0/0;	0	–	–	0	0-1;	2-3	0-1;	*Lr1 + Lr3 + Lr34*
L57	Viktoria 95/No. 1	*T. miguschovae*	0-5/0; and 1	0-1	0-1;	0-1;	0-1;	3	3	3	*Lr1 + Lr3 + Lr34*
L58	Viktoria 95/No. 1	*T. miguschovae*	0-20/0; and 3	0	0	3–	0-1;	3	3	3	
			RIA ES spring soft wheat varieties								
	Saratovskaya 29		70/3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4	*Lr10*
	Saratovskaya 55		70/3-4	3	3	3	3-4	–	–	–	
	Saratovskaya 68		40/3	3-4	3-4	3-4	3-4	–	–	–	*Lr10*
	Saratovskaya 70		70/3-4	3-4	3-4	3-4	3-4	–	–	–	
	Saratovskaya 73		50/3	3	3	2	3	–	–	–	*Lr10*
	Saratovskaya 74		60/3	3	3	3	3	–	–	–	*Lr10*
	Favorit	*Agropyron intermedium*	0/0;	0	0	0	0	0	0	0	*Lr6Agi*
	Voevoda	*Ag. intermedium*	0/0;	0	0	0	0	0	0	0	*Lr6Agi*
	Belyanka	*Ag. intermedium*	0/0;	0	0	0	0	0	0	0	*Lr6Agi*
	Dobrynya	*Ag. elongatum*	15/2-3	0	3	0	0	0	0	0	*Lr19*
	L503	*Ag. elongatum*	15/2-3	0	3	0	0	0	0	0	*Lr10 Lr19*
	L505	*Ag. elongatum*	15/2-3	0	3	0	0	0	0	0	*Lr10 Lr19*
	Prokhorovka	*Secale sereale*	30/3	0	0	3	0 и 3	0	0	3	*Lr10 Lr26*

Note. SH — species involved in hybridization, FD — damage under field conditions; populations and isolates of the pathogen: 1 — Test-clone1, 2 — Test-clone2, 3 — Test-clone3, 4 — Pop_Sar, 5 — Pop_Kr, 6 — Pop_Chel, 7 — Pop_Dag (for description of *Puccinia triticina* Erikss. populations and isolates see Table 1). S29 — Saratovskaya 29, S68 — Saratovskaya 68, S70 — Saratovskaya 70, S73 — Saratovskaya 73, S74 — Saratovskaya 74, Fav — Favorit, Dobr — Dobrynya, Bel — Belyanka, Voev — Voevoda, Prokh — Prokhorovka, Sar. zol — Saratovskaya zolotistaya, Zol. volna — Zolotaya volna. 0 — no signs, 0; — necroses without pustules, 1 — very small pustules surrounded by necrosis, 2 — medium-sized pustules surrounded by necrosis or chlorosis, 3 — medium-sized pustules without necrosis, 4 — large pustules without necrosis. Scores 0, 0;, 1, 2 mean plant resistance, 3, 4 mean plant susceptibility [13]. Dashes mean that the sample was not tested.

pheevii (*Lr18*, *Lr50*), *Ag. elongatum* (*Lr19, Lr24, Lr29*), *Ag. intermedium* (*Lr38*), *T. dicoccoides* (*Lr33*, *Lr53*, *Lr64*), *T. durum* (*Lr23, Lr61*) и *T. monococcum* (*Lr63*). Genes *Lr9*, *Lr19*, *Lr21*, *Lr23*, *Lr24*, *Lr26*, *Lr28*, *Lr37*, and *Lr39* were transferred to commercial varieties of common wheat [43, 44)]. Some of these samples (Thatcher lines with genes *Lr9*, *Lr24*, *Lr36*; varieties Trident and Milan with *Lr37*) we used to increase the genetic diversity of highly productive spring bread wheat varieties grown in the Lower Volga Region. Along with the known *Lr* genes, we used samples of alien species, presumably carrying new resistance genes [10, 11].

Group I. Lines L3 and L42 (*Lr37*), L52 (*Lr29*), L4 (*Lr24*), L10 (*Lr9*) and L30 (*Lr36*) were obtained using donors of known *Lr* genes (see Table 3). Molecular markers confirmed the presence of the resistance gene *Lr37* of adult plants of lines L3 and L42 produced with the participation of Milan and Trident varieties as donors of this gene. Also, the *Lr19* gene transferred from the Dobrynya variety was identified in these lines, and the additional *Lr6Agi* gene from the Favorite variety was identified in L3. Both lines were highly resistant in the field conditions of the Saratov region. Seedlings of the L3 line carrying genes *Lr19* + *Lr37* +*Lr6Agi*, when inoculated with clone No. 2 virulent to *Lr19* (see Table 3), responded significantly higher (score 0) than a susceptible L42 line (*Lr19* + *Lr37*), and were moderately resistant (score 1-2) upon inoculation with the Dagestan population and clone No. 3 virulent to *Lr26*.

Only the *Lr29* gene was identified in the L52 line, obtained on the basis of the brown rust susceptible variety Saratovskaya 70 and the Tc*Lr29* line. Seedlings of the L52 line, as well as the initial isogenic line Tc*Lr29*, were highly resistant to all geographical populations and clones of the pathogen (reaction type 0). In the field conditions, their response varied from 0; to 1. Until now, *Lr29* the donor of which is *Ag. elongatum* has not been used in Russian and foreign breeding programs [12, 42].

In lines L4 and L10, which pedigrees involve Tc*Lr24* and Tc*Lr9*, we did not identify these genes. Molecular analysis determined *Lr19* inherited from the varieties Dobrynya and L503. An additional *Lr26* gene introgression from the Prokhorovka variety was detected in the L10 line, and *Lr6Agi* from the Belyanka cultivar was found in L4. The high resistance of L4 and L10 seedlings and adult plants indicates the effectiveness of the combinations *Lr19* + *Lr26* and *Lr19* + *Lr6Agi* genes in wheat protecting against brown rust in the Volga region.

L30 line with Tc*Lr36* in the pedigree showed susceptibility during seedling phase when infected with test clone No. 2 virulent to *Lr19* carriers. Molecular markers identified L30 as the carrier of *Lr19* + *Lr10* genes. Under field conditions, the L30 showed 1 point response that was lower than that of Tc*Lr19*, but higher than that of Tc*Lr36*, which may be due to the additive interaction of the *Lr10*, *Lr19*, and *Lr36* genes.

Group II. Tetraploid wheat species are believed to be more resistant to brown rust than diploids and hexaploids [45]. However, only a few *Lr* genes were moved from them to common wheat. *Lr23* introduced from *T. durum* is the most frequently transferred [12]. The *Lr23* gene lost its effectiveness in the Volga region in the late 1990s. However, under field conditions bread wheat varieties with this gene show different residual resistance effects. The varieties of durum wheat Saratovskaya zolotistaya, Zolotaya volna and Nik involved in L8, L25, L13, L19, L39 and L43 development, are resistant to brown rust in the Lower Volga Region [46]. The genetic control of their resistance to this disease is undisclosed. However, in the pedigree of the Zolotaya volna and Nick varieties, there is Saratovskaya zolotistaya with a type of reaction to the leaf rust pathogen 1.1+.

In our study, most of the introgression lines produced with the partici-

pation of durum wheat varieties were characterized by high resistance during period of seedlings as well as adult plants. The exception was the line L43 which was attacked by pathogen clone No. 2 virulent to Lr19. For L8 and L19, we noted a segregation on resistance to disease in the field, which indicates the heterogeneity of these lines and the need for further selection.

Lr19 gene was detected in all lines based on Dobrynya variety (L13, L39, L43, L19) and *T. durum* Zolotaya volna and Nik varieties (see Table 3). In L13, *Lr26* gene was also identified the combination of which with *Lr19* can determine high resistance of this line. L19 carries ineffective *Lr10* gene. The *Lr19* gene was also detected in L25, while its donors were not in the pedigree. *Lr10* gene the source of which was the Saratovskaya 68 variety was also detected in this line. A high resistance of seedlings and adult plants in lines L39, L19, and L25 indicates the presence of additional genetic material from *T. durum* along with translocation from *Ag. elongatum*.

Lines L2, L8, L11 and L46 were obtained with the participation of L164 = L504/Saratovskaya 57//L504. Their durum wheat-derived genetic material could be translocated from L164, in the pedigree of which there is Saratovskaya 57 variety resistant to brown rust. All lines of this group carry *Lr19*, which is consistent with the analysis of the L2 and L1 pedigrees in creation of which line L505 participated. *Lr10* was identified in L11 and L2. The *Lr26* gene was not inherited from the Prokhorovka cultivar. High resistance of its seedlings and adult plants suggests the presence of an additional *Lr* gene from Saratovskaya 57 durum wheat. In L2, the Lr26 gene of CIMMYT (International Maize and Wheat Improvement Center) line Trap#1/Bow was determined.

The gene combination *Lr10* + *Lr19* + *Lr26* in the L2 line leads to high juvenile and adult resistance to brown rust. As already noted, the *Lr19* gene was identified in L8 and L46, but its origin, as per the pedigrees, is not clear. Nevertheless, according to the pedigree, L46 may have *Lr26* from the Prokhorovka cultivar, as it was confirmed by molecular analysis. Therefore, L46 carries *Lr19* + *Lr26* combination. L8 showed high resistance to all leaf rust samples, which cannot be caused by the presence of only *Lr19*, therefore there is reason to assume the additional genes from durum wheat (*LrTdur*) of the Zolotaya volna and Saratovskaya 57 which has L164 in the pedigree. Two recessive brown rust resistance genes transmitted from durum wheat Saratovskaya 57 were previously identified in L164 [47].

Along with durum wheat, tetraploid species *T. persicum*, *T. dicoccoides*, *T. dicoccum* of similar genomic composition (AuAuBB), as well as *T. timopheevii* (GGAtAt) were used to improve genetic diversity of Saratov spring bread wheat varieties. In the L38 line based on Dobrynya cultivar and *T. persicum* sample, one *Lr19* gene was established using DNA markers. Moreover, this line was high resistant throughout the growing season, which indicates the presence of an additional *Lr* gene from *T. persicum*. The gene symbol catalog [42] does not contain information on genes moved to common wheat from this species; therefore, it can be assumed that the L38 line has a new *Lr* gene, which in combination with *Lr19* provides high protection against brown rust.

Most lines based on susceptible varieties Saratovskaya 74 and Saratovskaya 73 and sample *T. dicoccum* k-7507 (Iran), the L24, L28, L29, L47, and L48, contain a combination of the *Lr19* and *Lr26*. A similar combination was identified in the L5 line obtained with the participation of *T. dicoccoides*. The L28 line is heterogeneous on *Lr19*, which probably causes its segregation of brown rust resistance in field tests. In L28 and L29, the *Lr10* gene was also determined. The L47 line differed from these lines in susceptibility to test clone No. 3 virulent to *Lr26*. Molecular markers revealed in this line a combination of

two ineffective genes, *Lr10* + *Lr26*. Moreover, this line, like other lines involving *T. dicoccum*, was high resistant in field tests, which indicates the presence of additional *Lr* genes. It was previously shown that resistance to brown rust in *T. dicoccum* k-7507 is controlled by one dominant *Lr* gene [10]. The reason for the presence of genes from *Agropyron elongatum* (*Lr19*) and rye (*Lr26*) is unclear. However, the combination of *Lr* genes can cause a high resistance. The gene symbol catalog [42] describes two genes, *Lr53* and *Lr64*, translocated to common wheat from *T. dicoccum*. The line with *Lr53* in our long-term investigations was high juvenile resistant to all *P. triticina* Russian populations, including the Saratov one (score 0, 0;, 1). Two alleles of 320 bp and 375 bp were amplified by cfd1 marker in the *Lr53*-bearig positive control (Fig. 1), while one 275 bp allele was amplified in L5, L24, L29, L47, and L48 lines, which indicates the lack of *Lr53* [29].

L49 was obtained via hybridization of *T. timopheevii* and bread wheat varieties Saratovskaya 68 (*Lr10*) and Dobrynya (*Lr19*). Molecular analysis revealed *Lr10* and *Lr28* in this line, whereas *Lr19* gene of Dobrynya variety was not detected. The detection of the SCS421 marker, in our opinion, indicates the presence of the *T. timopheevii* (*LrTtim*) genetic material in the sample. We showed earlier [48] that this marker is not strictly specific to determine *Lr28* gene from *Ae. speltoides*, and is also present in samples of *T. timopheevii*.

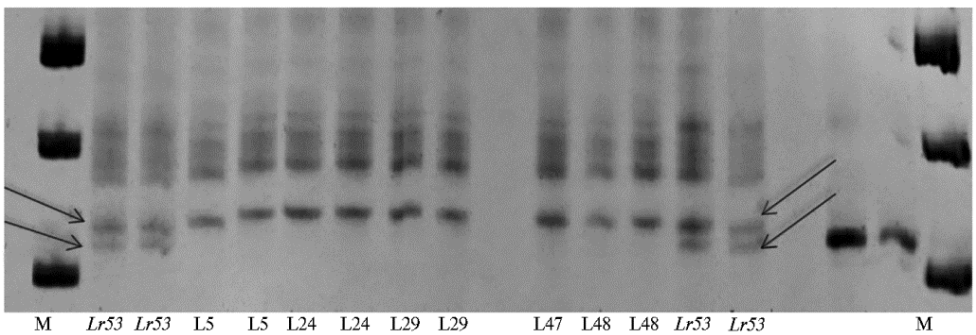

Fig. 1. PCR identification of cfd1 marker of *Lr53* in introgression lines (L) **of spring soft wheat (ARISER):** M — molecular weight marker (DNA length marker 50 bp, Diaem, Russia), *Lr53* — positive control (Tc*Lr53*). Arrows indicate 320 bp and 375 bp PCR products.

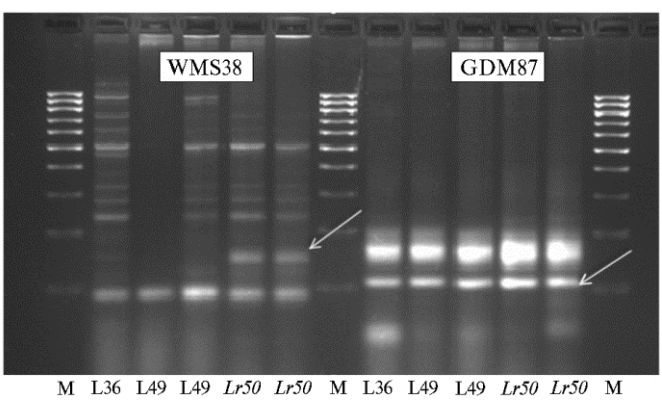

Fig. 2. PCR identification of microsatellite markers WMS382 and GDM87 of *Lr50* in introgression lines (L) **of spring bread wheat (ARISER):** M — molecular weight marker (DNA length marker 100 bp, Diaem, Russia), *Lr50* — positive control (line KS96WGRC36). Arrows indicate 139 bp (WMS382) and 110 bp (GDM87) PCR products.

In the gene symbol catalog [42], there are two genes, the *Lr18* and *Lr50*, moved to common wheat from *T. timopheevii*. The *Lr18* gene is ineffective in the Volga Region. Seedlings of the line with this gene are susceptible to brown leaf rust (score 3-4). A response of the line with *Lr50* upon inoculation with the Saratovskaya population of the pathogen varied from 0-1 to 2+ points and differed from that of L49. WMS382

marker of the *Lr50* gene is more closely linked to this gene (6.7 cM) than GDM87 (9.4 cM). The electrophoretic pattern we obtained indicated the absence of *Lr50* in this line (Fig. 2). The results for GDM87, which was detected in the L49 line and the L36 line, turned out to be false positive. Similar cases of inefficiency of this marker for screening *Lr50* are widely discussed in the literature (https://maswheat.ucdavis.edu/protocols/Lr50/index.htm), and therefore it is recommended for use in marker-assisted selection (MAS) only as optional to WMS382.

Group III. Diploid species *Ae. tauschii* is used worldwide to confer disease resistance and other economically valuable traits. In our work, synthetic amphidiploid Croc/*Ae. squarrosa* (205)//Weaver (CIMMYT) was involved to produce lines L6, L7, L9, L20, L40, L44, L45 and L51. This synthetic amphidiploid has a complex of economically valuable traits and is used in plant breeding in many countries [49, 50]. This group of lines did not have the *Lr21* and *Lr22a* genes transmitted from *Ae. taushcii*, while *Lr39*(= *Lr41*) was found in lines L40, L44, and L45 (Fig. 3). The *Lr19* gene was inherited by lines L6, L9, L44, L45 and was absent in L20, L40 and L51, despite the fact that varieties with this gene were present in the pedigrees of each of these lines. The *Lr6Agi* was identified in the L7 and L20 lines with the participation of Favorit and Belyanka varieties, as well as in L40 and L45, in the pedigree of which the indicated varieties were absent.

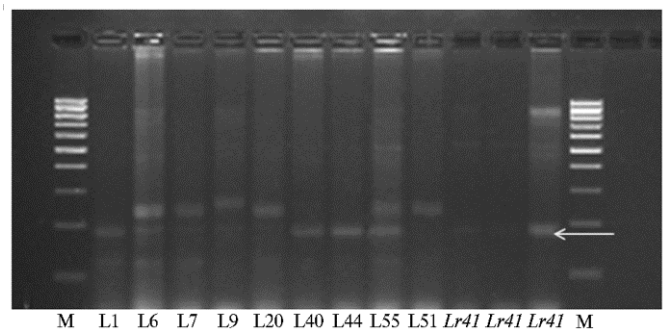

FIg. 3. PCR identification of *Lr39*(=*Lr41*) gene microsatellite marker GDM35 in introgression lines (L) **of spring bread wheat (ARISER):** M — molecular weight marker (DNA length marker 100 bp, Diaem, Russia), *Lr41* — positive control (line KS-90-WGRC-10). Arrow indicates 180 bp PCR product.

When analyzing plants individually, we revealed *Lr6Agi* gene segregation for L40 line and stable inheritance for L45 line. So additional cytogenetic analyzes or molecular PLUG markers-based analysis (PCR-based landmark unique gene) [51, 52] are necessary to finally confirm the presence of this gene. Additional *Lr26* gene was identified in L7, L9, and L44 lines, the probable source of which was the Weaver line of the synthetics pedigree.

All lines with the synthetic amphidiploid in pedigrees were highly resistant. For L7 *Lr10* + *Lr26* + *Lr6Agi*), L9 (*Lr19* + *Lr26*), L20 (*Lr10* + *Lr6Agi*), L40 (*Lr10* + *Lr39* + *Lr6Agi*), L44 (*Lr19* + *Lr26* + *Lr39*), L51 (*Lr6Agi*), and L45 (*Lr10* + *Lr19* + *Lr39* + *Lr6Agi*) this is consistent with the data on the genetic control. In L6, we can assume the presence of another gene, since upon inoculation with clone No. 2 virulent to *Lr19*, its resistance was observed (see Table 1).

Group IV. A susceptible sample of the hexaploid *T. petropavloskyi* and the resistant variety Voevoda participate in lines L21 and L3. Line L21 was characterized by susceptibility both of seedlings and adult plants, which indicates the absence of genetic material from the Voevoda variety. This is confirmed by molecular analysis. In L21, no markers of *Lr6Agi* gene were detected, but an ineffective *Lr10* gene was identified. The highly resistant L31 line carries the *Lr6Agi* gene which, probably, determines the L31 resistance.

In general, the *T. petropavloskyi* is characterized as highly susceptible to

fungal diseases [45]. However, this species is of interest for breeding as a donor of other economically important and biological traits. Cytological analysis will help to assess the presence of the genetic material from *T. petropavloskyi* in the lines of this group.

Group V. The lines of this group were produced with the participation of hexaploid species *T. kiharae* and *T. miguschovae*. *T. kiharae* is a homologue of *T. spelta* L., and *T. miguschovae* was created as a homologue of *T. aestivum*. These species are important for breeding common wheat as donors of high productivity [53]. The *T. kiharae* forms used to produce L17, L18, L22, L32, L33, L33, and L53 lines was resistant to brown rust and in preliminary studies showed one dominant gene for resistance [12].

In all lines of this group, we detected *Lr19* gene individually (L17, L32) or in combination with other genes, the *Lr19* + *Lr28* for L18; *Lr10* + *Lr19* for L22; *Lr3* + *Lr19* + *Lr28* for L33; *Lr3* + *Lr19* for L53. For lines L17, L22 and L32, these results are confirmed by a phytopathological test (susceptibility to clone No. 2). According to the genealogy of the lines L17, L18, L33, L53, the source of *Lr19* could be varieties Dobrynya and L503, while L22 and L32 were produced on the basis of varieties Saratovskaya 68 and Saratovskaya 70, in which this gene is absent.

Lines L18 and L33 had an SCS421 marker associated with *Lr28* [22]. As shown above, this marker is not strictly specific for the Lr28 gene translocation from *Ae. speltoides* and is detected in samples obtained with the participation of *T. timopheevii* [48]. Hypothetically, it can be assumed that the detection of this marker in L18 and L33 indicates the presence of the *T. kiharae* genetic material. This is confirmed by the high resistance to the disease in the field and lab tests. Since among the known *Lr* genes there are no transmitted from this species [42], we can assume that they are new and not identical to the known effective ones (*LrTkh*).

In lines L56, L57, L58 obtained with the participation of *T. miguschovae*, the resistance type varied upon infection of seedlings with populations and clones. Adult plants of L56 line were highly resistant, the other two lines showed resistance segregation. Molecular marker detected ineffective *Lr3* and *Lr1* genes and the partial resistance gene *Lr34* in L56 and L57. However, these genes were not found in the L58 line of similar origin.

An analysis of 42 promising wheat lines showed high genetic diversity in brown rust resistance. Among them there were carriers of known *Lr* genes not previously used in spring bread wheat breeding in Russia (*Lr29* in L4), and the carriers of presumably new *Lr* genes from *T. durum* (L8, L39 *Lr19* + *LrTdur*, L25, L19, L11 — *Lr10* + *Lr19* + *LrTdur*), *T. persicum* (L38 — *Lr19* + *LrTpers*), *T. timopheevii* (L49 — *Lr10* + *LrTtim*), *Ae. tauschii* (L6 — *Lr19* + *LrAtau*), *T. kiharae* (L33 — *Lr3* + *Lr19* + *LrTkh*). Moreover, we have identified carriers of effective combinations of *Lr* genes: *Lr19* + *Lr26* (L10, L13, L46, L24, L48, L5, L9), *Lr19* + *Lr37* (L42), *Lr10* + *Lr19* + *Lr26* (L2, L28, L29), *Lr19* + *Lr26* + *Lr39* (L44), *Lr19* + *Lr37* + *Lr6Agi* (L3), *Lr19* + *Lr6Agi* (L4), *Lr10* + *Lr26* + *Lr6Agi* (L7), *Lr10* + *Lr39* + *Lr6Agi* (L40), as well as *Lr10* + *Lr19* + *Lr39* + *Lr6Agi* (L45) and *Lr1* + *Lr3* + *Lr34* (L56, L57).

Most of the identified alien *Lr* genes are in linkage groups with effective disease resistance genes. In one translocation with *Lr19*, there is a highly efficient stem rust resistance gene *Sr25*. The rye translocation 1BL.1RS, along with the *Lr26* gene, contains genes for resistance to powdery mildew (*Pm8*), stem (*Sr31*) and yellow (*Yr9*) rust, and translocation with *Lr37* gene of *Ae. ventricosa* contains genes for resistance to stem (*Sr38*) and yellow (*Yr17*) rust, cercosporellose root rot (*Pch2*), and cereal cyst forming nematode (*Cre5*). The *Lr34* gene is

closely linked to the genes of resistance to powdery mildew (*Pm38*), stem rust (*Sr57*) and yellow rust (*Yr18*) [12, 42]. Lines with these translocations will have group resistance to several diseases.

Virulence of the pathogen. In 2017 and 2018, along with immunological studies of introgression lines of spring wheat, we monitored the virulence of the Saratov population of *P. triticina*. Infectious material was collected from susceptible varieties growing in the general crop with the studied set of lines. In both years, when the tester *Lr*-lines were inoculated with the combined population of *P. triticina*, the genes *Lr9*, *Lr24*, *Lr28*, *Lr29*, *Lr39*(= *Lr41*), *Lr42*, *Lr45*, *Lr47*, *Lr50*, *Lr51*, *Lr53*, *Lr6Agi* were high effective (score 0 and 0;). Lines with *Lr28*, *Lr29*, *Lr39*, *Lr51*, *Lr6Agi* were also characterized by high field resistance. The entire set of these genes may be of interest for breeding in the Volga Region and increasing genetic diversity of cultivated wheat varieties. The Thatcher lines with the *Lr44*, *Lr57* showed a moderate resistance of 2 to 2 ++. All other lines showed susceptibility with different intensities of the lesion.

In 2017 and 2018, 45 and 35 *P. triticina* monopustular isolates, respectively, were tested with 20 isogenic *Lr* lines. Isolates virulent to Tc*Lr19* line had a moderate frequency (16% in 2017 and 20% in 2018). The pathogen virulence rate for the Tc*Lr26* line was high (80% in 2017 and 77% 2018). All isolates virulent to *Lr19* carriers were avirulent to *Lr26*. Probably, this gene combination is "forbidden" for the pathogen. The confirmation could be the results of immunological studies and high resistance of the introgression lines L10, L13, L28, L46 and others carrying the *Lr19* + *Lr26* combination. A significant variation in the frequencies of the pathogen over the years was observed on lines with genes *Lr2a*, *Lr2b*, *Lr2c*, *Lr15* (20% in 2017 and 100% in 2018). Frequencies of the pathogen virulence to lines with *Lr1*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, and *Lr30* were consistently high in both years (100%). This explains the high damage to seedlings and adult plants of the varieties and line L10 with *Lr10* gene. However, the above *Lr* genes in combination with the adult resistance gene *Lr34* may have an additive effect on increasing field resistance. Such facts are described [54] and are noted in our study for lines L56 and L57. In 2017, the studied Saratov population (ARISER) was represented by three pathogen virulence phenotypes (races), the MHTKH, TGTTT, and THTTR, and in 2018 by two phenotypes, the TGTTT and THTTR.

The THTTR phenotype is widely distributed throughout Russia and is detected almost annually. All resistant lines in our study were immune to this phenotype. The TGTTT phenotype is most characteristic of the Volga populations, though also noted in other Russian regions [55]. Its unequal representation in the Saratov population by years can explain the variability in the damage to varieties and lines with *Lr19* gene.

Thus, we have characterized the genetic control of resistance to brown rust (*Puccinia triticina* Erikss.) in a new promising breeding material that combines resistance to leaf rust with adaptability to adverse environmental factors, productivity and grain quality. Its distinctive feature is the widespread use of leaf rust resistance genes from related species. Lines with resistance genes effective in the Lower Volga Region (*Lr29*), which are little used in breeding in Russia, were determined. Lines with effective combinations of known *Lr* genes and with combinations of known *Lr* genes with presumably new alien genes have been identified. Alien genes have been transferred from durum wheat varieties, *Triticum persicum*, *T. timopheevii* and *T. kiharae*, i.e., from both primary and secondary common wheat pool. The use of effective combinations of *Lr19*, *Lr26*, *Lr34*, *Lr37* genes linked to effective genes for resistance to other diseases will determine the resistance of new lines to a complex of diseases, which increases the

value of such combinations. The information we obtain on the composition of the brown rust pathogen population in the Saratov Region and its changes during 2017-2018 will be key for planning and conducting work on advanced selection for brown rust resistance.

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EVALUATION OF OAT GENOTYPES FOR THE CONTENT OF β-GLUCANS IN GRAIN ON THE BASIS OF ITS PHYSICAL CHARACTERISTICS

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Abstract

Due to the beneficial effect of oat β-glucans on human health and their negative role in the assimilation of feed by non-ruminant animals, the selection of oats for increased (cereal direction) and reduced (feed use) content of these polysaccharides in grain is an urgent task. To perform screening of breeding material for the specified biochemical indicator of oat quality, it is advisable to use simple, express and non-destructive methods of grain analysis. The aim of this work is the development of a rapid method to evaluate oat genotypes for the content of β-glucans in grain based on the measurement of physical characteristics of grain. For the first time, it was found that in oat samples with a high content of β-glucans, the film-free grain was characterized by a higher density ($r = 0.818$). Probably, the mechanism explaining the presence of this correlation is associated with the formation of thicker and more densely packed cell walls in the endosperm, which has a greater number of these chemicals. In the research, 16 accessions of hulled oats and 2 accessions of naked oats from the VIR collection were involved. Concentration of β-glucans in grain was measured by the conventional enzymatic techniques. Physical characteristics (nature, 1000-grain weight, density, volume fraction of water uptake) were studied on the whole and/or hull-less grain. Measuring grain density for each oat accession was performed by the sand replacement method described by D.C. Doehlert and M.S. McMullen (2008); the natural grain weight was measured by the techniques offered by C.K. Walker, and J.F. Panozzo (2011); water uptake by grain was determined by the vacuum infiltration methodology. It is shown that 1000-grain weight of the studied oat accessions was not associated with the level of β-glucans in grain. There is an insignificant positive dependence between β-glucan content on the one hand, and the natural weight, density of whole grain and the volume fraction of water uptake by grain on the other hand. The oat accessions with higher content of β-glucans had higher density of hull-less grain ($r = 0.818$, $p \le 0.05$). For an approximate calculation of the value of the content β-glucans in grain of genotypes of oats, you can use the formula: SBG = 4.16 × PZ, where PZ is the density of the film-free grain, g/cm^3; SBG is relative content of β-glucans in the grain, %; 4.16 is a coefficient of transition from the grain densities to the values of β-glucans. Minimum content of β-glucans in grain (3.2-3.8 %) and the lowest grain density (1.05-1.10 g/cm^3) were observed in the accessions Pushkinskii, Hondai 8473 and Privet. Maximum values of β-glucan content and grain density (5.7-6.7 % and 1.26-1.31 g/cm^3, respectively) were recorded in the accessions Pomor, Haruaoba and Marion. As a result, a rapid method was offered for evaluation of oat genotypes, which makes it possible to divide accessions into two contrasting groups: with maximum and minimum content of β-glucans in grain, considerably differing in both chemical and physical parameters. This method does not require expensive chemical agents or complex equipment, and may be implemented in any laboratory of a typical breeding center. The effect of possible introduction of the proposed technique involves saving financial and labor resources as well as avoiding complete grain damage, thus providing an opportunity for further utilization of the conserved breeding material in other analyses of grain for its quality.

Keywords: oats, grain, β-glucans, density, test weight, 1000-grain weight, water uptake, evaluation

Oat (*Avena sativa* L.) grain is not only high nutritional valuable, but also contains unique dietary fiber, the (1,3)(1,4)-β-D-glucans [1-3]. These polysaccharides have a beneficial effect on human health, as they can lower food glycemic index [4, 5], reduce blood cholesterol [6], including low-density cholesterol [7], improve liver function, and prevent excess body weight [8-10]. However, the fact that β-glucans act as a negative factor in nutrient assimilation by non-ruminant animals burdens the positive role of these compounds. Thus, experiments with feeding broiler chickens showed significant differences in grain nutritional value of oat varieties, which was negatively dependent on the content of β-glucans in the grain [11]. Since the proportion of barley and oat grain in Russian compound feeds for non-ruminant animals is more than half, a decrease in the concentration of β-glucans in the crop is necessary. In this regard, the selection of food oats for increased level of β-glucans and feed oats for reduced β-glucans in grain remains topical [12].

The content of β-glucans in grain is measured by a standard chemical analysis [13, 14]. The advantages of this method are its accuracy, and the disadvantages are its high complexity and the need to use expensive imported reagents and laboratory equipment. Another currently used physical method is based on the measurement of near-infrared reflection [15] using an automatic grain analyzer, for example, the Infratec™ 1241 Grain Analyzer (FOSS Analytical A/S, Denmark) [16]. Unfortunately, most chemical and physical methods require the complete grain destruction.

Screening of breeding material requires rapid and preferably non-destructive methods that can divide the hybrid population of oats into two extreme groups by the content of β-glucans in the grain. Available publications are mainly devoted to comparing the content of β-glucans in grain with the physical characteristics of whole grain [17, 18]. Since β-glucans are a part of the endosperm cell walls, the estimates of physical characteristics of not only the whole oat grains, but also of grains free from hulls are advisable.

In this work we have discovered that in oat samples having a high content of β-glucans, hulless grains are characterized by a higher density ($r = 0.818$, $p \leq 0.05$). Based on this, we suggest the technique of oat genotype evaluation which allows separation of samples with a maximum and minimum content of β-glucans in grain. Preliminary results were partially presented in our report at 10th International Oat Conference: innovation for food and health (St. Petersburg, Russia, July 11-15, 2016) [19].

The aim of the work was to analyze the relationship of the whole and hulless oat grain physical characteristics with the content of β-glucans.

Materials and methods. The 16 hulled oats and 2 hulless oats (the collection of the Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg) were investigated. To determine the physical characteristics of each sample, both hulled and hulless grains were used. In the latter case, floral glumes were removed manually without destroying fruit glumes and seed glumes.

A 1000-grain weight was calculated by weighing 250 grains. Bushel weight of grains was evaluated by a micro method of Walker et al. [20]. The volume of a known grain weight (about 10 g) was measured in a 50 ml cylinder. The density of hulled and hulless grains of each sample was determined by sand displacement method [21] with white fine sand from the South China Sea coast (Vietnam); the average sand density per 12 measurements was 1.55 ± 0.01 g/cm^3.

Air cavities in the grain deprived of floral films was estimated by vacuum infiltration with water (a 35 ml medical syringe at an air pressure of 50 kPa). The time of contact of grain with water in the syringe was about 1 min. After

removing from the water, grain surface was dried with filter paper. Then the grain was weighed and the volume of absorbed water was calculated as a ratio to the initial volume of dry grain.

All physical parameters of grain were determined in 3 replicates. The content of β-glucans was evaluated by the standard method [13] in 2-3-fold repetition.

Statistical processing was performed with Microsoft Excel 2003. The mean values (*M*), standard errors of the mean (±SEM), and the correlation coefficient (*r*) were calculated. The significance of differences was evaluated using Student's *t*-test at $p \leq 0.05$.

Results. Table 1 describes the oat samples we used.

1. Characterization of oat (*Avena sativa* L.) samples used to compare physical parameters of hulled and hulless grain (collection of Vavilov All-Russian Institute of Plant Genetic Resources — VIR)

No. in VIR catalog	Name	Variety	Origin
		Hulled oats	
k-9978	Marion	*A. byzantina*	USA
k-11840	Borrus	*A. sativa* var. *aurea*	Germany
k-13904	Ogle	*A. sativa* var. *aurea*	USA
k-13918	Kirovets	*A. sativa* var. *aurea*	Russia, Kirov Province
k-13943	Proat	*A. sativa* var. *aristata*	USA
k-13947	Tulancingo	*A. byzantina*	Mexio
k-14373	Fakir	*A. sativa* var. *aurea*	Россия, Kirov Province
k-14597	Sprint 2	*A. sativa* var. *aurea*	Russia, Ekaterinburg Province
k-14648	Argamak	*A. sativa* var. *mutica*	Russia, Kirov Province
k-14787	Privet	*A. sativa* var. *aurea*	Russia, Moscow Province
k-14858	Borot	*A. sativa* var. *mutica*	Russia, Leningrad Province
k-14872	Haruaoba	*A. byzantina*	Japan
k-14877	Hondai 8473	*A. sativa* var. *grisea*	Japan
k-14907	Vernyi	*A. byzantina*	Russia, Adygea
k-15126	Matilda	*A. sativa* var. *aurea*	Sweden
k-15176	Lev	*A. sativa* var. *mutica*	Russia, Moscow Province
		Hulless oats	
k-14717	Pushkinskii	*A. sativa* var. *inermis*	Russia, Leningrad Province
k-15117	Pomor	*A. sativa* var. *inermis*	Russia, Kemerovo Province

2. Correlation coefficients (*r*) of parameters in hulled grain and grain deprived from hulls in 18 oat (*Avena sativa* L.) samples (collection of Vavilov All-Russian Institute of Plant Genetic Resources — VIR)

Parameter	Hulled grains			Hulless grains		
	bushel weight	1000-grain weight	density	absorbed water	density	β-glucans
Hulled grain:						
bushel weight	1					
1000-grain weight	0.347	1				
density	0.780*	0.176	1			
Hulless grain:						
absorbed water	0.101	–0.197	0.082	1		
density	0.101	–0.239	0.278	0.382	1	
β-glucans	0.299	–0.096	0.495	0.237	0.818*	1

* *r* values are statistically significant at $p \leq 0.05$.

Moderate insignificant positive correlations occur between the content of β-glucans in grain and the density of whole grains, between the density of hulls and the volume of water absorbed by hulless grain, and between bushel weight of whole grains and 1000-grain weight (Table 2). Weak positive correlations are between the content of β-glucans in grain and its bushel weight and also water absorbed by hulless grain. The bushel weight and the whole grain density show strong positive correlation, while the dependence of the bushel weight on the density of de-hulled grain is practically absent. The density of the de-hulled grain and the content of β-glucans correlate strongly and positively.

The increased density of de-hulled grain of the oat samples with a higher content of β-glucans, apparently, could be due to the formation of thicker (and probably more densely packed) cell walls in the endosperm or a smaller volume of air cavities between the fruit and seed glumes and the hard part of the grain. Our results (see Table 2) indicate the absence of a negative relationship between the relative volume of absorbed water under negative pressure (and, consequently, the volume of air cavities) in grain deprived from floral glumes and the density of such grain. We assume the first mechanism to be more likely. This is indirectly supported by the close relationship between the density of barley grain and the hardness of its endosperm (20).

Based on statistically proven data that oat genotypes with a higher content of β-glucans had a higher density of de-hulled grain, the coefficients were calculated of transition from density values to the content of β-glucans in grains measured by the standard chemical method [13]. The average value of this dimensionless indicator is 4.16±0.12.

Therefore, for an approximate calculation of the content of β-glucans (CBG) in grain of oats genotypes, one can use the formula: CBG = 4.16 × GD, where GD is the density of de-hulled grain, g/cm^3, CBG is the relative content of β-glucans in grain, %, and 4.16 is a coefficient of transition from grain density to the content of β-glucans.

The obtained experimental data allowed us to distinguish among the examined 18 oat genotypes two contrasting groups of three samples each with a maximum and minimum content of β-glucans in grain, above 5.6% and less than 3.9%, respectively. These groups differed significantly not only in the content of β-glucans, but also in grain density. With an average density of de-hulled grain of 1.17±0.04 g/cm3 and 4.88±0.47% β-glucans, Pushkinskii, Privet and Hondai 8473 samples had the lowest indicators (1.07±0.02 g/cm^3 and 3.57±0.18%, respectively), while the maximum values (1.28±0.02 g/cm^3 and 6.13±0.30%) were characteristic of Pomor, Marion and Haruaoba samples. Differences in the density of de-hulled grains and the content of β-glucans between the genotypes of different groups were statistically significant at $p \leq 0.05$.

Currently, the possible relationships between the amount of β-glucans in grain and various physicochemical, morphological, and agronomic characters of oat genotypes are being actively investigated. A negative correlation was reported between the content of β-glucans and the total amount of dietary fiber and crude fiber, as well as a positive relationship with protein accumulation [22]. In oats, a close relationship between grain β-glucans and fat content has been demonstrated [16]. A significant positive correlation was found between grain β-glucan concentrations and bushel weight, on the one hand, and 1000-grain weight, on the other, while there were significant negative correlations of the β-glucan content with protein content and grain hoodness [17].

Our results on 1000-grain weight of whole grain for oats samples contrasting in the content of β-glucans support experimental data of C. Griffey et al. [18], demonstrating the absence of a relationship between these indicators for barley, as well as the results of M. Saastamoinen et al. [17] about a positive correlation between grain β-glucans and bushel weight. However, unlike the reports of the latest authors, our data show this correlation to be weak.

Over the years, in some countries, work has been done both on the search for oat samples with a high or low content of β-glucans in grain among existing varieties, and on the creation of forms with different accumulations of this polysaccharide depending on the intended use [23-25]. A few years ago, un-

der the auspices of the European Commission, the European project "Avena Genetic Resources for Quality in Human Consumption" was implemented, in which 658 varieties of oats were studied. It was confirmed that the genetic component has a significant effect on the content of β-glucans in oat grain [26]. In the Russian Federation, such studies are rare. Particularily, in a joint project between VIR (Russia) and the Nordic Gene Bank (NordGen, Nordic Genetic Resource Center, Sweden), the content of β-glucans in oats varieties was studied. The value of the considered indicator in the grain ranged from 3.3 to 6.2% [27]. The existing genotypic diversity of this biochemical trait (from 1.9 to 8.5%) [26-30] is quite sufficient for the progress of selection of oats for an increased or decreased content of β-glucans in grain for food and fodder use, respectively [31].

Thus, in oat samples with a high content of β-glucans, de-hulled grains had a higher density ($r = 0.818$, $p \leq 0.05$). Probably, the mechanism explaining the presence of this correlation is associated with the formation of thicker and more densely packed cell walls in the endosperm, which has a greater amount of these chemicals. Based on the statistically proven relationship between the content of β-glucans in grain and the density of de-hulled grain, an approach is proposed for evaluating oat genotypes, which allows us to divide the tested samples into two contrasting groups, with a maximum and minimum content of β-glucans. The method does not require expensive chemicals, sophisticated equipment and can be implemented in any conventional laboratory. In addition to saving material resources and labor costs when assessing oat genotypes, the proposed method avoids complete grain destruction. This makes it possible to further use the stored breeding material for grain quality analysis.

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ABOUT EFFECT OF WEEDS ON SPECTRAL REFLECTANCE PROPERTIES OF WINTER WHEAT CANOPY

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Abstract

Among the poorly studied factors affecting the spectral reflectivity of crops and, consequently, the success of detection of their condition based on remote sensing data is crop weedness. On the basis of field survey data, the effect of weed infestation on winter wheat spectral reflectance at different stages of vegetation was analyzed using the example of individual fields in the Tula region with chernozems, grey forest, and alluvial arable soils. Under field conditions, crop weedness, spectral reflectance of crops, weeds, winter wheat leaves and soil determined using FieldSpec® HandHeld 2™ field spectro-radiometer (ASD, Inc., USA) was assessed several times during the growing season, and the crop canopy surface was photographed. The decoding of the photos showed that the projective weed coverage on the crop canopy surface is low enough at the beginning and middle of the wheat growing season, but increases significantly since the beginning of leaves yellowing. At the same time, the projective coverage of weeds in the field with chernozems was minimal at the beginning and middle of the growing season, and maximal — by the end of the growing season. Projective coverage of weeds on fields with grey forest and alluvial arable soils did not differ statistically, but on alluvial soils it increased significantly by the end of wheat vegetation. Using the spectral mixing model, the contribution of weeds infestation to the integral reflection of light by crops in the visible and near infrared bands of the electromagnetic waves was estimated. It has been found that despite the rather high weedness of winter wheat canopy in the spring—summer growing season, its projective coverage on the surface of the crop canopy is small. The magnitude of the projective cover of weeds on the surface of crop canopy weakly depends on soil conditions, and is more determined by other factors (history of fields use, crop rotation, etc.). The effect of weed vegetation on the spectral reflectivity of crop canopy changes over time. It is minimal at the peak of the growing season, accounting for several percent for all wavelengths of the visible and near IR range. At the beginning of the post-winter vegetation period, the contribution of weed vegetation to the spectral reflectance of crop canopy can reach 10-20%, and at the end of the vegetation season, weed vegetation can predetermine the spectral appearance of crops at most wavelengths of the considered range. The greatest contribution is observed in all cases in the near IR (710-730 nm) and in the green (520-560 nm) spectral region, but at certain times there are local maxima of the contribution and in the blue spectral region of electromagnetic waves (400-420 nm). The data obtained open up the possibility for the development of new vegetation indices for remote monitoring of crops, which will be less affected by weedness than those traditionally used (for example, NDVI). Conversely, on the basis of the data obtained, special vegetation indices can be proposed for the remote detection of the weedness of winter wheat canopy.

Keywords: spectral reflectance, Tula region, crop weedness, winter wheat, remote sensing

An important aspect of agrometeorological service of crop production is the crop state monitoring. Its results are used in the planning of agricultural measures, required application of fertilizers and in predicting crop yields [1]. Traditionally, the monitoring of crop status is carried out at the registration sites in the field according to specially developed instructions [2]. Such approach is laborious and cannot be implemented quickly on large areas with good accuracy. Therefore, in recent decades, remote sensing with the use of satellite systems and unmanned aerial vehicles has been increasingly involved in crop monitoring. Remote sensing data help to determining the crops acreage [3, 4], to assess their state [5-7], to predict crop yield [8-10], and to assess soil fertility [11]. These approaches have no disadvantages of traditional methods, but they are still not well developed. The reasons are both the specifics of the remote sensing data (their generalization, the need for preliminary technical preparation for analysis, the significant dependence of information content on the date of the survey), and the specifics of monitored objects, the crops, in particular, poor knowledge of their spectral reflectance and its variability under the influence of certain factors [12].

Weediness of crops is among poorly studied factors affecting the crop spectral reflectance and, accordingly, the success of detecting their status by remote sensing [13, 14]. Crop weediness mostly appears as a result of low-tech farming and a lack of funds to purchase agrochemicals by land owners to control weeds. On the territory of the former USSR, a moderate and severe weediness occurs in more than 65% of croplands. This leads to significant yield losses [15] which, according to the Ministry of Agriculture of Russia, reach 30-40% yearly. The nature and degree of crop weediness, the phenology of weeds in coordination with development stage of cultivated plants varies significantly from season to season. This is due to the peculiarities of the meteorological conditions of the year, crop rotation, soil conditions, agricultural technology [16, 17].

The influence of weediness of crops on their spectral signature is still insufficiently studied. Such studies are underway, but mainly in connection with the development of precision farming and site-specific use of chemicals for weed control [18-20]. However, these studies are not involved remote recognition of weed species, since the accuracy of determining weed areas is of more practical importance, while weed species composition is determined directly in the field. Studies on the spectral reflectance of different weed species and its dynamics during the growing season are still very few [21-23].

The proposed article is the first to show the specificity of the weediness influence on the spectral reflectivity of winter wheat crops, accounting the stage of wheat development and soil conditions.

The aim of our research was to analyze field data on the spectral crop reflectance and its changes during the growing season by an example of test fields with winter wheat in the Tula region of Russia.

Materials and methods. The investigations were performed on three test fields with winter wheat of cv. Moskovskaya 39 (Yasnogorskii and Shchekinskii districts of the Tula Province, April-September 2018). The fields were contrasting in soils, i.e. gray forest arable soils dominated in the 1st field, arable alluvial soils in the 2nd field, and podzolized chernozemic arable soils in the 3rd (as per the prevailing soils). The pre-winter period of winter wheat vegetation in the fields studied lasted from early October to mid-November, snow cover disappeared in the last ten days of March, and crops were harvested during the first ten days of August. In all fields, weed control agents were not used during the research.

On each field, test sites of 4×4 m in size were arranged on the basic soils (the number of sites on each field is indicated in the tables below), on which the crop weediness was described as to the wheat phenological phases. From April to September, 3 surveys were carried out in fields with gray forest and alluvial soils (April 27, June 1, July 6) and 4 in a field with chernozems (April 20, May 25, June 29, August 17). In total, 95 descriptions of vegetation were performed on test sites at different times. The parameters recorded were total projective cover of all species, the total projective cover of the grass and moss layers, the specific projective cover (for segetal species), the weediness of crops in points according to Maltsev [24], species composition, aspect and aspect-forming species. For each species, the plant height (prevailing) and layering (stratification) as per Alekhine [25], abundance/coverage according to Brown-Blanca [26] were estimated, and a pronounced decrease in vitality was also a recorded parameter.

Phenophases were described with regard to the differences in life forms and systematic groups according to standard methods [27] separately for herbaceous plants growing on the sites (annual and perennial), as well as for horsetails and cereals. Wheat phenophases were also determined as per the BBCH scale [28]. The occurrence of species was calculated, systematized by groups (annual/biennial, perennial rhizomatous, root sucker perennials, etc.).

During plant descriptions, crops were also photographed (a 1.5 m vertical distance from the crop canopy, 5 replicates; a Nikon-D300s camera with a wide-angle lens, Japan). The spectral reflectance (SR) of the crop surface, of winter wheat leaves and of weeds appearing on the crop canopy surface (all from a 1 m height above plants), as well as of open soil surface (from a 15-20 cm height above soil) was measured using a FieldSpec® HandHeld 2™ spectroradiometer (ASD, Inc., USA). The device records reflection spectra in the 300 to 1025 nm wavelength range with a 2 nm interval. Before analysis, the spectral curves were smoothed using the Savitsky-Golay filter [29] in the R software package, and averaged.

The large format images were processed with CAN-EYE software (https://www6.paca.inrae.fr/can-eye/), the projective cover of the crops as a whole was determined [30], weed leaves on the crop canopy surface was visually delineated and their projective cover was measured. The projective cover was calculated as the relative area of objects (winter wheat leaves, weed leaves, or open soil surface) on the images acquired in nadir mode. For delineating, the ILWIS v.3.3 software package (https://www.itc.nl/ilwis/download/ilwis33/) was used.

The spectral reflectance of the crop surface was considered as a spectral mixture of reflections of winter wheat leaves, soil and weed leaves. According to the linear spectral mixture model, the contribution of each object is determined by the portion of this object in the mixture:

$$SR_m = S_1 \times SR_s + S_2 \times SR_{ww} + S_3 \times SR_{we},$$

where S_1, S_2, S_3 are the relative areas occupied by the corresponding classes (percent fractions), SR_m, SR_s, SR_{ww}, and SR_{we} are the spectral reflectance of crop canopy, soils, wheat leaves, and weed leaves, respectively. The contribution of each component to the spectral mixture is determined by its relative area in the image and the spectral properties. Thus, if the integrated crop reflection is equal to 1, then we can estimate the contribution of weeds and other components of the spectral mixture to the total SR. Quality of the crop SR simulation via linear spectral decomposition was evaluated by the determination coefficient R^2 between the simulation results and field measurements.

Using this approach, we estimated how the crop SR model based on the SRs of wheat plants, weeds and soil differ from the measured values. Also, given

the accuracy of modeling, we determined the contribution of weeds to the integrated SR for all sites and dates of the observation.

Statistical processing of data, i.e. calculation of average values, confidence intervals, estimation of statistical significance of differences ($t_{0.05}$), preliminary processing of spectral reflectance curves (smoothing and removal of outliers) was performed with stats and prospectr software in the R environment (https://www.r-project.org/).

Results. SR was measured for leaves of wheat and weeds that emerged on the surface of the crop. For weeds, the SR was determined only for a mixture of species dominating on the crop surface without accounting the indicator for species under the wheat canopy of. Thus, measurements were carried out only for those plants that could affect the SR of crop canopy on a specific date.

Agrophytocenoses of the field where gray forest soils predominate are characterized by a high diversity of vegetation. In three examinations, we identified one shrub species and 49 species of segetal herbs (25 perennials, 24 annuals and biennials) (Table 1).

1. γ-Diversity of vegetation in winter wheat Moskovskaya 39 variety agrophytocenosis in a field with a predominance of gray forest soils (Tula Province, Yasnogorskii District, 2018)

Indicator	Date		
	April 27	June 1	July 6
Number of sites	14	10	10
Beideman's wheat phenophase [27]	vegetation—tillering	vegetation—shooting	milk ripeness
BBCH wheat phenophase [28]	BBCH 23	BBCH 43	BBCH 77
Species per site, min-max (mean):			
segetal herbs	6-13 (8.5)	15-26 (18.6)	19-26 (22.4)
annual species	4-8 (6.1)	7-12 (9.7)	9-13 (11.4)
perennial species	1-7 (2.4)	6-14 (8.9)	8-14 (11.0)

During the observation period, species diversity was increasing. By the beginning of June, it was more than 2.0 times as much as at the beginning of the growing season, and in July it was 2.5 times higher compared to the initial value, while the proportion of annual and biennial herbs slightly decreased. In April at the sites they formed the main coverage and their share significantly exceeded half of the number of recorded species, and by June-August, due to the activation of growth of perennial grasses, the share of annual and perennial plants decreased to half.

In April, ephemers and annual/perennial weeds having wintering forms played the main role among weeds. These were *Capsella bursa-pastoris* (L.) Medik., *Consolida regalis* S.F. Gray, *Galium aparine* L., *Thlaspi arvense* L., *Tripleurospermum inodorum* (L.) Sch. Bip., *Viola arvensis* Murr.) (hereinafter, botanical plant names are given as per Mayevsky) [31]. In early June, the ephemera finished blooming and fructified, wintering annuals/perennials proceeded to full bloom and the beginning of ripening. Spring annuals (*Chenopodium album* L., members of genus *Galeopsis* L. etc.) and perennials joined them in the phase of seedlings and in vegetative phases preceding budding, as well as in the budding phase. The projective cover and the aspect of the field communities during this period were mainly formed by root sucker species *Equisetum arvense* L. and *Convolvulus arvensis* L., as well as *Consolida regalis*. By the beginning of July, the ephemera, as well as wintering biennials, completed ripening, many previously not observed annuals appeared on many sites, *Erigeron canadensis* L., *Fallopia convolvulus* (L.) Á. Löve, *Spergularia rubra* (L.) J. et C. Presl, biennial species *Picris hieracioides* L., *Cerastium holosteoides* Fr., *Myosotis arvensis* (L.) Hill became more abundant, new biennials (*Achillea millefolium* L., *Galium mollugo*

L., *Tanacetum vulgare* L., *Trifolium repens* L.) and green mosses emerged. The highest occurrence was observed in species typical for most sites throughout the observation period which made the core of this agrophytocenosis, namely *Arabidopsis thaliana* (L.) Heynh. (97.0 %), *Consolida regalis* (91.2 %), *Tripleurospermum inodorum* (91.2 %), *Viola arvensis* (82.4 %), *Capsella bursa-pastoris* (79.4 %), *Potentilla argentea* L. (73.5 %), *Myosotis micrantha* Pall. (70.6 %), *Poa compressa* L. (70.6 %), *Scleranthus annuus* L. (67.6 %), *Thlaspi arvense* (64.7%), *Vicea tetrasperma* (L.) Schreb. (58.8%). It is also important to note species abundant only in the summer, i.e. *Convolvulus arvensis* (50.1 %), *Equisetum arvense* (55.9 %), and *Hypericum perforatum* L. (58.5 %).

In the field with a predominance of alluvial arable soils, the γ-diversity of vegetation was high (41 species of segetal grasses, of which 20 were perennials, 21 were annuals and biennials) (Table 2).

2. γ-Diversity of vegetation in winter wheat Moskovskaya 39 variety agrophytocenosis in a field with a predominance of alluvial arable soils (Tula Province, Yasnogorskii District, 2018)

Indicator	Date		
	April 27	June 1	July 6
Number of sites	8	5	4
Beideman's wheat phenophase [27]	vegetation—tillering	vegetation—shooting	milk ripeness
BBCH wheat phenophase [28]	BBCH 23	BBCH 44	BBCH 77
Species per site, min-max (mean):			
segetal herbs	4-12 (8.1)	10-19 (14.0)	11-18 (14.0)
annual species	3-7 (4.4)	6-11 (8.2)	7-12 (8.7)
perennial species	0-10 (3.7)	3-8 (5.8)	4-7 (5.3)

During our observation, species diversity increased (see Table 2). In April, a lesser presence of annual and biennial grasses was recorded in the crops than on a field with gray forest soils, which can be explained by higher humidity and lower soil temperature, as well as the fact that the surveyed field was involved in agricultural use only in 2017 after fallowing, which, apparently, reduced drifts of annual and biennial weed species. Despite the relatively high species diversity, this field was characterized by lower weediness than the site on gray forest soils. In the summer months, the development of weeds was hindered by the high wheat plant density. Weeds of the 3rd and 4th layers extended because of light lack and were late in passing phenological phases.

In April, the dominating dorms were annual/biennial weeds having wintering (*Capsella bursa-pastoris*, *Galium aparine*, *Sisymbrium loeselii* L., *Tripleurospermum inodorum*, *Viola arvensis*), and seedlings of perennial species previously common on this site (*Artemisia campestris* L., *Potentilla argentea*, *Vicea cracca* L.). With the beginning of the summer period, spring annual species *Chenopodium album*, *Galeopsis speciosa* Mill., *Erigeron canadensis, Vicea tetrasperma* appeared, as well as *Carduus crispus* L., and mass growth of root sucker *Convolvulus arvensis* occurred. The most frequent species were *Galium aparine* (88.2 %), *Artemisia campestris* (76.4 %), *Capsella bursa-pastoris* (70.6 %), *Sisymbrium loeselii* (58.8 %), since the beginning of June the species which abundance increased were *Chenopodium album* (47.1 %), *Convolvulus arvensis* (47.1 %), *Vicea tetrasperma* (41.2 %), *Erigeron canadensis* (35.3 %), and *Galeopsis speciosa* (35.3 %).

On chernozem soils, the γ-diversity of segetal vegetation of the test field was also relatively high and comprised 44 grasses, of which 15 species are perennials, 29 are annuals and biennials (Table 3). An increase in species diversity was observed in May and August, during the months most favorable in terms of the ratio of heat and soil moisture.

In April, wintering forms of annual weeds (*Capsella bursa-pastoris*, *Ga-*

lium aparine, *Viola arvensis*, dominated, winter annuals *Raphanus raphanistrum* L., *Thlaspi arvense* and ephemer *Stellaria media* (L.) Vill. were rather frequent. In June, the drying of the soil led to the loss of a number of species (*Chenopodium album*, *Galeopsis* sp., *Fumaria officinalis* L.) from the stand. In August, spring forms *Capsella bursa-pastoris*, *Fumaria officinalis*, *Viola arvensis* developed, and late spring annuals, *Echinochloa crus-galli* (L.) Beauv., *Fallopia convolvulus*, *Chaenorrhinum minus* (L.) Lange, *Setaria glauca* (L.) Beauv., *Solanum nigrum* L., and *Sonchus asper* (L.) Hill. appeared. Abundance of annual species *Erigeron canadensis*, and the perennials *Artemisia vulgaris* (L.), *Cirsium arvense* (L.) Scop. increased significantly by the second half of summer. *Sonchus arvensis* L. was quite abundant in summer. The most frequent species were *Galium aparine* (97.7 %), *Viola arvensis* (95.5 %), *Capsella bursa-pastoris* (77.2 %) which were found on the vast majority of sites throughout the observation.

3. γ-Diversity of vegetation in winter wheat Moskovskaya 39 variety agrophytocenosis in a field with a predominance of chernozem soils (Tula Province, Shchekinskii District, 2018)

Indicator	Date			
	April 20	May 25	June 29	August 17
Number of sites	14	13	9	8
Beideman's wheat phenophase [27]	vegetation—tillering	vegetation—shooting	milk ripeness	stubble
BBCH wheat phenophase [28]	BBCH 23	BBCH 32	BBCH 73	BBCH 99
Species per site, min-max (mean):				
segetal herbs	3-6 (4.7)	5-14 (8.5)	3-11 (5.8)	8-19 (12.6)
annual species	2-5 (4.0)	4-10 (6.9)	2-8 (4.4)	6-12 (8.6)
perennial species	0-1 (0.7)	0-5 (1.6)	0-3 (1.4)	1-7 (4.0)

Table 4 shows the results of delineating of projective cover of crops from images averaged for each test field with different prevailing soils. It should be noted that in geobotany the term "projective cover" stands for the relative projection area of individual species or their groups, layers, etc. of a phytocenosis on the soil. In this article, by this term we mean the relative area of objects (winter wheat leaves, weed leaves or open soil surface) depicted in photo of the crop canopy surface acquired in nadir.

4. Projective cover of winter wheat Moskovskaya 39 variety crops (%) in fields with different soils (Tula Province, Yasnogorskii and Shchekinskii districts, 2018)

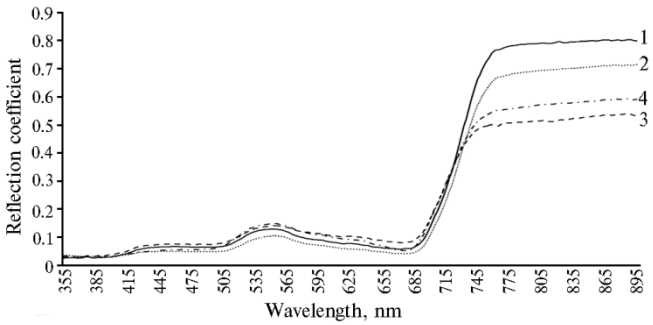
Soil	Date	Site number	Wheat	Weeds	Soil
Chernozem	04/20/2018	5	17.5±4.6	0.2±0.1	82.5±9.9
Gray forest	04/26/2018	14	22.0±2.7	5.9±1.7	72.6±2.7
Alluvial	04/26/2018	8	38.5±5.5	5.6±2.6	55.9±4.6
Chernozem	05/26/2018	13	93.9±1.6	0.5±0.1	5.6±1.6
Gray forest	06/01/2018	10	69.1±6.3	2.9±0.1	28.0±6.2
Alluvial	06/01/2018	4	83.6±2.0	2.9±0.1	13.5±1.8
Chernozem	06/29/2018	9	62.3±2.6	0.2±0.1	37.5±2.6
Gray forest	06.07.2018	10	69.0±2.9	6.4±2.5	24.6±2.4
Alluvial	07/06/2018	4	60.3±15.0	14.6±11.9	25.1±3.8
Chernozem	08/18/2018	8	28.4±9.5	25.4±12.1	46.2±7.2

N o t e. Confidence intervals $M \pm (t_{0,05} \times \text{SEM})$ are not more than ±5 %.

Decryption showed that the projective cover of weeds on the surface of the crop canopy is quite low at the beginning and middle of the wheat vegetation season, but significantly increases since the beginning of yellowing. Moreover, the projective cover of weed vegetation in the field with chernozems turned out to be minimal at the beginning and middle of the growing season and maximum at its end. The projective cover in fields with gray forest and alluvial arable soils did not significantly differ ($p > 0.05$), but on alluvial soils it significantly increased towards the end of wheat vegetation (differences between the periods of observation are statistically significant, $p \leq 0.05$).

SR of weeds differs from SR of wheat leaves (Fig. 1). These differences

were especially noticeable in the infrared (IR) and red regions of the spectrum. Interestingly, in different phenophases, the general shape of the SR curve and the patterns of light reflection were similar. But the nature of reflection in the IR and red regions of the spectrum during wheat and weeds development can vary in different directions. So, in the example shown in Figure 1, wheat leaves in a more mature state reflected less solar energy (especially in the IR region), and in weed vegetation, on the contrary, reflection was higher. Moreover, in weeds (unlike wheat), the difference in SR by the date of observation, depended more not on the phase of plant development, but on the change in the species composition of weeds exposed on the surface of the crop canopy. But even with this, the SR value of the weedy vegetation leaves that emerges on the crop canopy surface turned out to be very similar to each other (differences were not statistically significant, $p > 0.05$)



Fir. 1. Examples of spectral reflectance curves of winter wheat Moskovskaya 39 variety and weed vegetation leaves for two survey periods on a field with gray forest soils: 1 — wheat (04/26), 2 — wheat (06/01), 3 — weeds (04/26), 4 — weeds (06/01) (Tula Province, Yasnogorskii District, 2018; FieldSpec® HandHeld 2™, ASD, Inc., USA).

Modeling of the integrated SR by the SR of the spectral mixture components showed that, on the whole, the results adequately reflect the actual SR measurements in crops. The generalized data are presented in Figure 2.

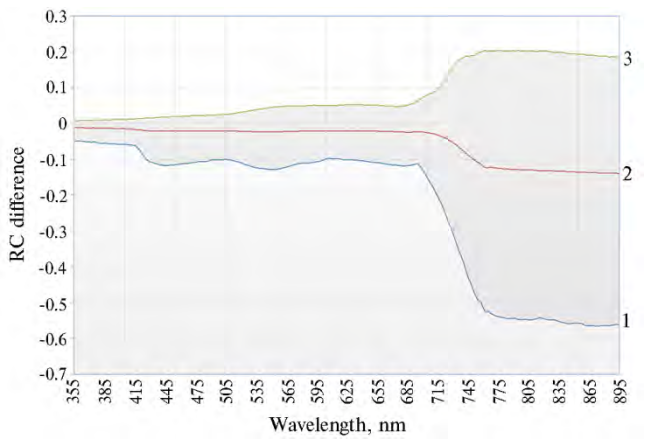


Fig. 2. Minimum (1)**, average** (2) **and maximum** (3) **difference between the values of the reflection coefficients obtained by SR field measuring and by linear spectral mixture model for each wavelength and all records** (winter wheat Moskovskaya 39 variety, Tula Province, Yasnogorskii and Shchekinskii districts, 2018).

The difference between the results of SR modeling and its measurements in the field was small for the visible spectrum (λ from 350 nm to 700 nm), but increased for the infrared range (λ = 700-900 nm). In the visible range, it does not exceed 0.1 of the reflection coefficient, while in the IR range for certain points it can reach 0.5-0.6 (see Fig. 2). The main reason probably is that the images used to evaluate the projective coverage were acquired in the visible spectral range, which does not reflect the heterogeneities characteristic of the IR range. In addition, both errors in weed identification based on wide-angle lens images and the presence (albeit in small quantities) of dead vegetation (stubble or leaves of trees form the nearest forest belts) at the beginning and end of the growing season could affect the result.

An assessment based on the modeling contribution of weed vegetation to the integral SR of crops showed the following (Fig. 3).

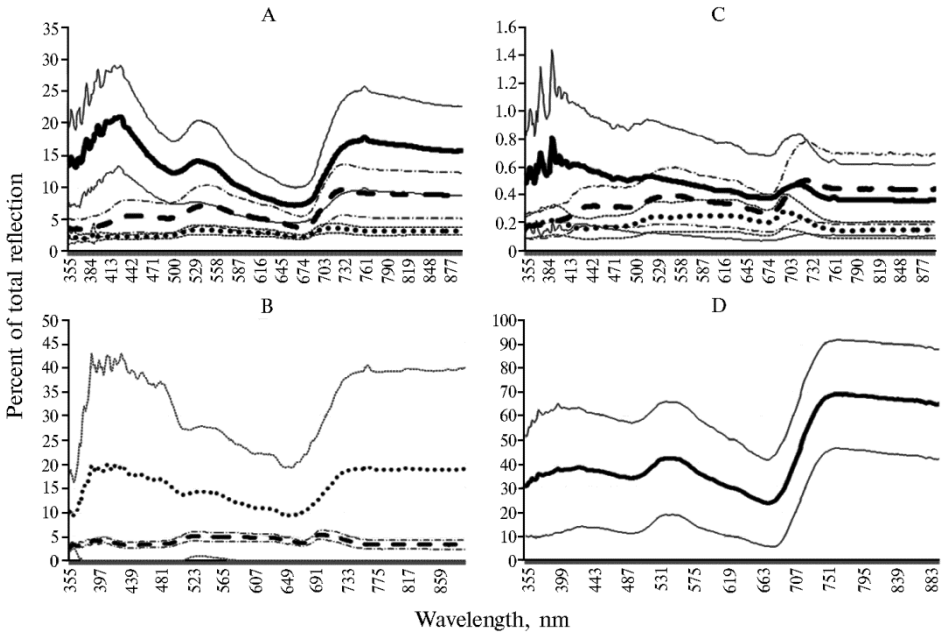


Fig. 3. Model-based means (*M*, bold lines) **with confidence intervals** ($t_{0,05}$ × SEM, thin lines) **of the contribution of weed vegetation to wheat crop integrated spectral reflectance on gray forest soils** (A, solid lines for 04/26/2018, dotted lines for 06/01/2018, point lines for 07/06/2018)**, on alluvial soils** (B, dotted lines for 06/01/2018, point lines for 07/06/2018) **and chernozem soils** (C, dotted lines for 04/20/2018, point lines for 05/26/2018, solid lines for 06/29/2018; D for 08/18/2018) (Tula Province, Yasnogorskii and Shchekinskii districts).

The weed contributions to crop SR were the largest in spring at the beginning of the growing season and also at its end. Moreover, at the end of the season, the contribution of weeds became maximal and after harvesting reached almost 100 % at certain wavelengths (see Fig. 3, D). In the middle of the growing season, the contribution of weed vegetation was minimal on all soils and did not exceed several percent. The contribution was always the greatest in the near IR (710-730 nm) and green (520-560 nm) spectral regions, but local peaks for certain dates also appeared in the blue region (400-420 nm).

Thus, the weediness of the examined winter wheat crops is generally quite high. It depends both on the type of soil and, apparently, on the field history and crop rotations, which is fully consistent with the regularities established earlier [16]. But weed vegetation in many cases does not reach the upper layer of crop canopy, especially with the maximum development of above-ground parts of wheat plants. This is clearly evidenced by the data on the projective cover of weeds on the crop canopy surface (see Table 4). To the greatest extent, weed vegetation emerges on the crop canopy surface at the end of the growing season and after wheat harvesting, which in principle confirms the previously established regularities [32]. This is due to the fact that yellowed wheat captures moisture and nutrients to a much lesser extent and also let more light to pass into the crops [33], which creates more favorable conditions for weeds development.

As mentioned above, the weed SR statistically significant (at $p \leq 0.05$) differs from wheat SR. The greatest differences occur in the IR and green spectral regions due to a greener color of weeds compared to wheat leaves (especially at the beginning of after-wintering vegetation and after passing the vegetation season

peak), which confirms the earlier data [19, 34]. Smaller, but in some cases quite noticeable differences are observed in the red range of the spectrum, where weed vegetation reflects solar energy slightly strongly than wheat leaves because of its slightly higher moisture content [35]. The noted variations in the spectra of weed vegetation during the growing season are related to the fact that both the color of the leaves of the weeds themselves and their amount in crops change, which confirms the data of earlier publications [13]. But these modifications are not of a cardinal nature, i.e. the local extremes of the reflection curve remain at fixed wavelengths, only their absolute value changes, but not by much. Thus, the change in the SR of weed vegetation that emerges on the crop canopy surface at all dates of our surveys on all soils and wavelengths did not exceed 5%.

It should be noted that the proportion of weed vegetation that emerges on the crop canopy surface is much lower than the total weediness. This proportion is associated with the prevailing soils and, apparently, with the meteorological conditions of the season, the specifics of agricultural technology of crop cultivation on different soils, and also with the predecessor [36]. Our surveys showed the smallest appearance of weed vegetation on the crop canopy surface for chernozems and the largest for arable alluvial soils. Note that the projective cover of segetal species in the mid-season on all soils, with rare exceptions, did not exceed several percent. Moreover, at the beginning of the season it can reach 10-20%, and at the end of the season, and especially immediately after harvesting wheat, it reaches 30-40%. This is due to the development of wheat plants and the density of crops, as well as to a lesser density of crops at the beginning of the growing season, wheat leaf yellowing after anthesis and the phenology of weed vegetation itself.

Due to the dynamics of SR and the projective cover of weeds and wheat during the growing season, the contribution of segetal vegetation to the integral SR of a crop is also changing. The quality of crop SR modeling by linear spectral decomposition was insufficient for reliable quantitative estimates in the IR spectrum (R^2 between model-based values and field measurements was 0.83 for visible spectrum and only 0.54 for the near IR range), but similar tendency was traced.

In general, the regularities of weed contribution to crop canopy reflectance are similar on different soils. At the beginning of the growing season, the contribution, as a rule, is minimal (from the complete absence to several percent), which was noted earlier [14]. In our studies, it turned out to be tangible (up to 20-30%) only on gray forest soils, which is most likely due to the peculiarities of using the field in the previous year. Consequently, the contribution of weeds at the beginning of the post-winter growing season is probably not determined by soil conditions, but depends on mode of using field in previous years. This contribution is maximal in the near IR and green spectral regions, which is quite expected for green vegetation.

In the course of plant development and the closure of crop canopy, the influence of weeds on the integral SR decreases and does not exceed several percent at all wavelengths on all soils. But in the mid-season on a field with alluvial soils at one site, we also recorded an increased influence of weed vegetation on the crop canopy SR (due to the abundant development of tall weeds that cover the wheat layer). This suggests that the contribution of weed vegetation may also increase and be statistically significant at the peak of the growing season, which is probably due to the specifics of intra-field variation in soil conditions and the measures to combat weed used in specific fields in past years.

At the end of the wheat growing (starting from leaf yellowing), the contribution of weeds to the SR of crop canopy increases on all soils, reaching a

maximum after harvesting when segetal plants which were under wheat canopy prior to harvesting appear on the crop canopy surface. Similar regularities were revealed earlier for spring barley crops [32]. In this period, in the near IR and green spectra, light reflection is due mainly to weed vegetation. Of interest is the fact that at this time the weed contribution to the winter wheat crop reflectance in the blue region increases significantly and often exceeds that in the IR region, which was not noted for spring barley.

So, our investigation showed that, despite the fairly high weediness of winter wheat crops in the spring-summer period, the projective cover of weeds on the crop canopy surface is small. The value of the projective cover of weed vegetation on the surface of crop canopy depends weakly on soil conditions, and is more determined by other factors (field history, crop rotation, etc.). The effect of weeds on the spectral reflectance of winter wheat crops varies over time. It is minimal at the peak of the growing season, amounting to several percent for all wavelengths of the visible and near infrared ranges. At the beginning of the post-winter growing, the contribution of weed vegetation to crop canopy SR can reach 10-20%, and at the end of the growing season weeds determine the reflectance of crops at most wavelengths of the considered range. In all cases, the contribution is the largest in the near IR (λ = 710-730 nm) and green (λ = 520-560 nm) spectrum, but local maxima are also noted in the blue spectral region (λ = 400-420 nm). Our findings open up the possibility to develop such vegetation indices for crop remote sensing that will allow researchers and practitioners to take into account the influence of weediness better than traditionally used indexes, for example, NDVI (Normalized Difference Vegetation Index). In addition, special vegetation indices can be offered for remote detection of weediness in winter wheat crops.

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PHOTOSYNTHETIC PIGMENTS AND PHYTOCHEMICAL ACTIVITY OF PHOTOSYNTHETIC APPARATUS OF MAIZE (*Zea mays* L.) LEAVES UNDER THE EFFECT OF THIAMETHOXAM

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Abstract

In the last decade, neonicotinoid insecticides have been actively used to protect plants from pests. Moreover, their effect on the plants, in particular on the state of photosynthetic pigments, has been studied paucity. In the present work, it was shown for the first time that treatment of maize (*Zea mays* L.) leaves with a thiamethoxam (TMX) insecticide leads to a decrease in the functional activity of photosystem II and a decrease in the energyzation of thylakoid membranes. In addition, the effect of thiamethoxam depends on the genotype of maize. The aim of the work was to study the effect of thiamethoxam pesticide on photosynthetic pigments and the photochemical activity of the photosynthetic apparatus of maize leaves of two genotypes. The experiments were carried out in 2018-2019. The object of the study was samples of maize leaves of the inbred line zppl 225 and hybrid zp 341 with high rates of germination, grain quality and yield (Institut za kukuruz "Zemun Polje", Belgrade, Serbia). The seeds were germinated until the roots appeared (length not less than 5 mm), after which they were planted in the soil (vermiculite: chernozem mixture, 1: 1) and grown under 16-hour daylight at a constant temperature of 25 °C. When the third true leaf appeared (more than 4 cm in length), the plants were sprayed with a TMX solution at a concentration of 0.2 mg/l. Plants grown under similar conditions without TMX spraying were used as controls. The measurements were carried out when the fifth true leaf reached a size of 12-14 cm. The content of photosynthetic pigments (chlorophyll a, b and carotenoids) was determined spectrophotometrically in 100 % acetone extract and calculated using the Holm-Wetstein formula. A change in the conformation of carotenoid molecules was recorded by Raman spectroscopy. Light-induced kinetics of prompt fluorescence (PF), delayed fluorescence (DF) and modulated reflection at λ = 820 nm (MR) were recorded simultaneously using a multifunctional plant efficiency analyzer M-PEA-2 (Hansatech Instruments, Great Britain). PF induction curves (OJIP curves) were analyzed using a standard JIP-test. It was found that in the phase of the fifth true leaf, in the inbred line zppl 225, the chlorophyll content in the presence of TMX decreased from 0.74 to 0.61 mg/g: the amount of chlorophyll a decreased by 17 %, chlorophyll b by 24 %. In contrast, no changes in pigment composition were detected in the leaves of the zp 341 hybrid when exposed to TMX. The OJIP curves of the control and TMX-treated leaves had a typical curve with characteristic O-J, J-I, and I-P phases, which reflected the processes of sequential reduction of carriers in the electron transport chain of photosynthesis (ETC) between two photosystems. The effect of the pesticide on the leaves of two maize genotypes was manifested in a decrease in the functional state of photosystem II, determined by the fluorescence parameter (PI_{ABS}), which was obtained based on the analysis of OJIP curves using the JIP-test. Comparison of PI_{ABS} in the control and under the influence of TMX revealed statistically sig-

nificant ($p < 0.05$) differences: in the leaves of zppl 225 and zp 341 samples treated with TMX, the PI_{ABS} parameter decreased by 29 and 24%, respectively. Changes in the fast phase of delayed fluorescence, associated with a decrease in the energyzation of the thylakoid membrane upon exposure to TMX, were detected in the leaves of maize. An analysis of the maximum oxidation and reduction rates of P700 (MR kinetics) indicates a decrease in the acceptor pool on the acceptor side of PSI in zppl 225 leaves when exposed to TMX. It was found that the reaction centers (RCs) of PSI zp 341 showed resistance to TMX (no change in the redox transformations of P700). TMX caused changes in the conformation of carotenoid molecules, but did not change their content in the leaf. The proposed combination of methods for prompt fluorescence, delayed fluorescence, modulated reflection at $\lambda = 820$ nm and Raman spectroscopy can be the basis for the formation of an effective technology for the diagnosis of early defects of photosynthetic pigments when pesticides enter an intact plant.

Keywords: *Zea mays* L., pesticides, Raman spectroscopy, thiamethoxam, chlorophyll, carotenoids, chlorophyll fluorescence

In the last decade, neuroactive nicotine-based insecticides (imidacloprid, acetamipride, dinotefuran, thiamethoxam) have been widely used to protect plants from pests. Neonicotinoids act as acetylcholine agonists by binding to nicotinic acetylcholine receptors (nAChRs) on the postsynaptic membrane, which causes blocking of synaptic transmission, inhibition of excitation and death of the insect [1]. However, there is no consensus on the effect of neonicotinoids on the state of the plant itself [2]. On the one hand, neonicotinoids are able to improve the morphological and physiological characteristics of some plants even under stressful conditions, which contributes to their growth and yield increase [3], and on the other hand, there is evidence of phytotoxicity of neonicotinoids [4, 5].

It is known that maize production is complicated by the influence of various abiotic and biotic factors, as well as the ability of phytophages to damage the plant at the very early stages of development. It is prospective to investigate changes in the state of pigment composition of maize plants under the action of neonicotinoids in both laboratory and field conditions

The photosynthetic apparatus (PSA) of higher plants, consisting of pigment-protein complexes, includes two types of pigments, chlorophylls and carotenoids. After absorption of light quanta, the molecules of the antenna pigments transfer energy to the reaction centers (RCs) of two photosystems, in an excited state, give electrons to acceptors, which then reduce $NADP^+$ to NADPH, synthesize ATP and organic substances. The state of PSA is one of the sensitive indicators of stress in plants [6]. In its study, fluorescence methods are effective, which are highly sensitive and allow one to detect violations of the state of pigments long before the appearance of morphological changes [7, 8]. In studying the state of carotenoid molecules, the Raman spectroscopy method is widely used, which makes it possible to determine changes in the conformation of the carotenoid molecule of photosystem II (PSII) antenna via evaluating the contribution of C=C bonds valence vibrations (I_{1520}/I_{1006}), transitions from the planar to bent configuration of the molecule (I_{960}/I_{1006}), as well as changes in the length of the polyene chain (I_{1520}/I_{1160}) [9].

In the present work, it was shown for the first time that treating maize leaves with a TMX insecticide leads to a decrease in the functional activity of photosystem II and a decrease in the energyzation of thylakoid membranes. In addition, the effect of thiamethoxam is shown to depends on the genotype of maize. So, in the inbred line zppl 225, a decrease is shown in the content of chlorophyll and the acceptor pool on the acceptor side of PSI. TMX causes opposite changes in the conformation of carotenoid molecules in the antenna, but does not change their content.

The aim of the work was to investigate the effect of the TMX pesticide

on the photosynthetic pigments and the photochemical activity of the leaf photosynthetic apparatus in the two maize genotypes.

Materials and methods. Experiments were carried out in 2018-2019. Samples of maize (*Zea mays* L.) leaves of the inbred line zppl 225 and hybrid zp 341 with high germination rates, grain quality and yield (Institut za kukuruz "Zemun Polje", Belgrade, Serbia) were used [10]. Seeds were germinated until roots appeared (length not less than 5 mm), thereafter the germinated seeds were sown in soil (vermiculite mixed with chernozem, 1:1) and grown under 16-hour daylight at a constant temperature of 25 °C. When 10 days after germination the 3rd true leaf appeared (longer than 4 cm), plants were sprayed with a 0.2 mg/l thiamethoxam [5-methyl-3-(2-chlorothiazol-5-ylmethyl)-1,3,5-oxadiazinane-4-ylidene-N-nitroamine]. Maize leaves grown under similar conditions without spraying with thiamethoxam were a control. The measurements were carried out when the 5th real sheet reached a size of 12-14 cm.

The content of photosynthetic pigments (chlorophyll a, b, and carotenoids) was measured spectrophotometrically in a 100% acetone extract (a Hitachi-557, Hitachi, Japan) with calculation according to Holm-Wetstein formulas [11]. A change in the conformation of carotenoid molecules was recorded by Raman spectroscopy (a DFS 24 Raman spectrometer, LOMO, Russia) equipped with a 473 nm laser (Ciel, Eurolase, Germany) and a MORC 1/3648 recording system (LOMO, Russia) based on a linear CCD matrix TCD1304DG (Toshiba, Japan) with an LPO2-473RS-50 filter (Shemrock, USA). In the experiment, a fragment of the leaf blade was fixed on a stage and a Raman signal was recorded for 5 s; the laser power on the sample was 3 mW.

Light-induced kinetics of prompt fluorescence (PF), delayed fluorescence (DF) and modulated reflection at $\lambda = 820$ nm (MR) were recorded simultaneously (a M-PEA-2 multifunctional plant analyzer, Hansatech Instruments, Great Britain). The intensity of the actinic light and the duration of illumination were 3000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ and 60 s, respectively. Measurements were performed on the adaxial surface of intact leaves placed into a measuring unit with a leafclip. Before measurement, the plants were adapted to darkness for 15 min so that the reaction centers (RC) of the photosystems went into an "open" state with oxidized Q_A. Three signals were recorded during alternating light and dark intervals, the duration of which is described in detail by Strasser et al. [12].

PF induction curves (OJIP curves) were analyzed by a standard JIP test, estimating the energy fluxes through different parts of the photosynthesis electron transport chain [13] at 20 μs (F_O), 270 μs ($F_{270\mu s}$), 2 ms (F_J), 30 ms (F_I) and maximum fluorescence (F_M). These characteristics were used to calculate the maximal quantum yield of PSII photochemistry as a ratio of variable fluorescence (F_V) to F_M as $F_V/F_M = 1 - F_O/F_M$, probability that an electron moves further than Q_A^- $\psi_{Eo} = 1 - (F_J - F_O)/(F_M - F_O)$, light energy (ABS) absorption flux per RC as

$$ABS/RC = 4 \times (F_{270\mu s} - F_O) \times (F_M - F_O)^{-3} \times (F_J - F_O)^{-1} \times F_M,$$

and an indicator of plant PSII performance per the absorbed energy (PSII performance index):

$$PI_{ABS} = (ABS/RC)^{-1} \times F_V/F_M \times (1 - F_V/F_M)^{-1} \times \psi_{Eo} \times (1 - \psi_{Eo})^{-1}$$ [13].

Kinetics of modulated reflection/absorption of light at $\lambda = 820$ nm (MR) characterize the redox transformations of the PSI pigment (P700) and plastocyanin [12]. In our study, the MR signal was normalized to MR_0 and calculated as $MR/MR_0 - 1$, where MR_0 is the signal intensity at 0.7 ms.

Data statistical processing was carried out using a nonparametric Krus-

kal-Wallis test for the set of independent variables (Kruskal-Wallis test) (Statistica v.10 software, StatSoft, Inc., USA). Values $p < 0.05$ were deemed statistically significant. The presented table shows the mean (*M*) values and standard error of the means (±SEM).

Results. The pigment composition of the leaves did not differ much between the two maize genotypes (zppl 225 and zp 341), but after the thiamethoxam (TMX) application, obvious changes were found which depended on the genotype. Thus, TMX decreased the content of chlorophyll from 0.74 mg/g to 0.61 mg/g (chlorophyll a decreased by 17%, chlorophyll b by 24%; $p < 0.05$) in leaves of the inbred line zppl 225. In contrast, in leaves of the zp 341 hybrid exposed to TMX there were no changes in the pigment composition.

OJIP curves in both control and TMX-treated leaves have a typical pattern with characteristic phases O-J, J-I, and I-P, which reflects the sequential reduction of carriers in photosynthetic ETC between two photosystems [13] (Fig. 1, A, D). The initial level O (origin) corresponds to the fluorescence intensity at "open" PSII RCs (F_O), when all acceptors in PSII are oxidized and can accept an electron. The increase in fluorescence in the O-J phase is due to the photoinduced reduction of Q_A^-. The next J-I-P phases reflect a further increase in fluorescence associated with the Q_A^- accumulation due to the reduction of Q_B electron acceptors, the quinone pool, and primary acceptors in photosystem I (PSI) [14].

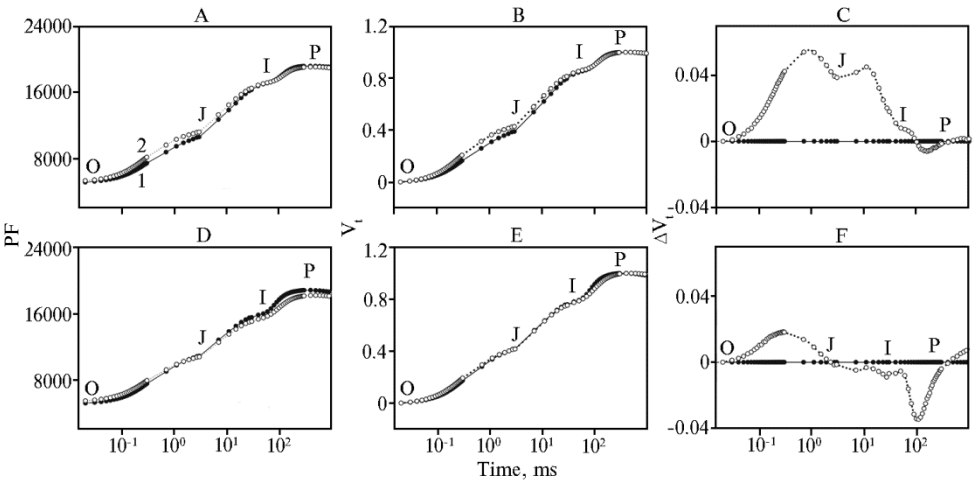

Fig. 1. OJIP curves of maize (*Zea mays* L.) leaves (A, D)**, the derived curves of relative variable fluorescence $V_t = (F_t - F_O)/(F_M - F_O)$ after normalization to O and P levels** (B, E) **and the difference in the values of the functions $\Delta V_t = V_{t(TMX)} - V_{t(control)}$** (C, E) **for the inbred line zppl 225** (A, B, C) **and the hybrid zp 341** (D, E, F) **in control** (1) **and upon thiamethoxam treatment** (2)**.** O, J, I, and P are 20 μs, 2 ms, 30 ms, and 300 ms peaks of the induction curve.

TMX exposure caused slight changes in the OJIP of both the inbred line zppl 225 and the hybrid zp 341 (see Fig. 1, A, D). For a more detailed analysis, the induction curves were normalized to O and P levels to derive a relative fluorescence variable (V_t) functions (see Fig. 1, B, E) and the difference in the values of the V_t functions between the control and samples processed with TMX (ΔV_t) (see Fig. 1, C, F). The effect of the pesticide on the leaves of two maize genotypes appeared as an increase in ΔV_t in the O-J phase, which indicates the accumulation of reduced Q_A as a result of an increase in the proportion of Q_B non-reducing centers of PSII, i.e. centers that are not capable of electron transfer along the ETC [15]. Changes in O-J phase were more apparent in leaves of the zppl 225 inbred line than in the zp 341 hybrid. In addition, the effect of

TMX in zppl 225 leaves was accompanied by an increase in the J-I phase amplitude, i.e. ΔV_t increased in the O-J and J-I phases, which indicates the accumulation of reduced Q_A forms and plastoquinone molecules that are not capable of electron transfer for dark photosynthesis reactions [8].

JIP-test parameters derived from analysis of OJIP induction curves for leaves of two maize (*Zea mays* L.) genotypes upon thiamethoxam (TMX) treatment (*M*±SEM)

Fluorescence parameter	zppl 225	zppl 225 + TMX	zp 341	zp 341 + TMX
F_V/F_M	0.740±0.004 (100 %)	0.740±0.004 (99 %)	0.740±0.010 (100 %)	0.710±0.010 (97 %)
ψ_{Eo}	0.69±0.01 (100 %)	0.65±0.01 (93 %)*	0.67±0.01 (100 %)	0.66±0.02 (100 %)
ABS/RC	2.62±0.09 (100 %)	2.93±0.13 (112 %)	2.57±0.12 (100 %)	2.95±0.18 (115 %)
PI_{ABS}	2.50±0.10 (100 %)	1.77±0.15 (71 %)*	2.24±0.23(100 %)	1.70±0.09 (76 %)*

N o t e. F_V/F_M — the maximal quantum yield of PSII photochemistry, ψ_{Eo} — probability that an electron moves further than Q_A^-, ABS/RC — absorption flux per reaction center (RC), PI_{ABS} — an indicator of plant PSII performance per the absorbed energy. The values in parentheses are a percentage of control.
* Difference from control are statistically significant ($p < 0.05$).

PF induction curves were analyzed by the JIP-test [13] (Table). As known, the most adequate parameter of the JIP-test is the F_V/F_M ratio which correlates with the maximal quantum yield of the PSII primary photochemical reaction and is used as an indicator of photosynthesis efficiency [6]. The F_V/F_M value in the control at 5th leaf phase of zppl 225 and zp 341 was 0.74±0.01. The obtained photosynthetic activity values derived from F_V/F_M were comparable with the F_V/F_M values reported for C4 plants [16]. There were no statistically significant differences in the F_V/F_M between control and TMX-treated leaves for zppl 225 and zp 341 ($p > 0.05$).

Unlike the F_V/F_M parameter, the PI_{ABS} performance index changed significantly when leaves were exposed to TMX. It is known that PI_{ABS} correlates with plant viability and reflects the current PSA functioning under stress [17]. Comparison of PI_{ABS} in the control and under the influence of TMX revealed statistically significant ($p < 0.05$) differences, i.e. in the zppl 225 and zp 341 leaves treated with TMX the PI_{ABS} decreased by 29% and 24%, respectively.

PI_{ABS} is an indicator that integrates three independent parameters, the fraction of active RCs (ABS/RC), the efficiency of exciton-captured electron transfer in the electron transfer chain beyond Q_A (ψ_{Eo}), and the probability that the exciton will be trapped in the RC (F_V/F_M) [13]. The decrease in PI_{ABS} in zppl 225 leaves exposed to TMX was due to a significant decrease in the electron transport at acceptor side of PSII (ψ_{Eo}), which is confirmed by the changes in the PF induction curves shown above ($p < 0.05$). In contrast, a decrease in PI_{ABS} in zp 341 leaves could be associated with a slight decrease in the fraction of active RCs (increase in ABS/RC) and PSII photochemistry (F_V/F_M).

We evaluated the redox transformations of the reaction centers of PSI (P700) molecules upon TMX treatment by modulated reflection at $\lambda = 820$ nm (MR) [18] (Fig. 2, A, C). It is known that the kinetics of the light-induced decrease of the MR signal in the first 15-20 ms reflects the P700 oxidation (fast phase) and reaches a minimum at ~ 20 ms (MR_{min}). MR_{min} is a transitory steady state with equal rate of P700 oxidation and re-reduction. Subsequently, the reduction rate prevails over the oxidation rate due to electron donation from PSII, leading to a decrease in the absorption at $\lambda = 820$ nm and an increase in the MR signal (slow phase) to a maximum at ~ 200 ms (MR_{max}) [12].

TMX causes changes in the redox P700 transformations in leaves of both the inbred line zppl 225 and hybrid zp 341 (see Fig. 2, A, C). Note that zppl 225 leaves showed changes in chlorophyll in the fast and slow phases of MR associated with a decrease in amplitude at ~ 20 ms (see Fig. 2, A). An analysis of

the maximum P700 oxidation and reduction rates (MR kinetics) indicates a decrease in the oxidized and an increase in the reduced PSI (P700) acceptors in zppl 225 leaves under the influence of TMX. This is probably due to a decrease in the pool of acceptors, such as P700, on the acceptor side of the PSI. On the contrary, PSI RCs of zp 341 hybrid leaves showed resistance to TMX (no change in MR kinetics) (see Fig. 2, B).

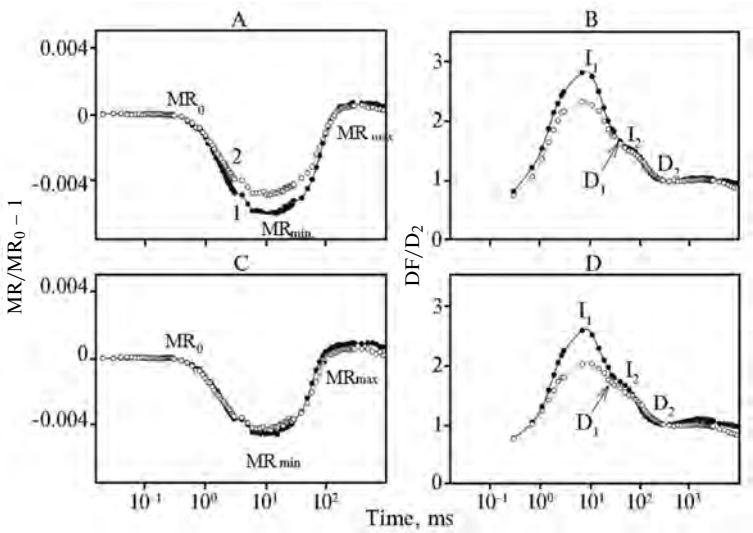

Fig. 2. Light-induced kinetics of modulated reflection at λ = 820 nm (MR) (A, C) **and delayed fluorescence** (DF) **normalized to the minimum signal level D2 at 200 ms** (B, D) **in leaves of maize (*Zea mays* L.) inbred line zppl 225** (A, B) **and hybrid zp 341** (C, D) **in control** (1) **and upon thiamethoxam treatment** (2)**.** MR kinetics are normalized and expressed as $MR/MR_0 - 1$, where MR_0 is the signal intensity at 0.7 ms. I_1, I_2, D_1, D_2 are maximums and minimums on the DF induction curve.

We estimated the degree of thylakoid membrane energization based on peaks I_1 and I_2 of delayed fluorescence measured in the microsecond time range (0-0.09 ms). It is known that DF is proportional to the rate of recombination reactions in PSII, which are affected by the energization of the thylakoid membrane [6]. We considered the fast phase of the DF associated with the membrane potential formation. It is known that the fast phase of the DF has two peaks, I_1 (~ 7 ms) and I_2 (~ 100 ms), after which it drops to a D_2 minimum (~ 200 ms) [19]. Figure 2 (B, D) shows the induction curves of the DF of zppl 225 and zp 341 leaves treated with TMX, normalized to the minimum fluorescence D_2. Under the TMX influence I_1 amplitude decreased in zppl 225 and zp 341 leaves by 23 and 21%, respectively, compared to control. A decrease in the I_1 peak in the microsecond time range could be caused by a decrease in the Q_A re-oxidation rate (disturbance of electron transport on the acceptor side of PSII) and/or a decrease in the Z^+ reduction from 4MnCa of oxygen-evolving complex (OEC) [19].

The state of OEC and its ability to donate an electron for $P680^+$ through Z^+ were monitored by the appearance of an additional peak on the PF induction curve. For this, we calculated the relative fluorescence between the O and J peaks was as $W_{OJ} = (F_t - F_O)/(F_J - F_O)$ (see Fig. 3, A, C) and the difference in the values of the functions between the control and the samples treated with TMX as $\Delta W_{OJ} = W_{OJ(TMX)} - W_{OJ(control)}$ (see Fig. 3, B, D). In TMX-treated leaves of zppl 225 and zp 341 an additional peak appeared between O and J at ~ 300 μs (peak K) (see Fig. 3, B, D) which is characteristic of high-temperature stress due to the limitation of electron transfer from OEC [13].

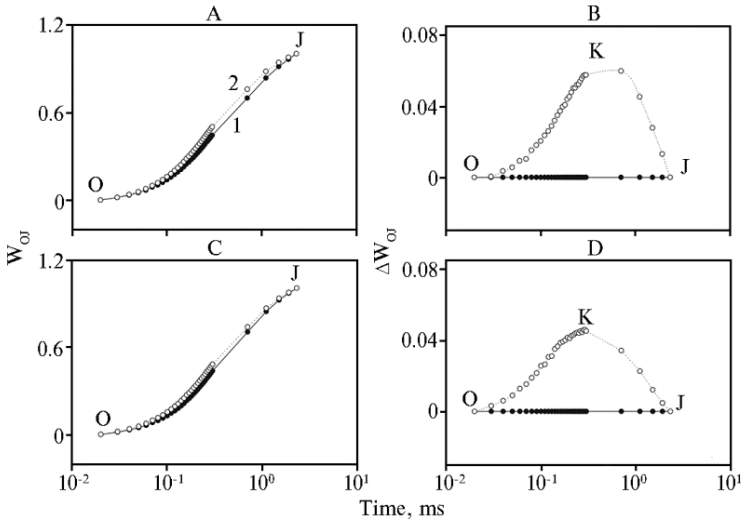

Fig. 3. The relative variable fluorescence $W_{OJ} = (F_t - F_O)/(F_J - F_O)$ between the O and J levels (A, C) **and the difference in the function values $\Delta W_{OJ} = W_{OJ(TMX)} - W_{OJ(control)}$** (B, D) **in leaves of maize (*Zea mays* L.) inbred line zppl 225** (A, B) **and hybrid zp 341** (C, D) **in control** (1) **and upon thiamethoxam treatment** (2)**.** O, K, J are the peaks on the induction curve at 20 μs, 300 μs, and 2 ms, respectively.

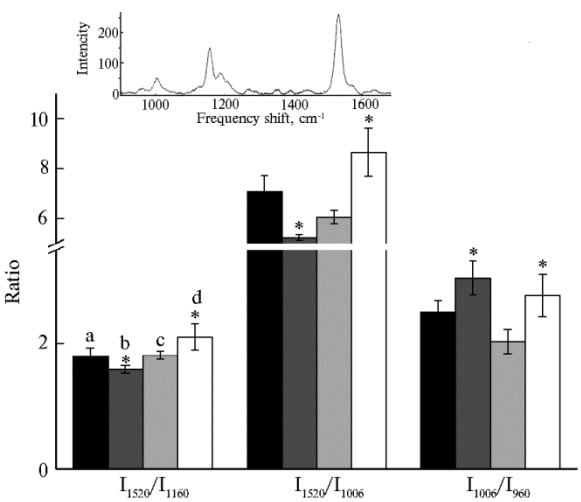

Fig. 4. Changes in Raman spectra of carotenoids in leaves of maize (*Zea mays* L.) of two genotypes upon thiamethoxam treatment (TMX)**:** a — inbred line zppl 225, b — zppl 225 + TMX, c — hybrid zp 341, d — hybrid zp 341+ TMX. Asterisks indicate statistically significant differences ($p < 0.05$). The inset shows the Raman spectrum of leaf carotenoid for the inbred maize line zppl 225.

In the Raman scattering spectra of maize leaves, we revealed the bands characteristic of carotenoids (960 cm^{-1}, 1006 cm^{-1}, 1156 cm^{-1}, 1190 cm^{-1}, 1200 cm^{-1}, and 1520 cm^{-1}) which are due to electron valence vibrations in the polyene chain of the molecule (Fig. 4). The maximum changes in the amplitude of the Raman spectrum bands were observed for I_{1520}/I_{1006} (reflects contribution of −C=C− bond valence vibrations of the carotenoid polyene chain), I_{1520}/I_{1160} (reflects changes in the contribution of −C=C− bonds vs. −C−C− bonds of the polyene chain) and I_{1006}/I_{960} (reflects the position of carotenoid polyene chain relative to the pyrrole rings) [20]. Peaks at 1190 and 1200 cm^{-1} in the Raman spectra are indicative of a 15-cis conformation of carotenoids (data not shown).

We found an increase in the ratio I_{960}/I_{1006} bands in maize leaves when TMX was applied (see Fig. 4). This may indicate a change in the conformation of carotenoid molecules in the PSA antenna. Under the influence of TMX, the conformation of the polyene chain of the carotenoid molecule changed in the leaves of the zppl 225 inbred line, with a 26% decrease in the contribution of the −C=C− valence vibrations (I_{1520}/I_{1006} ratio). In the leaves of the zp 341 hybrid

TMX caused a 16% and 43% increase in the ratios I_{1520}/I_{1160} and I_{1520}/I_{1006}, respectively, which indicates a change in the conformation of carotenoids in the leaves of the zp 341 hybrid, but not their content.

In this work, we investigated the effect of TMX pesticide on the photosynthetic pigments and the photochemical activity of the photosynthetic apparatus of leaves of two maize genotypes using modern spectral and fluorescence methods.

The obtained results show that the zppl 225 inbred line is subject to greater changes when spraying leaves with TMX than the zp 341 hybrid. The zppl 225 inbred line shows lower chlorophyll content, which probably indicates that the synthesis of these pigments decreases under the influence of TMX. Note that in the zppl 225 leaves a decrease in the ratio of chlorophylls (a + b) carotenoids are revealed, which, as a rule, correlates with an increase in the content of carotenoids under stress [21]. On the contrary, no changes in the pigment composition are revealed in the leaves of the zp 341 hybrid. Earlier on the example of cotton, neonicotinoids were shown not to affect the chlorophyll content, while in okra leaves, when exposed to acetamipride, a gradual increase in the total chlorophyll content was reported [22]. At the same time, TMX can act as a bioactivator of rice plants, increasing their physiological and metabolic activity [23].

In our work, the TMX effect on PSII, PSI, and electron carriers between photosystems in leaves of zppl 225 and zp 341 was evaluated based on a set of parameters obtained after analysis of the kinetic curves of PF, DF and MR. An analysis of the kinetic curves of PF by the JIP-test, which describes the energy fluxes through different parts of the electron transport chain of photosynthesis [13], revealed that TMX had an insignificant ($p > 0.05$) effect on PSII photochemistry (F_V/F_M) in leaves of both studied genotypes. Changes were detected on the acceptor side of PSII associated with a decrease in the probability of electron transport after the acceptor Q_A^- (ψ_{Eo}) in the leaves of the inbred line zppl 225. Lower probability of electron transport on the acceptor side of PSII (ψ_{Eo}) caused a decrease in the functionality of PSII (PI_{ABS}). Other neonicotinodine pesticides, such as acetamipride, also do not affect F_V/F_M and lead to a significant decrease in the functional activity of PSII due to a decrease in the pool size and electron transport on the acceptor side of PSII in the cyanobacteria *Synechocystis* sp. [24].

Thus, spraying maize plants with pesticide thiamethoxam (TMX) causes a decrease in the chlorophyll content in leaves of the zppl 225 inbred line compared to the hybrid zp 341. In addition, a decrease in the acceptor pool on the acceptor side of photosystem I (PSI) is found in leaves of zppl 225. It was established that TMX affects the functional activity of photosystem II (PSII), decreasing the PI_{ABS} parameter, and reduces the potential on the thylakoid membrane (I_1) in both zppl 225 and zp 341. TMX causes opposite changes in the conformation of carotenoid molecules in the antenna, but does not change their content, which, apparently, is associated with a more efficient dissipation of excess energy through the system of carotenoids in leaves of zp 341 compared to zppl 225. The proposed combination of methods makes it possible to efficiently assess the functional state of photosynthetic apparatus in leaves under field and lab conditions both during biomonitoring and when comparing different lines of crops.

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DEVELOPMENT OF NEW qPCR-BASED IDENTIFICATION SYSTEMS FOR NON-QUARANTINE POTATO (*Solanum tuberosum* L.) PATHOGENS DISTRIBUTED IN THE TERRITORY OF RUSSIA

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Abstract

Russia is among the largest potato producers in the world. According to statistics for 2018, the sown area of potatoes amounted to 310.7 thousand ha, which is 3.5 % more than in 2017, and the gross harvest in the industrial sector is 7157 thousand tons. At the same time, potatoes are susceptible to infection by various plant pathogens of different taxonomic groups. In modern potato growing, the progressive spread of viral and bacterial diseases, which, in addition to reducing the yield, causes a catastrophic deterioration of tubers' quality, leads to a serious problem for commercial production. Not all dangerous pathogens belong to quarantine organisms in Russia. However, the need for their accurate and highly specific identification is not less than for quarantine organisms. Currently, common diagnostic methods in potato growing are indicator plants, serological and cytological tests. They are relatively reliable, but not always sensitive enough, time-consuming and their use requires highly qualified personnel. A modern alternative to these methods are diagnostic systems based on polymerase chain reaction (PCR), in particular, its real-time modification (quantitative PCR, qPCR). In the present study, for the first time in Russia, qPCR-based tests were developed for six non-quarantine pathogens — necrotic strains of potato virus Y (PVY^{N-NTN} and $PVY^{N:O}$), tobacco rattle virus (TRV), and pathogenic bacteria *Dickeya solani*, *D. dianthicola*, and *Pectobacterium atrosepticum*. Primers and fluorescent-labeled probes were designed based on nucleotide sequences deposited in the NCBI GenBank international database for the amplicon size not more than 500 bp. The specificity of the proposed systems was shown in tests with the genetic material of pathogens that infect potatoes, which are taxonomically close or occupy similar ecological niches, such as the ordinary strain PVY, potato mop-top virus, *Pectobacterium carotovorum*, and *D. zeae*. The quality of the proposed test systems was also evaluated using plant material, presumably infected with the analyzed pathogens. Nucleic acids were isolated using Proba-NK (for RNA) and Proba-GS (for DNA) reagents (AgroDiagnostica LLC, Russia). For the reverse transcription reaction, the RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used. PCR was carried out in the Tertsik thermocycler (DNA-technology, Russia), and quantitative PCR was performed in the DT-96 detection thermocycler (DNA-technology, Russia). To assess possible inhibition, an internal control sample (IC, a plasmid with a specific 560 bp insert) was added to the reaction mixture. Positive control samples (PCs) were cloned using the Quick-TA kit (Evrogen, Russia). Plasmid DNA concentration was determined (a NanoVue spectrophotometer, GE HealthCare, USA). Specific amplification products were sequenced (an ABI PRISM 3730 automated sequencer, Applied Biosystems, USA). Analytical sensitivity was evaluated by quantitative PCR, in which sequential 10-fold dilutions of plasmid DNA (PC in four independent replicates) in the range of 10^7 to 10^0 copies per reaction were used as templates. High sensitivity of the developed test systems, ranging from 10 to 500 copies of specific DNA per reaction, as well as high reproducibility (*Cv* 1.5-2.0

%) were shown. The maximum fluorescence increase for the developed hydrolyzed probes ranged from 1200 to 2000 units of background. The universality of the proposed amplification profiles can serve as the basis for adapting test systems to the multiplex PCR format. The obtained results indicate that these systems detect the analyzed pathogens with high specificity and sensitivity and can be used as part of phytosanitary control and routine detection of 6 non-quarantine pathogens in plants, planting material and food products.

Keywords: diagnostics, quantitative PCR, sensitivity, specificity, *Pectobacterium atrosepticum*, *Dickeya solani, Dickeya dianthicola*, necrotic strains of potato virus Y, PVY, tobacco rattle virus

Potato is among main crops with a global production of about 375 million tons. Diseases caused by plant pathogens of different nature belonging to different taxonomic groups become an important factor reducing the quantity and quality of potato yields. Annual losses caused by viroses make up 7% of the crop, by mycoses and bacterioses 14% [1, 2]. At present, more than 50 viral and bacterial pathogens of potato ~~diseases~~ are identified in regions with diverse climatic conditions [3, 4]. At the same time, only some of them are tested during the certification of seed potatoes in Russia, and there is no integrated monitoring system for viral and bacterial diseases of this crop [5, 6].

Modern potato growing is characterized by the progressive spread of viral pathogens, such as causative agents of necrotic spotting disease of tubers and rattle virus, as well as potato blackleg ~~bacteriosis~~. In addition to reducing the yield, these agents catastrophically deteriorate the standard quality of tubers, which becomes a serious problem for commercial production [7].

Potato tuber necrotic ringspot disease (PTNRD, the emerging of rings and arcs of dark brown color on tubers) is caused by necrotic strains of the virus Y (potato virus Y, $PVY^{N\text{-}NTN}$). At the same time, PTNRD-causing isolates that possess serological properties characteristic of the ordinary strain of potato virus Y (PVY^{O}), were identified. These strains isolated in Europe are assigned to the subgroup $PVY^{N\text{-}Wilga}$ necrotic strains (by the name of the variety on which the virus with similar properties was first detected) [8-10].

The group of PVY isolates with similar properties, discovered later in North America, was designated $PVY^{N:O}$ [11]. The PVY genome is a single-stranded RNA about 9700 nucleotides long. To date, the whole genome sequences and their polymorphism have been determined for all of the listed groups of isolates, and this fact greatly simplifies the study and development of diagnostic systems [12]. Potato stem-mottle disease (corky ringspot) which manifests itself as arcs, rings or flecks that form on or within tubers is caused by the tobacco rattle virus (TRV) which is spread primarily by stubby-root nematodes of the order *Dorylaimida* genus *Trichodorus* [13, 14].

According to the report from Laboratory of Phytoparasitology of the Center for Applied Hygiene and Epidemiology RAS which studies the distribution of *Trichodorus* nematodes, rattle virus is detected in 75% plant samples invaded by nematodes [15]. TRV refers to typical pathogens of natural focal diseases. Already in the first year that a healthy potato plant enters the infectious area, the tuber yield becomes unmarketable [16, 17]. The disease caused by the virus is described in many countries, and in the mid-1970s it was detected in the territory of the former USSR [16]. The virus is widely present in the Netherlands, Germany, Belgium, England, France, Poland, Sweden, Austria, Finland, the USA, Japan, the Baltic countries and Russia [18-20]. Identification of TRV is difficult, since a mixed infection is frequent [15, 17]. The TRV genome is a linear single-stranded RNA which consists of two parts, RNA-1 and RNA-2, 9000 to 11500 nucleotides long in total [21]. At present, a significant number of structures of the complete genomes of this virus have been determined for isolates of different geographical origin [22, 23].

The spread of potato blackleg in recent years has been noticeably pro-

gressing. A number of new pathogens causing watery rot of stems and soft rot of tubers have been identified [24]. Symptoms caused by *Dickeya* spp. bacteria on tubers and potato plants in many ways are similar to those of *Pectobacterium* spp. infection [25].

However, the *Dickeya* spp. can cause the disease when the amount of the infectious agent is small. *Dickeya* spp. can spread more easily over plant vascular tissues, are more aggressive, and at elevated temperatures, wet rot damage that they cause is stronger than in the case of *Pectobacterium atroseptica* infection. *Dickeya* spp. bacteria are typical in regions with a hot climate, however, due to local climate changes, these agents are recently observed in regions with a temperate climate, traditional for the production of potatoes. An important step in the study of these bacterial pathogens was the sequencing of their genome structures [26-28].

The main way to radically combat potato viral and bacterial diseases is to identify pathogens in the early stages of seed production, followed by culling the infected material. Classical diagnostic methods are reliable, but often do not have sufficient sensitivity, and also require highly qualified personnel and a significant time [29]. In this regard, new domestic PCR-based assays are needed to identify pathogens that degrade the quality of marketable potatoes and cause significant economic damage.

In the present paper, we report on the first Russian quantitative PCR-based systems for diagnostics and identification of six non-quarantine pathogens (necrotic strains of Y potato $PVY^{N\text{-}NTN}$ and $PVY^{N:O}$, tobacco rattle virus, pathogenic bacteria *Dickeya solani*, *Dickeya dianthicola*, *Pectobacterium atrosepticum*).

The purpose of the work is the development of original domestic systems for identifying non-quarantine potato pathogens based on the quantitative polymerase chain reaction, assessing the sensitivity and effectiveness of these systems, as well as the possibility of their use in the practice of plant pathology.

Materials and methods. PVY^{NTN}, $PVY^{N:O}$, TRV, *Pectobacterium atrosepticum*, *Dickeya solani*, *Dickeya dianthicola* were received from Lorch All-Russian Research Institute of Potato (VNIIKH) collection. In addition, plant pathogens taxonomically close or similar in symptoms on potato plants were used, i.e. PVY (potato virus Y, common strain), potato mop top virus (PMTV), *Pectobacterium carotovorum*, *Dickeya zeae*. Their isolates were collected during testing seed material from the Central and Southern Federal Districts of the Russian Federation. Monoclonal antibodies (Bioreba, Switzerland) were involved in tests. In addition, the proposed test systems were validated on the genetic material of 9 samples of potato tubers (*Solanum tuberosum* L., various varieties from Moscow Province) which were presumably infected by the analyzed pathogens.

Nucleic acids were extracted with Proba-NK kit (for RNA) and Proba-GS kit (for DNA) (AgroDiagnostica LLC, Russia) as per the manufacturer's protocol. For reverse transcription, the RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used.

The primers and fluorescently labeled probes were designed by aligning nucleotide sequences of a number of loci of detected organisms deposited in the NCBI database (GenBank, https://www.ncbi.nlm.nih.gov/genbank/). A ClustalW algorithm [30] was used for alignment. The selection and assessment of the physicochemical properties of oligonucleotides was performed with Oligo v. 6.71 software (https://www.oligo.net/oligo_updates.htm).

PCR protocols (Tertsik thermocycler, DNA-technology, Russia) were as follows: for primer pairs Y-NTN(F-R), Y-NW(F-R), TRV(F-R), Dsol(F-R) — 90 s at 93 °C; 20 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (5 cycles); 1 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (40 cycles); for primer pairs Patr(F-R), Ddi(F-

R) — 90 s at 93 °C; 20 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (5 cycles); 1 s at 93 °C, 5 s at 60 °C (40 cycles). qPCR was performed according to similar programs (a detecting thermocycler DT-96, DNA-Technology, Russia). The results were analyzed by a threshold method [31]. Each DNA sample was tested in triplicate. The composition of PCR buffer and the protocol of separation of amplification products by agarose gel electrophoresis were described in earlier papers [32, 33].

An internal control (IC), the pTZ57R/T plasmid with a specific 560 bp insert, was added to the reaction mixture to assess possible inhibition, so the reaction format was multiplex. Positive controls (PCs) were cloned using the Quick-TA kit (Evrogen, Russia) according to the manufacturer's protocol. The plasmid DNA concentration was measured by NanoVue spectrophotometer, GE Healthcare, USA). DNA samples were sequenced at Evrogen JSC (ABI PRISM BigDye Terminator v. 3.1 kit, Applied Biosystems, USA), followed by analysis of the reaction products (an ABI PRISM 3730 automatic sequencer, Applied Biosystems, USA).

Analytical sensitivity was evaluated by qPCR with sequential 10-fold dilutions of plasmid DNA as a template (PCs in four independent replicates) in the range from 10^7 copies to 10^0 (single copies) per reaction. A linear dependence of the threshold cycle on the number of copies of specific DNA in the reaction was plotted for each PC.

Standard deviations ±SD calculated from the averaged Cq value for a series of dilutions were used to assess reproducibility. The variability of Cq values was evaluated by testing each dilution thrice in triplicate. The coefficients of variation (*Cv*, %) were calculated.

Results. The analysis of the nucleotide sequences deposited in GenBank for the analyzed pathogens allows us to selected the following loci for the design of primers and probes: coat protein gene (GU980964, AF321554, AJ535662, GU550076, AJ315774) for PVY^{NTN}, *VPg* gene (EF638893, EF638901, EF638902-638892) for $PVY^{N:0}$, RNA-dependent RNA polymerase gene (JX267264-267270, MF918561-918567) for TRV, RNA polymerase σ-factor gene (*rpoD*, MH118541, LC275948-275957) for *D. dianthicola*. For the remaining pathogens, marker fragments of sequences of complete genomes were used, CP024956, CP009125, CP007744 for *P. atrosepticum*, CP024710, CP017453, CP016928 for *D. solani*. When designing primers, the corresponding loci of the target pathogens and closely related taxa were aligned. The desired temperature of primer annealing at least 60 °C was also accounted. The resultant four pairs of primers had a calculated annealing temperature of 64 °C, two pairs of 60 °C. The structure and properties of the designed primers, as well as the size of the amplification products are shown in Table 1.

1. Characterization of primers designed for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens

Pathogen	Nucleotide sequence 5′→3′	T_o, °C	Amplicon, bp
PVY^{NTN}	Y-NTNF: TGAAACCAATCGTTGAGAAACA Y-NTNR: GTACTGATGCCACCGTCGT	60	290
$PVY^{N:0}$	Y-N0F: CAAGTCAAGCAGGAGGTTTG Y-N0R: CCCAGTCTGCCTTAGTTTA	60	350
TRV	TRVF: TTTCTTACATTCATGACTGGCT TRVR: TTGACCAACTCTCGCGGTAC	60	320
Pectobacterium atrosepticum	PatrF: CAGTAGGTTTGGGAGCAGCC PatrR: CCACTACCGATGATGCTCCC	64	280
Dickeya solani	DsolF: ATGTACTAATCAGACATGTTGCTT DsolR: TGTATCCTGATTAATTTGTGATCC	60	200
D.dianthicola	DdiF: TGTCCGATTTGATCACCGT DdiR: ATGCTGTTGTCATCATCGGAC	64	160

N o t e. PVY^{NTN} — potato virus Y (strain NTN), $PVY^{N:0}$ — potato virus Y (strain N:0), TRV — tobacco rattle virus; T_o — calculated temperature of primer annealing.

To assess the specificity of the developed primers, in addition to the DNA or cDNA of the analyzed pathogens, we used the genetic material of closely related or occupying similar ecological niches organisms, such as the PVY common strain, Potato mop-top virus (PMTV), bacteria *Pectobacterium carotovotum*, *Dickeya zeae*. Electrophoretic analysis of amplification products showed that all used primer pairs were strictly specific. In subsequent analysis we used qPCR format (Table 2).

2. Specificity of primers designed for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens by qPCR method

Pathogen	Primer					
	Y-NTNF-R	Y-NOF-R	TRVF-R	PatrF-R	DsolF-R	DdiF-R
PVY^{NTN}	+ (29. 1)	–	–	–	–	–
$PVY^{N:0}$	–	–	–	–	–	–
PVY	–	–	–	–	–	–
TRV	–	–	+ (24. 1)	–	–	–
PMTV	–	–	–	–	–	–
Pectobacterium atrosepticum	–	–	–	+ (25. 3)	–	–
P. carotovorum	–	–	–	–	–	–
Dickeya solani	–	–	–	–	+ (27. 4)	–
D. dianthicola	–	–	–	–	–	+ (29. 3)
D. zeae	–	–	–	–	–	–

N o t e. PVY — potato virus Y (common strain), PVY^{NTN} — potato virus Y (strain NTN), $PVY^{N:0}$ — potato virus Y (strain N:0), TRV — tobacco rattle virus, PMTV — potato mop-top virus. The average values of threshold cycles per three replicates are given in parentheses; "+" is a positive result, "–" is a negative result.

3. Probes and analytical sensitivity of designed qPCR-based systems for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens

Pathogen	Probe nucleotide sequence 5′→3′	T_o, °C	Sensitivity, DNA copies per reaction
PVY^{NTN}	(BHQ1)-TGCGGCCTTCAT(FAMdT)TGAATGTGCGC	82	1×10^1
$PVY^{N:0}$	(BHQ1)-GCAAGCCTTGGGCAG(FAMdT)AACACGACCA	81	1×10^2
TRV	(BHQ1)-GAACCGTGGCAGG(FAMdT)GAGAGGAGACAC	82	1×10^2
Pectobacterium atrosepticum	(BHQ1)-CGCGTCTTTTTT(FAMdT)GGGGTGTCGGCA	83	5×10^2
Dickeya solani	(BHQ1)-CGACGTGAAAATGTGA(FAMdT)GACTTCCATCC	82	5×10^1
D. dianthicola	(BHQ1)-TTCGTCTTCTTCGC(FAMdT)TTCGTCGTCGTCA	82	1×10^1

N o t e. PVY^{NTN} — potato virus Y (strain NTN), $PVY^{N:0}$ — potato virus Y (strain N:0), TRV — tobacco rattle virus; T_o — calculated temperature of probe annealing.

Another important feature for a diagnostic system is its analytical sensitivity. qPCR analyses with 10-fold dilutions of plasmids (PC) with known concentrations and copy numbers in the reaction mixture showed that the systems for PVY^{NTN} and *D. dianthicola* identification had the highest sensitivity (about 10 copies of the plasmid with the corresponding cloned DNA fragment per reaction), the system for *P. atrosepticum* identification was the least sensitive (500 copies per reaction) (Table 3).

The efficiency values for the developed test systems ranged from 91.2 to 98.0%. The values of standard deviations during testing of each of 10-fold dilutions in triplicate ranged from 0.09 to 0.53, and the *Cv* value did not exceed 2%. As an example, Figure 1 shows graphs of the threshold cycle vs. the number of plasmid copies with cloned DNA fragments of target objects (PC) per reaction, as well as graphs of fluorescence signal accumulation for PVY^{NTN} and *D. dianthicola* ~~test~~ systems as examples.

The quality of the developed test systems was also evaluated using potato tubers of different varieties presumably infected with the analyzed pathogens. In PCR, in addition to specific primers and probes, the IC (plasmid) with the corresponding pair of primers and probe were added to the reaction mixture. The presence of a band, corresponding to IC amplification product, was demonstrated on the electrophoregrams of all nine potato samples' cDNA/DNA analyzed by primer pairs to PVY^{NTN} and *D. dianthicola*. This indicates the absence of reaction

inhibition (Fig. 2). In turn, the absence of a specific band for negative control indicates the absence of contamination of the working area with specific amplicons. As follows from the electrophoregrams shown, PVY^{NTN} virus cDNA was revealed in samples No. 3 (cultivar Red Scarlett), No. 4 (cultivar Zhukovsky rannii), No. 7 (cultivar Favorit III), No. 8 (cultivar Pomdor), No. 9 (cultivar Floris), and *D. dianthicola* DNA in samples No. 2 (cultivar Red Scarlett), No. 3 (cultivar Red Scarlett), No. 6 (cultivar Romano), 8 (cultivar Pomdor), 9 (cultivar Floris).

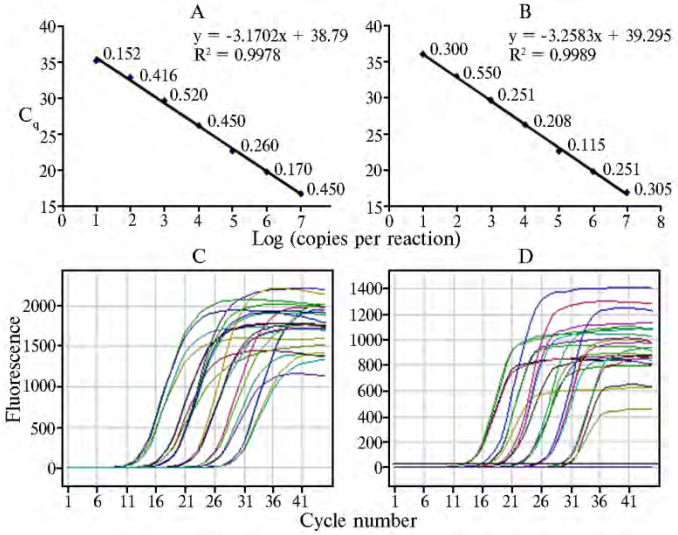

Fig. 1. Assessment of sensitivity and effectiveness of designed qPCR-based tests for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens with 10-fold dilutions of specific positive controls: A and B — qPCR threshold cycle dependence on plasmid copy number per reaction (with the standard deviations for four replicates indicated next to each point), C and D — corresponding graphs of fluorescence accumulation. Sensitivity for PVY^{NTN} is 10 copies per reaction, 98.0% effectiveness (A), for *Dickeya dianthicola* 10 copies per reaction, effectiveness 96.7% (B).

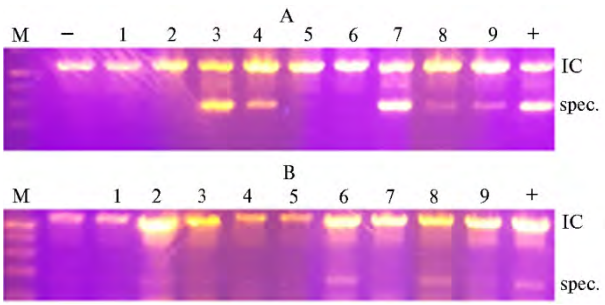

Fig. 2. Electrophoregrams of PCR amplification products (A — cDNA, Y-NTNF-R primer pair; B — DNA, DdiF-R primer pair) **in testing potato tubers presumably infected by non-quarantine pathogens.** IC — internal control, spec. — specific amplification product; 1, 2, 3 — Red Scarlett, 4 — Zhukovsky rannii, 5, 6 — Romano, M — molecular weight marker; "+" is a positive control sample, "–" is a negative control sample.

Note that several variants of systems for identification of the pathogens we examined in this paper have been currently described in special literature. Shvidchenko et al. [34] showed that the real-time PCR is significantly more specific than ELISA for detection of potato viruses, including PVY (the diagnostic sensitivity of ELISA relative to real time PCR is 68%). Also a number of multiplex PCR-based systems have been proposed, in particular for identification of the necrotic strains $PVY^{N:0}$ and PVY^{NTN} [35], as well as the main pectinolytic bacteria, including *P. atrosepticum* and *Dickeya* spp. (with minimum sensitivity of 0.01 ng/μl specific DNA) [36], however, these were not adapted to the qPCR format. Primers that specifically distinguish species of the genus *Dickeya* were described in 2013 [37] and successfully used to identify pathogens in vegetable crops in Northern Ireland [38]. For this test system a very high sensitivity, about 0.05 pg of DNA per reaction (about 10 copies of plasmid DNA), was shown.

Also, a number of systems of different sensitivity for detection of tobacco rattle virus have been described [39-41]. As per Holeva et al. [42], the sensitivity of the system was about 1 fg of plasmid DNA per reaction (approximately 10^2 copies).

Thus, the main characteristics of the proposed assays were close to those of the most effective foreign analogues or even exceeded them. The sensitivity of the systems ranged from 10 to 500 copies of specific DNA per reaction, and the amplification efficiency exceeded 90%. High reproducibility of the results is shown (*Cv* no more than 2%, standard deviations over dilutions no more than 0.53). The designed probes are shown to be high specific, and the internal controls are optimized. The quality of the test systems is confirmed by tests with plant material infected with the studied pathogens. The totality of the obtained results indicates that the developed ~~test~~ systems identify with high sensitivity and specificity six non-quarantine potato pathogens that cause significant economic damage. These systems can be important tools in monitoring plant pathogen infections of planting material, plants, and food products.

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WHOLE GENOME SEQUENCING OF *Bacillus thuringiensis* var. *darmstadiensis* 56 STRAIN AND THE STUDY OF INSECTICIDAL ACTIVITY OF THE BIOLOGICAL PREPARATION ON ITS BASIS

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Abstract

Multifunctional microbiological preparations are promising for use in plant protection due to their diverse effects including growth-promoting effect and complex antifungal and insecticidal activity. One of the key microorganisms used as the basis of biological preparations production is the gram-positive spore-forming bacterium *Bacillus thuringiensis* (Bt). The high specificity of the action and the environmental safety of Bt-based preparations contribute to maintain biocenosis balance and to reduce the number of treatments as well as to obtain environmentally friendly products. Previously, *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) strain was isolated and selected at the All-Russian Research Institute of Agricultural Microbiology. It possesses insecticidal effect for the larval stages of leaf-eating insect pests, growth-promoting activity for potatoes and antifungal effect against various phytopathogenic fungi. This paper presents the first data on sequencing and annotation of the whole genome of the BtH_{10} 56 industrial strain; the factors responsible for the insecticidal and antifungal activity of this strain are identified, and the high efficiency of the biological preparation based on this strain is demonstrated under the field conditions against the Colorado potato beetle (*Leptinotarsa decemlineata* Say.). The goal of the work was to identify the molecular determinants of the insecticidal properties of the industrial strain *Bacillus thuringiensis* var. *darmstadiensis* 56 as well as to test its activity in the field. Field trials of the effectiveness of the biological preparation based on *Bt*H_{10} 56 against the Colorado potato beetle was carried out on potatoes (*Solanum tuberosum* L.) of the Vineta and Rocco varieties in 2018 and 2019 (MTS-Agro LLC, Voronezh Province) in the area of 1 ha. To evaluate the entomocidal activity, we used a liquid form of the preparation based on the strain produced by the Ekos branch of ARRIAM (the spore titer was $2.12\text{-}2.3\times10^9$ CFU/ml) in yeast-polysaccharide medium in a 100 l bioreactor. The application rate of the preparation was 20 l/ha. The potato plantings were treated using an OPG-2000 sprayer (Zarya LLC, Russia). As a chemical standard, the insecticidal preparations Cepellin, EC and Colorado, SC (Agro Expert Group LLC, Russia) at 100 g/l and 0.1 l/ha doses, respectively, were used as the chemical standards. The counts were carried out in 5, 10 and 14 days after treatment. The biological effectiveness of the preparation was determined by analyzing a decrease in the number of pests according to the Abbott formula. According to the test results, the high efficiency of the developed preparation against the Colorado potato beetle was established. This efficiency varied from 83.8 to 87.8 % and did not differ

from the chemical standards. Using Illumina and Oxford Nanopore technology, we obtained the complete genome sequence of the BtH_{10} 56 strain. After assembly and annotation of the genome, a search for toxins was conducted. The CryProcessor and BtToxin_scanner programs were used to search and classify genes encoding the Bt insecticidal toxins. As a result, a gene belonging to the *cry1E* group, *cry1Ea7*, was found. The toxins belonging to this group are characterized by activity against various *Lepidoptera* pests. It was found that the genome of the strain does not contain genes encoding Vip, Sip and Cyt. toxins, however, it harbors several genes encoding synthetases of non-ribosomally synthesized peptides (*nrp*) that may explain its multifunctional properties. Thus, considering the data obtained the liquid form of the biological preparation based on BtH_{10} 56, can be recommended for use in the industry and organic farming.

Keywords: *Bacillus thuringiensis* var. *darmstadiensis*, insecticidal activity, exotoxin, endotoxin, Oxford Nanopore, Illumina, Cry toxin, *Bt*, Colorado potato beetle, *Leptinotarsa decemlineata*

Bacillus thuringiensis (Bt) is a spore-forming soil bacterium widely used as a biological plant protection agent. Currently, about 100 subspecies of this bacterium have been described, isolated all over the world from various sources - insects, soil, plant debris, water reservoirs [1, 2]. Successful commercial products were developed on the basis of the subspecies *kurstaki*, *aizawai*, *san diego*, *tenebrionis* for protection against insect pests [3, 4], as well as the subspecies *israelensis* against bloodsucking dipterans [5)]. Currently, Russian preparations are made only on the basis of two Bt subspecies, *kurstaki* and *thuringiensis* [6].

Biological products based on Bt strains contain a spore-crystalline complex and a number of other metabolites as an active substance. Strains of some varieties during growth and development form and secrete thermostable water-soluble exotoxin (β-exotoxin) into the nutrient medium. The spectrum of action of exotoxin is much wider than that of the spore-crystalline complex [7, 8]. Exotoxin can act not only when infected orally, but also contactly through the covers of insects, and in combination with a spore-crystalline complex can act as a synergist. Bt-based exotoxin-containing preparations are used to reduce the number of *Lepidoptera* insects and also the members of *Coleoptera* and *Diptera* orders. The presence of three main entomocidal components (spores, δ-endotoxin, and β-exotoxin) in the Bt preparation not only enhances entomocidal effect, but also expands the spectrum of action [9, 10].

The subspecies *darmstadiensis* was first isolated in Germany from the larvae of the bee moth *Galleria mellonella* in 1968 [11]. It is known that some of its strains contain insecticidal toxins of the Cry1 group (from “crystal”) that are active against representatives of the order *Lepidoptera*: *Bombyx mori*, *Lambdina fiscellaria*, *Malacosoma disstria*, *Choristoneura fumiferana* [12], *Anticarsia gemmatalis* [13]. Despite the promise of using the *darmstadiensis* subspecies in agriculture and bio-technology, there are no registered commercial preparations based on its strains

Earlier, we screened natural Bt isolates, which resulted in selection of virulent strains of *B. thuringiensis* var. *darmstadiensis* (BtH_{10}). BtH_{10} 56 was isolated from the corpses of the Colorado potato beetle in the Leningrad region, after which multistage selection was carried out for physiological and economically valuable properties [14]. Lab tests showed a high entomocidal activity of the strain against the larval stages of the Colorado potato beetle and potato ladybug, as well as antifungal activity against various plant pathogenic fungi, including *Botrytis cinerea*, *Pythium* spp., *Bipolaris sorokiniana*, *Rhizoctonia solani*, and *Fusarium oxysporum*. In addition, the strain BtH_{10} 56 exhibits a growth-promoting effect, increasing potato green mass and tuber yield, improving the germination of seeds in cabbage, tomato, cucumbers, zucchini, and beets. The growth-promoting effects of BtH_{10} 56 are higher than those of the BtH_{10} prototype

strain No. 25 [15].

Potato is one of the main commercial crops in the Russian Federation. Potato harvest losses caused by its most dangerous pest, the Colorado potato beetle, can reach 40-50% [16]. The need for environmentally friendly products makes biological low-hazard preparations for plant protection all the more attractive. The use of chemical pesticides has a negative effect on the environment and disrupts the ecological connections between organisms. Biological preparations specific in their action are a promising option for plant protection because of their safety for non-targeted biota, the presence of which, in its turn, simplifies the process of keeping the number of pests below the economic threshold of harmfulness. The use of natural regulatory mechanisms along with microbiological control provide environmentally friendly production of foods within sustainable agroecosystems. The biologicals based on the bacterial strain BtH_{10} 56, in addition to pronounced entomocidal activity against the larval stages of leaf-eating pests, has high antifungal activity against various fungal plant pathogens and also has a growth-promoting effect [15]

In the present work, the sequencing and annotation of the complete genome for the producer strain BtH_{10} 56 are performed for the first time, the factors responsible for its insecticidal and antifungal activity are identified, and the high field efficiency of the biopreparation based on this strain against the Colorado potato beetle *Leptinotarsa decemlineata* Say is shown.

The purpose of the work was to identify the molecular determinants of the insecticidal properties of the producer strain *Bacillus thuringiensis* var. *darmstadiensis* 56 and field testing of BTH10 56-based biopreparation.

Materials and methods. Complete sequencing of the BtH_{10} 56 genome was perfumed using Illumina (Illumina, Inc., United States) techniques and monomolecular nanopore sequencing (Oxford Nanopore, UK). NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, USA) was used to construct a genomic DNA library. Sequencing the library (Illumina HiSeq2500, HiSeq Rapid Run v2 sequencing reagents) resulted in 2735262 reads of 250 nt, 683.8 million nt in total. Primer sequences and regions of poor read quality (<q30) were removed with Cutadapt v. 1.17 software [17] and Sickle v. 1.33 (https://github.com/najoshi/sickle), respectively. Additionally, genomic DNA was sequenced on a MinION system (Oxford Nanopore, UK) using Ligation Sequencing kit 1D protocol with FLO-MIN106 cells. The resultant 31,234 reads with an average read length of 16540 nt were generated, 516.6 million nt in total, which were trimmed with Canu v. 1.6 software (parameter –correct) [18]. Then, a hybrid assembly of filtered Illumina reads and corrected MinION reads was performed with SPAdes v. 3.11.1 software [19]. The additionally obtained contigs were once more assembled by npScarf method [20] using MinION-generated raw reads. The gaps between the contigs were filled by consensus sequences from the Illumina reads using the SPAdes graph (––spadesDir parameter of npScarf). The search for genes and their annotation was performed using RAST server (http://rast.theseed.org/FIG/rast.cgi) followed by a comparison of the sequences of the predicted proteins with the NCBI databases. The CryProcessor program (https://lab7.arriam.ru/tools/cry_processor/) was used to search and classify Cry toxin genes, and the BtToxin_scanner program (http://bcam.hzau.edu.cn/BtToxin_scanner/index.php) was used to identify other Bt insecticidal toxins.

In field conditions, the effectiveness of a *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56)-based biological against Colorado potato beetle was as-

sessed on potatoes (*Solanum tuberosum* L.) Vineta variety in 2018 and Rocco variety in 2019 (1 ha, MTS-Agro LLC, Voronezh Province). The tested liquid biopreparation was produced in 100 l fermenters (Ekos branch of ARRIAM), (east-polysaccharide medium, spore titer of 2.12×10^9-2.3×10^9 CFU/ml). The quality of the biological was evaluated by standard methods [21].

An OPG-2000 sprayer (Zarya LLC, Russia), 20 m working width, was used to apply preparations. The biopreparation was applied at 20 l/ha. Insecticidal preparations Cepellin, CE (in 2018) and Colorado, BPK (Agro Expert Group LLC, Russia) (in 2019) at 100 g/l and 0.1 l/ha, respectively, were chemical standards. The pest numbers were counted immediately before and 5 and 10 days after treatment (in 2018 and 2019) and, in addition, 14 days after treatment (in 2018). Five potato plants adjacent to each other were collected diagonally at 20 points (100 plants in total). The biological effectiveness of the biopreparation was determined by the Abbott formula based on the decrease in pest numbers [22].

Results. A total of 6290617 bp was determined for BtH_{10} 56 genome using two technologies (Illumina and monomolecular nanopore sequencing) (Table 1). There were seven contigs in total of which chromosome and two plasmids were assembled as circular contigs; another 4 contigs represented plasmids in a linear form which may be due to the presence of extended repeats.

The *B. thuringiensis* BTH10 56 genome was found to comprise 13 copies of the operon of rRNA genes (16S—23S—5S) and 107 transport RNA genes (tRNAs) encoding all 20 amino acids. As per the annotation, 6611 potential protein-coding genes were predicted, the functions of 4517 (68%) proteins were predicted through comparison with NCBI databases. CRISPR loci (clustered regularly interspaced short palindromic repeats) were not found in the B. *thuringiensis* BTH10 56 genome.

1. Assembly and annotation of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) genome

Cjntig	Structure	Size, bp	Protein-coding genes	tRNA genes	rRNA genes
1	Circular	5553288	5755	107	39
2	Circular	349728	445	–	–
3	Linear	155294	173	–	–
4	Circular	140546	140	–	–
5	Linear	57038	68	–	–
6	Linear	24713	21	–	–
7	Linear	10010	9	–	–
Total		6290617	6611	107	39

N o t e. Dashes mean the absence of the genes.

In the *B. thuringiensis* BtH_{10} 56 genome, there were 6 clusters of *nrp* genes encoding nonribosomal peptide synthetases that could produce various peptides with antifungal and antimicrobial activity [23]. The presence of these sequences in the BtH_{10} 56 genome may explain its antifungal properties.

An insecticidal toxin gene located on one of the large plasmids (contig 3) was identified in the BtH_{10} 56 genome. Analysis of the amino acid sequence of the corresponding protein showed that the toxin belongs to the group Cry1E, subtype Cry1Ea7. Cry1E toxins are three-domain insecticidal toxins of *B. thuringiensis* that are active against various *Lepidoptera* insects [24, 25]. Cry1Ea toxins are characteristic of the *darmstadiensis* subspecies and, as per data published, are active against larvae *Lepidoptera* members *Conopomorpha cramerella*, *Manduca sexta*, *Spodoptera littoralis*, *Bombyx mori*, *Lambdina fiscellaria*, *Malacosoma disstria*, *Cacyreus marshalli*, *Anticarsia gemmatalis*, *Choristoneura fumiferana* [12, 13, 26, 27], which allows the strain BtH_{10} 56 to be deemed promising against these pests.

Cytotoxic proteins Cyt and vegetative toxins Vip are characteristic of some Bt subspecies [8]. However, we did not find the *Cyt* and *Vip* genes in the genome of the studied strain. The insecticidal activity of BtH10 56 against leaf-eating insect larvae, previously detected in lab tests [14], is apparently due to the production of Cry1Ea toxin.

Since an efficiency of a strain in lab tests can significantly differ from its effect on the natural population of insect pests, the next stage of our study was the field tests of the insecticidal activity of the biological.

2. Effectiveness of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56)-based liquid biological against Colorado beetle (Vineta variety, Voronezh Province**,** 2018)

Variant	Pest number				Effectiveness, %		
	before treatment	days after treatment			days after treatment		
		5	10	14	5	10	14
BtH_{10} 56	426	193	52	16	54.7	87.8	96.2
Cepellin (standard)	181	104	27	0	42.5	85.0	100.0
Control (no treatment)	229	254	298	288			

N o t e. Day 10 and day 14 correspond to double treatment.

3. Effectiveness of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56)-based liquid biological against Colorado beetle (Rocco variety, Voronezh Province)

Variant	Pest number			Effectiveness, %	
	before treatment	days after treatment		days after treatment	
		5	10	5	10
BtH_{10} 56	285	145	46	49.1	83.8
Colorado (standard)	171	28	18	83.6	89.5
Control (no treatment)	133	144	186		

N o t e. Day 10 and day 14 correspond to double treatment.

In 2018, surveys on Vineta potato crops prior to the treatment showed 30-40% plants to be populated by Colorado beetle at a 100 m^2 distance from the field edge. Insignificant focal distribution of the pest occurred over the remaining area. The pest population consisted of I (58.5%), II (28.7%) and III (12.8%) larval instars. Immediately after counting, potato plantings were treated with preparations. In 5 days the effectiveness of the BtH_{10} 56-based biological was 54.7%, being slightly higher compared to the chemical standard Cepellin (42.5%). Because of hot and dry weather (air temperature was 37 °C), the potato plants were re-treated. On day 10 after the first treatment (day 5 after the second one), the efficiency was 87.8% being comparable to that of the chemical standard. On day 14, the effectiveness of the BtH_{10} 56-based preparation reached 96.2% (Table 2).

In 2019, the chemical standard for Rocco variety was Colorado insecticide. On day 5, the effectiveness of the biological was 49.1%, being lower than that of the chemical standard. In hot weather (air temperature reached 38-40 °C), the pest developed intensively, so the treatments with BtH_{10} 56 and the chemical standard were repeated. On day 10 after the first application the effectiveness for the biological preparation was 83.8%, for the chemical standard 89.5% (Table 3).

In general, tests of a BtH_{10} 56-based biological preparation (Voronezh Province, 2018-2019) showed its high efficiency against Colorado potato beetle, 83.8-87.8% on day 10 for two different potato varieties. These field data are consistent with the previous lab findings [14]. However, the effects of Bt-based preparations are not limited to the role of an insecticide. There is reason to believe that the growth-promoting effect due to the production of siderophores, indole-3-acetic acid, 1-aminocyclopropane-1-carboxylate deaminase, and en-

zymes that dissolve mineral phosphate is significant [28-30]. The multifunctional properties of the BtH10 56 strain shown by us earlier [14, 15] may indicate this strain to be a promising plant protection agent due to its safety for non-targeted biota, growth-promoting action and antifungal activity. The antifungal effect of a number of Bt strains is associated with the production of short Nrp peptides (nonribosomally synthetized peptides) formed by special synthases via an alternative ribosome-independent pathway bypassing the translation apparatus [31]. Six clusters of such genes we have identified in the BtH_{10} 56 genome.

The insecticidal activity obtained in the field trials is consistent with sequencing and annotation of the BtH_{10} 56 genome, which indicate a gene encoding the Cry1Ea7 toxin. According to the literature, this type of toxin is active against members of order *Lepidoptera* [12, 13, 26, 27]. At the same time, Cry toxin can be effective against different orders of insects, which can be detected only experimentally, as a result of difficult long-term experiments [32]. It is also impossible to exclude the probable influence of other virulence factors on the diversity of insect pests affected by Bt bacteria [8]. In particular, this strain produces thermostable exotoxin [14]; however, we did not find genes for the biosynthesis of class I exotoxin in the BtH_{10} 56 genome. Thus, this strain could produce class II exotoxin. The genetic control of this toxin as not yet been clear, however, it has been previously shown to possess activity against insects of *Coleoptera* order [33]. Probably, the synergistic effect of this exotoxin together with Cry1Ea7 causes a strong toxic effect on the *L. decemlineata* larvae shown in field and lab tests.

At present, *Bacillus thuringiensis* var. *darmstadiensis*-based preparations are not offered in Russian market, however, Baciturin developed in Institute of Microbiology of the National Academy of Sciences of Belarus is successfully used in Belarus (the active substance is the spore-crystalline complex and thermostable β-exotoxin of *Bacillus thuringiensis* var. *darmstadiensis*). Baciturin in field experiments shows similar efficacy against Colorado beetle, 85-94% [34]. In addition to var. *darmstadiensis*, an action against Colorado potato beetle is characteristic of var. *thuringiensis*-based preparaions, for example, Bitoxibacillin® registered in the Russian Federation (LLC PO Sibbiofarm, Berdsk) [6]. Many of the known var. *thuringiensis* and var. *darmstadiensis* strains can produce both endotoxin and β-exotoxin, resulting in similar insecticidal activity of preparations based on these subspecies [35]. In other countries, biologicals based on *B. thuringiensis* var. *aizawai* and var. *tenebrionis* are used to control the Colorado potato beetle, however, only the insecticidal endotoxins Cry1Ia and Cry3Aa are the active components [4, 36].

Thus, the insecticidal activity of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) is caused by the presence of a gene encoding Cry1Ea7 toxin and also to an exotoxin, probably belonging to class II. Genes encoding protein toxins of the Cyt, Vip and Sip groups, as well as class I exotoxin, are absent in this strain. The presence of genes encoding a series of synthetases of the nonribosomally synthesized Nrp peptides determines antifungal properties of BtH_{10} 56. The results of two-year field trials indicate high entomocidal activity of the liquid form of the BtH_{10} 56-based biological under commercial farming (Voronezh Province) which is comparable to that of the chemical standards. Our data indicate the suitability of using BtH_{10} 56-based biological preparation in integrated plant protection systems and in organic farming.

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THE EFFECT OF AMARANTHINE ON THE STRESS-RESISTANCE OF TOMATOES (*Lycopersicon esculentum* Mill.) INVADED BY THE ROOT-KNOT NEMATODE (*Meloidogyne incognita*)

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Abstract

Root-knot nematodes of the genus *Meloidogyne* are sedentary parasites that infect the root system of plants, the annual damage from which in the world exceeds 80 billion EUR per year. Infection of plants with these nematodes causes biogenic stress, which is associated with changes in the respiratory processes of plants, a decrease in photosynthesis, the appearance of highly reactive oxygen radicals in the tissues with the formation of toxic intermediate products that cause oxidative stress. The high biological activity of amaranthine isolated from *Amarathus tricolor* L. combined with antioxidant properties, show the promise of its study as a factor resistance to stress of plant during invasion by parasitic nematodes. In this work, we first showed the adaptogenic properties of amaranthine towards tomato plants infected with root-knot nematode. We investigated the effect of the amaranthine beta-cyanine pigment extracted from *A. tricolor* on the parasite-host system of the tomato *Lycopersicon esculentum* Mill. and root-knot nematode *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949. Aqueous solutions of the amaranthine in concentrations from 0.1 to 2.0 mg/ml were tested. When studying the effect of amaranthine on nematodes in vitro, it was found that the analyzed compound has a nematostatic effect in the range of concentrations from 1.0 mg/ml and lower. The 2.0 mg/ml concentration was lethal for *M. incognita*. The effect of amaranthine on biometric and photosynthetic characteristics of tomato plants infected with the root-knot nematode *M. incognita*, and morphological and physiological parameters of nematodes from plants treated with amaranthine were evaluated in a lab greenhouse. Before planting, the seeds were soaked in amaranthine solutions (0.5 and 1.0 mg/ml) for 3 hours, and then seedlings reached the phase of 3-4 true leaves were sprayed with solutions at the same concentrations and infected with nematodes (three thousand larvae per plant). This experiment revealed stimulating effect of the amaranthine in the tested concentrations on plant development. The treated seeds sprouted 2-3 days earlier than the control seeds, the average root length of the seedlings treated with the 1.0 mg/ml preparation on day 7 significantly differed from the control (18 %, $p \le 0.05$). The effect of amaranthine on nematodes was evaluated on day 40 after invasion. A comparative analysis of nematodes from the test and control plants showed that the plant treatment with 0.5 and 1.0 mg/ml aqueous amaranthine solutions led to a decrease in the number of nematodes on the roots. The number of sexually mature females per gram of roots under the treatment with 0.5 and 1.0 mg/ml solutions was 2.1 and 1.3 times less compared to the control. Female nematodes from such plants were 1.2 times smaller in both variants; the number of eggs was also 15-20 % less ($p \le 00.05$) as compared to control. The complex of protective mechanisms in infected tomato plants induced by the action of exogenous amaranthine includes stabilization of photosynthetic processes disturbed by the nematode, accumulation of carotenoid antioxidants, switching of non-cyclic electron transport from water in the Photosystem I to pseudocyclic, stimulation of tomato plant growth. Out findings indicate that amaranthine exhibits adap-

togenic properties associated with the weakening of negative biochemical and functional changes in plants during nematode invasion. Amaranthine can be proposed as a novel biogenic inducer which provides protective effect due to activation of non-specific plant response to biotic stress under pathogen invasion in greenhouse conditions, and also possesses growth-promoting properties.

Keywords: amaranthine, antioxidant, adaptogen, *Amaranthus tricolor* L., variety Valentine, *Meloidogyne incognita*, tomatoes, chlorophyll, carotenoids, oxidative stress, electron transport

As per modern concepts, plant pathogens are stress factors that cause complex protective reactions of plants, including both non-specific (common to different types of stressors) and specific components [1]. At the same time, biogenic stress induced by plant pathogens has a number of features that distinguish it from stress caused by abiogenic extreme factors. This fully applies to plant-parasitic nematodes which exert physical and chemical multifactorial effect on plants [2].

Nematodes, especially sedentary plant-parasitic nematodes which include the *Meloidogyne* root-knot nematodes, are dangerous parasites of agricultural plants that cause damage of over 80 billion euros per year [3]. Symptoms of pants invasion are like those characteristic of the effects of such extreme factors as drought, cold, and mineral starvation. Nematode invasion affects gas exchange in respiration, leads to a decrease in photosynthesis and the appearance of highly reactive oxygen species in the tissues with the production of toxic intermediate compounds that cause oxidative stress [4-6].

The substances of specialized metabolism play an important role among the factors contributing to the survival of plants under biogenic stress, including those caused by damage by plant-parasitic nematodes. These compounds can act on the pathogen as toxins, affect growth, larvae hatching and development; these metabolites are known to possess antifeedant and adaptogenic properties, as well as ability to change intracellular membranes and to normalize photosynthesis and metabolism intensity in infected plants [7-10].

Amaranth is one of the crops for which a rich composition of low molecular weight metabolites, the protectors and immunostimulants of living organism, are characteristic [11, 12]. High content of deterrents, including the amaranthine alkaloid, makes amaranth unattractive for many plan pathogens [13]. Data on the damage of amaranth plants by plant nematodes are scarce and contradictory. Testing 10 species of amaranth for resistance to gall nematodes in South Africa reveal no samples resistant to *Meloidogyne incognita* race 2 and *Meloidogyne javanica* [14]. However, there is evidence of high resistance of amaranth hybrids to the root-knot nematode [15]. Introducing amaranth plants into the crop rotation to control the root-knot nematode *M. javanica* on nightshade and pumpkin crops gave a positive result [16]. The inconsistency of the data, apparently, is associated with a wide variety of species-specific properties, including the composition and content of secondary metabolites in plants of this genus which comprises up to 90 species.

The violet-red beta-cyanine pigment amaranthine outstands of the metabolites of red-colored *Amaranthus tricolor* L. which reduce the effects of plant oxidative stress. Amaranthine can neutralize superoxide anions $O_2^{\bullet-}$), free radicals and chelate Fe^{2+} ions [17]. Exogenous amaranthine increases electron transfer along a non-cyclic pathway (Hill reaction) in isolated chloroplasts [18]. Amaranthine participates in protective and adaptive response to photostress, stimulates plant growth and seed germination, and shows a positive effect under extreme temperatures and drought conditions [12]. Amaranthin also showed a protective effect upon treatment of cucumber leaves affected by thrips [13].

High biological activity of amaranthine of *A. tricolor* combined with antioxidant and antifeedant properties makes this metabolite a promising factor in

plant resistance to stress during invasion by parasitic nematodes

In this work, the adaptogenic effect of amaranthine during nematode invasion in tomato plants is first shown, which is expressed in the activation of energy metabolism and recovery processes.

Our goal was to evaluate the effect of amaranthine on the morphophysiological and photosynthetic parameters of tomato plants under root-knot nematode *Meloidogyne incognita* invasion and to evaluate morphological and physiological parameters of nematodes from plants treated with amaranthine.

Materials and methods. The investigations were carried out in 2016-2017 on Carlson tomato (*Lycopersicon esculentum* Mill.) heterotic hybrid F_1 with a 30% resistance to *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949. Amarantin was extracted from freshly picked leaves of amaranth (*A. tricolor* L.) Valentina varieties (bred in the All-Russian Research Institute of Selection and Seed Production of Vegetable Crops, authors V.K. Gins, P.F. Kononkov, M.S. Gins) [12]. In the experiments, freeze-dried amaranthine powder was used.

The effect of aqueous solutions (2.0; 1.0; 0.75; 0.5 mg/ml) of amaranthine on the viability of nematodes was assessed in vitro by motor activity of II instar larvae. Nematodes placed in distilled water served as a control. The experiment was repeated thrice for each variant (20 larvae each). Root-knot nematodes initially collected in the Teplichny state farm (Moscow) were thereafter lab-cultivated on roots of a susceptible tomato variety. Larvae were obtained from isolated egg sacs of nematodes that live in the roots of infected tomato plants [19]. Larvae were incubated in a thermostat in a humidity chamber at 25 °C, in 24 and 48 hours the motility of individuals was evaluated.

The effect of various amaranthine concentrations (1.0; 0.5 and 0.1 mg/ml) on tomato seed germination was determined in vitro. The seeds were soaked in aqueous solutions of the substance for 3 hours, and the seeds soaked in distilled water were a control (20 seeds per each variant). Seeds were germinated in a ~~a~~ humidity chamber at 25 °C. The seedling development was evaluated in 3, 5 and 7 days.

The effect of amaranthine on tomatoes and root-knot nematode was investigated in a lab greenhouse. Before planting, the seeds were soaked in amaranthine solutions (0.5 and 1.0 mg/ml) for 3 hours. At 3-4 true leaves, the seedlings were sprayed with amaranthine solutions in the same concentrations and simultaneously infected with a nematode (3 thousand larvae per plant). The control was healthy and invaded plants treated with water. Plants were grown in separate 1 l flowerpots (10 plants per variant). The effectiveness of amaranthine was evaluated on day 40 after root invasion with nematodes. Root infestation estimates corresponded to 5-point scale (1-10% for 1 point; 11-35% for 2 points; 36-70% for 3 points; 71-100% for 4 points). Also the estimates were the number of stem swellings (galls per 1 g root), the weight of plant aerial parts and roots, and morphophysiological indicators of the state of the parasite population, i.e. the size of the females and their fecundity, eggs per ootheca [20].

Chlorophylls and carotenoids were measured by absorption spectra of leaf ethanol extracts [21] on day 10 and day 40 after the plant invasion with root-knot nematodes and foliar application of 0.5 mg/ml amaranthine aqueous solution to the invaded plants (Solar PB2201 spectrophotometer, ZAO SOLAR, Belarus).

Chloroplasts were isolated from leaves (1 g) triturated in 0.3 M sucrose; 0.1 M NaCl; 0.01 M $MgCl_2$; 0.05 M Tris buffer; 1% bovine serum albumin (BSA) (pH 7.5). The homogenate was filtered through nylon net and clarified for 3 min at 250 g (MPW 251 centrifuge, MPW Med. Instruments, Poland) to precipitate intact cells and cell large fragments, with repeated centrifugation of

the supernatant at 1000 g for 10 min. The precipitate of chloroplasts was suspended in 0.3 M sucrose, 0.025 M NaCl, 0.01 M $MgCl_2$; 0.05 M Tris buffer (pH 7.5).

The potency of electron transport chain (ETC) to transfer water electrons to potassium ferricyanide or molecular oxygen was assayed by the rate of O_2 reduction under separate functioning of each of these acceptors. O_2 photoreduction was determined by absorption with the Möller reaction reagent (adrenaline), which is capable of interaction with the superoxide anion radical on the reduction part of the photosystem. Adrenaline (0.3 mM) (FSUE Moscow Endocrine Plant, Russia) or potassium ferricyanide (0.5 mM) was added to the reaction mix. The oxygen concentration was measured amperometrically (a Universal polarograf OH-105 polarograph, Radelkis, Hungary) in a 1.2 ml cell with a closed platinum electrode, the reaction mix pH 7.8. The chloroplast suspension was illuminated with white light (LETI-60, Kazan Optical and Mechanical Plant OJSC, Russia). The concentration of chlorophyll was calculated using Arnon's formula.

Data were processed using analysis of variance (ANOVA) with STATISTICA 6.0 software (StatSoft, Inc., USA). The tables show the mean values (*M*) and standard errors of the means (±SEM). Significance of differences was evaluated by Student *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Results. The seed treatment with 1.0 and 0.5 mg/ml amaranthine stimulated germination. Seeds in the experiment germinated 2-3 days earlier than the control. On days 5 and 7, the average root length of seedlings when 1.0 mg/ml was applied exceeded the control by 10 and 18% ($p \leq 0.05$) (Table 1), cotyledon leaves also appeared and developed earlier.

1. Germination and root formation in tomato (*Lycopersicon esculentum* Mill.) heterotic F_1 hybrid Carlson after soaking seeds in different concentrations of amaranthine (*n* = 20, in vitro)

Variant	Days after seed soaking				
	day 3	day 5		day 7	
	germinated seeds, %	germinated seeds, %	root length, cm (*M*±SEM)	germinated seeds, %	root length, cm (*M*±SEM)
0.1 mg/ml	0	40	0.95±0.220	100	3.34±0.510
0.5 mg/ml	0	50	1.22±0.280	100	2.86±0.090
1.0 mg/ml	40	60	1.55±0.430	100	3.49±0.280*
Control (water)	0	40	1.40±0.470	100	2.96±0.460

* Differences with control are statistically significant at $p \leq 0.05$.

2. The number of moving II instar larvae root-knot nematode (*Meloidogyne incognita*) exposed to different concentrations of amaranthine (*M*±SEM)

Variant	Hours after exposure			At the end of the test when transferred to water
	0	24	48	
0.5 mg/ml	20±0.6	20±0.6	11±1.8	19±0.6
0.75 mg/ml	20±1.2	18±3.0	9±3.6	17±3.0
1.0 mg/ml	20	2±1.2	0	15±4.1
2.0 mg/ml	20	0	0	2±1.2
Control (water)	20	20	19±0.6	19±0.6

We also revealed the nematostatic effect of 0.5 to 1.0 mg/ml amaranthine (Table 2). Larvae in these solutions lost their mobility, but restored it after keeping in distilled water. In a 1.0 mg/ml amaranthine, loss of nematode mobility occurred after 24 hours. Nematode exposure to 0.5 mg/ml amaranthine solution for 48 hours resulted in a mobility loss of ~ 50% larvae. Amaranthine concentration of 2 mg/ml was lethal for larvae.

Similar properties were previously noted for physostigmine alkaloid with

a structure similar to amaranthine. This compound extracted from *Calabar bean* (*Fabaceae*) had a nematostatic effect on the migrating nematode *Ditylenchus dipsaci* at a concentration of 1.0 mg/ml. Pretreatment of pea seedlings with physostigmine sulfate (0.03 mg/ml) significantly protected plants from nematode infestation. It is possible that the mechanism of action of amaranthine on nematodes is similar to the action of physostigmine [22].

Foliar treatment of vegetative plants with 0.5 and 1.0 mg/ml amaranthine led to a decrease in the number of parasitic nematodes on the roots, and also influenced their morphophysiological parameters. On plants treated with 0.5 and 1.0, respectively mg/ml amaranthine, the number of sexually mature females per 1 g root on day 40 was 2.1 and 1.3 times less, respectively, the control. Female nematodes from the roots of the treated plants were 1.2 times smaller, and the number of eggs per ootheca was 15-20% less as compared to the control (Table 3).

3. Growth of invaded tomato (*Lycopersicon esculentum* Mill.) heterotic F_1 hybrid Carlson and morphophisiological parameters of root-knot nematode (*Meloidogyne incognita*) on day 40 after spraying vegetating plants with amaranthine (n = 10, M±SEM, pot test)

Variant	Weight, g		Infection ball	Females per 1 g root	Female size (length × wigth), mm^2	Eggs per ootheca
	aerial parts	roots				
Amaranthine, 0.5 mg/ml	32±1.1*	2.8±0.40	2	324±39.0*	0.300±0.0100*	93±12.0*
Amaranthine, 1.0 mg/ml	27±2.4	2.9±0.60	3	544±54.0	0.298±0.0070*	90±17.0*
Control (invaded plants. water)	24±1.1	3.1±0.70	4	684±46.0	0.354±0.0110	146±22.0
Control (healthy plants. water)	29±2.8	2.4±0.30	–	–	–	–

N o t e. Dashes mean absence of the data.
* Differences with the invaded control are statistically significant at $p \le 0.05$.

The infection ball in plants treated with amaranthine was significantly lower than in control. Especially effective concentration was 0.5 mg/ml, resulting 2 times less gall number compared to the roots of control plants. Weight of the aerial parts of invaded plants treated with 0.5 and 1.0 mg/ml amaranthine exceeded the control by 32% ($p \le 0.05$) and 10%, respectively. The weight of roots in infected plants, on which a significant number of galls were found, was noticeably greater than in healthy ones. Their weight ratio to the aboveground organs (stem and leaves) also differed from that in healthy plants. Under the influence of amaranthine, the ratio of root to aboveground parts was comparable to that of uninfected plants, which indicates the normalization of metabolic processes and the physiological state of tomatoes.

4. Dynamics of chloroplast pigments in leaves of tomato (*Lycopersicon esculentum* Mill.) heterotic F_1 hybrid Carlson after treatment with amaranthine at root-knot nematode (*Meloidogyne incognita*) invasion (n = 10, M±SEM, pot test)

Variant	Day 0		Day 10		Day 40	
	chlorophylls a + b	carotenoids	chlorophylls a + b	carotenoids	chlorophylls a + b	carotenoids
Amaranthine, 0.5 mg/ml	2.35±0.087	0.80±0.025	2.63±0.121	0.90±0.018	2.31±0.074*	0.77±0.011*
Control (invaded plants. water)	2.29±0.030	0.79±0.042	2.50±0.089	0.83±0.045	2.11±0.144	0.60±0.035
Control (healthy plants. water)	2.30±0.139	0.81±0.035	2.65±0.145	0.91±0.057	2.40±0.108*	0.80±0.014*

* Differences with the invaded control are statistically significant at $p \le 0.05$.

Important indicators of plant physiological status include the content of photosynthetic pigments in chloroplasts. On day 10 in the leaves of tomato plants invaded with nematodes, the chlorophylls increased by 13%, carotenoids

by 10% (Table 4). Perhaps the increase in the content of photosynthetic pigments was a response to oxidative stress caused by the gall nematode invasion into the roots. The total content of chlorophylls and carotenoids in healthy and amaranthine-treated plants was almost the same. On day 40, a decrease in leaf photosynthetic pigments occurred in all studied plants ($p \leq 0.05$). The content of chlorophylls in nematode-invaded plants decreased by 12%, carotenoids by 15%, and in the invaded plants after amaranthine treatment these indicators were 8 and 5% lower, respectively, compared to healthy plants.

The lower amount of chlorophylls and carotenoids in chloroplasts upon root-knot nematode infestation (biogenic stress) indicates changes in the photosynthetic electron transport chain in tomato C3 plants. In photosynthetic systems, in addition to the main chain of electron transfer from water to NADP or an artificial acceptor (non-cyclic electron transport), the chain of pseudocyclic electron transport works as $H_2O \rightarrow PSII \rightarrow PSI \rightarrow O_2 \rightarrow H_2O$. Pseudocyclic transport acts as an alternative electron transfer pathway, which leads to the reduction of molecular oxygen and the formation of superoxide anion and H_2O_2 [23]. It is known that the enhancement of electron transfer to oxygen can occur under a decrease in the oxidized NADP or inhibition of dark photosynthesis reactions, as well as under the adverse factors. For example, at water deficit in leaves, in chloroplasts there is a redistribution of flow of electrons associated with the reduction of CO_2 and O_2 [24, 25].

5. Non-cyclic (Hill reaction) **and pseudocyclic** (Møller reaction) **electron transport in tomato (*Lycopersicon esculentum* Mill.) heterotic F_1 hybrid Carlson at root-knot nematode (*Meloidogyne incognita*) invasion (day 40) after treatment with amaranthine** ($n = 3$, $M \pm$SEM, pot test)

Variant	Hill reaction, μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$	Møller reaction, μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$
Amaranthine, 0.5 mg/ml	26±3.0*	17±2.0*
Control (invaded plants. water)	13±2.0*	22±3.0*
Control (healthy plants. water)	64±4.0	12±2.0

* Differences with the healthy control are statistically significant at $p \leq 0.05$.

In chloroplasts from the leaves of invaded plants, electron transfer to molecular oxygen along the pseudocyclic path increased sharply, up to 67% at $p \leq 0.05$, and decreased along the non-cyclic path, up to 80%, compared to the uninvaded plants (Table 5). The 0.5 mg/ml amaranthine solution applied to leaves of the invaded plants led to a 36% decrease in the electron transfer to molecular oxygen compared to healthy plants, and, therefore, in the chloroplasts electron transport via non-cyclic path (Hill reaction) increased up to 30%.

Amaranthine has an auxin-like effect on plants invaded by root-knot nematodes, including stimulation of aboveground mass and root growth, the influence on the content of chlorophylls and carotenoids, and also on the electron transfer via non-cyclic and pseudocyclic pathways. This fact indicates that the amaranthine molecule is unique in its functional properties [12, 26].

Infestation of tomato plants by root-knot nematodes can weaken photosynthesis, including primary processes in thylakoid membranes. When oxidative stress strengthens, photooxidation in chloroplasts intensifies, which probably causes the electron flux in the PSI region to be switched from non-cyclic to pseudocyclic pathway of transport electrons from water. Generation of reactive oxygen species (ROS), i.e. a superoxide radical and H_2O_2, is associated with pseudocyclic electron transport, which enhances oxidative stress in chloroplasts. Free radical oxidation leads to destruction of organic molecules, which may result in metabolic disturbance and even death of plants [27]. The natural antioxi-

dant system which includes low- and high-molecular-weight compounds, takes part in the regulation of the ROS amount. We revealed that in invaded tomato plants, the complex of protective mechanisms induced by exogenous amaranthine includes the accumulation of antioxidants carotenoids, switching non-cyclic electron transport from water in the PSI region to the pseudocyclic pathway, and stimulation of plant growth. All this in general leads to an increase in the resistance of tomato plants to root-knot nematodes.

Similar results were reported about the action of natural adaptogens, the furostanol glycosides extracted from *Dioscorea deltoidea* Wall cell culture, on the *M. incognita*—tomato parasite-~~and-~~host system. Application of furostanol glycosides to tomato seeds and vegetating plants markedly reduced susceptibility to parasitic nematodes [7]. The preparation influenced pigment pool of the photosynthetic apparatus, the peroxidase activity, and stimulated lipid peroxidation. The pattern of changes in the chloroplast pigment composition revealed upon treatment with amaranthine and furostanol glycosides indicates that these compounds support cell homeostasis via plant immunity stimulation.

Note that the search for natural compounds with nematicidal properties is a fairly wide research area. Data on the effect of plant-derived substances of different classes on parasitic nematodes and plant resistance are given in the Chitwood's review [10]. The induced plant systemic resistance to root-knot nematodes was noted for aqueous extracts from fresh leaves of lemon grass *Cymbopogon flexuosus* Steud. [28] and upon treating the aerial parts of plants with an oil extract from of *Argemone mexicana* L. seeds [29]. Watercress, as well as ordinary horseradish exhibit antagonistic properties against *M. incognita*. Preparations based on these plants have a high nematostatic activity, namely inhibit hatching of larvae from eggs, paralyze invasive larvae, act also as stimulants and can stabilize cultivated plant development. Preparations containing substances of specialized plant metabolism with nematicidal properties positively influence growth and photosynthesis of plants and negatively affect gall nematode development.

Thus, amaranthine has adaptogenic properties, namely weakens negative biochemical and functional changes in tomato plants under stress caused by nematode invasion, and activates synthesis of the compounds which provide improved energy metabolism and recovery processes. The 1.0 and 0.5 mg/ml amaranthine concentrations, on the one hand, stimulate tomato seed germination, plant growth and synthesis of chlorophylls and carotenoids, and, on the other hand, inhibit the mobility of nematode larvae and their development in plants, including morphometric and population indicators. Therefore, amaranthine can be a novel biogenic inducer which is capable of activating general non-specific stress response systems at plant pathogen invasions. This bioagent can provide both protective and growth-promoting effect in greenhouses.

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EVALUATION OF FACTORS HAVING AN EFFECT ON CANNABIDIOL AMOUNT IN *Cannabis sativa* L.

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Abstract

Industrial hemp is a multipurpose crop, supplying fibers, seeds, and pharmaceuticals. The non-psychotropic cannabidiol (CBD) derived from hemp is a promising pharmaceutical raw material. It shows no psychotropic effects, is not listed in UN Single Convention on Narcotic Drugs, but demanded for the production of medicine products. Regretfully, there are no domestic cultivars of hemp in Russia specialized in phytocannabidiol. Currently, there is a big need in industrial varieties of pharmaceutical specialization (CBD-cultivars). This paper is the first to report on selection of the accessions with high CBD content (above 9%) and trace amounts of Δ^9-tetrahydrocannabinol (THC), the main psychotropic cannabinoid, among the studied genotypic diversity of hemp plants. The objective of the study was the assessment of the effects of field watering, lighting conditions, sexual type of plants and stage of ontogenesis on CBD and THC accumulation on a broad in situ genotypic diversity of *Cannabis* L. germplasm accessions in order to form the optimal morpho-physiological and agronomical model of CBD-producing cultivar (ideotype) well adapted to field growing. Cannabis populations in situ, genotypically original, spatially separated (and, thus, not undergone to random cross pollination), were surveyed in 2008-2011 in four Russian regions. A total of 128 populations were selected for the research; among them, 52 populations were studied for the effect of shading, and 58 for the moisture excess/deficit. For each population, plants (not less than $n = 10$ per each variant, i.e. shading vs. lighting, and moisture excess vs. deficit) were collected randomly and representatively to form summarized sample, and then analyzed to reveal the effect of the said factors. The plants were collected from initial budding and flowering of male plants, up to the moment when fimbles have just begun to dry out and the first seeds at the basal parts of female inflorescences have started to ripen. Air-dry samples (with and without inflorescences, female plants, male plants) were crushed and biochemically analyzed. Inflorescences (generative parts) and only leaves (vegetative parts) of the two conventional sexual types were analyzed separately. The analysis of the CBD and THC contents in the studied accessions revealed a statistically significant ($p = 0.05$) genotypic variability for CBD between the samples. Natural sexual polymorphism in the content of CBD and THC is statistically unreliable. Both male and female plants contain approximately equal CBD concentrations from the budding time until the seed ripening. THC amount was insignificantly increased in female plants. From the budding phase until the start of seed ripening, plants increase their CBD content more than twice. From the budding phase until the seed ripening, CBD amount in inflorescences is significantly thrice more than in vegetative parts. The difference in THC content is significant as well, but not so noteworthy. By the start of budding of different sexual types CBD content in generative parts is twice as high as that of THC. CBD accumulation reaches its maximum in generative plant parts by the time when seed ripening starts initially. The effect of shading on plants of any sexual type has shown that CBD content significantly responds to the exposure and intensity of natural lighting. Any breach in daylight illumination will reduce the CBD content. The factor of excessive/deficient natural moistening has no significant effect on CBD and THC accumulation in plants, regardless of the development phase of an adult plant of any sexual type. Unlike to dioecious cultivars specialized for seed/oil production, both female and male plants may be used for CBD production. Female plants must have extended time of budding—full flowering in conditions of

reduced amounts of pollen in air and deferred seed ripening. Flowering and maximum pollen production from male plants must be deferred, because the lack of pollen in field promotes CBD formation in female inflorescences. CBD cultivar plants should have a maximum inflorescence size, a maximum budding and flowering period, and a minimum foliage.

Keywords: *Cannabis sativa* L., *C. ruderalis* L., hemp, plant sexual types, phytocannabinoids, cannabidiol, Δ^9-tetrahydrocannabinol, CBD-cultivar ideotype, breeding

Hemp (*Cannabis* L.) is one of the oldest crops [1]. Due to various factors, industrial hemp crops in the USSR and the Russian Federation regressed from 1 million hectares in the 1940s to 150 thousand hectares in the 1980s and currently are about 15 thousand hectares. Along with a decrease in hemp cultivation in the Russian Federation, there was a tendency towards a structural increase in synthetic drugs in illicit trafficking, under which various psychoactive substances were masked [2].

Nowadays, hemp is used not only to produce different textiles and shive, but also for innovative seed- and oil-based components for functional nutrition and pharmacy. From 1994 to 2017 in the EU, the area of hemp varieties grown for seed, oil and fiber increased from 8 to 33 thousand hectares. In France alone, in 2017, 18 thousand hectares were occupied by hemp. In the Baltic States of the EU in 2018, this area amounted to about 14 thousand hectares. In Canada, hemp is sown annually on more than 15 thousand hectares. In 2011, in the United States, the annual volume of officially imported commercial hemp products reached USD11 million [3, 4], and since 2018, the domestic hemp production was resumed after a period of limiting cultivation.

Inflorescences and leaves of hemp contain over 60 different phytocannabinoids, the terpenphenol compounds which are derivatives of 2-substituted 5-amylresorcinol. The precursor of all phytocannabinoids is cannabigerolic acid. It is transformed into cannabichromene, cannabidiol and Δ^9-tetrahydrocannabinol acids, which are converted to cannabinoids. Cannabidiol and Δ^9-tetrahydrocannabinol acids are the main ones. Their synthesis in a plant is genetically determined. Δ^9-Tetrahydrocannabinol (THC) is responsible for the psychotropic effect of drugs selectively targeted to cannabinoid receptors in brain. Unlike THC, cannabidiol (CBD) has a pronounced therapeutic and sedative effect on humans. The effect of its concentration is inversely proportional to the THC psychotropicity [5].

In a number of European countries and in the USA, the cannabidiol is practiced as a suppressant of the symptoms of vomiting and nausea provoked by anti-cancer treatment, and also as a remedy against weight loss in AIDS patients. Cannabidiol is used to treat neuropsychiatric disorders, rheumatism, glaucoma, multiple sclerosis, alcoholism, asthma, to achieve an analgesic effect [1, 6], under a decrease in appetite, impaired gestation in pregnant women, to stimulate lactation, under neurodegenerative processes [7, 8] and in antitumor therapy [9-11]. Cannabinoids are promising as a substitute for opiates [12, 13]. New data have been published regarding their use in the treatment of oncological diseases and in Alzheimer's disease, as well as information on the relationship of the endocannabinoid system and regulation of intraocular fluid flow, and on the vasodilating and neuroprotective effects of cannabinoids [14].

The literature provides the results of chemotyping cannabis lines vegetatively propagated in greenhouses for the content of one of the cannabinoids, the information on the genotyping varieties to differentiate technotypes and chemotypes, on differences in the metabolism of cannabinoids in varieties used for oil and fiber purposes and in chemotypes (type Purple Kush) [15, 16]. Biochemical markers are used to select plants with a maximum content of terpenes and CBD (chemotype III) cultivated in greenhouses [17].

The dynamics of the main cannabinoids in hemp plants was previously studied on ten zoned varieties during the sproutig—flowering phase on leaves of different layers, which is more likely important for forensic work [18, 19]. Other studies [20] focused on minimizing the THC content (less than 0.01%) in hybrid populations F_1 and F_2 in breeding monoecious hemp. Information on the cannabidiol accumulation during ontogenesis of technical (commercial) hemp plants is based on theoretical calculations and the study of only three monoecious varieties [21]. The works had a narrow specificity, were aimed at changing the zoned monoecious variety of different specialization by breeding methods and were not relevant for other technotypes of commercial varieties, and also did not cover the available genotypic diversity of the genus *Cannabis* L.

Hemp genetic resources in most research centers are very few, genotypically homogeneous [22, 23] and poorly studied with respect to economically significant traits. The lack of data about genetically diverse donors becomes a limitation when creating hemp cultivars for cannabidiol production (CBD cultivars).

Success in hemp selection for high CBD and reduced THC requires an effective strategy to ensure the development of varieties with biochemically, morphologically, technologically specialized and genetically stable traits. An essential element of such a strategy is the selection of initial samples and an adequate assessment of target characteristics during selection.

In this paper, among the genotypic diversity of the studied natural hemp populations, we first identified hemp forms for pharmaceutical use (with a contrastingly high content of CBD, more than 9%, and a trace amount of the main psychotropic cannabinoid THC) which are suitable for field cultivation. The greatest accumulation of CBD is shown to occur by the beginning of ripening, and shading leads to a decrease in this phytocannabinoid level.

The purpose of our study was to evaluate the effects of moisturizing, lighting, sex, and ontogenesis stage on the content of cannabidiol and Δ9-tetrahydrocannabinol in genotypically diverse hemp samples to form the optimal morphophysiological and agricultural model of a variety specialized for producing CBD in field conditions.

Materials and methods. Samplings were carried out in 2008-2011, with accounting natural pollination as an important criterion for the separation of populations [24]. The hemp (genus *Cannabis* L.) plants were collected from genotypically original, spatially separated (beyond the cross-pollination distance) populations in situ in the four federal districts of Russia.

A total of 128 populations were selected, of which 52 were examined for the effect of the shading factor, and 58 for the excess moisture factor. In the first case, in each of the 52 populations growing under the forest canopy, at least 10 plants were collected from unshaded areas and combined into a single sample for the population. In similar manner, a combined sample was also formed from plants vegetating under the crowns of trees. In second case, in 58 populations where part of the plants grew on water edge of a natural reservoir (river), and the rest on the hills beyond the reach of excess water, combined samples contrasting with respect to were excess moisture factor were also collected. The plants were collected randomly and representatively from the beginning of budding and flowering to the early seed ripening in order to neutralize the climatic and temporal varieties in accordance with the “ceteris paribus” principle.

To assess cannabinoid levels, air-dry samples (with and without inflorescences, female plants, male plants) were crushed and biochemically analyzed. Separately, inflorescences (generative parts) and only leaves (vegetative parts) were analyzed in two sex types. The dynamics of accumulation of cannabidiol and Δ^9-tetrahydrocannabinol was studied from the beginning of flowering of

male (fimble) hemp, budding of which usually occurred earlier than in female (pistillate) hemp, to the beginning of fimble drying and the first seed maturation in a basal part of inflorescences on female plants.

Samples for gas chromatographic analysis of cannabidiol and Δ^9-tetrahydrocannabinol were prepared according to the methods adopted in forensic practice. The measurements were carried out on a CHROM 5 chromatograph (Bruker, Czech Republic) of a standard configuration with a flame ionization detector according to the manufacturer's recommendations. Concentration of cannabinoids in the sample was measured in 3 analytical repetitions.

For statistical interpretation, analysis of variance (ANOVA) and correlation analysis with Statistica 10.0 software (StatSoft, Inc., USA) were performed. The figures show mean values (M) and standard deviations ($\pm$SD) at a 95% significance level. The calculated correlation coefficients (simple linear correlation) were considered significant at $p < 0.05$.

Results. The studied hemp samples significantly ($p = 0.05$) differed in the content of cannabinoids. From the beginning of the budding stage of the samples to the beginning of seed maturation, the CBD content in plants increased more than twice (Fig. A), and the generative parts of plants contained 2 times more CBD than the vegetative ones (see Fig. D). In terms of the amount of THC, the differences between the parts of plants were less contrast (see Fig. E).

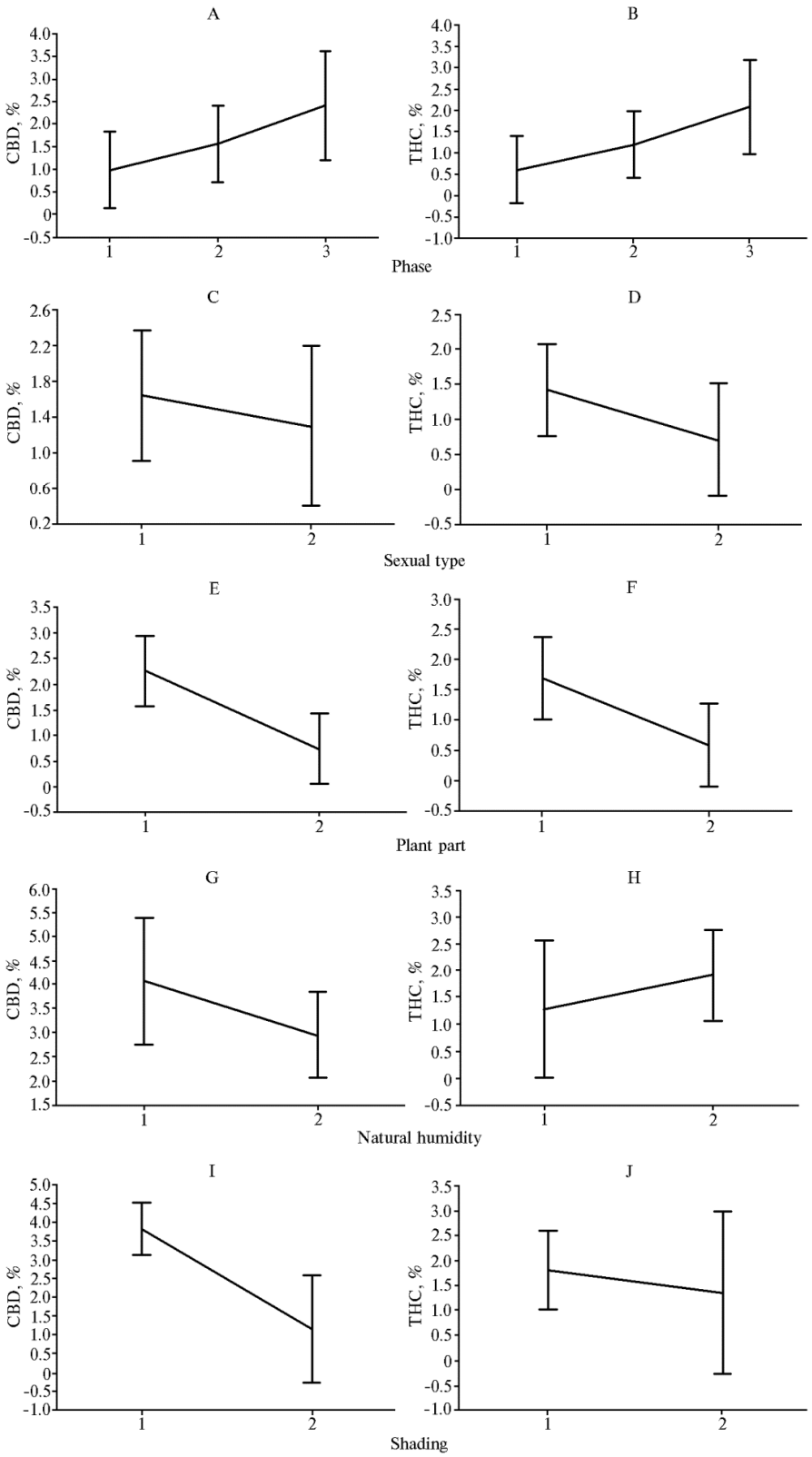
The diversity of cannabis sex types, usually up to seven types in a population, we conventionally reduced to two groups, i.e. the pistillate type (unisexual female hemp, monoecious female hemp, masculinized female hemp) and the fimble type (ordinary unisex male hemp, monoecious male hemp, feminized male hemp). The effect of gender on the CBD and THC levels turned out to be statistically unreliable (see Fig. C, D). In both groups (pistillate and pistillate types), the CBD level was approximately equal. Taking into account the influence of external factors, it can be noted that by the beginning of budding in cannabis plants of different sexual types, the CBD accumulation in the generative parts was 2 times higher than THC. However, the maximum CBD accumulates in the generative parts at the beginning of seed maturation (see Fig. A, B).

The excess or deficiency of natural moisture (edaphic factor) did not significantly affect the amounts of THC and CBD (see Fig. G, 3), while shading led to a significant ($p = 0.05$) decrease in CBD level (see Fig., I), but did not have a significant effect on the THC concentration (see Fig., K).

A weak negative correlation ($r = -0.16$) was established among the tested genotypes between the content of CBD and THC in hemp inflorescences. Consequently, the prospects of hemp selection for a contrastingly high level of cannabidiol at a significantly low (trace) THC amount are obvious. From budding to seed ripening, the cannabidiol content increased ($r = 0.35$). The correlation between the CBD level and the sexual type of plants turned out to be weak ($r = -0.1$).

Currently, the State Register of Breeding Achievements Allowed for Use in the Russian Federation does not include specialized hemp varieties for the production of physiologically active functional ingredients and products. There are no commercial varieties for the production of cannabidiol, the source of which for the time being are hybrids vegetatively propagated in greenhouses. Obviously, this may not be the only way to grow hemp to produce phytocannabidiol. Monoecious and dioecious specialized hemp varieties propagated by seeds for the CBD production can be successfully cultivated in field crops. Out findings indicate that the violation of natural light (shading) of hemp plants reduces the CBD concentration by more than 2 times. Therefore, cost-effective hemp cultivation for CBD in greenhouses is limited by the quality and quantity

of lighting.



The content of cannabidiol (CBD) **and Δ^9-tetrahydrocannabinol** (THC) **in hemp (Cannabis L.) plants**

depending on various factors: A, B — vegetation phase (1 — budding, 2 — flowering, 3 — ripening), C, D — sexual type (1 — female plants, 2 — male plants), E, F — generative and vegetative parts (1 — inflorescences , 2 — vegetative mass), G, H — natural moisture (1 — deficiency, 2 — excess), I, J — shading (1 — absent, 2 — present) (ANOVA, *M*±SD, different Russian regions, 2008-2011; for description of plant sampling, see "Materials and methods" section.

In Russia and neighboring countries, hemp breeding was carried out exclusively for the elimination of all cannabinoids, including CBD, in 97.8-99.6% plants of the variety. Laiko et al. [25] argue that the lack of CBD in commercial varieties may be a promising trend orientation. Our point of view does not coincide with this opinion. As was shown earlier [5], cannabidiol is not significant in psychotropic effect and is antagonistic to the psychotropic effect of THC. The decrease in the total cannabinoids in cultivars in 1980-2000 and the ongoing selection to achieve trace amounts of cannabinoids, up to their complete elimination in commercial hemp, did not prevent a sharp drop in commercial hemp areas in Russia and an increase in the number of drug addicts.

In this work, we searched for a promising breeding material with a functionally oriented composition of cannabinoids and plant morphology (maximum CBD, minimum THC, minimum foliage, maximum inflorescence size, adapted periods from budding to seed ripening), whereas previous works [21] were carried out using sample intended to select a monoecious universal variety.

Maintaining the monoecious trait in zoned varieties (0% of common fimbles) requires significant costs and is considered one of the main breeding and seed-growing tasks for monoecious hemp [20]. However, we believe that to realize the possibility of productive use of both female and male plants in a dioecious industrial hemp variety, the concept of functionality of various sex types of a variety, their biochemical and agronomic properties should be rearranged. Unlike the existing dioecious universal varieties (a combines fiber, seed and oilseed use), plants of conventional sexual types can be equally used for the maximum production of CBD.

Assessment of breeding material and differentiation of hemp samples by the CBD accumulation should begin with the budding phase. However, forms with a contrasting THC content can be distinguished with the onset of the seed ripening phase at the basal part of inflorescences. The phenological phase of the beginning of seed maturation in CBD varieties should be maximally shifted to the harvest time.

Our data differ from previously published results of experiments on varieties of Central Russian monoecious hemp [18]. Zelenina et al. [18, 19] investigated hemp leaves from germination to flowering. We showed that the maximum CBD accumulates with the onset of full flowering when the THC content is still low. The THC concentration may increase later, with the approach of seed ripening. During flowering of female plants, inflorescences continue to grow and increase in size, seed ripening begins in its lower parts, while budding is noted in the middle parts. In these periods of growth, plant foliage is already almost formed. With the beginning of budding, a significant amount of resinous substances is secreted formed in inflorescences, the function of which is to capture pollen migrating in the air, which is necessary for seed setting. At this time, the cannabidiol concentration in the inflorescence reaches a maximum, and from the beginning of seed maturation, it decreases. Based on the above facts, we concluded that a specialized variety it is necessary to selectively increase the size of the long-blooming inflorescences of both female and late blossoming male types. Blooming and maximum pollen production in male plants should be late, since pollen deficiency contributes to the CBD accumulation in female plants. Transcriptome analysis of the of glandular trichomes in hemp flowers

[26], which the authors indicate as the main site for the synthesis of cannabinoids, showed high activation of polyketide cyclase-like enzymes during mass budding and flowering. The corresponding transcript was found in a highly expressive state in trichomes, which confirms the peak of enzymatic activity in inflorescences in this period. This information is consistent with our findings which indicate that a prolonged flowering of a large number of inflorescences ensures a high accumulation of phytocannabidiol.

For breeding hemp for fiber and seed use, the ratio of sex types is of priority importance, since the conditional groups of male and female plants usually differ in a number of traits. We have shown that in hemp cultivars oriented to the maximum CBD yield, both conditional sexual groups can equally be producers of this cannabinoid. From the results of studying the dynamics of the CBD and THC accumulation in hemp plants of various sex types, it follows that in order to create a specialized CBD variety, it is necessary to select forms in which the male (fimble) plants bloom late and do not die off before harvesting, remaining equal to the CBD source with the female (pistillate) plants. Moreover, pistillate plants must also have a long period from the beginning of budding to full blooming, and a later start of seed maturation. Plants of specialized varieties should be harvested immediately at the onset of full blooming of sex types, but before the seeds ripen, since during the full bloom CBD concetration in inflorescences is already quite high while THC is low. In addition, the amount of THC may increase as the seed formation phase approaches. The seed ripening phase in CBD cultivars should occur as late as possible, approaching the harvest time.

The concept of the plant morphology for CBD hemp varieties is also different from that for varieties of universal and double (fiber) use. Plants of a specialized CBD variety should have a maximum weight and size of inflorescences and reduced vegetative part as much as possible. This is in line with previous studies [27], where the author comes to the conclusion about an increase in the reproductive parts of inflorescences while minimizing the stems of specialized varieties for oil and chemical compounds. The habitus of plants of a specialized variety is long-flowering inflorescences of large size with minimal foliage, since the content of CBD in the leaves is significantly less.

Concentration of cannabidiol in inflorescence (%) of hemp (*Cannabis* L.) plants selected for breeding varieties of pharmaceutical use from different populations (the Russian Federation, 2008-2011)

No. in VIR introduction catalog	Species	Ecotype	Cannabidiol
141445	*C. sativa*	Northern	2.40
141446	*C. sativa*	Northern	1.91
141447	*C. sativa*	Northern	1.87
141448	*C. sativa*	Central Russian	2.99
141855	*C. sativa*	Central Russian	3.96
141856	Presumably *C. sativa* × *C. ruderalis*	Central Russian	9.78
141451	Presumably *C. sativa* × *C. ruderalis*	Central Russian	2.76
141864	Presumably *C. sativa* × *C. ruderalis*	Central Russian	2.53

Our studies showed that samples that can be attributed to different hemp species and ecotypes accumulate cannabidiol in significant quantities. For example, sample 141855 contains 3.96% CBD, and 141856 contains more than 9% (Table).

Our data are fundamentally different from those published previously. We propose a model of industrial pharmaceutical-type hemp varieties intended for field cultivation rather than growing in greenhouses and growboxes, as it is practiced abroad for varieties and vegetatively propagated hybrids not included in the official list of the European Union. Importantly, specialized CBD varieties

for cultivation in field conditions are still absent but the need for them is quite high. Existing universal varieties for field growing do not meet the challenges of production of both phytocannabidiol and functional food ingredients. Revision of a specialized variety model is necessary to set selection strategy for pharmaceutical-type varieties, to specify their significant traits, and to study hemp genotypic diversity to form trait-based donor collections and, eventually, to produce the advanced breeding material. Given these circumstances, we plan to continue breeding and genetic research to create hemp varieties for pharmaceutical use.

Thus, from the beginning of hemp budding, plants of different sexual types accumulate in the generative parts 2 times more cannabidiol (CBD) than Δ^9-tetrahydrocannabinol (THC). The maximum CBD is detected by the beginning of ripening. Both conventional groups of plants (male "fimble" and female "pistillate" hemp) contain approximately equal amounts of CBD. Excess or deficiency of natural moisture from budding to seed maturation does not significantly affect the accumulation of THC and CBD while shading leads to a decrease in the CBD content. The plants of CBD-specialized varieties should have minimum foliage and produce inflorescences large in size and weight. As a result of the study, accessions with a valuable CBD/THC ratio are identified that will be used in breeding for specialized hemp varieties with no specific psychotropic activity.

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PROPERTIES OF CREZACIN AS A GROWTH STIMULANT OF VEGETABLE AMARANTH (*Amaranthus* L.)

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Abstract

Crezacin, tris(2-oxyethyl)ammonium ortho-cresoxy acetate-based adaptogen of humans and animals, is applied in Russia as a stimulant of growth and productivity of crops (wheat, oats, spinach, potatoes, etc.). In other countries, crezacin is not used for these purposes. There is no information about its use in the cultivation of food amaranth (*Amaranthus* L.), a source of high-quality protein and other useful substances. In this paper, we first report data on the effect of pre-sowing treatment with crezacin on seed germination, development and biometric parameters of amaranth plants during ontogenesis, and on their productivity and nutritive value. Our findings indicate the ability of crezacin to increase nitrate reductase activity, to influence the nitrite nitrogen content in the early stages of vegetation, to increase the electron transport rate ATP synthesis. The aim of the work was to assess the effect of different crezacin concentrations on seed germination, seedling quality, growth parameters, and activity of photosynthesis and nitrogen assimilation apparatus. Seeds of *Amaranthus caudatus* L. (sample K173) and *Amaranthus cruenthus* L. (sample K185) were soaked for 1 day in crezacin solutions (test) or in distilled water (control), and used in the experiments after air-drying at room temperature. In experiment 1, 10^{-10} до 10^{-5} M aqueous crezacin was applied to seeds then allowed for germination on wet filter paper in Petri dishes for 72 h at 24 °C. The proportion of germinated seeds was calculated. In experiment 2, we studied the effect of crezacin on the growth and physiological and biochemical parameters of plants. Seeds were treated with 10^{-7} M crezacin, germinated, and calibrated seedlings were transplanted into sand- filled container. Biometric parameters were measured every 15 days until harvest (120 days), productivity was estimated by the green mass increase. Chlorophyll concentration was assessed in the leaves of 45-day-old plants. The photochemical activity of isolated chloroplasts was evaluated by the rate of electron transport and photophosphorylation. From day 15 to day 45, the activity of nitrate reductase, the concentrations of N-NO_2 and total protein were measured in the leaves. The net photosynthesis (NP) for the period from day 45 to day 60 was calculated by A.A. Nichiporovich's method. Experiment 1 revealed a change in seed germination depending on the concentration of the preparation in both studied samples. A 10^{-8} concentration increased seed germination capacity by 10 % compared to control ($P = 0.95$), 10^{-7} M had maximum stimulating effect (by 25 %), and at 10^{-5} M the germination rate decreased by 22 %. Other concentrations had no significant effect. In experiment 2, in both varieties during latent growth stage the seedlings from the treated seeds were twice as high as the control, and the length of the main root was 1.6 times as much as in control. During later stages, the green mass of plants in the experiment exceeded the control 1.3-2.0-fold depending on the phase of ontogenesis. The treatments did not affect the height of plants. The NF value in leaves after treatment exceeded the control by 26 % ($P = 0.95$). At the same time, the chlorophyll content in the leaves did not change, and the electron transport rate in chloroplasts increased by more than 30 % while photophosphorylation by 60 %. The nitrate reductase activity in leaves on day 45 increased by almost 60 %, the total protein level by 20 %, and nitrite nitrogen amount by 16 % ($P = 0.95$). These findings indicate the stimulating effect of crezacin on amaranth seeds, plant growth, photosynthesis and protein synthesis, which leads to an increase in the productivity and nutritional value of plants.

Keywords: tris(2-oxyethyl)ammonium ortho-cresoxy acetate, crezacin, amaranth, seed germination, plant growth regulation, photophosphorylation, electron transport, protein content,

In the 1970s, a group of scientists led by M.G. Voronkova synthesized the biologically active chemical compound tris(2-oxyethyl)ammonium ortho-cresoxy acetate, or crezacin. A highly purified crezacin called trekrezan was originally intended for use in medicine as an adaptogen and immunostimulant, as well as in animal husbandry and veterinary medicine [1]. Later it was found that it has a stimulating effect not only on animal organisms, but also on plants, and undergoes natural degradation in the soil with the formation of water and carbon dioxide [2].

Currently, crezacin (triethanolammonium salt of ortho-cresol oxyacetic acid) and as part of Krezatsin, Energia-M, KPP, TAB, Mival preparations is registered in the State catalog of pesticides and agrochemicals approved for use in the Russian Federation [3] as a growth stimulant of many crops (wheat, corn, oats, cabbage, spinach, potatoes, etc.). Methods of crezacin application and its effects (increasing seed germination, enhancing growth processes, increasing yields, improving product quality, and increasing resistance to adverse environmental factors) are described in detail. However, we did not find information on the effect of crezacin on the light-dependent processes of photosynthesis, as well as on individual components of the nitrogen assimilation system and protein synthesis in plants. There are also no data on the use of crezacin in the cultivation of plants of the genus *Amaranthus* L. either in Russian or in foreign literature.

Members of the genus *Amaranth* (more than 100 species) are unique in their properties. For millennia, they have been used on the continents of South America, Asia, and Africa as food, medicine, feed, and decorative crops [4, 5]. Though all parts of plants are edible [6], they are subdivided into pseudo-grain and leaf (vegetable) forms [7, 8]. The high nutritional and medicinal value of amaranth has been scientifically substantiated by numerous modern studies of the chemical composition of the organs and tissues of these plants. All parts of amaranth plants are characterized by high protein content [4-7], and seeds (grain) surpass even legumes in its quantity and quality [9-11]. Amaranth protein enriched in lysine and other essential amino acids [10, 11] is close to animal protein in nutritional value and surpasses it in digestibility [10-12].

In addition, representatives of the genus serve as a rich source of mineral elements — iron, copper, zinc, selenium, phosphorus, calcium [12, 13]. They contain an increased levels of vitamins C [11, 15], A, E, group B [11-15] and other useful compounds such as flavonoids, anthocyanins, carotenoids, rutin [8-11], squalene [16] and antioxidants possessing antitumor, antibacterial and anti-inflammatory properties [6, 14, 17]. The use of amaranth extracts, leaves, seeds, oil or meal as a part of dishes and as a medicine helps in the prevention and treatment of diseases of the cardiovascular system [18] and the digestive system, diabetes mellitus, and obesity [13-17]. Amaranth plants are used for the preparation of baby food and diet food [14, 17].

Due to its beneficial properties, this southern culture is gaining popularity in many countries of the world [4, 5, 19], however, its introduction can cause difficulties both because of environmental conditions [19, 20] and as a result of the physiological peculiarities [4, 5, 7-10]. Amaranth has very small seeds that germinate unevenly, and small shoots with thin stems, which, after 5-7 days after germination, enter a state of hidden growth for 2-3 weeks. During this period, only the root system is actively developing, and the aerial part stops growing. Such seedlings suffer greatly from wind, lack of moisture and light, are easily clogged by weeds and die [7, 21]. In the countries of Europe and Central Russia, the development of amaranth plants is slowed down [19-21], since the condi-

tions of higher insolation and temperature are optimal for this southern culture.

It seems necessary to improve the quality of sowing material, strengthen the habit of seedlings by accelerating development at the stage of latent growth and increasing resistance to environmental factors, as well as increasing the productivity of adult plants and imptoving their nutritional value. For this, presowing seed treatment with various stimulants is widely used [20, 21].

In the context of the latest European rules on limiting the use of pesticides [22], environmentally friendly stimulants that improve the growth and adaptive qualities of plants are especially attractive. In previous works, we showed high efficiency of the use of a number of preparations for growing *Amaranthus caudatus* (cultivar K173) and *A. cruenthus* (cultivar K185), i.e. 2-(4-hydroxy) phenyl ethanol, an exometabolite of the purple bacterium with cytokinin activity [23], gibbersib, a fungal-based gibberellin preparation [24], and para-aminobenzoic acid, a component of folates [25].

Many papers describe the properties of crezacin, which are especially important for the culture of amaranth. Crezacin was found to increase the germination capacity of oat seeds [26], the weight and height of potato plants [27], the net productivity of winter wheat photosynthesis [28], the total protein content in spring wheat grain [29], and the amount of chlorophyll in leaves of *Isatis tinctoria* L. [30]. A special advantage of crezacin in cultivation of leaf amaranth may be its ability to cause significant accumulation of green mass, shown on potato and spinach plants [31, 27].

In this study, we first obtained data on the effect of presowing treatment with crezacin on seed germination, development, biometric parameters of food amaranth plants depending on the ontogenesis phase, productivity and nutrition value. The previously undescribed properties of crezacin have been disclosed, namely the ability to increase nitrate reductase activity, to influence the nitrite content in early vegetation, and to increase the electron transport rate in the chain of their transfer and ATP synthesis.

The aim of the work was to assess the effectiveness of different crezacin concentrations on seed germination, seedling quality, growth parameters of food amaranth plants, activity of photosynthesis and nitrogen assimilation apparatus.

Materials and methods. Seeds of amaranth *Amaranthus caudatus* L. (variety K173) and *Amaranthus cruenthus* L. (variety K185) with a 70% germination rate were provided by the All-Russian Institute for Selection and Seed Production of Vegetable Crops (Moscow Region). Seeds were treated by soaking for 1 day in distilled water (control) or in crezacin (a crystalline powder with an active substance content of 95%, Flora-Si LLC, Russia) solutions of different concentrations (test). Then they were dried at room temperature in a weak stream of air and used in experiments.

In determining the effect of crezacin on seed germination (experiment 1), aqueous solutions 10^{-10} до 10^{-5} M were used for soaking. The percentage of sprouted seeds was calculated after germination on wet filter paper in Petri dishes for 72 h at 24 °C.

To assess the effect of crezacin on the growth and physiological and biochemical parameters of plants (experiment 2), a 10^{-7} M solution was used for seed treatment; after germination, calibrated seedlings were transplanted into sand cuvettes, 3 cuvettes per variant, 10 seedlings per cuvette. The cuvettes were placed in a temperature chamber for plant growth LCC-1000MP Daihan Labtech (Daihan Labtech Co., Ltd, South Korea) at 150 W/m^2 illumination, 24 °C, and 14-hour photoperiod.

Watering was carried out once a day with Knopp nutrient medium. Bi-

ometric indicators were recorded every 15 days until harvesting (day 120), productivity was evaluated by the increase in green mass. The content of chlorophyll was determined in the leaves of 45-day-old plants [32]. The photochemical activity of isolated chloroplasts [33] was evaluated by the rate of electron transport [34] and photophosphorylation [35]. From day 15 to day 45, the activity of nitrate reductase (NR), the content of nitrite nitrogen [36], and total protein [37] were measured in the leaves. The net productivity of photosynthesis (NPP) from day 45 to day 60 was calculated by Nichiporovich's method [38].

The article presents the results of one typical experiment out of five. Biometric parameters were measured in 30 plants, biochemical analyzes were performed in three repetitions.

Statistical processing was performed in Microsoft Excel. The tables and figures show the arithmetic means (M) and standard errors of the means (±SEM). The significance of differences was evaluated by Student's t-test at $P = 0.95$.

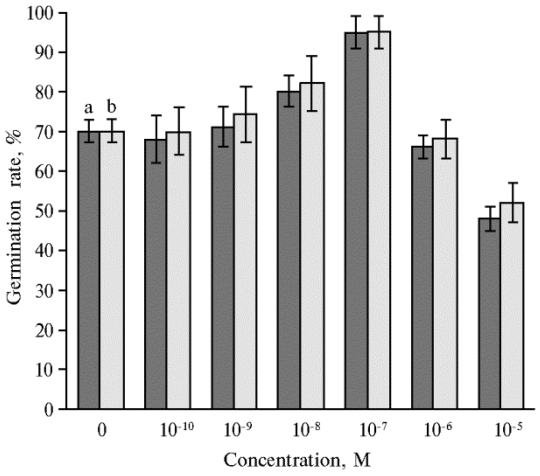

Fig. 1. Seed germination in amaranth *Amaranthus caudatus* L. (variety K173) (a) **and *Amaranthus cruenthus* L. (variety K185) (b) upon treatment with different concentrations of crezacin** (aqueous solutions, lab test).

Results. Upon presowing treatment of amaranth seeds with aqueous solutions of crezacin, the germination rate depended on the concentration of the preparation, and the effect turned out to be the same for both studied variety samples (Fig. 1). At a concentration of 10^{-8} M, the germination rate significantly ($P = 0.95$) increased by 10% compared to the control; at 10^{-7}, the maximum stimulating effect occurred, i.e. a 25% increase in germination for each variety specimen ($P = 0.95$), whereas at 10^{-5} M the indicator fell by 22% ($P = 0.95$). At other concentrations, the effect was insignificant.

In experiment 2, 10^{-7} M crezacin was used, since this concentration was optimal for seed germination. The weight of the aerial parts of the plants of both varieties throughout life exceeded the control by more than 20% (Fig. 2), and the effect of the preparation was associated with the phases of ontogenesis. Thus, 15- and 30-day-old seedlings, which are at the stage of latent growth, exceeded the control by 60% and 100% in weight, respectively ($P = 0.95$) (see Fig. 2). At this, an increase in the length of the main root of 15-day old seedlings averaged 60% in both cultivars ($P = 0.95$).

During active vegetation (days 45-60), green mass accumulation decreased compared to the previous phase, but remained on average 45% higher than in the control. The weight of leaves on day 60 was 46% higher compared to the control for K173, and 52% higher for K185 ($P = 0.95$). On day 90, the aerial biomass accumulation decreased, but nevertheless significantly ($P = 0.95$) exceeded the control by 27%. On day 120, the aboveground biomass of plants grown from crezacin-treated seeds again almost doubled the control.

The presowing treatment of seeds with crezacin did not affect the growth of amaranth plants in height throughout their life, with the exception of day 75 when the test plants lagged behind the control by almost 20% ($P = 0.95$).

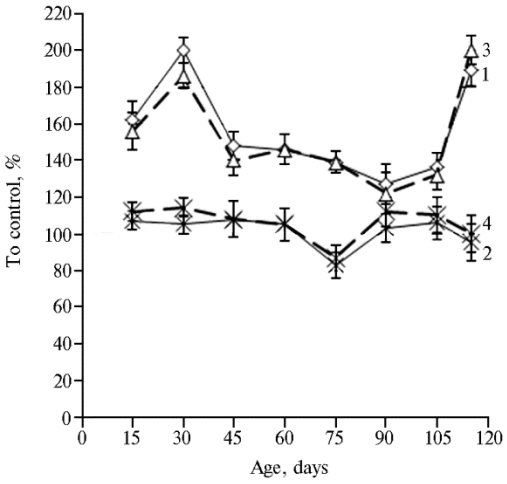

Fig. 2. The increase in weight (1, 3) **and height** (2, 4) **in growing plants of amaranth *Amaranthus caudatus* L. (cultivar K173)** (1, 2) **and *Amaranthus cruenthus* L. (cultivar K185)** (3, 4) **upon seed treatment with 10^{-7} M crezacin** (water solution, lab test**).**

Investigation of the light-dependent reaction in photosynthesis in 45-day-old K173 plants revealed an increase in the performance of the electron transport chain of isolated chloroplasts and photosynthetic phosphorylation (Table 1). The chlorophyll content in the leaves did not change significantly. The leaf NPP calculated over the period from day 45 to day 60, increased by 26±6% (P = 0.95) compared to the control.

The effect of seed treatment with crezacin on elements of the nitrogen assimilation system we assessed in K173 amaranth plants in early growing period (days 15-45). All parameters showed changes with a degree which was determined ontogenetically (Table 2). The activity of nitrate reductase increased 1.5 times only on day 45, the total protein content in leaves exceeded the control by 20-30% on average, and the nitrite N level fluctuated throughout this entire period of development (P = 0.95)

1. Chlorophyll content, electron transport and photophosphorylation in chloroplasts from leaves of 45-day-old amaranth *Amaranthanthus caudatus* L. (cultivar K173) plants upon seed treatment with 10^{-7} M crezacin (*M*±SEM, water solution, lab test)

Variant	Chlorophyll		Rate			
			electron transport		photophosphorylation	
	mg/g dry weight	to control, %	μmol $K_3[Fe(CN)_6]$	to control, %	μmol ATP	to control, %
Control	9.9±0.2	100.0±2.0	110.4±7.7	100.0±7.0	112.0±4.3	100.0±3.8
Test	10.5±0.9	106.1±9.1	148.9±7.0	134.9±6.1	184.5±9.4	164.7±4.5

N o t e. The electron transport rate is calculated per 1 mg chlorophyll per 1 h.

2. Nitrogen assimilation parameers (to control, %) **in leaves of amaranth *Amaranthanthus caudatus* L. (cultivar K173) plants upon seed treatment with 10^{-7} M crezacin** (*M*±SEM, water solution, lab test)

Plant age, days	Nitrate reductase activity	$N\text{-}NO_2$	Total protein
15	109±5	135±2	132±9
30	102±6	106±4	129±4
45	158±7	116±4	120±5

Thus, we established the dependence of the germination capacity of amaranth seeds on the dose of crezacin applied a wide range of low concentrations, 10^{-10}-10^{-5} M. In plants, such a dose-dependent response to a chemical agent is characteristic of the phytohormones [39]. The phytohormones exhibit biological activity in extremely low doses and act as stimulants in a narrow range of concentrations, being inhibitors when exceeding the range. Due to this property of crezacin, which we established for the first time, its hormone-like properties can be assumed. Note that some authors compare the manifestation of crezacin activity with the action of auxins and gibberellins [2, 28]. However, the hypothesis

about certain elicitoring properties of many small molecules with biological activity to which crezacin can be attributed seems to be more substantiated [40]. We supported this suggestion earlier in the study of the effects of p-aminobenzoic acid and 4-hydroxyphenethyl alcohol [27, 29].

We did not find the effect of crezacin on the growth of amaranth plants in height, although this property was described [30, 35], with the exception of day 75 (panicle formation). However, we confirmed another property of crezacin, established earlier, i.e. the ability to enhance the accumulation of green mass [30, 35]. In this case, the effect of the crezacin was also associated with the phases of ontogenesis (see Fig. 2). The strongest treatment effect (a 1.5-2.0-fold increase in weight) occurred at the stage of latent growth (days 15-30). Since in this period the growth of the aerial part of seedlings in the control practically ceases, it can be argued that due to the crezacin application the stop did not occur. During active vegetation (days 45-60), the positive effect of the crezacin visually decreased almost by half compared to the previous phase, but this actually happened due to a sharp increase in seedling growth in the control after they emerged from the hidden growth phase, whereas the effect of crezacin remained significant. Since the beginning of the generative phase (day 75), with initiation and formation of panicles, the energy and plastic resources of plants were mainly spent on the development of generative organs. As a result, during these periods a noticeable lag of plants from the control in height was observed, but there was no decrease in the green mass accumulation. With the onset of the heading stage (day 90), the aerial biomass further decreased. On day 120, the weight of the aerial parts of plants grown from treated seeds was almost 2 times higher than the control, mainly due to panicles with ripened seeds but not the green biomass.

Crezacin applied to seeds resulted in an increase in the aerial biomass at all stages of plant growth compared to control whereas plants remained constant in height or slightly retarded in stem elongation, which led to a positive overall effect, i.e. the habit of the plants became stronger than in the control. Due to this, plant resistance to mechanical damage and low humidity increased, which is especially important for seedlings at the stage of hidden growth

It is especially worth to note the crezacin as a stimulant of root growth which occurs due to an increase in the length of the main root. Despite the fact that during latent growth period, the root system development is quite active even without external stimulation and only the aboveground part practically stops growing, the stimulating effect of crezacin during the critical period for seedlings contributes to their stronger rooting, fixing in the soil and, therefore, better survival.

It was reported that under the action of crezacin the content of chlorophyll increases [27, 30]. We did not find such a change in amaranth leaves. In studying the effects of crezacin on the light-dependent reactions of photosynthesis, we first found an increase in the electron transport rate and the photophosphorylation rate in isolated chloroplasts. As a result, the total energy pool of cells increases, which ensures an increase in the NPP of amaranth leaves on day 45 to day 60 (see Table 1). A similar effect has been described for other plants [28].

It is known that crezacin can affect the metabolism of nitrogen compounds [29] and causes an increase in the total protein content [28, 30]. As a result of the crezacin application to seeds, we found changes in the activity of nitrate reductase, nitrite nitrogen $N\text{-}NO_2$ content and total proteins during stage of early vegetation of amaranth plants (days 15-45) compared to the control. There is no obvious interdependence in these changes, however, some studies

indicate that these parameters are not always in a direct relationship [41]. We can definitely say that under the influence of crezacin, the amount of leaf protein useful for humans is significantly higher, which, consequently, improves the nutritional value of the culture. The observed fluctuations in the nitrite nitrogen content were possibly due to its different use in synthetic processes during definite phases of this stage of ontogenesis.

So, soaking seeds of *Amaranthus caudatus* L. (variety K173) and *Amaranthus cruenthus* L. (variety K185) in 10^{-7} M aqueous solution of crezacin significantly increases seed germination capacity and significantly affects properties of the resultant plants, primarily seedling quality. Their shoots at the stage of latent growth significantly exceed the control in weight, not exceeding the control in height. Due to this, the plants possess a stronger habit, which can contribute to resistance to damaging environmental factors. An additional beneficial effect is a significant lengthening of the main root, due to which the seedlings are rooting better and become more resistant to wind, lack of moisture, and weeds. During active vegetation, productivity of test plants increased due to an increase in the biomass of the edible aboveground part. We did not reveal species-specific changes in seed germination and growth parameters of resultant plants in response to crezacin application. The nutritional value of K173 variety plants is due to an increase in leaf protein. It is established that the described effects are due to stimulation of the photochemical activity of chloroplasts, accompanied by an increase in the photosynthetic phosphorylation rate and the energy pool of cells. Crezacin can be recommended for leaf amaranth cultivation to improve seed quality, productivity, nutrition value, and to facilitate the crop introduction in the middle latitudes of Europe and Central Russia.

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GENERATION OF *Rubus arcticus* L. HAPLOIDS THROUGH in vitro MICROSPORE CULTURE TECHNIQUE

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Abstract

Arctic bramble (*Rubus arcticus* L.) is a valuable small-fruit crop used as a plantation crop for a relatively short time. *R. arcticus* is a remontant donor in interspecific hybridization with *Rubus idaeus* L., though conditioning low yields to hybrids. So *R. arcticus* is primarily bred for yield enhancement; therefor, the acceleration of the breeding process is of great importance. This can be achieved using plants with a doubled haploid set of chromosomes. This paper is the first to describe the technique of production haploid plants of *R. arcticus* via in vitro microspore culture. In the experiments we used Finnish cultivars Pima and Mespi and Swedish cultivar Astra. To obtain donor explants, the method of forcing generative shoots was used throughout the year. Microspores were isolated from anthers with the use of manual homogenizer into a 1.5 ml micro test Eppendorf tube. The homogenate was added with 0.5 ml sterile water containing 30 g/l glucose, centrifuged at 4500 rpm, and the microspores were transferred with a microdoser to nutrient medium for morphogenesis initiation. To obtain microspores, the anthers were isolated from buds of 9 to 12 mm long 4-5 days before the flower bloomed. The concentration of the microspores in the suspension was about 40,000 per 0.5 ml sterile aqueous solution with glucose; for this, 60 anthers were crushed. To induce embryoidogenesis, we used the Murashige and Skoog (MS) plant growth medium supplemented with 0.50 to 2.00 mg/l growth regulator 6-benzylaminopurine (BA). After the appearance of embryoids, we used MS, 75 % MS, or 50 % MS growth media, and also the effect of carbohydrate sources, i.e. glucose, sucrose and maltose at a 20, 30 and 40 g/l dosage, was investigated. We have identified the following microspore development stages: tetrads, non-vacuolated microspore, strongly vacuolated microspore, three-cell pollen. It was found that MS nutrient media containing 1.5 mg/l BA provides for 23±3 embryoids on day 51±2 of culture. We have also found the effect of MS concentration and the source of carbohydrate nutrition on the growth of embryoids. The combination of 0.75 MS and 30 g/l glucose was the most effective leading to embryoid growth on day 12±2 and the appearance of leaflets on day 44±1. On day 40 of culture the embryoids reached 5±0.2 mm in length. The ploidy control of regenerant plants, by counting chromosomes and chloroplasts in the stomata guard cells, confirmed the haploid set of chromosomes ($n = 7$). These findings allow the use of the proposed technique to generate *R. arcticus* haploids which, after doubling the chromosome set, may be involved in breeding.

Keywords: *Rubus arcticus*, haploid, diploid, microspore culture, embryoid, morphogenesis, regenerant plant

Arctic bramble (*Rubus arcticus* L.) is a valuable berry plant. Despite the relatively short period of its use as a plantation crop, several highly productive

varieties were obtained. Arctic bramble is a donor of remontance and excellent taste traits in interspecific hybridization with *Rubus idaeus* L., however, the offspring inherits low productivity from arctic raspberry. That is why the *R. arcticus* is mainly bred for increased plant productivity. Therefore the acceleration of the selection and the use of haploids for breeding cross-pollinated plants are of particular importance [1-3]. The doubled haploid technology overcomes a number of breeding difficulties. Homozygous lines based on haploids can be obtained in 2-3 years [4]. At the same time, according to some researchers, the use of androcline regenerants is the only way to maintain the heterosis effect of a valuable hybrid line [5].

Obtaining haploid plants has a common experimental design plan, i.e. exposure to elevated and/or lower temperatures, chemical treatment of donor plants, change in the nutrient medium composition of growth regulators, in carbohydrate components, in anther and microspores age to better induce tissue culture and regeneration. To date, many papers describe methods for obtaining haploid plants of wheat [5, 6], cabbage [7-9], rapeseed [10], rice [11], carrots [12-14] but there are no works on production of haploid plants of *R. arcticus* L. in microspore culture.

There are a lot of factors influencing the activation of switching microspores from the gametophytic to the sporophytic pathway of development, for example, the types and concentrations of growth regulators [15-17] and carbohydrate nutrition [18-20].

Earlier, we have proposed a technique for *R. arcticus* clonal micropropagation [21]. To continue these studies, in the present work, we first obtained haploid regenerated *R. arcticus* plants in a microspore culture via optimizing the 6-benzylaminopurine concentration and carbohydrate nutrition to induce embryoidogenesis

Our goal was to develop a method for producing haploid plants of *Rubus arcticus* L. in microspore culture.

Materials and methods. The research was carried out in 2018 on Arctic bramble varieties Pima, Mespi from Finland and Astra from Sweden. Microspores were isolated from pollen, which were selected from buds of different ages and sizes.

To reduce contamination when introduced into in vitro culture and for year-round production of buds, plant forcing method was used [22]. Plants, after flowering was completed, were kept in pots for 30 days at 4 °C. After cold exposure, such plants again formed generative shoots.

The buds were sterilized 1 min in 70% aqueous solution of ethanol, then 15 min in 5% aqueous solution of sodium or potassium hypochlorite, and washed in sterile distilled water at least three times. The extracted anthers were collected in 1.5 ml Eppendorf microtubes, 60 anthers per each, and crushed with a manual homogenizer. Sterile water (0.5 ml, 30 g/l glucose) was added to the microtubes using a Proline 20-200 single-channel microdoser (Sartorius Proline, Finland). The tubes were centrifuged (Microspin FV-2400 minicentrifuge-vortex, SIA BioSan, Latvia) for 15 s at 4500 rpm. From each tube, a microspore suspension was transferred by microdoser into a 10 ml culture flask (a suspension from one tube was placed in one culture flask; approximately 80,000 microspores/ml. To isolate the anthers and microspores, 9-12 mm long buds were collected 4-5 days before blooming.

At each stage of in vitro microspore culture, the composition of the nutrient medium and the growth regulators were changed. To activate in vitro

morphogenesis, microspores were implanted on a Murashige-Skoog medium (MS) [23] supplemented with 100 mg/l mesinositol, 2 mg/l glycine, 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, 30 g/l glucose and 5.0 g/l agar, pH of 5.8. As a growth regulator, 0.50 to 2.00 mg/l 6-benzylaminopurin (BA, AppliChem GmbH, Germany) was used. After the emergence of embryoids, MS medium (AppliChem GmbH, Germany) was used (full and reduced to 75% and 50% concentration of macro- and micronutrients according to the recipe) [23]. The influence of the carbohydrate source on the growth and development of embryoids was assessed using 20, 30 and 40 g/l glucose, sucrose and maltose.

Microspores, embryoids, and regenerants were grown under illumination of 1500 lux at a 16 h (day)/8 h (night) photoperiod and a temperature of about 25 °C. The 10 ml culture flasks were used for in vitro culture and during growth and development of embryoids, and 100 ml culture flasks for the regenerated plant growth.

Chromosomes were counted in root meristemic zone of the regenerated plants. The roots were placed in Carnoy's fixative [24] and allowed for 24 h, then washed for 20 min under running water, immersed in an enzyme mixture (0.3% pectinase, 0.3% macerase, 0.3% cellulase + citrate buffer) and incubated at of 37 °C for 2 h. Afterwards, the roots for 3 min were immersed in 60% acetic and crushed with a dissecting needle, then circled with a 3:1 fixative, shaken, washed in absolute alcohol, dried, stained with methylene blue, and washed in distilled water. Chromosomes were counted as per description [24] (Light Microscope Biomed-3, Biomed, Russia). To count the chloroplast number, 5 cuts from leaves of each plant, each cut of 5 mm in diameter, were used. The cuts were stained with iodine dye. In vitro growing regenerants were photographed (a Samsung NX-10 digital camera, Samsung, South Korea).

The arithmetic mean values (*M*) with standard error of the mean (±SEM) and coefficient of variation (*Cv*) are shown. The calculation of the confidence interval based on Student's *t*-distribution at a significance level of $p \leq 0.05$ was performed using Microsoft Excel 2010 statistical program.

Results. To start with, the number of microspores in the anthers was counted and the stages of microspore development were established depending on the bud length. Buds of 1.0 to 1.4 mm in length contained 70 to 86 anthers with 590 to 710 mature pollen grains per anther. We identified four main stages in microspore development (Fig. 1). Stage 1 is a tetrad (in buds from 4 to 5 mm long), stage 2 is an unvacuolated microspore with a central location of the nucleus (buds of 5 to 7 mm long), stage 3 is a strongly vacuolated microspore with a thickened wall, a large vacuole and a small nucleus located laterally (in buds of 7 to 8 mm long), and stage 4 is three-cell pollen (in buds longer than 8 mm) (see Fig. 1).

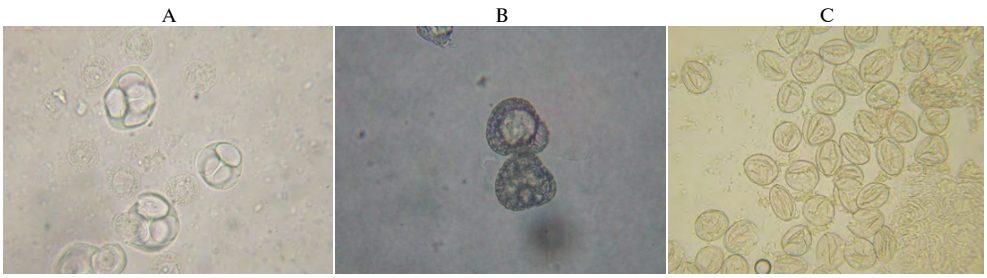

Fig. 1. Microspores of Arctic bramble (*Rubus arcticus* L.) Astra cultivar on Murashige-Skoog medium (30 g/l sucrose, 1.5 mg/l 6-benzylaminopurin, pH 5.8): A — tetrads, B — highly vacuolated microspore, C — mature pollen (magnification ×600, light microscope Biomed-3, Biomed, Russia).

1. Morphogenesis of microspores of different Arctic bramble (*Rubus arcticus* L.) cultivars on Murasige-Skoog medium at different concentration of 6-benzylaminopurine (BA)

Cultivar	BA, mg/l	Emergence of embryoids, days		Number of embryoids on day 60	
		M±SEM[1, 2]	*Cv*, %	*M*±SEM[1, 2]	*Cv*, %
Astra	0.50	63±2[a]	7.8	5±1[c]	11.2
	0.75	58±1[ab]	4.5	12±2[ac]	4.5
	1.00	55±1[bc]	10.0	14±2[ac]	6.3
	1.25	51±2[c]	9.2	23±3[b]	5.6
	1.50	51±3[bc]	7.8	20±1[b]	7.2
	1.75	51±3[bc]	8.2	9±2[c]	8.7
	2.00	0	0	0	0
Pima	0.50	65±3	2.6	4±1[a]	9.1
	0.75	54±2[a]	4.6	7±2[ab]	12.4
	1.00	53±2[a]	7.1	8±2[ab]	9.3
	1.25	53±1[a]	8.6	18±1	9.7
	1.50	54±2[a]	5.8	9±1[b]	6.5
	1.75	54±2[a]	4.3	8±2[ab]	7.7
	2.00	–	–	–	–
Mespi	0.50	63±2[a]	12.2	10±2[a]	5.4
	0.75	60±1[a]	12.1	13±2[ab]	6.5
	1.00	57±2[ab]	10.1	15±2[ab]	6.4
	1.25	53±2[b]	9.3	20±1	7.6
	1.50	52±1[b]	6.5	14±1[b]	3.8
	1.75	53±2[b]	3.4	14±2[b]	5.1
	2.00	63±2[a]	7.8	5±1[c]	11.2

N o t e. The sucrose concentration is 30 g/l, pH 5.8; 40,000 microspores per 0.5 ml. Each experiment was performed in a 3-fold repetition. Dashes mean the death of explants. 1 — a confidence interval based on Student's *t*-distribution at $p \le 0.05$; 2 — indicators in the column marked with the same letters (a, b, c) have no statistically significant differences at $p \le 0.05$ as per the Student's *t*-test.

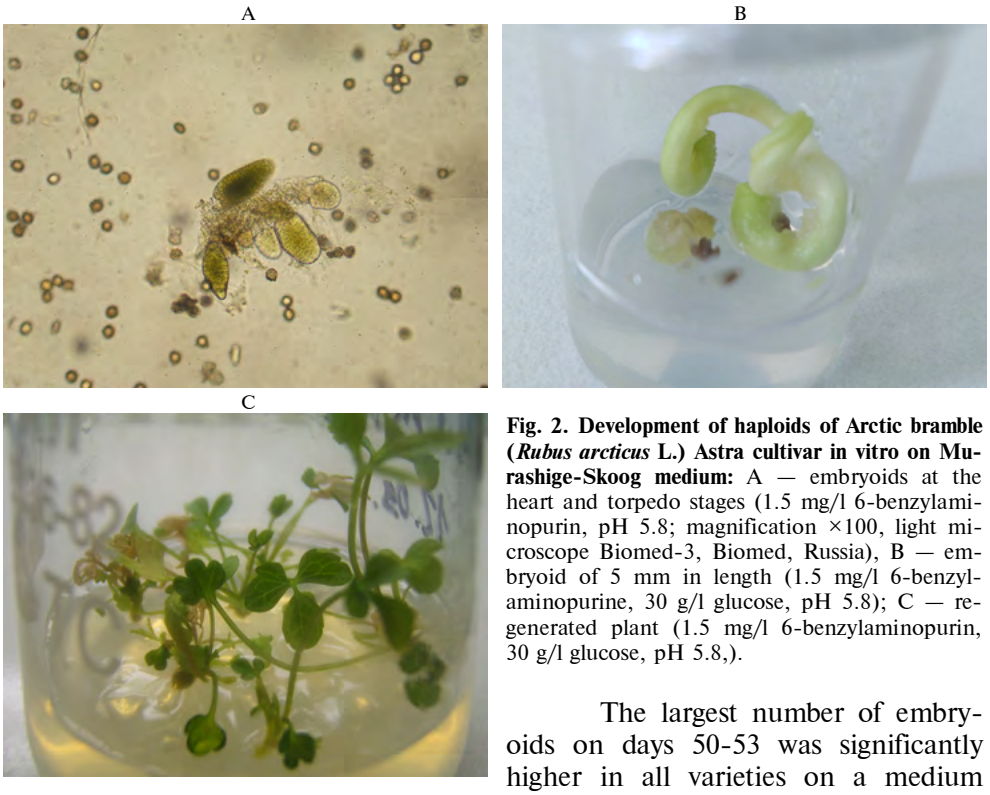

Fig. 2. Development of haploids of Arctic bramble (*Rubus arcticus* L.) Astra cultivar in vitro on Murashige-Skoog medium: A — embryoids at the heart and torpedo stages (1.5 mg/l 6-benzylaminopurin, pH 5.8; magnification ×100, light microscope Biomed-3, Biomed, Russia), B — embryoid of 5 mm in length (1.5 mg/l 6-benzylaminopurine, 30 g/l glucose, pH 5.8); C — regenerated plant (1.5 mg/l 6-benzylaminopurin, 30 g/l glucose, pH 5.8,).

The largest number of embryoids on days 50-53 was significantly higher in all varieties on a medium containing 1.5 mg/l BA (Table 1). Visually, embryoids were detected starting from the heart stage, when their size reached 0.5 mm. We did not reveal a reliable effect of the genotype on the morphogenesis of microspores in vitro. Though

we did not perform a special investigation of the influence of the stage of development of microspores on the morphogenetic activity, it is only necessary to note that the microspores that were placed on the nutrient medium were overwhelmingly at the stage of vacuolization.

In studies on the induction of embryogenesis and the production of secondary embryoids, various concentrations of BA, as a growth regulator, are actively used [25]. In our work, BA induced direct embryoidogenesis.

We also found out that more intensive growth processes occurred on a nutrient medium with a 75% macro- and microelements and 30 g/l glucose. In this variant, embryoid growth occurred on day 12, which was significantly different from other variants. On day 40, their size reached 5 mm (Fig. 2). However, the smallest death rate of embryoids was observed on a MS medium with a complete macro- and microelements (Table 2).

2. Growth of embryoids of Arctic bramble (*Rubus arcticus* L.) Astra cultivar in vitro on the Murashige-Skoog medium under different concentrations of macro- and microelements and carbohydrates

Micro- and macro-elements, %	Carbohydrate, g/l	Start of embryoid growth, days		Leaf emergence, days		Length in 40 days of growth, mm	
		$M\pm SEM^{1,2}$	*Cv*, %	$M\pm SEM^{1,2}$	*Cv*, %	$M\pm SEM^{1,2}$	*Cv*, %
100	Sucrose, 20	55±3	13.2	–	–	1.0±0.5^{a}	29.1
	Sucrose, 30	41±2^{a}	10.3	–	–	1.0±0.5^{a}	11.3
	Sucrose, 40	40±1^{a}	5.4	63±3^{a}	3.4	2.0±0.6^{ab}	7.1
	Maltose, 20	37±2^{a}	12.3	60±3^{a}	7.6	3.0±0.3^{b}	5.3
	Maltose, 30	19±1^{b}	5.4	49±2^{b}	2.1	3.0±0.5^{b}	5.6
	Maltose, 40	20±1^{b}	4.2	49±3^{b}	6.4	4.0±0.5^{b}	8.0
	Glucose, 20	25±3^{b}	14.1	49±4^{ab}	11.9	2.0±1.0^{ab}	14.6
	Glucose, 30	17±2^{b}	10.2	47±4^{ab}	9.5	3.0±0.3^{b}	7.5
	Glucose, 40	20±2^{b}	9.8	48±4^{b}	8.7	4.0±0.4^{b}	5.9
75	Sucrose, 20	–	–	–	–	–	–
	Sucrose, 30	43±3^{a}	6.8	60±3^{a}	13.2	3.0±0.3^{a}	11.2
	Sucrose, 40	40±2^{a}	8.2	60±4^{a}	12.5	3.0±0.3^{a}	8.4
	Maltose, 20	–	–	–	–	–	–
	Maltose, 30	21±2^{b}	5.5	46±2^{b}	5.6	4.0±0.4^{abc}	9.8
	Maltose, 40	20±2^{b}	4.3	46±2^{b}	8.1	5.0±0.5^{bc}	12.3
	Glucose, 20	25±3^{b}	11.2	46±3^{b}	10.3	4.0±0.3^{ab}	5.3
	Glucose, 30	12±2^{c}	5.2	44±1^{b}	7.0	5.0±0.2^{c}	7.9
	Glucose, 40	15±2^{c}	8.7	45±2^{b}	6.2	5.0±0.5^{bc}	9.2
50	Sucrose, 20	–	–	–	–	–	–
	Sucrose, 30	–	–	–	–	–	–
	Sucrose, 40	52±2^{a}	10.3	80±5^{a}	12.5	3.0±0.4^{a}	13.9
	Maltose, 20	–	–	–	–	–	–
	Maltose, 30	58±1	5.4	73±2^{a}	6.7	3.0±0.4^{a}	9.4
	Maltose, 40	50±2^{a}	7.9	70±1^{a}	8.3	3.0±0.5^{a}	6.7
	Glucose, 20	41±2^{b}	6.3	–	–	–	–
	Glucose, 30	39±2^{b}	7.5	–	–	–	–
	Glucose, 40	35±3^{b}	4.3	62±2	5.9	4.0±0.3^{a}	5.7

N o t e. The 6-benzylaminopurin concentration is 1.5 mg/l, pH 5.8. Each experiment was performed in a 3-fold repetition. Dashes mean the death of explants. 1 — a confidence interval based on Student's *t*-distribution at $p \le 0.05$; 2 — indicators in the column marked with the same letters (a, b, c) have no statistically significant differences at $p \le 0.05$ as per the Student's *t*-test.

Many authors note the positive effect of increased osmotic pressure, which is created at sucrose concentrations from 10 to 17%, on the emergence of embryoids [26-28], however, in our experiments, embryoidogenesis in *R. arcticus* actively occurred at a 3% concentration, and already at 4%, the rate of development decreased. This discrepancy in the results can be explained by the fact that we studied the transition from the embryoid stage to the regenerant plant, and not the stage of embryoid formation from the microspore.

The ploidy of regenerated plants was determined by counting the number of chromosomes and the number of chloroplasts in the stomata guard cells (Fig. 3). The number of chloroplasts in the stomata guard cells varied from 8 to 12 in

diploid plants, and from 2 to 6 in haploid plants. The possibility to use this indirect method to confirm ploidy has been proved in a number of works [29-31].

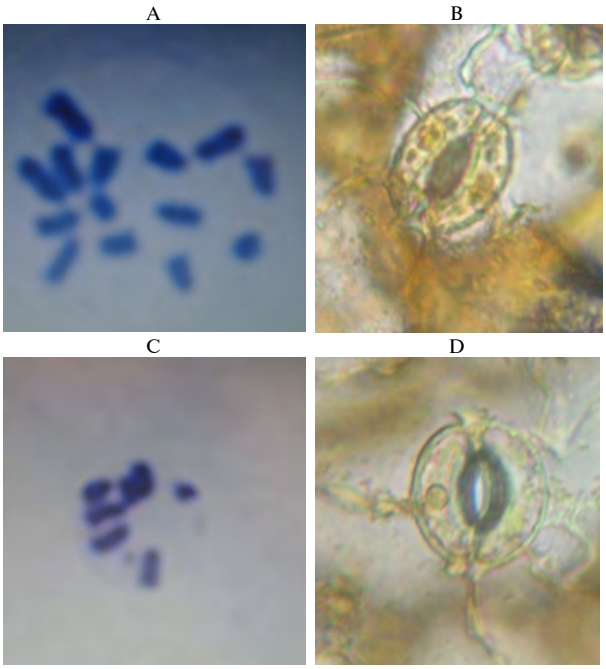

Fig. 3. Chromosomes of dividing cells in root meristem at the metaphase stage (A, C, magnification ×1000) **and chloroplasts of stomata guard cells** (B, D, magnification ×630) **in regenerants of Arctic bramble (*Rubus arcticus* L.) Astra cultivar:** A, B — the diploid ($2n$ = 14, 10 chloroplasts in the guard cells), C, D — the haploid (n = 7, 4 chloroplasts in the guard cells). Light microscope Biomed-3 (Biomed, Russia).

This investigation does not allow us to identify which particular stage of microspore development is better to produce haploid plants of Arctic raspberry, however, this is important for determining the factors that influence the switching of the development program of microspores from gametophytic to sporophytic [17, 19, 28, 32] . It can only be noted that most of the microspores were at the stage of vacuolization. One of the main factors of switching to the sporophytic pathway of development in the Arctic bramble microspores was the use of the growth regulator 6-benzylaminopurine and glucose as a carbohydrate source. The crucial point in obtaining haploid plants is not only the production of embryoids, but also the preservation of their viability and morphogenetic activity when transplanted to a fresh nutrient medium [33]. Out findings indicate that for a success, it is necessary to reduce the concentration of macro- and microelements in MS nutrient medium to 75% of their full amount, while a decrease to 50% leads to a decrease in viability.

Thus, to obtain haploid regenerants of *Rubus arcticus* L. varieties Pims, Mespi and Astra, it is advisable to use full Murashige-Skoog nutrient medium enriched with 1.5 mg/l 6-benzylaminopurine, which gives from 18±1 to 23±3 embryoids per 40,000 microspores. To regenerate plants from embryoids, one should use a 75% Murashige-Skoog medium supplemented with 30-40 g/l glucose and 1.5 mg/l 6-benzylaminopurin, which provides the shortest time of induction resulting in 5±0.2 mm embryoids by day 40. These findings and the developed technique can be used to create doubled haploids of other *R. arcticus* varieties for inclusion in breeding programs.

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ANALYSIS OF MICROBIOME OF RECULTIVATED SOILS OF THE KINGISEPP AREA OF PHOSPHORITE MINING

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Abstract

The microbial composition of reclaimed disturbed soil covers may indicate the degree of their recovery and the processes occurring in them, as well as their suitability for further use in agriculture. Kingisepp phosphorite quarry was developed in the 1960s, and at the end of the 1970s reclamation was performed. This object is unique because its soil physical parameters were monitored for 29 years and the reclamation was performed with the planting of three plant cultures — spruce, larch and pine. In the area with spruce it was leveled with an addition of peaty-mineral mixture, and in areas with larch and pine only mineral substrate without peat was added. However, the analysis of the microbiome composition of the soil cover at the reclamation sites has not yet been carried out. Our study showed that the structure of the studied soil microbiome did not depend on the physicochemical parameters of the soil, the diversity of the soil microbiome did not correlate with the main mineral nutrients, and the dominant plant species did not significantly affect the structure of the microbiome. The aim of the work was to study the microbiome of these sites using high-throughput sequencing of amplicon libraries of the 16S rRNA gene, as well as to search for the connection between the microbiome composition and the type of remediation and physical and chemical parameters of the soil. For three plots, descriptions of vegetation cover and soil cuts were made, and soil samples were taken to determine their physical and chemical parameters and DNA extraction. The granulometric composition of the samples, pH levels, substrate induced and basal respiration, as well as the content of organic carbon, mobile compounds of phosphorus and potassium, exchangeable ammonium and nitrates were measured. Quantity of bacteria, archaea and fungi was determined using real-time PCR. For the analysis of microbial communities, the level of their alpha and beta diversity was measured, their taxonomic structure was determined, as well as their relationship with the soil biochemical parameters and vegetation cover. According to the results of the studies, the soil parameters were similar for all plots, and the levels of basal and substrate-induced respiration were very low (around 0.02-0.05 μg CO_2/g per hour). The plot under the spruce showed a more acidic soil extract reaction (pH 6.5) than the plots under larch and pine (pH 7.6 and 7.1, respectively). The type of vegetation was not a sufficiently strong ecological factor and microbial communities turned out to be close in structure. The quantitative composition of microorganisms did not differ significantly between the three experimental plots, except for the lower content of archaea in the plot with spruce. The level of alpha-diversity of the prokaryotic community in all three plots was also

similar, but the area under the spruce differed from others by a higher diversity of actinobacteria. *Proteobacteria*, *Actinobacteria* and *Acidobacteria* phyla were dominant in all samples. The most numerous taxon in all plots was *Pseudomonas*, in the plot with spruce dominated *Actinobacteria*, *Rhizobiaceae*, *Kouleothrixaceae*, Ellin6529, N1423WL, with pine — *Rhodoplanes* and *Sinobacteraceae*, with larch — IS-44. Pine plot was also characterized by a relative low content of *Micrococcaceae* and Ellin6075, and spruce plot — of RB41. In general, in the studied microbiomes, bacteria are identified that belong to both oligotrophic slow-growing forms characteristic of stabilized soil communities with a full carbon cycle, and to fast-growing copyotrophic, often associated with a rhizosphere niche. In this regard, this stage of overgrowing of reclaimed soils of the Kingisepp phosphorite deposit can be attributed to pre-climax.

Keywords: reclamation, soil microbiome, alpha and beta diversity, high throughput sequencing, 16S rRNA

The widespread increase in territories with disturbed soil and plant cover which require land reclamation is an urgent environmental problem of our time. The formed mining complexes need restoration and costly reclamation, at the same time they are good sites for studies of soil formation and primary succession processes [1]. Such observations are important in the applied aspect, since reclaimed soils can again acquire agricultural significance [2], and in fundamental aspects, the study of pedogenesis will allow us to approach understanding of soil evolution.

As a rule, during restoration of ecosystems, the main attention is paid to studying the physicochemical parameters of soils [3] or to vegetation cover [4, 5]. However, the soil microbiome remains the key factor in soil formation processes [6, 7]. The widest understanding of the microbiome is provided by high-throughput sequencing methods [8-10], which allow the most complete description of the microbial composition of samples [11]. In this case, the composition of the microbiome can vary to a significant extent depending on various biotic and abiotic influences [12]. Soil moisture and pH are among the strongest factors affecting soil microbiomes [13, 14]. An important role is given to the diversity of the vegetation cover [15-17]. In particular, we previously showed that with a relatively small scatter in the values of physicochemical parameters within the same soil type, the formation of a certain soil microbiome profile is largely determined by the structure and composition of phytocenosis [18], with differences being observed mainly at a low taxonomic level (orders, families and genera).

Thus, the issue on how the rehabilitation of the soil cover occurs and how its microbiome changes in this case remains relevant. At present, the formation and diversity of microbial communities in disturbed territories is largely associated precisely with the decisive role of plant communities [19]. Since the rehabilitation of disturbed soil cover takes a long time, for soil studies it is necessary to choose sites that are under long-term monitoring. An example is Kingisepp quarry, where phosphate rock was mined in the 1960s, and mining reclamation was carried out in the late 1970s. The uniqueness of this object is that the monitoring of soil indicators has been carried out here for 29 years, and the restoration was carried out using three different tree species [20]. However, no microbial composition analysis has yet been carried out at the reclamation sites, which would make it possible to assess the degree of restoration of the soil cover.

In the present paper, we showed that in the reclamation areas of the Kingisepp phosphorite deposit, the structure of the soil microbiome did not depend on the physicochemical parameters of the soil, the diversity of the soil microbiome did not correlate with the main mineral nutrients, and the dominant plant species did not significantly affect the microbiome profile.

The aim of our work was profiling microbiomes of reclaimed soils and to search for patterns that link the microbiome structure with physicochemical soil

parameters and the dominants of woody vegetation.

Materials and methods. The surveys were carried out in 2016 in quarry No. 3 of Phosphorit Production Association (Kingisepp phosphorite deposit, west of the Leningrad Region, Kingisepp District, between Kingisepp and Ivangorod). Samples were collected at three 20×40 m sites (Nos. 1, 2 and 3) subjected to restoration in the 1970s. The age of the test plots was 37, 32, and 28 years, respectively. In 2014, geobotanical descriptions of the sites were carried out according to the dominant scheme; in 2016, studied the recent (new) soil formation was studied.

At each site, 3 soil sections were laid. When describing soil profiles, a substantive-morphological approach was used, based on the identification of soil taxa by the totality of morphological characters. From each horizon, three probes were taken, 100 g each, for laboratory analyzes. A 15 g sample from each upper organomineral horizon was used for microbiological analysis.

The particle size distribution was determined using the Kaczynski pipetting method with pyrophosphate peptization of microaggregates [21], the organic carbon content was determined by Tyurin's method based on dichromate oxidizability [22], and the pH of the aqueous extract was determined potentiometrically in a soil:water ratio of 1:2.5. The concentration of mobile phosphorus and potassium compounds was measured as per Kirsanov in TsINAO modification [23], exchangeable ammonium by TsINAO method [24], and nitrates ionometrically [25]. The substrate-induced respiration (SIR, the measurement is based on the registration of additional CO_2 emission in response to glucose as the introduced substrate) and basal respiration (BR, determined by the same method as SID, but without enriching soil with glucose) was evaluated as described [26].

DNA was extracted in 4 replicates for each plot by previously developed method [27] with glass balls of different diameters as abrasive. Soil samples were homogenized (a Precellys 24 devise, Bertin Technologies, France). The purity and amount of DNA in the preparation was assayed electrophoretically in 1% agarose with 0.5× TAE buffer (DNA concentration in a sample averaged 50 ng/ml). Purified DNA was used for quantitative PCR (qPCR) and preparation of amplicon libraries as per the instructions of the sequencing protocol (Illumina, Inc., USA).

To identify three main taxonomic groups of microorganisms in the soil, quantitative qPCR was performed with the following primer pairs: to the 16S rDNA fragment of bacteria — EUB338 (5′-ACTCCTACGGGAGGCAGCAG-3′) and EUB518 EUB518 (5′-ATTACCGCGGCTGCTGG-3′) [28, 29], to the archaea 16S rDNA fragment — ARC915f (5′-AGGAATTGGCGGGGGAGCAC-3′) and ARC1059r (5′-GCCATGCACCWCCTCT-3′) [30], to the fungal ITS fragment — ITS1f (5′-TCCGTAGGTGAACCTGCGG-3′) и 5.8S (5′-CGCTGCGTTCTTCATCG-3′) [31]. qPCRmix-HS SYBR kit (Eurogen, Russia) was used to prepare the reaction mixture according to the manufacturer's instructions. The standards were the series of 10-fold dilutions of fragments of the 16S rRNA gene of *E. coli* and *H. pilori*, as well as the ITS1 fragment of *S. cerevisiae*. Each sample of the PCR mixture, including standards, was analyzed in triplicate. The measurements were carried out on a CFX96 thermocycler (Bio-Rad, Germany) according to the following protocol: 3 min at 95 °C; 20 s at 95 °C, 20 s at 50 °C, and 20 s at 72 °C (40 cycles). For replicates of both PCR and different DNA samples in a site), the mean values (*M*) and standard errors the mean (±SEM) were calculated. After processing, the results were expressed as the number of ribosomal operons per 1 g soil

In preparing libraries of 16S rRNA gene fragments for each soil DNA sample, PCR was performed with universal primers for the variable region V4, the F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACVSGGGTATCTAAT-3′) (a T100 thermocycler, Bio-Rad, Germany) according to the

following protocol: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (35 cycles) [32]. Sequencing and primary processing of data was performed (an Illumina MiSeq instrument, (Illumina, Inc., USA) in the ARRIAM Genomic Technologies and Cell Biology Center for Collective Use of Research Equipment. Sequenced fragments of the 16S rRNA gene were processed using the Trimmomatic [33] and QIIME [34] software. All adapter sequences were trimmed, paired-end reads were assembled, and the quality of nucleotide sequences was checked. All non-bacterial and chimeric sequences were removed, and data were normalized. Sequences with more than 97% similarity were combined into operational taxonomic units (OTUs) using the de novo UCLUST-based algorithm [35]. One sequence was selected from each OTU to compile a set of representative sequences. The representative sequences were classified using RDP naïve Bayesian rRNA Classifier program, and then aligned according to PyNast algorithm [36], using the Green-genes coreset database [37] as a matrix. After sequence alignment, distance matrices were constructed within QIIME using the Euclidean distances.

For a comparative analysis of communities, the indices of α- and β-diversity were calculated. α-Diversity was evaluated using species richness indices (the number of OTUs in the sample, Chao 1 abundance index, Faith's PD phylogenetic diversity index) and the Shannon index of diversity H [38-40].

The significance of differences between microbiomes by α-diversity indices was evaluated using Student's *t*-test. To determine β-diversity, the Weighted unifrac method was used, which allows one to estimate the percentage of similarities/differences between all compared pairs of microbiomes [41]. The results were analyzed using multivariate statistics (analysis of the main components) with Emperor software [42]. A Mantel test (Pearson correlation, 100 permutations) for combined replications was performed with QIIME to assess the correlation of bacterial community composition with concentration of basic soil macroelements [43]. To assess the significance of differences between abundance of individual taxa in analyzed samples, a script we wrote using the Mann-Whitney U-test with a significance threshold of 0.05 in Python programming language [44] was applied in addition to the QIIME software.

The paper presents the mean values of indicators (M) and their standard errors (±SEM). The revealed differences were considered statistically significant at $p < 0.05$.

Results. The Kingisepp quarry was intended for mining phosphorites confined to Obolus sandstones of the Upper Ordovician over which limestones and dolomites lie, a little above which there are Middle Devonian clays, marls, sands, and mudstones. Lacustrine-glacial sands and sandy loams, moraine loams and peat are the Upper Quaternary sediments deposits [3]. All these rocks to one degree or another compose quarry dumps. The phosphorite deposit has been developed open pit since the 1960s. As a result, dump areas of different ages were formed, on which, after several years of self-growth and spontaneous subsidence of the rocks, mine reclamation was applied. This territory is one of the largest in the north-west of Russia in terms of area of violation and the scale of remediation work.

During mine technical restoration at site No. 1 in 1979, after leveling the dumps, a peat-mineral mixture (quaternary loamy-sandy rocks with an impurity peat content of 20-30%) was applied to the blocky material, then biological recultivation was used (planting of spruce seedlings). In sites No. 2 and No. 3, after dump leveling, a mineral substrate consisting of the so-called loose rock that does not contain peat impurities was distributed along the surface. Larches were planted in site No. 2, and pines were planted in site No. 3.

The soils of the reclaimed sites are carbo-lithozems. In general, the main soil parameters turned out to be similar in all sites. However, we note that the content of mobile phosphorus and nitrates is more than 2 times higher in site No. 2 under larch compared to similar horizons in other areas (Table 1). The pH of the aqueous soil extract in the site No. 1 (under the spruce) is weakly acidic, while in the rest it is slightly alkaline. As per granulometric composition, replantozems were characterized as medium loams.

1. Soil biochemical and granulometric parameters of various phytocenoses in the sites of long-term restoration (quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016)

Parameter	Replantozem		
	peat-mineral mixture under the spruces 37 years, site No. 1	on mineral rock	
		under the larches, 32 years, site No. 2	under the pines, 27 years, site No. 3
Soil horizon type	AY (	AY	AY
Depth, cm	3-18	1-18	1-10
pH_{BWater}	6.5	7.6	7.2
$C_{org.}$, %	2.0	2.4	1.9
N_{total}, %	0.20	0.31	0.46
C/N	11.6	8.9	4.8
N (NH_4), mg/kg	31.1	27.2	27.4
N (NO_3), mg/kg	0.24	0.82	0.37
P (P_2O_5), mg/kg	2043.5	4198.5	1731.5
K (K_2O), mg/kg	338.3	242.7	214.7
Particle fraction, %:			
< 0.001 mm	15.0	22.0	24.3
< 0.01 mm	33.2	36	36

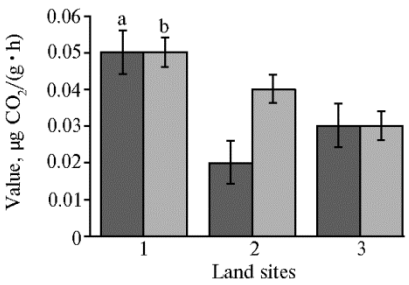

Fig. 1. Basal (a) **and substrate-induced** (b) **soil respiration of organomineral horizons** (AY) **in various phytocenoses at sites of long-term restoration** (n = 3, M±SEM, quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016). For description of sites by type of reclamation, see the section Materials and methods.

In all sites, we revealed very low rates of basal and substrate-induced soil respiration (Fig. 1) with maximum of 0.05 μg CO_2/(g·h) and minimum of 0.02 μg CO_2/(g·h). Additional CO_2 emissions were observed only in site No. 2 (under larch). In general, the soils formed on dumps under pines showed the least respiratory activity. It should be noted that there were no significant differences between the two indicators, although they are usually observed for mature soils of climax ecosystems.

The microbial biomass varied from 1.6±0.1 μg C g (under pines, site No. 3) to 2.4±0.09 μg C g (under spruces, site No. 1). The values of the microbial metabolic coefficient (the ratio of basal respiration to the carbon content of microbial biomass) were extremely small, the maximum value of 0.021±0.002 μg CO_2-C/(mg $C_{mic.}$·h) was also recorded for the soil under spruces, the minimum 0.010±0.005 μg CO_2-C/(mg $C_{mic.}$·h) for soil under larches (site No. 2).

Right after the measures of mining recultivation were applied, self-growth of typical explerents occurred, including *Tussilago farfara* L., *Chamaenerion angustifolium* (L.) Scop., and *Calamagrostis epigeios* (L.) Roth. By 2014, spruce forest was formed in site No. 1, where we identified 11 species of higher vascular plants, 2 ligneous and 9 grassy species, from 8 families. The total projective cover of grassy vegetation here was 5%. In site No. 2 under larches, it reached 25%. In total, 14 species of higher plants from 10 families, 4 ligneous and 10 grassy spe-

cies, grew in site No. 2. In site No. 3 with planted pines, 12 species of higher plants, 3 ligneous and 9 grassy, were revealed at a total projective grass cover of 25%. Approximately the same number of species was accounted for a variety of life forms of herbaceous plants, namely long- and short-rhizomatous plants, tap-root plants, tap-fibrous-root plants, sod perennial grasses. As per the Shannon index H and the inverse Simpson index 1/D (Table 2), the smallest diversity was characteristic of the spruce forest with dead soil cover.

2. Diversity of plant communities in various phytocenoses at sites of long-term restoration (quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2014)

Site No.	Shannon index H	Inverse Simpson index 1/D	Number of species	
			per site	total
1	1.3	2.1	11	
2	2.0	4.2	14	22
3	1.9	4.2	12	

N o t e. For description of sites by type of reclamation, see the section Materials and methods.

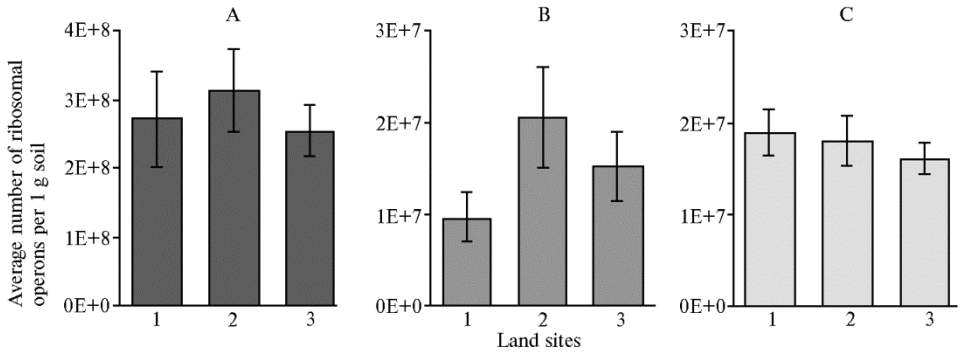

Fig. 2. qPCR-based quantitation of three groups of microorganisms in soil samples from various phytocenoses at sites of long-term restoration: A — bacteria, B — archaea, C — fungi (n = 4 for sites Nos. 1 and 2, n = 3 for site No. 3; 3 measurements for each repetition, M±SEM; quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016). For description of sites by type of reclamation, see the section Materials and methods.

qPCR analysis of abundance of three groups of microorganisms in three test sites revealed some differences in the number of ribosomal operons of bacteria and archaea (Fig. 2). Their counts turned out to be the largest in site No. 2 under larch, $(3.13\pm0.6)\times10^8$ and $(2.06\pm0.55)\times10^7$, respectively. For bacteria this indicator did not significantly differ from that in other sites, $(2.73\pm0.67)\times10^8$ for site No. 1 and $(2.55\pm0.37)\times10^8$ for site No. 3, whereas for archaea it was significantly ($p < 0.05$) higher than in two other sites, $(0.9\pm0.27)\times10^7$ in site No. 1 and $(1.52\pm0.39)\times10^7$ in site No. 3. The fungal abundance in the soil of different sites did not statistically significantly differ, $(1.88\pm0.26)\times10^7$ for No. 1, $(1.8\pm0.27)\times10^7$ for No. 2, and $(1.6\pm0.17)\times10^7$ for No. 3.

3. Diversity of soil microbiomes in various phytocenoses at sites of long-term restoration (M±SEM, quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016)

Site No.	Shannon index H	Faith's PD index	Chao 1	OTU number	Library coverage, %
1	9.3±0.1	171.1±9.7	2559.5±181.9	2163.8±165.5	84.5
2	9.4±0.1	169.6±10.5	2368.8±117.8	1978.8±176.6	83.5
3	9.3±0.1	188.3±10.1	2672.3±208.7	2322.3±176.8	86.9

N o t e. OTU — operational taxonomic unit; n = 4 for sites Nos. 1 and 2, n = 3 for site No. 3. Для участков №№ 1 и 2 n = 4, для участка № 3 n = 3. For description of sites by type of reclamation, see the section Materials and methods.

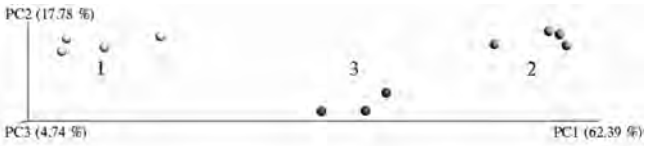

Fig. 3. Comparison of β-diversity of soil metagenomes with PCoA (Principal Components Analysis of the matrix of pairwise comparisons constructed by the weighted_unifrac method) **in various phytocenoses at sites of long-term restoration:** 1, 2, 3 — sites Nos. 1, 2 and 3 (quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016). Axes are the projections of multidimensional data with the indicated values of the explained variation (%), the number of circles corresponds to the number of repetitions. For description of sites by type of reclamation, see the section Materials and methods.

For analysis of the soil microbiome of each site, DNA was isolated in 4 replicates. However, after sequencing for site No. 3 (pine), only 3 replicates were processed. Further, all measurements for sites No. 1 and No. 2 were in 4 replicates, and for section No. 3 in 3 replicates.

The indices of α-diversity and the number of microbiomes in all sites were comparable (Table 3). Figure 3 shows a β-diversity graph representing a three-dimensional projection of data from the matrix of pairwise distances between the microbiomes of soil samples constructed by weighted_unifrac method. It is seen that microbiomes clearly differentiate by the site of soil sampling.

Analysis of the diversity of the dominant bacterial phyla, *Proteobacetria*, *Actinobacteria*, and *Acidobacteria*, several patterns appeared (Table 4). Actinobacteria were the most diverse in microbiomes formed under dead cover in spruce forest (site No. 1), and the number of OTUs of this group of bacteria significantly decreased as spruce (site No. 1) > pine (site No. 3) > larch (site No. 2).

4. α-Diversity among bacterial groups of soil microbiomes in various phytocenoses at sites of long-term restoration (*M*±SEM, quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016)

Site No.	Shannon index H	Faith's PD index	Chao 1	OTU number
		Actinobacteria		
1	7.2±0.1[a]	23.9±0.5[a]	558.2±8.0[a]	311.9±5.1[a]
2	6.9±0.1[b]	21.9±0.4[a]	379.5±23.4[a]	272.1±5.8[a]
3	6.8±0.1[a]	23.1±0.7[b]	454.0±10.4[a]	291.5±6.4[b]
		Proteobacteria		
1	7.2±0.1[a]	43.5±1.3[ab]	702.0±37.6[a]	528.9±18.2[a]
2	7.7±0.1[a]	48.1±0.8[a]	742.6±31.7[b]	564.5±14.5[b]
3	7.3±0.2[b]	49.7±1.0[b]	850.6±44.3[c]	595.1±14.3[a]
		Acidobacteria		
1	6.4±0.1[a]	18.6±0.3[a]	343.7±16.9[a]	275.7±5.6[a]
2	6.0±0.3[b]	19.3±0.2[b]	389.0±16.8[b]	275.9±5.3[b]
3	6.3±0.4[c]	20.0±0.2[a]	455.3±21.4[a]	293.7±15.0[c]

Note. OTU — operational taxonomic unit; $n = 4$ for sites Nos. 1 and 2, $n = 3$ for site No. 3. For description of sites by type of reclamation, see the section Materials and methods. In the columns within the taxonomic group, the same letters (a, b, c) indicate statistically significant difference ($p < 0.05$).

Diversity of proteobacteria, on the contrary, was the highest in larch plantings (site No. 2) and the lowest in the spruce phytocenosis (site No. 1). It is also worth noting the reliable ($p < 0.05$) minimum phylogenetic diversity of proteobacteria (Faith's index) in the microbiome of the spruce forest, the reliable maximum ($p < 0.05$) of the number of detected OTUs under planted pine trees (site No. 3) and the maximum Shannon index for site No. 2 (larch) compared to site No. 1. The latter may indicate the most even appearance of various proteobacteria in the phytocenosis under the pine canopy. For members of *Acidobacteria* phylum, the general trend of increasing diversity and abundance under pine also remained (site No. 3). There were significant ($p < 0.05$) maxima of the phylogenetic diversity of *Acidobacteria* (Faith's index and Chao 1 index), characterizing the maximum richness of this group in site No. 3 compared to

site No. 1.

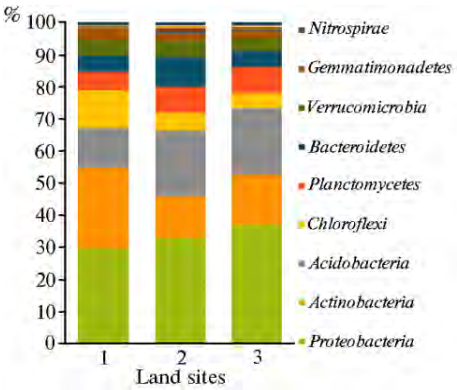

Fig. 4. Taxonomic profile of soil microbiome (procariote phyla) in various phytocenoses at sites of long-term restoration (quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016). For each site, replicates are combined. Phyla representing > 1% are indicated. For description of sites by type of reclamation, see the section Materials and methods.

Analysis of the normalized 16S rRNA gene amplicon libraries for bacteria and archaea identified 220,910 nucleotide sequences. The dominant phyla were *Proteobacteria* (29.6-37%), *Actinobacteria* (12.8-25.2%) and *Acidobacteria* (12.3-20.8%), followed by members of phyla *Chloroflexi* (4.6-11.8%), *Planctomycetes* (5.7-8.2 %), *Bacteroidetes* (4.9-9.2%), *Verrucomicrobia* (3.9-5.0%), *Gemmatimonadetes* (2.1-3.8%), *Nitrospirae* (0.2-1.0%); the rest made less than 1% (Fig. 4). It is worth noting the relatively low representation (0.1%) and low diversity of archaea from the groups *Crenarchaeota* and [*Parvarchaeota*]. In general, the quantitative composition of phyla in different sites was similar, but it should be mentioned that in site No. 1 the abundance of *Actinobacteria* and *Chloroflexi* groups was higher compared to others, and the *Acidobacteria* were lower.

5. Heatmap of the major OUT percentage in soil microbiomes in various phytocenoses at sites of long-term restoration (quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016)

Site No.			OTU	Phylum	Class	Order	Family	Genus
1	2	3						
			52697	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	–
			9881*	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Rhodoplanes*
			39913	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Rhodoplanes*
			39246	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Rhodoplanes*
			36288*	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	–
			2794	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*
			54700	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	–
			44729	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	–
			9787	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Delftia*
			18503*	*Proteobacteria*	*Betaproteobacteria*	IS-44	–	–
			18585	*Proteobacteria*	*Betaproteobacteria*	SC-I-84	–	–
			7276*	*Proteobacteria*	*Betaproteobacteria*	SC-I-84	–	–
			29307	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	–
			33418	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
			15473*	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Sinobacteraceae*	–
			35545	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	–
			46306	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Agromyces*
			18325*	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	–
			32722*	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Promicromonosporaceae*	*Promicromonospora*
			37122*	*Actinobacteria*	MB-A2-108	0319-7L14	–	–
			45581	*Actinobacteria*	*Thermoleophilia*	*Gaiellales*	*Gaiellaceae*	–
			6537*	*Actinobacteria*	*Thermoleophilia*	*Gaiellales*	*Gaiellaceae*	–
			36062*	*Actinobacteria*	*Thermoleophilia*	*Solirubrobacterales*	–	–
			17100	*Actinobacteria*	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteraceae*	–
			6252*	*Acidobacteria*	*[Chloracidobacteria]*	RB41	–	–
			9076*	*Acidobacteria*	*[Chloracidobacteria]*	RB41	–	–
			20749*	*Acidobacteria*	*[Chloracidobacteria]*	RB41	Ellin6075	–
			22604*	*Acidobacteria*	*[Chloracidobacteria]*	RB41	Ellin6075	–
			54563	*Acidobacteria*	*Acidobacteria*-6	iii1-15	–	–
			52756	*Acidobacteria*	*Acidobacteria*-6	iii1-15	–	–

Continued Table 5

37100	*Acidobacteria*	*Acidobacteria*-6	iii1-15	–	–
43517	*Acidobacteria*	*Acidobacteria*-6	iii1-15	mb2424	–
4897	*Chloroflexi*	*Chloroflexi*	*[Roseiflexales]*	*[Kouleothrixaceae]*	–
32350	*Chloroflexi*	*Chloroflexi*	*[Roseiflexales]*	*[Kouleothrixaceae]*	–
57060*	*Chloroflexi*	*Chloroflexi*	*[Roseiflexales]*	*[Kouleothrixaceae]*	–
23485	*Chloroflexi*	Ellin6529	–	–	–
46192*	*Chloroflexi*	Ellin6529	–	–	–
11945	*Bacteroidetes*	*[Saprospirae]*	*[Saprospirales]*	*Chitinophagaceae*	–
54731	*Bacteroidetes*	*[Saprospirae]*	*[Saprospirales]*	*Saprospiraceae*	–
49979	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Adhaeribacter*
9879	*Verrucomicrobia*	*[Spartobacteria]*	*[Chthoniobacterales]*	*[Chthoniobacteraceae]*	*Chthoniobacter*
24870	*Verrucomicrobia*	*[Spartobacteria]*	*[Chthoniobacterales]*	*[Chthoniobacteraceae]*	DA101
20531	*Gemmatimonadetes*	Gemm-1	–	–	–
27129*	*Gemmatimonadetes*	*Gemmatimonadetes*	*N1423WL*	–	–
28624	*Planctomycetes*	*Phycisphaerae*	*CCM11a*	–	–

N o t e. OTU — operational taxonomic unit. OTU with a representation of > 0.5% in at least one of the options is considered major. For each OTU, the phylum and the lowest permitted taxon are indicated. An asterisk (*) marks OTUs, which representations differ more than 5-fold between at least a pair of sites ($p < 0.05$). Dashes mean that OTU is not identified at the marked taxon level. For description of sites by type of reclamation, see the section Materials and methods.

In total, among all replicates of the 16S rRNA gene libraries for the three studied sites, 5760 OTUs were identified. Of these, only 45 OTUs accounted for more than 0.5% of all sequences for at least one library (Table 5). They covered all 9 of the most represented bacterial phyla. The OTU taxonomic positions were determined mainly up to the levels of genera and families, but in some cases only up to classes and orders. Most OTUs belonged to the phylum *Proteobacteria* associated with the rhizosphere. The genus *Pseudomonas* which prevailed in soils under the pine trees was the most numerous. OTUs of the *Rhizobiales* and *Sphingomonadales* orders, closely associated with plants in the soil, are also common. For one of the OTUs of the *Rhizobiaceae* family, the excess in the soil under spruces was 5-fold compared to pines. Representatives of *Sinobacteraceae* are 5 times more abundant under pines than under spruces. The genus *Rhodoplanes* is associated with the decomposition of wood in forest soils [45]. OTUs belonging to these orders were more often found in site No. 1, which was recultivated earlier than the others.

The genus *Delftia*, on the contrary, was more characteristic of younger replantozems in sites No. 2 and No. 3. Its appearance is associated with active bioremediation processes in soils [46, 47]. The second most numerous OTU group was the *Actinobacteria* phylum (classes *Actinobacteria*, *Thermoleophillia* and MB-A2-108). The most numerous OTUs belonged to the families *Micrococcaceae* and *Microbacteriaceae*, whose members, including the genus *Agromyces* of biodestructors [48]. Moreover, in the soils under spruces and larches *Micrococcaceae* significantly (10-fold) increased compared to the soil under pines. In site No. 1, the count of *Promicromonospora actinobacteria* was significantly higher (20 times, $p < 0.05$) than in sites No. 2 and No. 3. Among acidobacteria, the *Chloracidobacteria* class dominated in all sites; the second largest class was *Acidobacteria*-6. OTUs of the order RB41, dominant in sites No. 2 and No. 3, are often described in metagenomes of disturbed and stressed soils [49-51]. Among the minor phyla, OTUs of classes *Chloroflexi* and Ellin6529, which dominate in site No. 1, should be noted. Bacteria of Ellin6529 class can fix atmospheric nitrogen [52]. Also, in site No. 1, bacteria of the genus DA101 from *Verrucomicrobia* phylum prevailed.

In our study, we did not find any correlation between the soil microbiome composition and soil-forming processes, in particular, the decomposition of organic residues in the soil and their mineralization. The Mantel test with

weighted_unifrac-derived community distance matrices did not reveal a statistically significant correlation of the composition of the microbiome with pH or the main biogenic soil elements (C, N, P, and K) (Table 6).

6. Correlation of soil microbiome β-diversity with main chemical soil parameters in various phytocenoses at sites of long-term restoration (quarry No. 3 of PO Phosphorite, Kingisepp phosphorite deposit, Leningrad Province, Kingisepp District, 2016))

Parameter	*r*	p-value
pH	0.99064	0.1638
Concentration:		
C	–0.02333	1.0000
N_{total}	–0.67638	0.6840
N (NH_4)	0.76821	0.4973
N (NO_3)	0.48269	0.8311
P (P_2O_5)	0.08478	1.0000
K (K_2O)	0.51337	0.6560

N o t e. All replicates for all sites are combined. For description of sites by type of reclamation, see the section Materials and methods.

In the phosphate rock quarry in Kingisepp district, forest communities were formed that are typical of the taiga zone of the European Russia. The organomineral horizon of site No. 1 was more acidic which may be due to the introduction of a substrate with an admixture of peat at reclamation. As per the data on basal and substrate-induced respiration, the microbiological activity of soils is low, which may indicate a reduced sustainability of microbial communities.

In all site we revealed a rather low α-diversity. This may be a consequence of the low intensity of soil formation on crushed-stone dumps, which is in good agreement with the earlier data for podzolic and sod-podzolic soils of the Northwest region [53]. The indices of α-diversity were similar for microbiomes of all sites, while in site No. 3 (under the pine), a significant increase in the species and phylogenetic diversity of the microbial community was recorded. But despite the fact that for the sites the indices of α-diversity were generally comparable, their values significantly changed for the three dominant phyla in the soil microbiomes formed under different plant communities (see Table 4). So, for site No. 1, a relative increase in diversity, as well as the proportion of actinobacteria, was characteristic, while the diversity and abundance of proteobacteria was maximum in pine plantings (site No. 3). Actinobacteria and proteobacteria often turn out to be antagonists in a soil microbiome [54, 55]. An interesting fact is that the soil in site No. 1 had the lowest pH value, although actinobacteria in most cases inhabit soils of neutral or closer to alkaline pH [56]. In the sites, most likely, it is the composition of the litter and the availability of organic matter for microbial decomposition, which, apparently, differs due to the uneven application of peat in different areas, have a more decisive effect on the structure of the microbiome than pH. It is likely that the soil formed under the spruce canopy differed from soils of other sites in organic matter of low accessibility for the microbial community. This may explain the relative increase in the diversity and abundance of actinobacteria, most of which are hydrolytic with oligotrophic nutrition type [57]. In site No. 3, on the contrary, the diversity of higher plants and their life forms is the greatest. This suggests a wider spectrum and a wider variety of organic substances, including readily available ones, capable, in turn, to affect the microbiome, in particular, the copiotrophic microorganisms, to which the majority of *Proteobacteria* phylum members belongs [58].

Taxonomic analysis we performed for the reclaimed soils showed the formation of a microbial community characteristic of acidified sod-podzolic and

podzolic soils of the Northwest [53] with prevalence of acidobacteria of orders RB41, iii1-15, as well as actinobacteria (*Gaiellaceae* and *Solirubrobacteriaceae* families). The appearance of these groups, as well as a significant proportion of bacteria from the phyla *Bacteroidetes*, *Verrucomicrobia*, and *Planctomycetes* (their members are mainly oligotrophs) [59, 60], indicate the completeness of the carbon cycle and stabilization of the microbial community composition. However, the observed stage, apparently, should be considered as pre-climax, since in all analyzed communities there are many copiotrophic bacteria, in particular, pseudomonads (on average 5.5%) [61]. Noteworthy, proteobacteria (family *Comamonadaceae*, genus *Delftia*) are detected in all sites. In the literature, this microorganism is often found in communities of contaminated and technologically disturbed soils, as well as in activated sludge microbiomes [62]. Apparently, this bacterium takes part in the soil formation in the reclaimed sites under examination.

So, the study we undertook in the reclamation areas of Leningrad Province (Kingisepp phosphorite deposit) did not reveal a relationship between the structure of the soil microbiome and the physicochemical parameters of soils. In addition, the diversity of soil microbiome does not correlate with soil concentration of the main mineral elements. The factor of the plant dominant also does not have a significant effect on the microbiome structure. It is possible that in the case under discussion, other environmental factors are of decisive importance for microorganisms. The supposed factors may be different composition of plant waste and litter and, as a consequence, the diversity of organic substrates. Analysis of the α-diversity and taxonomic composition of microbiomes at three test sites suggests a pre-climax stage of the soil cover development. At the same time, the soil parameters and the high content of mineral phosphorus turn out to be too strong abiotic factors that impede the complete restoration of the microbiome of disturbed soils.

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MICROBIOLOGICAL AND ECOPHYSIOLOGICAL PARAMETERS OF SOD-PODZOLIC SOIL UPON LONG-TERM APPLICATION OF STRAW AND MINERAL FERTILIZERS, THE CORRELATION WITH THE YIELD

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Abstract

In modern agriculture, top priority is given to requirements for environmentally friendly application of fertilizers, providing for the intensification of use of biological sources of soil fertility recovery, primarily bioresources of farming ecosystems. One of significant, easily renewable biological resources is field residues of agricultural crops, which, according to many researchers, are the key to sustainable crop production and biosphere preservation. In this regard, one of main requirements for biologically based resource-saving methods and agrotechnologies is the returning of afterharvesting residues back into the soil without alienating the ones from the field, which ensures the enhancement of organic carbon input, improvement of biological status of soils, and their fertility and productivity, in general. A number of domestic and foreign papers prove the plant residues (PR), the structure of which consists of over 80 % of straw of cereals and leguminous crops, to be important for the preservation of favorable microbiological state of the soil. However, there is uncertainty in the qualitative and quantitative evaluations of the effect of the straw on the soil microbial community since the PR burial that may have both positive and negative consequences, which is often noted when introducing straw with a wide C to N ratio. Experimental data available in the scientific literature were obtained mainly when conducting research in laboratory and short-term field experiments with single use of straw as a fertilizer. The data of long field experiments with the repeated introduction of straw in crop rotation are useful for a more complete understanding of the straw effect on the microbial community and the use of this knowledge for the development of effective methods for managing the plant residues. The purpose of this study was to assess the effect of long-term use of straw of cereals and leguminous crops and mineral fertilizers (MF), separately and in combination, on the biological status of sod-podzolic sandy-loam soil. The indicators characterizing the composition, structure and metabolic activity of the microbial community of sod-podzolic soil were determined at the end of the 4th rotation of the 5-course row-crop rotation in a long field experiment: microbial biomass ($C_{mic.}$), microbial number and ratio of ecotrophic groups of microorganisms (ETGM), basal respiration (BR), and ecophysiological coefficients. It has been established that the return of afterharvesting residues in combination with medium doses of MF provides a balanced supply of nutrients and organic carbon to the microbial community and contributes to the reduction of mineralization processes and to the accumulation of C_{mic}. The microbial biomass closely correlated with the content of total ($r = 0.94$, $p < 0.05$) and easily decomposable carbon ($r = 0.89$, $p < 0.05$) and nitrogen ($r = 0.95$, $p < 0.05$) in the soil, and the yield of annual grasses closely correlated with the most part of indicators being determined in the experiment.

Keywords: microbial community, microbial biomass, soil, straw, mineral fertilizers

Soil fertility and its rational use are largely determined by the intensity and orientation of the biochemical activity of microorganisms involved in the destruction and mineralization of soil organic matter (SOM). Soil microbial biomass (MB) is a very sensitive pool of SOM, and any change in the management of agroecosystems affects the structure and content of MB much faster

than the content of total organic matter [1-3].

Agricultural land use, including regular mechanical tillage, the use of various agro-chemicals, the annual alienation of most of the phytomass, often leads to a decrease in MB reserves, significant violations in its structure and functions compared to natural ecosystems [4, 5]. The unfavorable effect of agricultural production on the microbiological quality of the soil is becoming a global challenge [6]. A number of studies [7, 8] show the negative effect of mineral fertilizers (MF), primarily nitrogen fertilizers, on MB, which is explained by acidification, as well as a decrease in bioavailable carbon reserves.

Organic matter is the main limiting factor for microbial activity in arable soils [7, 9, 10]. Methods which ensure high exogenous carbon entry into the soil, including by returning post-harvest plant residues (PR) as a trophic and energy source for microorganisms [11], contribute to MB conservation and growth. Thus, the return of straw provided more favorable microbiological and biochemical soil conditions compared to its removal [12, 13]. In the scientific literature there also data on the absence or negative effect of grain straw on biological activity and effective soil fertility [14]. However, little is known about the consequences of prolonged use of straw for the microbial community.

This paper is our first report on long-term (20 years) application of straw in combination with mineral fertilizers. As a result, the biological state of sod-podzolic soil have become more favorable, approaching natural fallow lands in a number of microbiological parameters with prevailing microbial and soil carbon accumulation and yield increase.

The aim of the study was to assess the effect of repeated application of straw of cereals and leguminous crops in grain crop rotation (separately and in combination with mineral fertilizers) on biological parameters f sod-podzolic soil and the yield of annual grasses.

Materials and methods. The studies were carried out in a long-term, since 1996, field experiment of the All-Russian Research Institute of Organic Fertilizers and Peat (sod-podzolic soil, crop rotation — winter wheat, grain lupine, potatoes, barley, annual grasses lupine + oats). The experimental design included the following treatment: no fertilizers (control), MF (annual average dose $N_{54}P_{51}K_{57}$ applied before sowing), straw of winter wheat, lupine, and barley (3 t/ha each applied in the fall after harvesting grain and leguminous crops), straw of winter wheat, lupine and barley (3 t/ha each). In total, for four crop rotation, 36 t/ha straw was incorporated into the arable layer of the soil. The experiment was arranged in 2-fold temporal repetition and 3-fold spatial repetition (42-47 m^2 plots).

Soil samples were collected at the end of the 4th crop rotation (2016-2017) with a reed drill (0-20 cm layer) 2 weeks after harvesting the green mass of annual grasses (lupine of Crystal variety, oats of Amigo variety). Sets of 20-30 individual samples from each plot were combined into single sample. At the same time, samples of natural bare fallow soils and grass fallow soils (0-20 cm layer) located close to arable soils and being their genetic analogues were collected.

The microbiological and ecophysiological parameters most common in domestic and foreign studies were determined [7-9, 15]. The number of proteolytic microorganisms was counted on meat peptone agar (MPA), amylolytic microorganisms on starch-ammonia agar (SAA), oligotrophs on minimal agar (MA), oligonitrophic microorganisms on Ashby nitrogen-free medium [16].

The carbon of microbial biomass ($C_{mic.}$) was quantitated by the rehydration method. Basal respiration (BR, mg $C\text{-}CO_2 \cdot kg^{-1}$ dry soil $\cdot day^{-1}$) was deter-

mined by the rate of soil CO_2 release for 24 hours of lab incubation at 22 °C and 60% humidity. For CO_2 absorption, 0.5 N NaOH was used followed by titration using 0.2 N HCl in the presence of phenolphthalein. The coefficients of oligotrophicity ($C_{oligt.}$) and oligonitrophilicity ($C_{olign.}$) were calculated as the ratio of the number of microorganisms on MA to MPA (MA/MPA), and on Ashby to MPA (Ashby/MPA), respectively [16]. Microbial factor was calculated as $C_{mic.}/C_{org.}$ (%). Microbial metabolic coefficient (specific respiration of MB) qCO_2 was calculated as the ratio $BR/C_{mic.}$ (mg $C\text{-}CO_2 \cdot kg^{-1} \cdot C_{mic.}^{-1} \cdot h^{-1}$). We determined total organic carbon ($C_{org.}$) by wet "burning" method ($K_2Cr_2O_7 + H_2SO_4$, 20 min in a drying cabinet at 160 °C) with a photometry (λ = 590 nm); cold water extractable organic carbon (C_w) in extracts after shaking for 3 minutes (soil:water = 1:20); hot water extractable organic carbon (C_{hw}) after 1-hour boiling soil suspension (soil:water = 1:5) in a water bath (80 °C) followed by C determination in filtered extracts after evaporation of aliquots (similar to the $C_{org.}$ analysis). Total nitrogen ($N_{tot.}$) was measured by the indophenol greens photometric method; hydrolyzable alkaline (N_{ha}) as per Kornfield's method using 1.0 N NaOH for hydrolysis of the soil in Conway plates (48 h at 28 °C), 2% boric acid solution to absorb the released NH_3 and titration using 0.02 N H_2SO_4. Assays were performed in 3-6-fold repetition; microbiological tests were carried out with fresh samples on the day of soil sampling, chemical analyses were performed with air-dry samples and re-calculated for dry soil.

The green mass yield of annual herbs (lupine-oat mixture) was recorded for each plot (16.8 m^2 area) and expressed as dry matter.

Statistical processing was performed with STAT.EXE software (Pryanishnikov All-Russian Research Institute of Fertilizers and Soil Science, Moscow) by the method of univariate analysis of variance ($p = 0.05$) with calculation of mean (*M*) and standard deviations (±SD) using the Fisher *F*-test and LSD to assess the significance of the difference between the means. Correlation coefficients were calculated with Statistica 6.0 software (StatSoft, Inc., USA) ($p \le 0.05$).

Results. Plant residues are a complex nutrient and energy substrate, the main source of bioavailable carbon for heterotrophic microorganisms [9, 11]. Nutrients entering the soil from MF, especially nitrogen, are also important for their active life

During the experiment, 14.4 t/ha of organic carbon (about 90% of the initial reserves) were delivered to the arable layer in treatment with straw. At the end of the 4th rotation, $C_{org.}$ in treatment MF + straw supplement was 0.567%, which was 1.22 and 1.13 times higher ($p < 0.05$) compared to the control and MF application, respectively (Table 1).

Easily degradable, rapidly transformed components of organic matter are the most essential to maintain microbial activity [17]. Water-soluble organic compounds, i.e. simple amino acids, monosugars, partially fulvic and humic acids, are the most available carbon source for soil microorganisms [18]. The concentration of C_w in the control and upon MF application was very low, 47.1 and 49.7 mg/kg soil, respectively (comparable to fallow). The highest C_w level was characteristic of arable layer with straw incorporation, 59.4 mg/kg, which was 1.26 and 1.20 times higher ($p < 0.05$) than without fertilizers and with MF, respectively. Highest C_{hw} value (188 mg/kg) was noted under MF + straw (1.20 and 1.08 times higher than without fertilizing and with MF), but the differences between the variants were not significant ($p > 0.05$). C_w and C_{hw} of the natural fallow amounted to 122 and 363 mg/kg, which was 2.05 and 1.93 times higher ($p < 0.05$) even compared to the maximum values for these fractions in the crop

rotation soil (see Table 1).

1. Biological and agrochemical estimates of soz-podzolic soil upon multiple straw incorporation and application of $N_{54}P_{51}K_{57}$ mineral fertilizers (MF) **in grain-tillage crop rotation** (*M*±SD; Vladimir Province, Sudogodskii District, 2016-2017)

Option	$C_{org.}$	$C_{mic.}$	C_w	C_{hw}	BR	$C_{mic}/C_{org.}$	qCO_2
No fertilizers	0.463±0.030^a	317±42^a	47.1±0.9^a	157±9^a	3.7±0.8^a	6.85	0.48
MF	0.501±0.029^b	346±38^a	49.7±0.9^a	174±17^a	7.5±2.1^{bc}	6.91	0.90
MF + straw	0.567±0.009^c	465±42^b	52.3±4.1^{ab}	188±9^a	8.1±1.4^{bc}	8.20	0.73
Straw	0.524±0.037^{bc}	383±29^a	59.4±5.6^b	182±29^a	6.7±1.2^b	7.31	0.73
Bare fallow	0.400±0.008^d	187±12^c	48.1±3.1^a	116±11^b	4.2±0.1^{ab}	4.68	0.94
Grass fallow	0.778±0.052^e	609±68^d	122±8.8^c	363±26^c	9.2±1.5^c	7.83	0.63

N o t e. $C_{org.}$ — total carbon, %; $C_{mic.}$ — microbial carbon, mg/kg soil; C_w — cold water extractable organic carbon, mg/kg soil; C_{hw} — hot water extractable organic carbon, mg/kg soil; BR — basal respiration, mg C-CO_2/kg soil; $C_{mic.}/C_{org.}$ — microbial factor, %; qCO_2 — microbial metabolic coefficient, mg C-$CO_2 \cdot kg^{-1} \cdot C_{mic.}^{-1} \cdot h^{-1}$. For the experiment design description, see Materials and methods section. Identical letter indices indicate the absence of a statistically significant difference ($p > 0.05$).

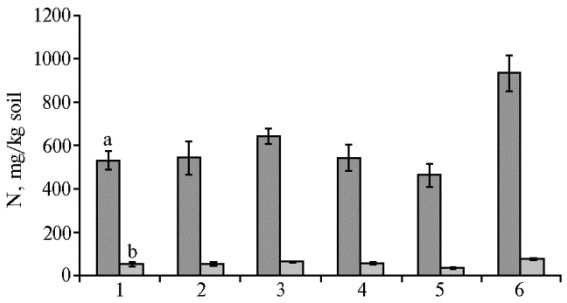

Fig. 1. Total (a) **and hydrolyzable alkaline** (b) **nitrogen in sod-podzolic soil upon multiple straw incorporation and application of $N_{54}P_{51}K_{57}$ mineral fertilizers in grain-row crop rotation:** 1 — no fertilizers, 2 — $N_{54}P_{51}K_{57}$, 3 — $N_{54}P_{51}K_{57}$ + straw, 4 — straw, 5 — bare fallow, 6 — grass fallow (*M*±SD; Vladimir Province, Sudogodskii District, 2016-2017). For the experiment design description, see Materials and methods section.

Nitrogen is a factors significantly limiting soil microbial activity. The $N_{tot.}$ was the highest upon long-term incorporation of straw in combination with MF (640 mg/kg), though the differences with other options were not significant ($p > 0.05$). In this treatment, the concentration of easily hydrolyzable nitrogen N_{ha} was significantly higher and reached 62.5 mg/kg which was 1.2 times as much as the same indicator without fertilizers and with MF. However, in the soil of grass fallow these indicators were significantly ($p < 0.05$) higher, 930 mg/kg soil for $N_{tot.}$ and 75.6 mg/kg soil for N_{ha} (Fig. 1).

Soil microbial biomass is a living component of SOM, mainly due to archaea, bacteria and eukaryotes, with the exception of roots and animals [19]. MB measurement is widely used as a relatively simple means of assessing the impact of environmental and anthropogenic changes on soil microorganisms [20]. As per meta-analysis of reported data (414 observations), the content of $C_{mic.}$ in arable soils of various types ranged from 43 to 2155 mg/kg with an average value of 365 mg/kg soil [7]. As per our observation, the microbial carbon content was minimal in long-fallowing soil (187 mg/kg) and maximal in grass fallow (609 mg/kg). In our field experiment, $C_{mic.}$ value was the maximum (465 mg/kg) upon application of MF + straw, being higher than in the other variants ($p < 0.05$). This is consistent with the results of studies [3, 10, 21, 22] reporting an increase in $C_{mic.}$ when incorporating straw in the soil. The annual use of MF did not contribute to the growth of $C_{mic.}$, providing only 9% growth of the value compared to control (see Table 1). Generalization of data array by Kallenbach et al. [7] showed that most studies also revealed a negative effect of MF on microbial communities, which is explained by their acidifying effect and the deficit of available carbon sources after an initial increase in mineralization activity. Different reports note that in post-agrogenic soils of natural fallow status the conditions (absence of mechanical treatments, constant soil

cover with vegetation, and accumulation of easily decomposable organic matter) contribute to an increase in MB to a greater extent than in arable soils [23]. Overall increase in $C_{mic.}$ in soils is regarded as an absolutely positive fact, increasing their biological status [20].

$C_{mic.}/C_{org.}$ ratio is an indicator of the availability of soil organic carbon [24]. Moreover, a high proportion of $C_{mic.}$ indicates the fixing of organic carbon in MB and favorable conditions for the microbial community functioning. Low microbial factor indicates a decrease in the supply of microflora with available organic matter. $C_{mic.}/C_{org.}$ ratio in soils most often varies between 1-5% [7]; some authors give values up to 10% and higher [17]. In our research, the value of $C_{mic.}/C_{org.}$ ranged from 6.85 (control) to 8.20% (MF + straw), which indicates an improvement in SOM quality, accessibility to microflora and a large accumulation of carbon in MB during long-term use of straw in combination with MF. Kallenbach et al. [25] and Miltner et al. [26] also emphasize that the carbon accumulated in MB makes an important contribution to the humus soil pool formation.

Microbial respiration is an integral parameter that quantitates the overall metabolic activity of heterotrophic soil microflora. Microbial CO_2 production, determined in laboratory conditions, should be evaluated as the potential activity of MB under optimal temperature and humidity [15]. The lowest BR value (3.7 mg C-CO_2/kg soil) we recorded where crops in the rotation were grown without fertilizers, using only basic soil resources. As per the BR values, straw used separately had a significant effect on microbial metabolism, increasing the respiratory rate 1.8-fold ($p < 0.05$). Mineralization processes proceeded most actively and at approximately the same rate in the MF and MF + straw treatment, where the BR rate was 2.03 and 2.20 times higher ($p < 0.05$) than in the control (see Table 1).

Metabolic coefficient qCO_2 which characterizes the efficiency of substrate use by microorganisms is an informative indicator of the ecophysiological state of the soil microbial community [27]. For arable soils, qCO_2 most often varies from 0.5 to 2.0 mg C-$CO_2 \cdot kg^{-1} \cdot C_{miv.}^{-1} \cdot h^{-1}$ [28]. High values indicate a very significant need for energy sources or low efficiency of the use of organic substrate. The qCO_2 value in our experiments depended on the land use conditions and applied fertilizers and varied from 0.48 mg C-$CO_2 \cdot kg^{-1} \cdot C_{miv.}^{-1} \cdot h^{-1}$ in the control to 0.90 C-$CO_2 \cdot kg^{-1} \cdot C_{mic.}^{-1} \cdot h^{-1}$ upon MF and 0.94 C-$CO_2 \cdot kg^{-1} \cdot C_{mic.}^{-1} \cdot h^{-1}$ in the bare fallow soil (see Table 1). According to Zhang et al. [22], ecologically, the high microbial metabolic coefficient qCO_2 reflects the need for heterotrophs in carbon, and if the carbon that is lost by respiration is not replenished in the soil, MB decreases. When straw was used in combination with mineral fertilizers, qCO_2 value was 1.5 times higher than in the control, but 1.2 times lower ($p < 0.05$) compared to MF alone. That is, the high supply of microorganisms with nutrients during the annual introduction of MF without a sufficient amount of easily decomposable organic matter did not contribute to efficient consumption of carbon, which was lost to a greater extent during respiration than on biomass synthesis, leading, in turn, to a reduced pool of the soil carbon. The low qCO_2 value without fertilizers may indicate better consumption of carbon by soil biota when root residues of crop in the rotation are only sources of nutrition and energy. The high qCO_2 value (0.94 mg C-$CO_2 \cdot kg^{-1} \cdot C_{mic.}^{-1} \cdot h^{-1}$) in the soil of fallow, indicating a loss of carbon, can be explained by stimulation of the respiratory activity of the soil due to regular mechanical treatments.

The abundance and structure of the community of soil microorganisms are of paramount importance for understanding microbiological processes in the soil [4] and can be characterized by the number and ratio of microorganisms

from various physiological or ecological trophic groups. It must be borne in mind that, given the size of a particular group, it is possible to conclude only about the physiological potential of soil microorganisms, but not about its performance in natural conditions [29]. The highest abundance of ecotrophic groups of microorganisms was characteristic of MF and MF + straw variants. However, lower $C_{oligt.}$ (0.95 and 1.09) and $C_{olign.}$ (1.33 and 1.21), when straw was repeatedly used together with MF, testified to the relative dominance of copyotrophic microflora under these conditions as compared to oligotrophic microflora (Table 2).

2. Abundance and structure of microbial community in sod-podzolic soil upon multiple straw incorporation and application of $N_{54}P_{51}K_{57}$ mineral fertilizers (MF) **in grain-row crop rotation** (*M*±SD; Vladimir Province, Sudogodskii District, 2016-2017)

Option	Number of microorganisms, mln CFU/g soil				$C_{oligt.}$	$C_{olign.}$
	proteolytic	amylolytic	oligotrophic	oligonitrophilic		
No fertilizers	6.0 ± 0.9^{ab}	7.9 ± 2.8^{a}	8.6 ± 2.2^{a}	9.0 ± 2.1^{a}	1.43	1.50
MF	14.1 ± 4.1^{c}	19.6 ± 3.6^{b}	18.4 ± 2.9^{b}	21.7 ± 4.1^{b}	1.31	1.54
MF + straw	12.3 ± 2.2^{bc}	14.1 ± 1.5^{c}	11.7 ± 2.8^{a}	16.4 ± 3.9^{ab}	0.95	1.33
Straw	11.3 ± 3.2^{bc}	13.3 ± 2.2^{c}	12.3 ± 2.5^{a}	13.7 ± 3.2^{a}	1.09	1.21
Bare fallow	2.8 ± 0.2^{a}	3.1 ± 0.7^{a}	3.8 ± 0.9^{c}	2.2 ± 1.1^{c}	1.36	0.79
Grass fallow	7.9 ± 2.5^{b}	12.8 ± 4.2^{c}	10.8 ± 3.4^{a}	12.5 ± 4.8^{a}	1.37	1.58

N o t e. $C_{oligt.}$ and $C_{olign.}$ — coefficients of oligotrophicity and oligonitrophilicity. For the experiment design description, see Materials and methods section. Identical letter indices indicate the absence of a statistically significant difference ($p > 0.05$).

According to Kolodyazhny et al. [30], the incorporation of straw into sod-podzolic soil contributed to a 2.5-fold increase in the number of copyotrophs (ammonifying and amylolytic microorganisms) and a 1.5-fold decrease of oligotrophs and pedotrophs. Higher indices of the absolute and relative abundance of oligotrophs under annual introduction of MF indirectly confirm the prevalence of humus destruction. In general, in most cases, the presence of crop residues in the soil had a beneficial effect on microbial communities [6, 7, 13].

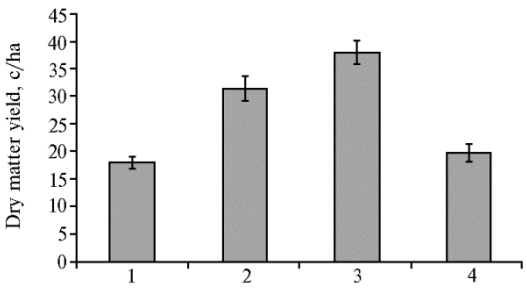

Fig. 2. Yield of annual grasses (lupine + oats) on sod-podzolic soil upon multiple straw incorporation and application of $N_{54}P_{51}K_{57}$ mineral fertilizers in grain-row crop rotation: 1 — no fertilizers, 2 — $N_{54}P_{51}K_{57}$, 3 — $N_{54}P_{51}K_{57}$ + sraw, 4 — straw (*M*±SD; Vladimir Province, Sudogodskii District, 2016-2017). For the experiment design description, see Materials and methods section.

Long-term incorporation of straw in combination with MF provided the maximum yield of leguminous grass herbs, 38.2 c/ha dry matter, which was significantly higher ($p < 0.05$) compared to not only the non-fertilized variant, but also to the MF application (Fig. 2). This is consistent with research data [21, 31] which also established a positive effect of straw on soil fertility and crop productivity.

In this work, we evaluated a quantitative relationship of biological indicators with the total C and N content, as well as with easily metabolized fractions. The yield of annual grasses significantly ($p < 0.05$) and positively correlated with $C_{org.}$, $N_{tot.}$, C_{hw}, $C_{mic.}$, $C_{mic.}/C_{org.}$ and also with the counts of microorganisms on MPA ($r = 0.88$-0.93). A rather close, but insignificant negative correlation was revealed between productivity and $C_{oligt.}$ ($r = -0.60$). Microbial carbon significantly ($p < 0.05$) and closely correlated with $C_{org.}$ ($r = 0.94$), $N_{tot.}$ ($r = 0.95$), and C_{hw} ($r = 0.89$), as it is often observed in long-term field experiments. In a long

field experiment on loamy loess soils of Germany in grain-cultivated crop rotation with mineral and organic fertilizers a significant positive correlation was found between $C_{mic.}$ and $C_{org.}$, and also between $C_{mic.}$ and C_{hw}, $r = 0.71$ and $r = 0.65$, respectively [32].

Thus, the long-term (12-fold over a 20-year period of field test) application of straw and mineral fertilizers in five-course crop rotation significantly affected microbial activity in sod-podzolic soil. With the annual application of mineral fertilizers (MF) and the removal of all post-harvest residues, the high functional activity of microorganisms was maintained, as evidenced by the high values of basal respiration and the microbial metabolic coefficient qCO_2. However, with regard to low of $C_{mic.}$, $C_{mic.}/C_{org.}$, $C_{org.}$ values, destructive mineralization prevails that is not favorable for carbon accumulation in the microbial biomass and in the soil. Under such conditions, microorganisms use the organic substrate less efficiently, spending C on respiration and increasing the loss of carbon from the soil. The introduction of straw without MF in the crop rotation does not have a negative effect on the biological properties and yield. This is possibly due to alternation of cereal straw and lupine straw, which differ in biochemical composition, during crop rotation. Regular incorporation of straw in combination with medium doses of mineral fertilizers maintains a balanced supply of microbial community with nutrients and carbon, increasing carbon accumulation in microbial biomass. The $C_{mic.}/C_{org.}$ value in this variant was comparable with that for natural fallow soil. Our findings confirm the importance of regular application of grain straw and leguminous straw for favorable C regime in soil. This contributes to providing soil microbial community with biologically available organic matter and an increase in soil microbial biomass.

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INFLUENCE OF BIOCHAR ON THE TAXONOMIC COMPOSITION AND STRUCTURE OF PROKARYOTIC COMMUNITIES IN AGRO SODDY-PODZOLIC SOIL

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Abstract

Currently the scientific literature is actively discussing the feasibility of biochar using in agriculture. Biochar is one of the new types of organic meliorants. It is obtained by pyrolysis of wood or other plant waste in an inert atmosphere converting carbon compounds to a stable state. Its use is recommended to increase the soils biological activity and the agricultural crops productivity and it is actively implement in agricultural technologies of foreign countries. However many aspects of the biochar influence on the agrocenoses properties and state have been poorly studied. There is information about both positive and negative processes occuring in soils under biochar. The main concern is the data on the biochar influence on humus mineralization, since dehumification can lead to loss of soil fertility and ecological stability. This is especially important for soddy-podzolic soils characterized by a low humus content and a weak degree of humification. Such soils initially have low ecological stability and are quite vulnerable to human impact. Therefore using soddy-podzolic soils in agriculture considerable attention should be paid to the microbiological and biochemical transformation of soil organic matter. Studies on the biochar influence on the soil microbiota composition and state in our country are isolated, and for soddy-podzolic soils of the North-Western region of Russia are conducted for the first time. The aim of this work was to assess the influence of biochar on the features of the agro soddy-podzolic soils prokaryotic community. The research was carried out in incubation experiments on well-cultivated agro soddy-podzolic sandy loam soil of the Leningrad region. The biochar was produced by fast pyrolysis of birch and aspen wood at 550 °C. Its concentration in the experiment was 1%. The incubation time was 7 and 90 days. The repeat of the variants of the experiment was 3-fold. The content of total organic carbon and nitrogen, mineral forms of nitrogen, and soil suspension pH were determined in soil samples using methods commonly used in agrochemical practice. The method of sequencing the variable region of the 16S rRNA gene was used to determine the taxonomic composition of soil prokaryotes. The sequence clustering and the taxonomic identification of the taxonomic units (OTU, Operational Taxonomic Unit) were performed using the QIIME program. The diversity and evenness of agro soddy-podzolic soil bacterial communities was estimated by the OTU number and Shannon index. Statistical data processing was performed using IBM SPSS Statistics, Version 25 (IBM, USA). The reliability of the differences between the variants was measured by one-factor variance analysis using the Duncan's or Student-Newman-Keuls test at $p < 0.05$. The intensification of the processes of mineralization of soil organic matter occurred under biochar. The humus content in the soil decreased from 4.41 to 3.83 % which is 11 % more than in the control during the observation period. Activation of organic matter transformation processes took place simultaneously with changes in the state of the prokaryotic community. This community was represented mainly by the bacteria phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Chloroflexi*, *Acido-*

bacteria, *Planctomycetes*, *Verrucomicrobia*. The biochar application was accompanied by an increase in the total bacteria diversity and by the abundance of phyla *Planctomycetes*, *Verrucomicrobia*, *Proteobacteria* and FBP representatives but also by a decrease in the abundance of phyla *Actinobacteria*, *Nitrospirae, Firmicutes* and *Fibrobacteres* representatives. In general biochar application leads to increase in the oligotrophs abundance and to reduce the copiotrophic proportion in prokaryotic community. The inhibition of bacteria from phylum *Nitrospirae* can be explained by a decrease in the concentration of available ammonium. In addition biochar application leads to increase in the abundance of some taxa containing active hydrolytics of natural polymers (orders *Myxococcales* and *Xanthomonadales*, class *Sphingobacteriia* etc.). Most likely this is due to the intensification of the difficult mobilizing organic substances transformation in agro soddy-podzolic soils under biochar.

Keywords: sequencing, structure of microbocenosis, bacteria, prokaryotes, biochar, soddy-podzolic soil, fertility

During recent decades, the search and development of new types of fertilizers and ameliorants which can maintain a deficiency-free balance of soil humus and ensure high soil fertility are being actively carried out. Biochar is a promising organogenic ameliorant that is actively used in world agricultural production [1-3]. Biochar is produced by pyrolysis of wood or other plant matter in an inert atmosphere. The use of biochar is an opportunity to solve a number of environmental challenges, i.e. the utilization of organic waste [4, 5], carbon sequestration [6], restoration of disturbed soils [5-7] and increase in crop productivity [4, 8, 9].

The composition and properties of various types of biochar, in particular, the feedstock for its production [10], the physical and physicochemical characteristics [11, 12], have been studied in sufficient detail. Its influence on the agronomically valuable properties of some types of soils was studied, including elements of plant mineral nutrition [4, 9, 13], the reaction of soil medium, and water-physical properties [5, 6, 12, 13]. However, the mechanisms responsible for such effects are not fully understood. Changes in microbocenosis in soils at different doses of biochar application [14-16], incubation periods [15, 17] and different biochar quality [18] are described. But this did not reveal clear patterns of modulation of the microbiota profile under the influence of biochar. Information about the associated changes in soil biota and soil organic matter, the most important components of agrocenoses which largely determine their fertility and environmental sustainability, is limited and often contradictory [19-22]. Thus, there is evidence that under the influence of biochar, the microbial biomass and biological activity of soils increase, and dehumification processes begin [21-23]. According to other data, biochar does not stimulate soil microorganisms, so the intensity of mineralization decreases [24-27]. The transformation of humus composition when biochar is incorporated into soils is also poorly investigated. Our recent studies have shown that the introduction of biochar into sod-podzolic soil leads to both negative and positive modifications of humic substances [28, 29]. Intensive mineralization of humus (with losses up to 20%) is accompanied by an increase in the proportion of its stabilized forms which, in turn, increases the stability of humus as a whole [28].

Soil prokaryotes are actively involved in the transformation of organic matter. Therefore, studying the effect of biochar on the prokaryotic community of agro soddy-podzolic soil is of interest both for understanding the fundamental processes of soil fertility formation and for farming practice.

This paper is the first to report about changes in the profile of prokaryotic community of agro soddy-podzolic soil, accompanying its dehumification under the influence of biochar, i.e. an increase in the abundance of oligotrophs and a number of taxa, representatives of which are involved in the decomposition of complex natural biopolymers, and a decrease in the proportion of copiotrophs. In addition, here we present data on the metagenome composition of the sod-podzolic soil microbiota in the northwestern part of European Russia, infor-

mation on which for this region is still extremely limited.

The purpose of the present study was to assess the effect of biochar on parameters of prokaryotic communities of well-cultivated agro soddy-podzolic sandy loam soil of the Leningrad Province.

Materials and methods. Samples of high-humus agro soddy-podzolic sandy loam soil (Menkovsky branch of the Agrophysical Institute, Leningrad Province, Gatchinsky District) were taken from the arable horizon (0-20 cm) in June 2017. Biochar was obtained by rapid pyrolysis from birch and aspen wood at 550 °C. A detailed description of the soil and biochar is given earlier [28, 29].

A short-term incubation experiment was performed at room temperature (20-22 °C). The soil weight in a pot was 300 g dry matter, the content of biochar was 0% (control) and 1.0%. Soil moisture throughout the experiment remained equal to 60% of the total moisture capacity. Soil samples were analyzed on day 7 and day 90, with a 3-fold repetition per variant.

Agrochemical parameters of the soil were assessed by standard methods [30]: pH potentiometrically, soil organic carbon according to Tyurin, organic nitrogen by Tyurin's microchromic method, nitrates with disulfophenolic acid, ammonium with Nessler reagent.

DNA was extracted from 0.25 g portions of soil samples and purified with the NucleoSpin® Soil kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufacturer's instructions.

Universal PCR primers to 16S rRNA marker gene variable region V4, F515/R806 (5′-GTGCCAGCMGCCGCGGTAA-3′/5′-GGACTACVSGGGTATCTAAT-3′) modified to contain adapters and unique barcodes were used to construct amplicon libraries (Illumina, Inc., USA). PCR (a T100 Thermal Cycler, Bio-Rad Laboratories, Inc., USA) was carried out in a 15 μl reaction mixture containing Q5® High-Fidelity DNA Polymerase (0.5-1.0 units) and 1× Q5 Reaction Buffer (New England BioLabs Inc., UK), 5 pM of forward and reverse primers, 10 ng of matrix DNA and 2 nM of each dNTP (Thermo Fisher, Inc., USA). The template DNA denaturation (94 °C, 1 min) was followed by 35 cycles of elongation (94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s) with final elongation at 72 °C for 3 min. The library preparation and sequencing was performed on the Illumina MiSeq platform using MiSeq ReagentKit v3 (600 cycles) with 2×300 nt paired-end reads (Illumina, Inc., USA) as per the Illumina MiSeq Reagent Kit Preparation Guide for metagenome sequencing of 16S amplicon libraries. Illumina software (Illumina, Inc., USA) and Trimmomatic software packages [31], fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), fastq-join (https://github.com/brwnj/fastq-join) and QIIME software [32] were used for read demultiplexing, removing alien sequences, assessing read quality, trimming, joining paired-end reads, checking for chimeras and homopolymers. Clustering and taxonomic identification of resultant operational taxonomic units (OTU) were performed with QIIME program.

The diversity and evenness of the bacterial communities of agro soddy-podzolic soil was estimated by the number of OTUs (an analogue of species richness) and the Shannon index $H = \Sigma p_i \ln(p_i)$, where p_i is the fraction of the ith species in the community [33].

Statistical processing was performed with the IBM SPSS Statistics, Version 25 software (IBM, USA). The significance of differences was estimated by one-way analysis of variance with the Duncan's test or Student-Newman-Keuls (SNK) test at $p < 0.05$ ($n = 3$). The tables show mean values (M) with a confidence interval at $p < 0.05$ ($t_{0.05} \times$ SEM).

Results. Carbon in biochar, despite the high content, is mainly inert, dif-

ficult to oxidize, and labile C fractions in biochar are small. In the organic matter of biochar, the carbon, determined by wet oxidation method makes less than 1.5%, and the amount of water-soluble carbon compounds is negligible (0.008%) [27]. Bio-char did not affect the content of soil organic carbon (humus), since it is a highly stabilized compound.

Tested agro soddy-podzolic soil was slightly acidic (pH_{H2O} 6.7), with high humus (4.45%), mineral and organic nitrogen content (Table 1).

1. Mineralization of organic matter in agro sod-podzolic soil upon incubation with biochar ($n = 3$, $M \pm t_{0.05} \times$ SEM)

Variant	$C_{org.}$, %	N forms			$C_{org.}:N_{org.}$
		$N_{org.}$, %	$N\text{-}NO_3$, mg/kg	$N\text{-}NH_4$, mg/kg	
		Day 0			
Soil (initial sample)	2.56 ± 0.05^{c}	0.22 ± 0.00^{c}	11.2 ± 0.7^{a}	14.8 ± 0.6^{e}	11.6 ± 0.3^{a}
		Day 7			
Control	2.50 ± 0.05^{bc}	0.21 ± 0.00^{c}	18.9 ± 1.5^{b}	10.4 ± 0.6^{d}	11.9 ± 0.4^{a}
Biochar	2.46 ± 0.04^{b}	0.21 ± 0.01^{c}	17.0 ± 0.6^{b}	9.3 ± 0.04^{c}	11.7 ± 0.3^{a}
		Day 90			
Control	2.48 ± 0.04^{b}	0.19 ± 0.01^{b}	17.9 ± 0.6^{b}	6.8 ± 0.05^{b}	13.1 ± 0.2^{b}
Biochar	2.22 ± 0.04^{a}	0.16 ± 0.01^{a}	12.6 ± 0.7^{a}	4.7 ± 0.07^{a}	13.9 ± 0.4^{c}

Note. Different letters denote mean values that are statistically significantly different from each other at $p < 0.05$ (belonging to different subsets).

During incubation, the soil organic matter mineralization intensified under the influence of biochar. By the end of the experiment (90 days), the content of organic forms of nitrogen and carbon in soil with biochar was lower 16 and 10%, respectively, than in the control. Moreover, the loss of humus in the soil with biochar during the incubation period was 0.57%. These data are consistent with the results of our previous studies on the effect of biochar on the humus content and its fractional group composition in agro soddy-podzolic sandy loam soils [21]. Thus, our short-term (up to 90 days) experiments found out that incubation of sandy-loam agro soddy-podzolic soils with biochar can cause their dehumification.

During the incubation, the mineralization of N-organic compounds was higher compared to C-organic compounds, leading to a significant ($p < 0.05$) increase in C/N upon biochar application, i.e., the soil organic matter humification decreased. In the control soil, the mineralization of organic matter was accompanied by a decrease in the content of ammonium nitrogen and an increase in nitrate nitrogen, i.e., an increase in nitrification occurred. By the end of the experiment (90 days), $N\text{-}NH_4$ concentration decreased sharper in the soil with biochar compared to the control, whereas $N\text{-}NO_3$ content remained unchanged compared to its starting value. The $N\text{-}NO_3$ level in the soil with biochar was higher and comparable to the control only at the beginning of incubation (after 7 days). Apparently, during longer composting, ammonium cations generated due to mineralization of organic matter can be absorbed by negatively charged functional groups of biochar [6]. As a result, the amount of extractable ammonium forms in the soil declined, and nitrification was limited by the amount of substrate (ammonium) available to nitrifying bacteria.

The changes in the soil organic matter occurred together with taxonomic profile modification soil prokaryotes.

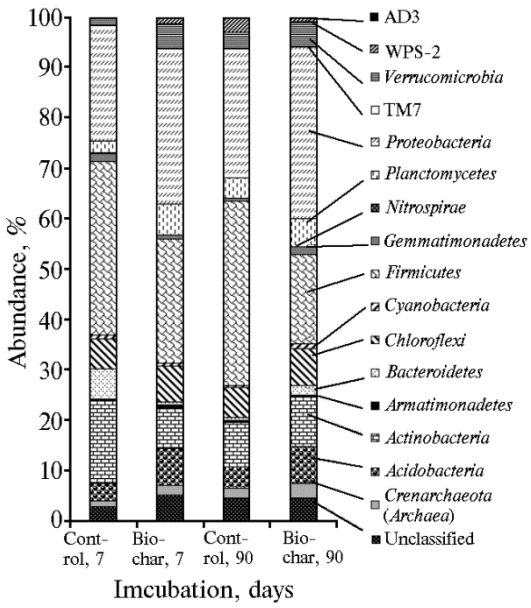
Clustering sequences of the 16S rRNA gene variable region revealed 6392 OTUs for a taxonomic analysis (Table 2). The soil bacterial communities without and with biochar did not significant differed in the OTU number. The Shannon index testified that there were no differences between samples at the beginning of the experiment, but by the end the H value slightly (by 7.5%) but significantly ($p < 0.05$) increased with biochar, which could indicate an increase in

diversity and greater uniformity of the community under the influence of biochar.

2. Operational taxonomic units (OTUs) **and evenness of bacterial community in agro sod-podzolic soil upon incubation with biochar** ($n = 3$, $M \pm t_{0.05} \times$ SEM)

Indicator	Control, 7 days	Biochar, 7 days	Control, 90 days	Biochar, 90 days
OUT number	3211±1085[a]	3710±285[a]	3335±467[a]	3498±185[a]
Shannon index H	9.49±0.35[ab]	9.84±0.45[ab]	9.23±0.20[b]	9.91±0.11[a]

N o t e. Different letters denote mean values that are statistically significantly different from each other at $p < 0.05$ (belonging to different subsets).



Richness of prokaryotic phyla (%) in agro sod-podzolic soil during incubation with biochar ($n = 3$). The figure shows phyla the abundance of which exceeds 0.1% at least in one test variant.

The ratio of *Archaea* and *Bacteria* did not change significantly when biochar was used. There was only a tendency to an increase in the abundance of archaea in the soil with biochar (Fig.). A metagenomic analysis of the prokaryotic community showed that *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Planctomycetes*, *Verrucomicrobia* dominated in all samples (and also *Bacteroidetes* in the soil composted for 7 days without biochar) (see Fig.). The predominance of these phyla is generally characteristic of soddy-podzolic soils, except the phylum *Planctomycetes* which is usually not abundant in these soils [34, 35].

After 7-day incubation, the abundance of *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes* phyla significantly increased ($p < 0.05$), and *Actinobacteria*, *Nitrospirae*, *Fibrobacteres*, *Gemmatimonadetes* phyla decreased in soil with biochar compared to soil without it. We found significant changes in the abundance of a number of classes, orders, families and genera, mainly related to the phyla *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Proteobacteria*, and *Firmicutes* (Table 3). After 90-day incubation, the differences in the bacterial community profiles in the soil with and without biochar were much weaker, and changes in abundance were noted mainly for other taxa. Thus, counts of *Proteobacteria*, *Gemmatimonadetes*, and FBP phyla increased, but the abundance of phylum *Firmicutes* decreased.

Currently, no unified regularity has been established for the effect of biochar on the abundance of bacterial phyla in different soils. For example, the decrease in the abundance of *Actinobacteria* phyla we observed as a result of the biochar introduction is consistent with data from other authors [16]. At the same time, some works described an increase in the abundance of this phylum in the presence of biochar [14]. There is also no consensus on the effect of biochar application on the abundance of phyla *Firmicutes* [14, 18, 36], *Verrucomicrobia* [14], *Planctomycetes* [14, 18], *Proteobacteria* [14, 37]. Probably, modification of the prokariotic community is associated indirectly with soil physicochemical changes (pH, sorption ability, and cation exchange capacity) when biochar is applied [4, 37].

3. Richness of prokaryotic taxa (%) in agro sod-podzolic soil during incubation with biochar ($n = 3$)

Taxon	Control, 7 days	Biochar, 7 days	Control, 90 days	Biochar, 90 days
		Phylum		
Actinobacteria	16.29^{a}	8.20^{b}	9.10^{b}	9.90^{b}
Firmicutes	34.35^{a}	24.41^{ab}	29.20^{a}	17.50^{b}
FBP	0.05^{b}	0.05^{b}	0.03^{b}	0. 12^{a}
Fibrobacteres	0.03^{b}	0^{a}	0.01^{b}	0.13^{ab}
Gemmatimonadetes	1.6^{a}	0.84^{b}	0.62^{b}	1.64^{a}
Nitrospirae	0.15^{a}	0.03^{b}	0.01^{b}	0.01^{b}
Planctomycetes	2.28^{b}	5.98^{a}	4.20^{ab}	5.56^{ab}
Proteobacteria	22.81^{b}	30.83^{ab}	26.22^{b}	33.83^{a}
Verrucomicrobia	1.50^{b}	5.00^{a}	3.35^{ab}	4.88^{ab}
		Class		
Sva0725	1.11^{a}	0.18^{b}	0.04^{b}	0.07^{b}
TM1	0.001^{b}	0.01^{a}	0.02^{a}	0.02^{a}
[Chloracidobacteria]	0.90^{a}	0.07^{b}	0.06^{b}	0.02^{b}
DA052	0.007^{b}	1.01^{a}	0.44^{a}	0.91^{a}
Actinobacteria	11.02^{a}	3.29^{c}	3.91^{bc}	4.90^{b}
Nitriliruptoria	0.01^{a}	0^{b}	0^{b}	0^{b}
Sphingobacteriia	1.93^{a}	0.17^{b}	0.15^{b}	0.72^{a}
Chloroflexi	0.85^{a}	0.03^{b}	0.02^{b}	0.01^{b}
Ktedonobacteria	0.07^{b}	4.08^{a}	4.13^{a}	4.5^{a}
TK10	0.41^{c}	1.72^{a}	0.96^{bc}	1.45^{b}
Bacilli	33.38^{a}	21.00^{b}	24.93^{b}	15.05^{c}
Planctomycetia	1.68^{b}	5.08^{a}	3.61^{ab}	4.41^{ab}
		Order		
RB41	0.90^{a}	0.07^{b}	0.0^{b}	0.02^{b}
Actinomycetales	11.01^{a}	3.29^{c}	3.90^{bc}	4.90^{b}
KD8-87	0.5^{a}	0^{b}	0^{b}	0^{b}
Thermogemmatisporales	0.004^{c}	1.04^{b}	1.05^{ab}	2.21^{a}
AKYG1722	0.35^{a}	0.001^{b}	0.004^{b}	0.004^{b}
JG30-KF-AS9	0.01^{b}	0.97^{a}	1.22^{a}	0.56^{a}
Nitrospirales	0.15^{b}	0.03^{a}	0.02^{a}	0.01^{a}
Rhizobiales	4.51^{b}	9.72^{a}	7.90^{ab}	11.79^{a}
Rhodospirillales	1.34^{b}	3.67^{a}	3.18^{ab}	4.37^{a}
Ellin6067	0.06^{b}	0.43^{a}	0.30^{ab}	0.19^{ab}
Myxococcales	2.12^{ab}	2.68^{ab}	1.80^{b}	2.97^{a}
Spirobacillales	0.31^{a}	0.02^{b}	0.04^{b}	0.04^{b}
Pseudomonadales	0.44^{a}	0.04^{b}	0.04^{ab}	0.02^{ab}
Xanthomonadales	2.23^{b}	2.35^{b}	2.02^{b}	3.23^{a}
		Family		
Actinospicaceae	0.001^{b}	0.04^{a}	0.04^{a}	0.04^{a}
Dermabacteraceae	0.05^{a}	0^{b}	0^{b}	0^{b}
Dermacoccaceae	0^{b}	0.01^{b}	0.13^{a}	0.02^{b}
Intrasporangiaceae	0.29^{a}	0.06^{b}	0.04^{b}	0.04^{b}
Microbacteriaceae	1.86^{a}	0.16^{b}	0.43^{b}	0.14^{b}
Nakamurellaceae	0.07^{a}	0.05^{a}	0.03^{b}	0.07^{a}
Propionibacteriaceae	0.02^{a}	0.001^{b}	0.004^{ab}	0.01^{ab}
Chthonomonadaceae	0.01^{b}	0.17^{a}	0.16^{ab}	0.09^{ab}
Gemmataceae	0.07^{b}	1.71^{a}	0.94^{ab}	1.47^{ab}
Burkholderiaceae	0.08^{c}	0.70^{b}	0.49^{b}	1.21^{a}
Comamonadaceae	0.88^{a}	0.23^{b}	0.22^{b}	0.26^{b}
Coxiellaceae	0.08^{b}	0.34^{a}	0.27^{ab}	0.14^{ab}
Xanthomonadaceae	1.80^{a}	0.56^{b}	1.04^{ab}	1.52^{a}
[Chthoniobacteraceae]	0.42^{b}	3.88^{ab}	2.72^{b}	3.96^{a}
		Genus		
Actinotalea	0.22^{a}	0^{b}	0.002^{b}	0.01^{b}
Brachybacterium	0.05^{a}	0^{b}	0^{b}	0^{b}
Agrococcus	0.11^{a}	0.001^{b}	0.004^{b}	0.003^{b}
Actinoplanes	0.15^{a}	0.02^{b}	0.02^{b}	0.05^{ab}
Catellatospora	0.03^{a}	0.001^{b}	0.01^{b}	0.004^{b}
Pontibacter	0.03^{a}	0.001^{b}	0^{b}	0^{b}
Dyadobacter	0.06^{a}	0.001^{b}	0^{b}	0.02^{ab}
Ammoniphilus	1.05^{a}	0.21^{b}	0.27^{b}	0.29^{b}
Coprococcus	0.03^{b}	0.03^{b}	0.10^{a}	0.02^{b}
Symbiobacterium	0.03^{a}	0.01^{b}	0.01^{b}	0.01^{b}
Gemmata	0.02^{b}	0.49^{a}	0.28^{ab}	0.54^{a}
Nostocoida	0^{b}	0.0006^{a}	0.0004^{a}	0.0004^{ab}
Asticcacaulis	0.02^{ab}	0.01^{b}	0.01^{b}	0.06^{a}
Devosia	1.17^{a}	0.10^{b}	0.16^{b}	0.26^{b}

Continued Table 3

Hyphomicrobium	0.11[b]	0.29[a]	0.20[ab]	0.35[a]
Sphingomonas	0.37[a]	0.01[b]	0.13[b]	0.25[a]
Burkholderia	0.02[c]	0.66[b]	0.47[b]	1.16[a]
Methylibium	0.05[a]	0.01[b]	0.02[b]	0.01[b]
Janthinobacterium	0.01[b]	0.02[ab]	0.03[a]	0.01[b]
Pseudomonas	0.07[a]	0.01[b]	0.01[b]	0.01[b]
Rhodanobacter	0.25[a]	0.06[b]	0.25[a]	0.26[ab]
Pedosphaera	0.001[b]	0.05[a]	0.02[ab]	0.03[ab]

N o t e. The table shows taxa for which there were statistically significant difference in abundance at least at least at one period during the experiment. Different letters denote mean values that are statistically significantly different from each other at $p < 0.05$ (belonging to different subsets).

In general, the abundance of oligotrophic bacteria significantly increased on day 7 upon biochar application, the phyla FBP and *Verrucomicrobia* more than 3-fold, and *Planctomycetes* more than 2-fold. Oligotrophic bacteria of the genera *Hyphomicrobium* increased significantly on day 7 of incubation, and *Asticcacaulis* on day 90. At the same time, there was a significant decrease ($p < 0.05$) in the counts of *Actinobacteria* (7 days of incubation) and *Firmicutes* (90 days of incubation). The members of these taxa are copyiotrophs or hydrolytics that can exist under conditions of high concentrations of nutrients. In particular, the abundance of the *Bacilli* class, which includes active soil copyiotrophs, decreased. Thus, biochar provides more favorable conditions for bacteria that are unable to survive at high concentration of available organic compounds. Bacteria that grow well on rich nutrient media (*Actinobacteria*, *Bacilli*), in contrast, were somewhat inhibited. Perhaps the reason is that readily available organic matter is adsorbed on the biochar, decreasing concentration of organic compounds in the soil solution, which creates advantages for more oligotrophic bacteria.

In addition, biochar caused a rapid and significant (5-fold) reduction in the abundance of bacteria of the phylum *Nitrospirae*, namely the nitrification bacteria of the genus *Nitrospira* mainly found in the studied soil, which is consistent with the suppression of nitrification observed in this soil when applying biochar (see Table 1).

A noticeable modification also occurred in the community of soil hydrolytics. The abundance of *Actinobacteria* phylum, the destructors of many difficultly-hydrolyzed organic substances, decreased after a short incubation with biochar as compared to the control, unlike active hydrolytics of difficultly-decomposable polymers from the order *Myxococcales* and cellulolytics of the *Sphingobacteriia* class which, on the contrary, became more abundant [38, 39]. In addition, there was a significant increase in the counts of members of the order *Xanthomonadales*, *Burkholderiaceae* family (almost 10-fold on day 7) and the genus *Asticcacaulis* which was recently shown to be involved in the decomposition of cellulose or products of its degradation [38]. Consequently, the addition of biochar elevates abundance of several bacterial groups responsible for the hydrolysis of difficultly-decomposable organic substances.

So, metagenomic analysis revealed that biochar incorporation into agro soddy-podzolic soil quickly changes the profile of the soil prokaryotic community. We did not observe its fundamental restructuring, nevertheless, the proportion of oligotrophic bacteria increased, copyiotrophs decreased, and in addition, the structure of hydrolytic bacteria community was modified. The latter, probably, explains intensive transformation of organic substances that we identified under the influence of biochar. Of course, profiling modification of microbial community accompanying such a transformation is of interest as a special case of changes in microbiocenosis during soil humification and dehumification. This issue should be be studied, since it is the activity of specific microorganisms that leads to the intensification of these processes and determines their balance which directly affects

soil fertility and sustainability of agrocenoses.

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BIOECOLOGICAL FEATURES OF CHERRY FLY *Rhagoletis cerasi* (L. 1758) (*Diptera*: *Tephritidae*) DEVELOPMENT IN THE CENTRAL NON-CHERNOZEM ZONE OF RUSSIA

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Abstract

Cherry fruit fly (*Rhagoletis cerasi* (L. 1758) (*Diptera*: *Tephritidae*)) in Central non-Chernozem zone of Russia appeared in the late 1990th—early 2000th, which is associated with global warming and the significant expansion in the acreage of forage plants in this region. Due to ecological plasticity the fly has quickly adapted to local conditions of habitat, and annually flies in a large number. This paper is the first to investigate dates of the flight start, dynamics and duration depending on weather conditions, the periods of egg laying, hatching and feeding of larvae, terms of cocoon formation in soil, and also the peculiarities of the pest diapause under the conditions of Central non-Chernozem Russia. Damage of fruits in cherry varieties of different time of ripening was also assessed. Based on these data, effective methods for monitoring the phytophage are proposed. The studies were carried out in 2016-2018 in the cherry plantations of the All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery (ARHIBAN, Moscow Province, Leninskii District, 55.47° n.l., 37.7° e.l., 124 m above sea level) on cherry (*Prunus cerasus* L.) varieties of early (Sania, Bagryanaya), middle (Molodezhnaya, Volochaevka) and late (Malinovka, Apukhtinskaya) ripening periods. To the end, the research is targeted to improve monitoring methods, increase the efficiency and environmental safety of protective measures. The harsh conditions of the northern horticultural zone had a significant impact on the bioecology of the northern phytophage population. Depending on the weather conditions, the beginning and duration of flying, egg laying, hatching of larvae, and pupation vary greatly from year to year. The beginning of flies in different years was observed when the sum of effective temperatures (SET) above 10 °C from 191.9 °C to 268.6 °C, with the difference in dates from 3 to 33 days; oviposition occurred at 227.4 °C to 285.1 °C, with the difference in dates to 27 days; hatching of larvae occurred at 290.3 °C to 347.1 °C, with the difference in dates from 2 to 24 days; the pupation occurred at 481.4 °C to 559.9 °C, with the difference in dates from 5 to 22 days. The feeding period of the larvae ranged from 18 to 26 days, and the imago flying period from 40 to 69 days. In the conditions if Central non-Chernozem Russia, both one-year and two-year diapauses *R. cerasi* are possible. In keeping larvae in special cages (under trees in the garden), after the first winter diapause, only 42.0 % of the overwintered individuals turned into adults. After the second winter diapause, those were 4.8 % of the initial number of larvae gone on diapause. In the third year, no flies were recorded. In two years, only 46.8 % of the individuals who went into diapause turned into adults, the rest died for various reasons. Also, the damage of cherry fruits varied from 7 % to 21 % for the early cultivars, from 38 % to 57 % for the middle-ripening cultivars, and from 61 % to 75 % for lthe ate-season maturing cultivars. Determination of *R. cerasi* phenophases based on calendar dates and phenophases of the host plant gives contradictory results in different years. Bilateral yellow glue traps combined with SET estimates and visual control of fruit ripening can improve *R. cerasi* monitoring to enable effective protective measures.

Keywords: insect, *Diptera*, *Rhagoletis cerasi* L., pest, bio-ecology, diapause, sum of effective temperatures

The European cherry fruit fly *Rhagoletis cerasi* L. (1758) (*Diptera*: *Tephritidae*) is the most economically significant phytophage of cherries and cherries

in Europe [1-3] and Asia [4], including Russia [5-7]. In recent years, its invasion into North America has been noted [7, 8], where this phytophage causes significant damage along with other species of the genus *Rhagoletis* (*R. cingulata*, *R. indifferens*, and *R. fausta*) [9-11]. Studies conducted in different countries and geographical areas revealed several races of *R. cerasi* [12-14], however, the southern and northern races are more often mentioned [15-17]. Females of the northern race, when crossed with males of the southern, are barren, females of the southern race, when mating with males of the north, give offspring [16-18]. A number of scientific papers emphasize that this phenomenon is due to the presence of an intracellular bacterial infection of Wolbachia [19-21], maternally inherited and spreading in the host populations by the mechanism of cytoplasmic incompatibility (CI), which leads to embryonic mortality during mating infected males with uninfected females or females with another strain of Wolbachia [20-22].

Possible differences in the bioecology of the southern and northern populations of *R. cerasi* are of particular importance. In recent decades, due to global warming, the advancement of cherry plantings in the northern regions, creation of new frost-resistant varieties and an increase in cultivation areas, the area of the phytophage in the northern gardening has significantly expanded. Cherry fly is an oligophage. In addition to *Prunus* sp. (*P. cerasus*, *P. avium*, *P. serotina*, *P. mahaleb*), *R. cerasi* damages *Lonicera* sp. (*L. xylosteum*, *L. tatarica*) [23, 24]. The bioecology of *R. cerasi* is closely depends on agroecological conditions, including air and soil temperatures at different periods, precipitation, as well as the phenology of the host plants, i.e. cultivar peculiarities and fruit quality, since the survival of the phytophage depends on synchronization, due to pupal diapause, of the appearance of adults with the presence of fruits [25, 26].

In the late 1990s and early 2000s, individuals of a cherry fly were quite rare found in the Central Non-Chernozem Zone (CNZ). Currently, in this region there is an annual massive damage by this pest. On untreated plantations, the damage to fruits is constantly increasing. Now it reaches up to 70-75%, and, according to reports, in the absence of protective measures, fruit damage can reach 100% [27, 28).

In the present work, we first described the phenology and high harmfulness of *R. cerasi* in the Central Non-Chernozem Zone. The start dates of the flight, the dynamics and the duration of the fly's flight, depending on weather conditions, egg laying, hatching and feeding of the larvae, their departure to the soil for coconing, and also the diapause of the pest in the CNZ conditions are determined. Based on the assessment of damage to cherry fruits, effective methods for monitoring the phytophage are proposed.

The aim of the work was to study the bioecological features and development of cherry flies on cherry varieties that ripen at different times in the Central Non-Chernozem Zone of Russia to improve monitoring and measures for more effective and environmentally friendly plant protection against the pest.

Materials and methods. The investigations were carried out in 2016-2018 in the cherry plantations of the All-Russian Institute of Horticulture and Nursery (ARHIBAN, Moscow Province, Leninsky District, 55.47° N, 37.7° E, 124 m above sea level) on early (Sania, Bagryanaya), mid-season (Molodezhnaya, Volochaevka) and late (Malinovka, Apukhtinskaya) cherry varieties in the lab plot and in the demonstration garden.

To detect the beginning and dynamics of *R. cerasi* flight, 5 days after the end of the early ripening cherry flowering periods, yellow double-sided adhesive traps were hung at 1.7 m height on trees from the southern and southeastern

sides on the outer projection of a tree crown in 10 sites (one tree per site), trap dimensions 20×10 cm. Before the first flying flies and after July 25 (until the end of the flight), traps were inspected daily, the rest of the time 2-3 times a weekю. Five days after the detection of flies in the traps, 300 fruits were picked daily (30 from each test site) and examined (a binocular microscope MBS-10, LZOS OJSC, Russia), and in 10 females were daily dissected to determine if off-laying eggs. Two days after the detection of eggs, the fruits were selected and examined daily in search of hatching larvae.

Two weeks after the detection of hatching larvae, daily, 1000 ripening or ripened fruits (100 per site), without picking from the trees, were examined with a magnifying glass for the presence of outlet holes of larvae. When revealing holes, the fruits were torn off, cut into two parts and examined under the binocular to establish damage typical for *R. cerasi*.

To determine the damage to different varieties, saline solution was used [3]. Before mass harvesting, 100 fruits were randomly collected from trees of each cultivar, the seeds were separated from the pulp, and the fruits were cut into several smaller parts and placed in saturated saline (350 g salt/l water). After 10 min, the emerged larvae were counted.

To establish the diapause period, during full ripeness, damaged fruits were collected three times with a 2 day interval, placed on dry sand layer 2 cm thick [29] and left in a room out of direct sunlight. After 1 week, the sand was sieved, 500 puparia were collected, placed in cages, 50 individuals per each, at a 5 cm depth. The cages were 20×30 cm boxes, lined on the inside with a plastic film filled with a mixture of soil from the garden, peat and sand (1:1:1, 10 cm thick layer). Cages were buried in the garden to the edges of the box at ground level on the south side of the trees. Cages were covered from three sides and from above (roof height 30 cm, rectangular shape) with a fine mesh net (meshes about 0.8 mm), and from the northern side a tight-fitting window was made of polyethylene. During the growing season, weeds growing in cages were cut off with secateurs through a window and removed (the plants were not torn out so as not to disturb pupae). Shortly before the flight period of *R. cerasi*, yellow glue traps were installed inside the cage through a window. Traps with flies adhering to them were removed every week after counting.

Mean sample value (M) and the standard deviation of the mean ($\pm\sigma$) were calculated from the dates of observations for each site, the significance of the differences was evaluated by the Fisher F-test. Statistical processing of tabular data was performed according to Dospekhov [30] using the Microsoft Excel software.

Results. Weather during the years of research (according to the weather station of Domodedovo Airport) are presented in Table 1.

Despite the significant influence of sharply changing weather conditions in the CNZ, *R. cerasi* individuals, due to environmental plasticity and adaptive capabilities, were able to synchronize their development with fruit formation and ripening of the host plant. The damage to varieties of different ripening periods varied over the years (Table 2), but ensured the survival of the *R. cerasi* population.

On the one hand, this was due to the possibility of prolonged diapause of the pupal stage in the soil (up to 10-11 months, with about 6 months at temperatures below 5-7 °C), and on the other, due to the ability of larger pupae to pause more than 1 years [29, 31, 32], as well as with an unequal response to the effects of prolonged low temperatures in different pupae (in some this process ends at the end of December, in others in March) [14]. According to the litera-

ture, in the post-diapause development of the pupa, stage II is reversible, i.e., a return is possible to stage I to remain in soil until the next season, which may be due to a response to environmental signals or to metabolic stimuli [33]. Soil composition may also affect the proportion of pupae diapausing for 1 year or more: in heavy clay soils, the percentage of additional diapausing pupae increases [34].

1. Air temperature and rainfall during observations of *Rhagoletis cerasi* L. development on cherry (*Prunus cerasus* L.) varieties (Domodedovo Airport weather station)

Parameter	Year	Month							
		III	IV	V	VI	VII	VIII	IX	X
Long-term average air temperature, °C		–1.4	5.8	13.2	17.0	19.2	17.0	11.3	5.1
Deviation from long-term average air temperature, °C	2016	+1.9	+2.3	+1.8	+1.2	+1.7	+2.5	–0.2	–1.1
	2017	+3.4	–0.5	–2.3	–2.5	–1.3	+1.8	+1.2	-0.5
	2018	+0.5	+1.4	+1.4	+0.3	+1.2	+2.3	+0.9	+0.6
Long-term average rainfall, mm		35	37	50	80	85	82	65	59
Deviation from long-term average rainfall, %	2016	114	92	126	76	144	204	91	90
	2017	126	214	168	175	124	83	43	115
	2018	83	108	142	87	108	54	89	66

In our experiments, out of 500 puparia that went to wintering in 2015, only 210 flies flew out the following year, which amounted to 42.0% of the original population. In 2017, 4.8% flew out, in 2018, that is, after the third season of diapause, no *R. cerasi* flies were found in cages. Over two seasons, 234 flies flew (46.8% of the puparia leaving for diapause in cages). This is a high percentage of flight compared to the natural conditions of diapause, where the percentage of flying adults varies greatly from year to year depending on environmental conditions and sometimes may not exceed 5-15% of the initial number of individuals leaving for wintering [35, 36].

2. Damage to cherry (*Prunus cerasus* L.) fruits caused by *Rhagoletis cerasi* L. fly during observation (Moscow Province, Leninsky District)

Variety	Ripening type	Damage, %			$M\pm\sigma$
		2016	2017	2018	
Sania	Early	17	8	15	13.3±4.7
Bagryanaya	early	21	7	12	13.3±7.1
Molodezhnaya	Mid-season	57	38	49	48.0±9.5
Volochaevka	Mid-season	53	41	43	45.7±6.4
Malinovka	Late	61	75	63	66.3±7.6
Apukhtinskaya	Late	64	73	68	68.3±4.5
$M\pm\sigma$		45.5±20.9	40.3±29.8	41.7±23.6	

Note. $F_{variety} > F_{01}$, $F_{year} < F_{05}$.

Depending on weather conditions, the beginning of the flight of flies fluctuated strongly over the years. As a rule, flies were found in traps a few days later than the actual departure date. The difference in dates ranged from 3 to 33 days (Table 3), and in terms of the sum of effective temperatures (SET) above 10 °C from 26.6 to 76.7 °C. At the same time, the choice of the threshold temperature for calculating the SET at a 10 °C level was more appropriate. In contrast to Southern and Western Europe, where winters are mild and spring temperatures rise evenly, winter is more severe in the central temperature zone, there are sharp jumps in air temperature in the spring. So, in 2016-2018, in March, the temperature ranged from –20 to +10 °C, in April — from –5 to +24 °C. A short-term increase in the average daily temperature above 7 °C could alternate with a sharp decrease (below 0 °C) for a long time, which did not contribute to the stable development of the pupa. Therefore, the calculation of SET above 5 or 7 °C, adopted in Southern, Western and Central Europe [1, 28, 37, 38], is less suitable in our zone and demonstrates a much larger fluctuation over the years (from 58.7

to 132.2 °C) in comparison to SET above 10 °C (from 26.6 to 76.7 °C) (Fig. 1). The calculation of SET above 10 °C for *R. cerasi* was carried out in other scientific studies conducted in Russia [14, 39].

3. Sums of effective temperatures (SET) **above 10 °C to start the *Rhagoletis cerasi* L. fly development on cherry plants during observation** (Moscow Province, Leninsky District)

Stage of development	2016		2017		2018	
	date	SET, °C	date	SET, °C	date	SET, °C
First flies in traps	06/03	191.9	07/06	268.6	06/06	218.5
Beginning of egg laying	06/14	227.4	07/11	285.1	06/14	280.8
Beginning of larvae hatching	06/20	290.3	07/14	310.2	06/22	347.1
Beginning of pupation	07/12	530.3	08/01	481.4	07/17	559.9

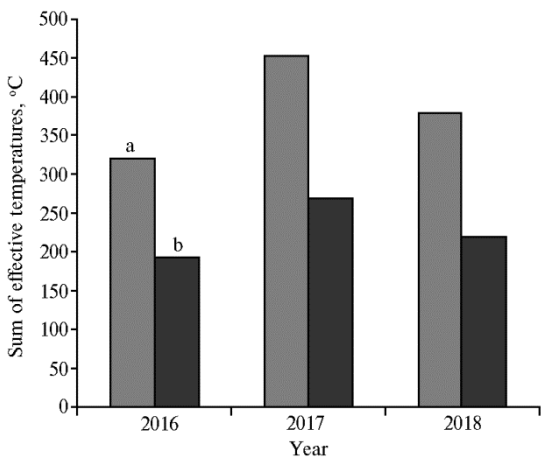

Fig. 1. Sums of the effective temperatures at appearance of *Rhagoletis cerasi* L. flies on the cherry (*Prunus cerasus* L.) at different thresholds during observation: a — above 7 °C, b — above 10 °C (Moscow Province, Leninsky District).

The period of additional feeding of flies before the laying of the first eggs (*R. cerasi* is a synovigenic species) took from 5 to 11 days. The difference in the SET accumulated by the beginning of the laying of eggs ranged from 4.3 to 57.7 °C, while the minimum difference in SET was between 2017 and 2018 and amounted to 27 days. The embryo development took from 3 to 8 days. The difference in the SET by the beginning of larvae hatching also varied significantly over the years, from 19.9 to 56.8 °C, the minimum value of this indicator was between 2016 and 2017 (24 days). The period of feeding of the first larvae leaving for pupation took from 18 to 26 days with a difference in SET to start the movement of larvae into the soil from 29.6 to 78.5 °C. The minimum SET was recorded in 2017 with the abnormally cold first half of the growing season.

The dynamics and intensity of the *R. cerasi* flight (Fig. 2) did not differ significantly in all 3 years of observation, although in 2016-2017 the number of flies increased evenly and, reaching a peak, also decreased evenly, and in 2018 there were three peaks of abundance, which was the result of a sharp fluctuation in air temperature. However, it should be emphasized that the maximum number of flies in all years of observations was noted in the middle of the flight period, which took from 8 to 14 days. The duration of the flight period of *R. cerasi* depended on weather conditions, food supply (including the duration of the fruit ripening period, thoroughness of harvesting) and ranged from 40 (2017) to 69 days (2018). In 2016, the flight lasted 55 days.

In the CNZ, the onset of *R. cerasi* release from winter diapause and the development of subsequent stages strongly fluctuated both in time and in SET (see Table 3). Since the microclimate of a particular location has a significant influence on the development of cherry flies, SET cannot guarantee the estimate of the exact time of the *R. cerasi* development individual phases. However, SET can be a good guideline in planning protective measures against the pest accounting changes in the fly activity as influenced by air temperature (at tempera-

tures below 15-16 °C, mating and eggs laying ceases, embryonic development lengthens) and rain intensity (heavy rain can lead to the death of adults) [4, 14, 39]. More reliable information on the flight start and dynamics can give, along with SET calculation, the use of yellow glue traps and visual observation of maturity and quality of fruits which also affect phytophage development. This fact was noted by other researchers [32, 40] and also confirmed by our data, indicating that on merry trees, the fly outbreaks and development are about 1 week ahead of those on cherries [7, 41, 42]. Note that more accurate timing of plant protection is especially important when using biologicals that have a significantly shorter action than chemicals [3].

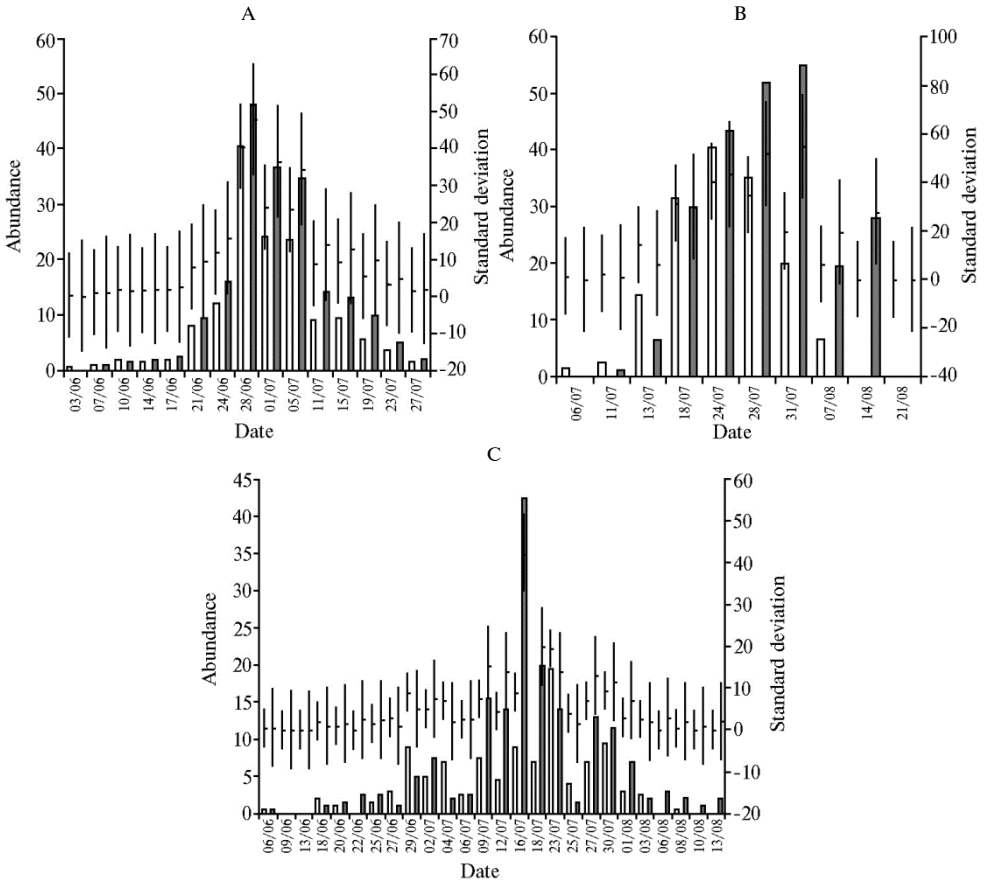

Fig. 2. Flight dynamics (abundance per trap on average) **of *Rhagoletis cerasi* L. fly on cherry (*Prunus cerasus* L.) plants in 2016** (A)**, 2017** (B)**, and 2018** (C) (light columns for the lab plot, dark columns for the demonstration garden) (Moscow Province, Leninsky District)**.**

In contrast to the southern regions, where early varieties, as a rule, go away from damage by a cherry fly, since the fruits ripen by the beginning of the mass egg laying by the phytophage [25, 39], in the CNZ the damage to these varieties, depending on the year conditions, reaches 7-21%. However, most of the larvae do not have time to finish feeding and are removed from the plantations together with the collected fruits. The highest damage is observed in varieties of late ripening (61-75%), but even in these varieties, as in mid-season varieties, some larvae do not have time to pupate before harvesting as the period when the flies exit diapause is uneven and quite extended. Despite the peak of flies in the garden occurs by the ripening fruits of late cherry variety (see Fig. 2), the flight, though much less intensive, continued even after the mass harvesting

was completed, within 2-5 days. It is known that the reproductive potential of females by this time sharply decreases, in the last days of flight they do not lay eggs [29], but improper harvesting allows larval to complete feeding significantly increases the number of wintering (diapausing) pests.

It should be noted that under the semi-artificial conditions that we created in our experiments, 53.2% puparia did not emerged as adults. This is significantly more than in lab growing with 5-25% death of pupae [33], but less than the indicators for natural habitats in other regions [35, 36].

Thus, in the climate conditions of the Central Non-Chernozem Zone (CNZ) of the Russian Federation, there is both a one-year and a two-year diapause of *Rhagoletis cerasi* L. The 89.7% flies emerged after a one-year diapause, 10.3% after a biennial diapause as per the total number of flies flown out. In the third year, no flies were detected in cages, which could be due to both bioecological features of their northern population and the influence of biotic (accumulation of predators, parasites, and pathogens in cages) and an abiotic stress factors. In the CNZ, *R. cerasi* damages cherry varieties of all ripening types, 7-21% fruits in early varieties, 38-57% in mid-season varieties, and 61-75% in late varieties. The emergence of adults after winter diapause fluctuates strongly both in dates (from 3 to 33 days) and in the sum of effective temperatures (SET from 191.9 to 268.6 °C). The tendency for other stages of pest development is the same, i.e. within 27 days (from 227.4 to 285.1 °C) to start laying eggs, from 2 to 24 days (from 290.3 to 347.1 °C) to start hatching of larvae, and from 5 to 20 days (from 481.4 to 559.9 °C) to start the larvae to pupate. The flight period of adults during our observation took from 40 to 69 days. A reliable method of monitoring the dynamics of *R. cerasi* flight is the use of yellow glue traps corrected to SET estimates and visual control of fruit quality and maturity.

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ROLE OF METEOROLOGICAL FACTOR IN LONG-TERM POPULATION DYNAMICS OF THE EUROPEAN CORN BORER, *Ostrinia nubilalis* Hbn., IN KRASNODAR AREA: THE ANALYSIS OF LIFE TABLES

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Abstract

The European corn borer, *Ostrinia nubilalis* Hbn. is one of the most dangerous pests of maize. Though weather, mostly air temperature and humidity, demonstrate their important influence on distribution, population level and development terms of the European corn borer, the accuracy of forecasting models remains rather low to predict pest population dynamics. The aim of our work consists in the comparison of meteorological data with the demographic indicators characterizing long-term population fluctuations in the European corn borer to identify the most significant meteorological criteria for the forecast of pest population dynamics. Observations of insect population dynamics conducted during 1994-2018 on maize fields in vicinity of village Botanika (Gulkevichi District, Krasnodar Area; 45°12′51″ N and 40°47′41″ E) during 1994-2018, and data obtained in 2018 were only used for verification of the dependence established on data array for 1994-2017. We estimated density of insect population at all stages of life cycle from egg to adult. The meteorological information was obtained from the Otrado-Kuban meteorological station located in the center of the territory of the test field location. Mortality for the different periods of insect development (eggs, I-II and III-V instar larvae, pupae, adults) was estimated as $K = \log N_1 - \log N_2$, where N_1 and N_2 are density of insects for the corresponding periods. Mortality for generation was calculates as $K = \log N_o - \log N_f$, where N_o and N_f is density of eggs and egg-laying females of the current generation. The breeding index per generation was calculated as $I = N_{t+1}/N_t$, where N_t and N_{t+1} is density of eggs for the current (t) and the following (t + 1) generation, respectively. Two generations of the European corn borer annually develop in Krasnodar area, and size of population during the first generation usually grows, while those in second generation decreases. The demographic characteristics of insect statistically significant correlate with meteorological factors only during time interval from the third decade of May to the first decade of June when the peak of egg oviposition and mass hatching of I instar larvae of the first pest generation usually occur. Egg mortality shows the closest negative correlation with air temperatures and positive correlation with moisture (water drops and vapor). No association of demographic indicators with meteorological factors during oviposition and hatching of second-generation larvae was found probably due to formation of high and dense maize crops with a microclimate favorable for insect development. Thus, the data obtained demonstrate that the last decade of May — the first decade of June is the only critical period during the European corn borer development in the region. Based on the findings, we drown a 3D linear contour diagram of the European corn borer first generation egg mortality as dependent on mean daily air temperature and relative atmospheric humidity during the last decade of May. This dependence is of interest in terms of an improved model for short-term forecast of pest population dynamics in Krasnodar Area.

Keywords: European corn borer, population dynamics, demography, egg mortality, air temperature, air humidity, Krasnodar Area

The European corn borer, *Ostrinia nubilalis* Hbn. is one of the most dangerous pests of maize, the loss of grain yield from which in Eastern Europe is still very high [1-4]. Despite a significant number of publications devoted to this insect both in Russia [5-7] and abroad [8-11], many features of its ecology remain insufficiently disclosed [12]. The significant role of meteorological condi-

tions, primarily temperature and humidity, in the distribution and abundance, as well as the timing of the corn borer development, has been convincingly shown [13-17]. However, the accuracy of the forecast, which is based on meteorological information, remains low [18].

The domestic theory of phytosanitary forecasting is underlaid by the Victor E. Shelford's law of tolerance which is based on the effects of limiting environmental factors during critical periods of the life cycle of a harmful species [19]. The population genetics study of genus *Ostrinia* members [1] distinguishes the following critical periods in the corn borer life cycle: pupation of overwintered larvae; mating and oviposition by butterflies; start of larval feeding [20]. It is understood that the viability of the population during these periods is determined by meteorological factors: the average daily temperature and the amount of precipitation over a period from a 11 °C threshold transition to the start of pupation of hibernating larvae; the amount of rainfall during the oviposition; the absence of showers, strong winds, high temperatures and other extreme meteorological phenomena in the initial period of larval feeding [20].

Disclosing patterns of harmful insects' population dynamics is among the most important aspect of plant protection, since the reliability of forecasts of the harmful species reproduction depends on a completeness of knowledge about all affecting factors and the accuracy of the models used [19].

In the present work, we first proved the decisive contribution of the death of corn borer eggs in the population dynamics of its first generation in the season. A reliable relationship between insect mortality and meteorological factors is found in the interval limited by decade III of May to decade I of June, when mass egg laying and hatching of the first instar larvae of the first generations usually occur.

Our aim was to compare meteorological data with demographic indicators characterizing long-term population fluctuations of corn borer local population, and to identify the most significant meteorological criteria for predicting the dynamics of the pest population.

Materials and methods. Observations were carried out in the vicinity of the village Botanika (Krasnodar Territory, Gulkevichsky District; 45°12′51″N and 40°47′41″E) in 1994-2018. Data for the 2018 season were used exclusively to verify dependencies established on the data array for 1994-2017. The scientific crop rotation of the VIR Kuban experimental station (VIR KES), a total area of 284 hectares, was the territory to estimate corn borer *Ostrinia nubilalis* population dynamics. Since 2014, the surveys also covered fields adjacent to the scientific crop rotation (Scientific Production Association NPO KOS-MAIS), a total area of 500 hectares. Standard meteorological information was received daily from Otrado-Kuban weather station located at the center of the VIR KES crop rotation.

Corn borers were counted annually in 6 maize crop fields on average (minimum 3 fields and maximum 14 fields with a total area of 18.4 to 175.0 ha, where zoned and promising hybrids and their parental forms were grown. The range of cultivated maize genotypes varied according to the State Register of Selection Achievements Allowed to Use [21]. All agricultural techniques adopted in the zone were used (tillage, sowing, application of herbicides, 1-2 inter-row cultivations). Seed sowing time, as a rule, was optimal. The stand density in the fields varied from 2.8 to 7.8 plants per 1 m^2, the insect population density was estimated per 1 m^2 crops. It is this method of calculating the density that seems to be the only possible to describe the full cycle of the insect seasonal dynamics, since the development of individuals of the 2nd generation ends after their hibernation in post-harvest plant debris in the fields.

Periodic counting (21-23 times per season) covered the life cycle of the

insect from egg to adult. The counts of overwintered individuals (that is, the 2nd generation of the previous season) were carried out in the fields that were occupied by maize last year. In such areas, the density and mortality of overwintered larvae, pupae, and adults in plant residues were evaluated twice (before pupation and at the end of adult eclosion). The distribution of the overwintered and hibernating larvae was determined at randomly selected 0.7-1.0 m^2 plots (20-25 plots per field). The density of egg masses of the 1st (May-June) and 2nd (July-August) generations was estimated at permanent (during the season) plots, consisting usually of 10 plants, the first and last of which were labeled. The number of such plots per field varied from 9 to 25, depending on the area of the latter. During the flight of adults, plants on the sites were carefully examined every 3-5 days, and the location of each found egg mass was marked, the number of eggs per mass was counted using a handheld magnifier, then the number of eggs from which the larvae hatched out was estimated [22]. The egg density per field was calculated from the total number of eggs during a series of periodic surveys. At the end of the oviposition period, a series of larval counts on plants was carried out, for which plants from randomly selected 0.7-1.5 m^2 plots, 5-10 plants per each, were dissected. The number of such plots varied from 15 to 35, depending on the field size. Weeds found at the plot were also inspected, as they could be infested by pest larvae.

Adult population density was calculated from number of pupal skins. The number of egg-laying females was calculated as the average density of eggs of the next generation divided by 225 [22]. This value characterizes population density of adults normalized to the sex ratio 1♀:1♂ and the average fecundity of the female. According to the results of long-term (1975-2004) counts of eggs laid by moths in labs, the average fecundity of a fertilized female is taken equal to 450 eggs. The average generation density of corn borer eggs, larvae, pupae, adults, and egg-laying females was calculated as weighted average over the surveyed acreage of fields occupied by maize.

Mortality rates of insects for developmental periods (eggs, larvae of I-II and III-V instars, pupae, and adults) were represented as the difference of density logarithms: $K = \log N_1 - \log N_2$, where N_1 and N_2 are the density of insects for relevant accounting periods [23, 24]. Mortality for generation was represented in the form $K = \log N_o - \log N_f$, where N_o and N_f are the density of eggs and ovipositing females of the current generation. Before the logarithm calculation, the density values were recalculated per 1000 m^2 of maize crops in order to avoid the appearance of negative logarithms at a low phytophage density. The breeding index per generation was calculated as $I = N_{t+1}/N_t$, where N_t and N_{t+1} are egg density of the current (t) and next (t + 1) generations [6].

All calculations were performed with Statistica 10.0 software (StatSoft, Inc., USA). The tables show means (*M*), maximum (max) and minimum (min) values, standard deviations (±SD), coefficients of variation (*Cv*) and correlation (*r*). Dependencies between the effects were assessed using correlation and regression analyzes, the reliability of correlations at a $p < 0.05$ significance level was evaluated using the Student's *t*-test and Fisher's *F*-test.

Results. A long-term trend was a gradual but steady increase in the degree of host plant resistance of new maize hybrids to the pest during development of first generation [21]. So, if in 1994-1997, the average estimation of leaf feeding damage by the pest on a scale of 1-9 scores [25] for maize entries grown on 18 fields varied within 3-7 scores, with an average value of 5.4 score and 27.1% *Cv*, then in 2014-2018 in 14 entries it ranged from 2 to 5 scores, 3.2 score on average with a *Cv* of 34.9%.

1. Density of corn borer (*Ostrinia nubilalis* Hbn.) individuals of the first and second generations during observations (neighborhood of the village Botanika, Krasnodar Territory, 1994-2017)

Periods	Density		
	per 1 m^2		Per plant, *M*±SD
	M±SD	min-max	
	First generation		
O	32.81±30.70	2.87-133.60	6.31±5.95
L1	20.14±16.81	1.94-67.35	3.87±3.25
L2	4.24±4.00	0.09-17.07	0.82±0.78
P	2.52±2.66	0.05-12.22	0.48±0.52
A	1.62±1.62	0.04-6.90	0.31±0.33
FO	0.55±0.50	0.02-1.82	0.11±0.12
	Second generation		
O	108.77±107.22	4.92-408.45	20.92±20.66
L1	47.02±43.77	2.32-163.20	9.04±8.48
L2	17.56±16.13	1.17-57.77	3.38±3.12
P[a]	1.52±1.41	0.13-5.11	–
A[a]	0.97±1.08	0.10-4.11	–
FO[a]	0.17±0.15	0.01-0.59	–

Note. O — eggs, L1 and L2 — larvae emerged from eggs and larvae of III-V instars feeding on plants, respectively, P — pupae, A — imago, FO — ovipositing females, [a] — the next season after overwintering. Dashes mean that there were no corresponding plants on the field.

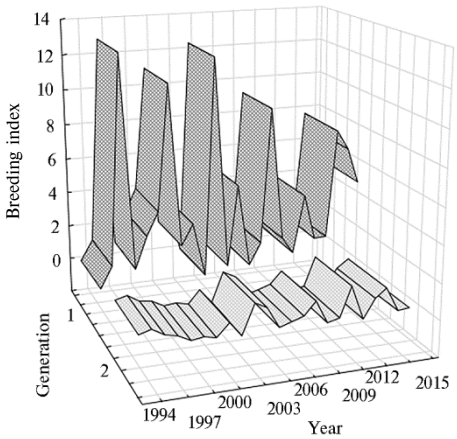

Fig. 1. Breeding index (I) of the corn borer (*Ostrinia nubilalis* Hbn.) of the first and second generations during observations (neighborhood of the village Botanika, Krasnodar Territory, 1994-2017).

Observations during 1994-2017 indicated a wide variation in the number of corn borers on maize, also fluctuations in the density of insects of the first and second generation significantly varied (Table 1). So, in the first generations, the density of eggs and larvae feeding on plants, as a rule, was significantly lower than in the second generations, while the densities of overwintered pupae, adults and egg-laying females were significantly higher, which was due to the additional mortality of insects of the second generations during harvesting, autumn and spring tillage, and overwintering (see Table 1). The insect breeding index also varied widely (Fig. 1): from 0.34 to 13.04 (an average of 4.24) for the first generation and from 0.02 to 1.85 (an average of 0.65) for the second generation. In other words, if in the first generation the corn borer usually increased in counts, then in the second generation it decreased.

Although the number of adult larvae (the harmful stages of corn borers) in the first generation is usually lower than in the second, the damage caused to plants by individuals of the first generation is usually significantly higher [26-28]. Thence the practical interest arises to analyze the dynamics of the pest abundance precisely for the first pest generation, when the abundance is subject to sharp fluctuations and the impact of meteorological factors on insect development during critical periods is quite expected [20].

Depending on the weather conditions of the year, the timing of certain stages of corn borers development shifted significantly. So, the time of the beginning of pupation among larvae from overwintered generation varied over a very wide range (from the first dates to the end of April). Oviposition by over-

wintered females, the beginning of which on mid-season maize genotypes (FAO 350-400) is confined to the phase of the middle leaf whorl (5-6 leaves), as a rule, began from decades I-III of May and ended in the middle June—early July with a peak in decade III of May—decade I of June. The development of the first generation larvae usually ended by the beginning of August, pupation of the main part of the population covered the period from early July to mid-August, the mass flight of the first generation adults occurred from mid-July to mid-August. Egg laying by first-generation females began on maize plants from the second half of July to mid-August with a peak in late July—early August. Almost every year, some of the second generation larvae (usually within 0-5%, up to a maximum of 30%) pupated, giving rise to individuals of the third generation. These larvae mostly died because of feed shortage and cold weather, therefore in the total mass of larvae ready for wintering, individuals of the third generation usually amounted to no more than 2.0% of those of the second generation with seasonal variations from 0 to 7.6%.

2. Statistically significant ($p < 0.05$) **correlations** (*r*) **between mortality of the first corn borer (*Ostrinia nubilalis* Hbn.) generation** (K effects) **and meteorological factors** (neighborhood of the village Botanika, Krasnodar Territory, 1994-2017)

Period	Mortality, K		
	eggs	I-II instar larvae	generation from egg to adult
	Air temperature, °C		
May, decade III	0.634		0.564
	Air humidity, %		
May, decade III	–0.568		–0.462
June, decade I	–0.542	–0.437	–0.590
	Precipitation, mm		
June, decade I	–0.435		–0.618

For 1994-2017, a correlation was analyzed of insect mortality (K effects) with average decadal values of air temperature, humidity and total precipitation for two periods (March—end of June and decade II of July—decade II of August) (Table 2).

3. Main statistical parameters of corn borer (*Ostrinia nubilalis* Hbn.) mortality (K effects) **during different stage of development as correlated to the mortality of a generation** (neighborhood of the village Botanika, Krasnodar Territory, 1994-2017)

Indicator	Mortality effects (K) during periods of development					
	generation from egg to adult	eggs	larvae		pupae	adults
			I-II instars	III-V instars		
M±SD	1.862±0.436	0.207±0.229	0.726±0.367	0.268±0.184	0.199±0.138	0,463±0,412
min-max	1.225-2.815	0.035-0.876	0.039-1.366	0.053-0.703	0.007-0.466	–0,173-1,505
Cv, %	23.42	110.96	50.55	68.64	69.39	89,08
r (correlation with mortality over generation)		0.70*	0.23	0.23	–0.09	0.39

* The value of the correlation coefficient is statistically significant ($p < 0.05$).

Significant correlations of the insect demography parameters with meteorological factors were found only in the interval from decade III of May to decade I of June, that is, when mass egg laying and larval hatching of the first generation occurred (see Table 2). Moreover, most often, a reliable relationship between meteorological factors and demographic indicators was found during egg development period. Correlation analysis indicates a significant negative effect of elevated air temperatures and the positive effect of moisture, both drop-liquid and water vapor, on egg survival (see Table 2). The effect of air humidity on the survival of younger larvae was less pronounced, and there was no statistically significant correlation at all between variation of meteorological factors and the

survival rate of older larvae, pupae or adults.

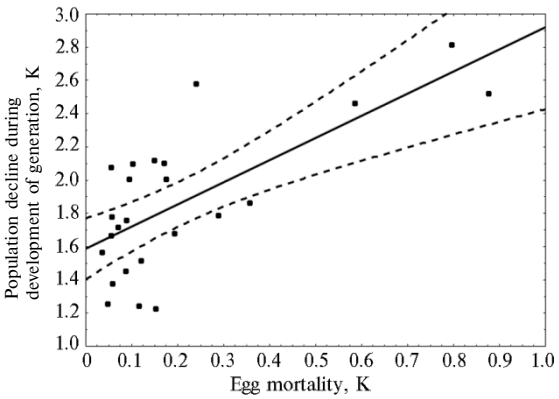

Fig. 2. The relationship between mortality (K effects) during egg development and from egg to adult of the first generations of corn borer (*Ostrinia nubilalis* Hbn.) per season (neighborhood of the village Botanika, Krasnodar Territory, 1994-2017).

The intra-stem lifestyle of the pest is undoubtedly one of the factors mitigating the weather impact on the viability of the insect. In the second critical period, that is, during the end of the development of the first generation and the beginning of the second one, we did not reveal reliable relationship of meteorological factors with the demographic indicators of the insect, probably, due to powerful stands with a microclimate favorable for insect reproduction, which maize plants form by this time [29]. In addition, in the second half of season, a pest population often is experiencing powerful press of parasitic insects [30], which could smooth the effects of meteorological factors. The results indicate that decade III of May—decade I of June can be deemed the only critical period in the development of the pest in the eastern part of the Krasnodar Territory. During this critical period, weather conditions had a strong effect on the development of eggs and, to a lesser extent, on the viability of hatched larvae. Correlation (Table 3) and regression (Fig. 2) analyzes indicate that egg mortality the most significantly contributes to the overall mortality of the first generation. This conclusion is quite obvious, since the role of a factor in the population dynamics is determined not so much by the factor intensity as by variability of its effect [24].

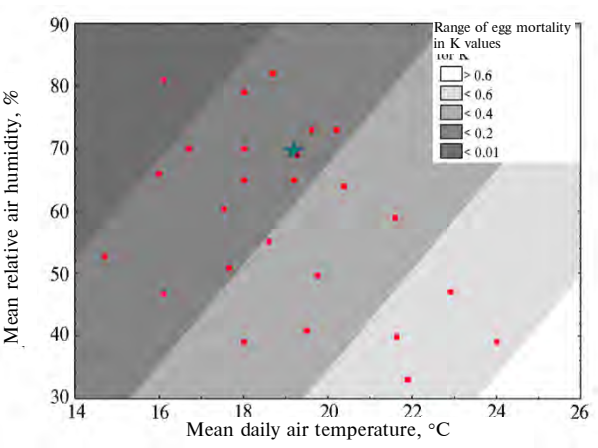

Fig. 3. 3D contour linear diagram of the relationship between egg mortality (K effects) **of the first generation of corn borer (*Ostrinia nubilalis* Hbn.) and meteorological conditions of decade III of May.** The actual air temperatures and humidity used to construct the diagram are indicated by red dots. An asterisk indicates a combination of the average daily air temperature of 19.3 °C and 70% relative humidity at which K = 0.093 (neighborhood of the village Botanika, Krasnodar Territory, 1994-2018).

The development of eggs during the first generation is important in terms of the dynamics of insect abundance. Thence a constructing 3D-contour linear model of K effects of pest mortality plotted vs. meteorological factors (average daily temperature and relative humidity in decade III of May) is of interest from the point of view of developing refined models for predicting the pest population. The provisional verification of the model with the use meteorological data and the demographic indicators of the pest for 2018 gave a positive result (Fig. 3).

In detail, the long-term dynamics of corn borer abundance was studied in North

America in Minnesota (USA) in 1948-1970 and in the Ontario province (Canada) in 1957-1965. The key factors in the dynamics of insect abundance in these regions were the portion of larvae pupating in summer (Minnesota) and capacity of females to realize their egg production (Ontario) [6]. Importantly, in both cases, a strict relationship was revealed between dynamics of the pest abundance and meteorological factors during critical periods of insect development. As for corn borer populations living in Europe, the dynamics of their abundance also significantly depends on abiotic factors, primarily temperature [31, 32]. It is known that temperature has a decisive effect on insect phenology, and this allows expectation of developing mathematical models to predict the timing of control measures against pests [33]. In addition, based on temperature dependences, models are developed that predict changes in the range and zones of damage of the corn borer, as well as its abundance under conditions of global climate warming [34]. In this context, our findings reveal additional aspects of causal relationships between fluctuations of weather and climate factors and the dynamics of insect abundance. The compilation of life tables allows researchers and practitioners to identify characteristic variation of pest abundance and the factors most significantly affecting pest population. Understanding how and why the number of living organisms changes in time and space is one of the most important tasks of theoretical and applied ecology [35, 36].

Thus, the key factors of corn borer population dynamics vary significantly depending on environmental conditions. In the eastern part of the Krasnodar Territory, the only biological factors affecting abundance of this pest, showing statistically confirmed relationship with meteorological factors, are the egg mortality and, to a lesser extent, the mortality of hatching larvae in the first generations of the season. The constructed contour diagram of first-generation corn borer egg mortality vs. average daily air temperature and relative air humidity over the last decade of May is of interest for improving technologies for short-term prediction of the pest reproduction in the Krasnodar Territory.

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DEVELOPMENT OF REAL TIME PCR KIT FOR DIAGNOSTICS OF GRAPEVINE BLACK WOOD CAUSATIVE AGENT *Candidatus* Phytoplasma solani

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Abstract

Today, phytoplasmas are causative agents of about three hundred different plant diseases. The greatest damage in European vineyards is due to two types of phytoplasmas, *Candidatus* Phytoplasma vitis Marzorati et al. 2006, the pathogen of flavescence dorée of grapevine, and *Candidatus* Phytoplasma solani Qualino et al., 2013, the causative agent of black wood of grapevine. Phytoplasma damage of vineyards can lead to crop losses of up to 25-30 %, and when infected up to 70 %, the vineyards should be completely uprooted. Symptoms of various phytoplasma diseases in grape are similar with each other and with viral and bacterial diseases that makes their visual differentiation to species impossible. The wide spread and high damage by phytoplasma diseases require deeper research of phytoplasma epidemiology and relevant molecular genetic diagnostic methods for monitoring phytopathogen in planting material. We have developed the first Russian kit for detection these pathogens by real-time polymerase chain reaction (Real-Time PCR) which allows effective identification of *Candidatus* Phytoplasma solani. A comparison of the developed kit with the recommended primers and probes for Real-Time PCR has shown a higher sensitivity and specificity as compared to existing diagnostic PCR systems. The goal of this work was to develop and test a kit for the detection of *Candidatus* Phytoplasma solani by the real-time polymerase chain reaction (qPCR). *Candidatus* Phytoplasma solani DNA samples and infected grape vines, roots and leaves of Chardonnay, Pinot noir and Bastardo Magarachsky varieties with visual signs of infection collected in the autumn of 2018 from the vineyards of the South Coast region of the Crimean peninsula were tested. Phytoplasma DNA was extracted as recommended by EPPO, with modifications, as well as with Cytosorb reagent kit (Syntol LLC, Russia). A pair of primers, SolaSeq_F 5′-AACTTAACCTTTTAACTAGGGC-3′ and SolaSeq_R 5′-CATCAAGGCATTTGCC-3′, was designed for *Candidatus* Phytoplasma solani DNA sequencing. To estimate the analytical sensitivity of the kit, a vector construct based on the Pal2T plasmid (Evrogen, Russia) was created with the insertion of the *Candidatus* Phytoplasma solani target 119 bp fragment of *SecY* gene. The sequence of *SecY* gene is conservative, unlike other genes recommended for diagnosis. The designed primers allow identification of all *Candidatus* Phytoplasma solani strains which sequences we found in the GenBank NCBI Nucleotide database on January 16, 2019. The developed kit was tested using various Real-Time PCR instruments. We have assessed the main characteristics of the kit, i.e. sensitivity, specificity, and reproducibility. Analytical sensitivity of the developed kit isn't less than 15 copies per PCR reaction. The analytical specificity was 100 % when tested with 37 closely related and accompanying microorgan-

isms, as well as four samples of grapes suspected to be infected by *Candidatus* Phytoplasma solani. There were no false-positive results in the analysis of other types of phytoplasmas and related microorganisms. Also, in analyzing target organism DNA samples, false-negative results were not found. The developed kit was tested on 194 samples of grapes suspected of being infected by *Candidatus* Phytoplasma solani. The specificity of *Candidatus* Phytoplasma solani detection was confirmed in all cases by DNA sequencing of positive samples. The developed kit allows rapid, accurate and high sensitive DNA identification of *Candidatus* Phytoplasma solani in plants at all stages of their vegetative development, including planting material, and can also be used for full-scale screening studies.

Keywords: phytoplasma, *Candidatus* Phytoplasma solani, grapes, real-time PCR, diagnostics, qPCR test kit, specificity, sensitivity, reproducibility, repeatability

In 1967, unicellular plant pathogens that were previously mistakenly assigned to the jaundice virus group due to the similarity of symptoms and the inability to culture on a nutrient medium [1-3] were classified as mycoplasma-like organisms due to morphological similarities and sensitivity to tetracycline antibiotics [4-6]. With the development of molecular biology methods based on the nucleotide sequencing ribosomal RNA genes [7, 8], these microorganisms were designated as a separate taxonomic group with the generic name *Candidatus* Phytoplasma [9, 10].

Phytoplasmas affect a wide range of plants, including grapes [11, 12]. Two types of phytoplasmas are most harmful for grapes, the grapevine yellow pathogen *Candidatus* Phytoplasma vitis Marzorati et al., 2006 (a quarantine object) [13, 16-18] and the black wood pathogen *Candidatus* Phytoplasma solani Qualino et al., 2013 [19-21]. The main host plant for the grapevine yellow pathogen *Candidatus* Phytoplasma vitis is a grape of the European-West Asian group, which includes almost all cultivated grape varieties. The yield loss of susceptible varieties can reach 100%, such plants die off 2-3 years after infection.

Phytoplasma *Candidatus* Phytoplasma solani also affects sugar corn, fruit crops and members of the *Solanaceae* family. Many wild-growing plants, in particular, stinging nettle and field bindweed, can be a reservoir for phytoplasmic infection [22, 23]. The distribution of phytoplasma in plants is not uniform. Phytoplasma is mainly localized in the phloem, so this part of the plant is preferable for the nucleic acid extraction and subsequent diagnosis. Some types of plants are tolerant to phytoplasma infection; therefore, when taking samples for analysis, attention should be paid to collecting diverse biomaterial (root fragments, vines, leaves) even without visual signs of infection.

Phytoplasmas lead to a yield decrease which varies from insignificant to almost complete loss [1, 12]. In grapes, the infection leads to yield losses of up to 25-30%, and at 70% infection the grape plants are completely uprooted. The prevalence of grape phytoplasmoses in the areas of cultivation can reach 70-80%, and the damage can exceed 40-80% [23].

According to EPPO (European and Mediterranean Plant Protection Organization, https://gd.eppo.int), as of November 2, 2019, *Candidatus* Phytoplasma solani is ubiquitous in Western and Eastern Europe, except for several countries in central and northern Europe [24]. Data to establish the really affected vineyards in Russia and other countries of the former USSR are insufficient [11]. For a comprehensive screening, it is necessary to develop a specific and easy-to-use kit for identification of *Candidatus* Phytoplasma solani.

For the first time in Russia, we developed a qPCR-based test system for detecting the *Candidatus* Phytoplasma solani and compared its quality with existing diagnostic systems for the main characteristics (specificity, sensitivity, reproducibility, repeatability).

Our goal was to develop and test a kit for detection of *Candidatus* Phytoplasma solani, the causal agent of the black wood of grapevine with real-time polymerase chain reaction (qPCR).

Materials and methods. *Candidatus* Phytoplasma solani DNA samples were received from the collection of the All-Russian Plant Quarantine Center (VNIIKR)-and contaminated grape material was provided by the Magarach All-Russian National Research Institute of Viticulture and Winemaking (VNNIIIV Magarach). Fragments of the vine, roots and leaves of Chardonnay, Pinot noir and Bastardo Magarachsky varieties with visual signs of *Candidatus* Phytoplasma infection were collected in the autumn of 2018 from the vineyards of the South Coast agroclimatic region of the Crimean Peninsula.

Samples for DNA extraction were ground manually and using a Precellys Evolution rotational homogenizer (Bertin Technologies, France). Phytoplasmic DNA was extracted according to EPPO recommendation [24] with modifications [25], and also with Cytosorb reagent kit (Syntol LLC, Russia) which provides cell lysis with a chaotropic agent guanidine hydrochloride followed by DNA sorption on silicon particles as described in patent application RU No. 2019111081/10 (021521).

Conventional PCR and qPCR were performed in the reaction buffer (3 mM $MgCl_2$, 0.25 mM dNTP, 2.5 units of SynTaq DNA polymerase with antibodies inhibiting enzyme activity) (Syntol LLC, Russia). The selected primers should meet the following criteria: G or C nucleotide at the 3′-end ("GC clamp"), the average annealing temperature 65 °C for primers, 2-5 °C higher for a probe. The presence or absence of secondary structures and the annealing temperature were checked via online Oligo Calc primer analysis software: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html), ThermoFisher Multiple Primer Analyzer (https://www.thermo-fisher.com), Promega Biomath Calculators (5×PCR Buffer, 3 mM $MgCl_2$) (https://worldwide.promega.com/resources/tools/biomath/tm-calculator/). Fluorescent labels in qPCR were 6FAM and 6ROX dyes attached to the 5′-end of the probe. Fluorescence dampers were dyes RTQ1 and BHQ2 at the 3′-end of the probe. The concentration of primers and probes in the reaction mixture was 450 nM and 200 nM, respectively. qPCR was performed using four devices, ANK-32 (IAI RAS, Russia) [26], CFX-96 (Bio-Rad, USA), Rotor-Gene 6000 (QiaGen, USA), and DTprime 5 M1 (DNA Technology, Russia), and the following amplification protocol: 5 min at 95 °C; 15 s at 95 °C, 40 s at 60 °C (50 cycles). Results were deemed positive if the fluorescence signal exceeded the threshold which was set as a 10% difference between the moduli of the highest and lowest fluorescence).

A pair of primers SolaSeq_F 5′-AACTTAACCTTTTTAACTAGGGC-3′ and SolaSeq_R 5′-CATCAAGGCATTTGCC-3′ was designed for sequencing DNA of *Candidatus* Phytoplasma solani. Amplification program for conventional PCR was as follows: 5 min at 95 °C; 20 s at 95 °C, 20 s at 60 °C, 1 min at 72 °C (36 cycles); 5 min at 72 °C. DNA sequencing was performed on a Nanophor 05 instrument (Institute for Analytical Instrumentation RAS, Russia). The results were analyzed using DNA analysis software version 5.0.3.2 (Institute for Analytical Instrumentation RAS, Russia).

Bioinformatic analysis and data processing were performed using the software UGENE (UNIPRO, Russia) and AliView (NBIS, Department of Cell and Molecular Biology, Uppsala University, Sweden). The search for target DNA sequences was carried out in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) [27].

Target DNA in developing primers and probe was *SecY* gene with 119 bp specific fragment, positions from 513 to 632 bp (KU600099.1, NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) length) [27].

To determine the analytical sensitivity of the reagent kit, a vector con-

struct based on the Pal2T plasmid (Evrogen, Russia) was created with the insertion of the 119 bp target nucleotide sequence of *Candidatus* Phytoplasma solani. Ligation was carried out for 24 hours at 4 °C using T4 DNA ligase (ThermoFisher Scientific, USA). The transformation of *Escherichia coli* (Migula 1895) was done using heat shock method. The presence of the vector was checked by PCR colony method with standard M13 primers. Plasmid was isolated using a PlasGen kit (Syntol LLC, Russia). To increase the efficiency of PCR, the plasmid was cleaved with NotI restriction endonuclease (ThermoFisher Scientific, USA). The concentration of plasmid DNA was measured on a Quantus fluorimeter (Promega Corporation, USA) in triplicate. To determine the sensitivity of the developed kit, qPCR was run in 2-fold repetition with a series of 10-fold dilutions with a known plasmid concentration [28].

Efficiency of the kit was tested on 37 samples of closely relative and accompanying objects: *Vitis vinifera*, *Xanthomonas campestris*, *Cercospora beticola*, *Fusarium culmorum*, *Fusarium* sp., *Fusarium tricinum*, *Gibellina cerealis*, *Botrytis cinerea*, *Pleospora betae*, *Phomopsis helianthi*, *Pseudomonas fluorescens*, *Alternaria tenuissima, Aspergillus niger* (collection of Syntol LLC, Russia); *Candidatus* Phytoplasma vitis (20.9 Ct), *Candidatus* Phytoplasma rubi (18.9 Ct), *Candidatus* Phytoplasma pyri (21.4 Ct), *Candidatus* Phytoplasma mali (18.4 Ct), *Candidatus* Phytoplasma convolvuli (15.9 Ct), *Candidatus* Phytoplasma asteris (15.0 Ct), *Candidatus* Phytoplasma cirsii (33.5 Ct), *Candidatus* Phytoplasma taraxacum (14.0 Ct), *Candidatus* Liberibacter solanacearum (23.6 Ct), *Xanthomonas oryzae* 0227, *Cercospora kikuchii* (VNIIKR collection); *Xanthomonas euvesicatoria* DSM 19128, *Xanthomonas gardneri* DSM 19127, *Xanthomonas perforans* DSM 18975, *Xanthomonas vesicatoria* DSM 22252, *Xanthomonas translucens* pv. *translucens* DSM 18974 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany); *Xanthomonas phaseoli* CFBP 2534 (CIRM-CFBP, France); *Fusarium sambucinum* F139, *Fusarium graminearum* F877, *Fusarium verticillioides* F43, *Fusarium avenaceum* F623 (All-Russian collection of industrial microorganisms, State Research Institute of Genetics and Selection of Industrial Microorganisms of the National Research Center Kurchatov Institute, Moscow); *Fusarium oxysporum* F840, *Phytophthora cinnamomi* F3332, F3333, *Phytophthora cactorum* 985 (All-Russian collection of microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms (IBPM) RAS, Pushchino, Moscow Province).

In qPCR, grape, bacteria, and fungi DNA concentration was 50 ng per reaction. Threshold cycle of *Candidatus* Phytoplasma sp. was determined with UniRT primers [13]. For *Candidatus* Liberibacter solanacearum, primers recommended by EPPO [14] were used.

Results. Table 1 shows primers and probes published by E. Angelini with co-workers and C. Pelletier with co-workers and currently used [1, 11, 16, 29, 30], as well as the primers and probe we developed for qPCR (*BN*).

1. Primers and probes used to identify *Candidatus* Phytoplasma solani with their corresponding amplification protocols

Name, reference	Target	Amplification mode	Sequence
BNrt [16]	*16S rRNA*	5 min at 95 °C; 15 s at 95 °C, 40 s at 60 °C (50 cycles)	F 5′-GGTTAAGTCCCGCAACGAG-3′ R 5′-CCCACCTTCCTCCAATTATCA-3′ Pb 5′-(6FAM)AACCCTTGTTGTTAATTGCCATCATTAAG(RTQ1)-3′
mapBN [29]	*adk*	5 min at 95 °C; 15 s at 95 °C, 20 s at 50 °C, 20 s at 72 °C (50 cycles)	F 5′-ATTTGATGAAACACGCTGGATTAA-3′ R 5′-TCCCTGGAACAATAAAAGTYGCA-3′ Pb 5′-(6ROX)AAACCCACAAAATGC(BHQ2)-3′

Continued Table 1

BN (own data)	*SecY*	5 min at 95 °C; 15 s at 95 °C, 40 s at 60 °C (50 cycles)	F 5′-AATACCAGTACAATACGCTCGC-3′ R 5′-AAAGGTTGCATCAAGGCATTTGC-3′ Pb 5′-(6FAM)AACACTGCTGGAGTAATGCCTGTAATT(RTQ1)-3′

N o t e. F — forward primer, R — reverse primer, Pb — probe.

To compare the analytical sensitivity of recommended systems and that developed by us, qPCR was run with a series of 10-fold dilutions of *Candidatus* Phytoplasma solani DNA. Although the *BNrt* primers were not inferior in the threshold cycle to that we developed, a 1:100 dilution did not give a positive result, which indicates a lower sensitivity of *BNrt* compared to *BN*. The fluorescence signal of positive samples with *BN* and *BNrt* was 1,500 and 500 relative fluorescence units (RFU), respectively. In both cases, the concentration of primers in the reaction mixture was 450 nM and the probe concentration was 200 nM. The difference in threshold cycles of *mapBN* with *BNrt* and *BN* was +3.0-3.5 Ct. In addition to the lower sensitivity of the *mapBN* system, a 1:100 dilution showed a complete absence of fluorescence signal growth (Fig. 1).

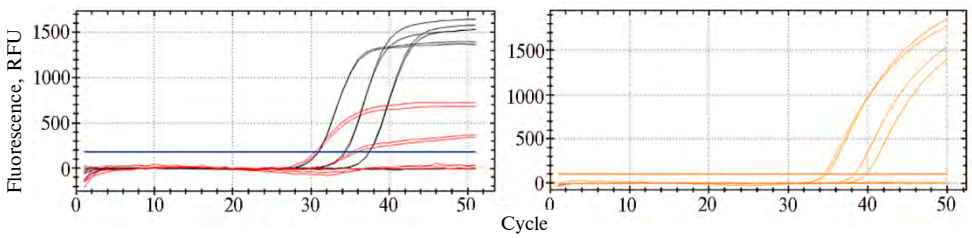

Fig. 1. Real-time polymerase chain reaction (qPCR) with a series of three 10-fold dilutions of *Candidatus* Phytoplasma solani DNA and different primers and probes: *BN* — black, *BNrt* — red, *mapBN* — orange (RFU — relative fluorescence units, a CFX-96 device, Bio-Rad, USA).

To estimate PCR specificity with *BNrt* primers, we used AliView software to align target fragments corresponding to primers and a probe for qPCR detection of *Candidatus* Phytoplasma solani. More than 100 DNA fragments were found in the NCBI database, 69 of which were of *Candidatus* Phytoplasma solani. Their identity with the target fragment for *BNrt* primers [16] ranged from 98 to 100%. Alignment showed a 100% coincidence of sequences of the primer and probe binding sites. This system, in addition to *Candidatus* Phytoplasma solani, also detected other 16SrXII phytoplasmas that cause witches' broom and potato purple top diseases [31, 32] on potatoes. In accordance with bioinformatic analysis, the *BNrt* system of primers and a probe for qPCR did not provide high specificity for the Candidatus Phytoplasma solani DNA detection.

The target gene for *mapBN* primers is *adk*. Sequence alignment showed a high specificity for attaching primers and a probe; however, only 10 sequences of this gene were found in the GenBank NCBI database (http://www.ncbi.nlm.nih.gov/BLAST), 7 of which were attributed to *Candidatus* Phytoplasma solani. Due to the small sample, *mapBN* primers were also rejected by us as candidates for use in the kit under development.

As a result, we chose the *SecY* gene, which encodes a protein involved in transmembrane transport, as a target to assess specificity of the of *Candidatus* Phytoplasma solani DNA detection. This gene is recommended by EPPO for the diagnosis of phytoplasma of wood blackening [24]. The *SecY* sequence analysis in the GenBank NCBI revealed 190 sequences, including 110 attributed to *Candidatus* Phytoplasma solani strains. The binding sites of the primers and a probe for qPCR we developed (*BN*) turned out to be strictly specific and allowed us to

detect all strains of *Candidatus* Phytoplasma solani, the DNA sequences of which were in the GenBank NCBI Nucleotide database on 01/16/2019 [27].

The results of DNA sequencing six grape samples, presumably infected by *Candidatus* Phytoplasma solani, carried out with primers to *SecY* gene to confirm the species affiliation of *Candidatus* Phytoplasma solani are presented in Figure 2.

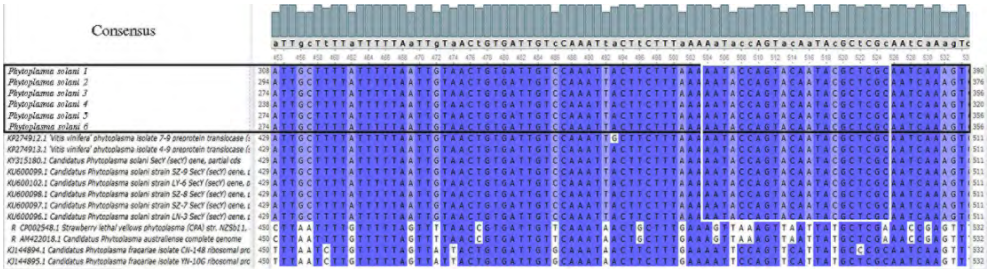

Fig. 2. Alignment of *Candidatus* Phytoplasma solani DNA sequences from the GenBank NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) with those from DNA sequencing of infected grape samples with primers to *SecY* gene. Alignments were generated in the Unipro UGENE program (Russia) version 1.31.1, the attachment zone for the forward primer *BN_F* is highlighted with a white frame. Phytoplasma solani 1-6 — samples collected in six different habitats. For a complete figure, see http://www.agrobiology.ru.

The analytical specificity of the reagent kit was assessed on 37 samples of closely related and accompanied organisms and six grape samples, presumably infected by *Candidatus* Phytoplasma solani according to visual signs. In determining the analytical specificity, positive results were obtained for all samples containing *Candidatus* Phytoplasma solani DNA, which was confirmed by DNA sequencing using *SolaSeq_F* and *So-laSeq_R* primers. We did not reveal false positive results with DNA of other phytoplasmas, including *Candidatus* phytoplasma convolvuli belonging, like *Candidatus* Phytoplasma solani, to the 16SrXII group, as well as with any associated organisms. The analytical specificity of the qPCR primers and probe to *SecY* gene for studied 37 samples was 100%.

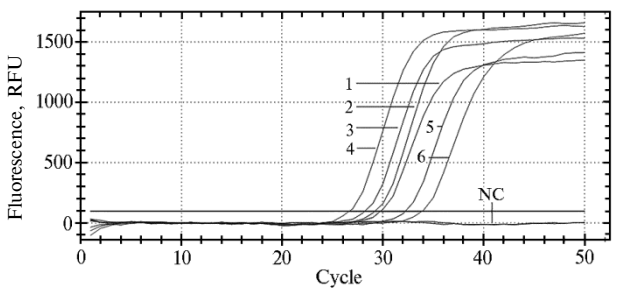

Fig. 3. Detecting *Candidatus* Phytoplasma solani DNA in six grape samples (1-6) **by real-time polymerase chain reaction (qPCR) with primers and probe to *SecY* gene** (NC — negative control; FAM detection channel, RFU — relative fluorescence units, a CFX-96 device, Bio-Rad, USA).

The analytical sensitivity of the reagent kit was evaluated by diluting plasmid DNA with the target insert of *Candidatus* Phytoplasma solani DNA. The initial plasmid concentration was 2×10^7 copies/μl. In PCR running, each reaction was duplicated. The calculated qPCR efficiency for a series of six 10-fold dilutions of the plasmid, from 10^7 to 10^2 copies per reaction, was E = 98.7%, with the slope of the kinetic curve A = –3.355, and the correlation coefficient $R^2 = 0.999$. Starting with the number of 50 copies of the plasmid per reaction, qPCR was performed in 24 replicates at dilutions of 50, 20, 15, 10, 5, and 1 plasmid copy per reaction. When diluting up to 10 copies per reaction, the result was unstable, and when diluting up to 1 copy of the plasmid per, a specific qPCR signal was not observed. The sensitivity of the reagent kit was established if at least 95% of the reactions gave a positive result. So the analytical sensitivity

of the reagent kit with *BN* primers was at least 15 copies per reaction

To test the developed kit for detecting *Candidatus* Phytoplasma solani DNA, we analyzed 194 grape samples from six territories (farms of the South-coastal agroclimatic region of the Crimean Peninsula). All tested samples yielded positive results for *Candidatus* Phytoplasma solani phytoplasm DNA (Fig. 3). DNA sequencing confirmed the obtained data.

The results for the developed system were confirmed with four devices for qPCR (Table 2). The qPCR efficacy was E = 97-100%, the slope of the kinetic curve was A = 3.31-3.39, the correlation coefficient R^2 = 1.000. Differences in threshold values averaged ±2 cycles, which is associated with a difference in the structure of optical modules, the heating and cooling rates of reactors, individual software for each device, and an algorithm for determining threshold cycle values.

To design primers and probe, we have chosen the *SecY* gene, encoding a translocase protein subunit which is characterized by high conservatism and specificity. This ensures differentiation of *Candidatus* Phytoplasma solani from closely related species. According to Davies et al. [9] and Lee et al. [10], this gene is the best marker to differentiate and classify strains of *Candidatus* Phytoplasma solani species. Target genes in existing and currently used diagnostic systems are the *16S rRNA* and *adk* gene regions [15]. The ribosomal RNA gene is inferior in specificity to both the gene encoding adenosine kinase (*adk*) and *SecY*. The analytical sensitivity of the kit we developed was at least 15 copies per reaction that is 10 times higher compared to the recommended diagnostic systems.

2. Reproducibility of qPCR results with primers and probe to *SecY* gene of *Candidatus* Phytoplasma solani on devices of different manufacturers

Samples, 10-fold dilutions	Threshold cycle, Ct			
	CFX96 (Bio-Rad, USA)	RG 6000 (QiaGen, USA)	DTprime 5 M1 (DNA Technology, Russia)	AHK 32 (IAI RAS, Russia)
10^{-1}	18.61	18.37	16.2	19.21
10^{-1}	18.73	18.26	16.2	19.27
10^{-2}	22.05	21.57	19.3	22.61
10^{-2}	21.94	21.72	19.3	22.75
10^{-3}	25.51	24.98	23.0	25.77
10^{-3}	25.45	24.92	22.8	26.05
10^{-4}	28.75	28.37	26.0	29.24
10^{-4}	28.66	28.18	26.1	29.10
10^{-5}	32.05	31.34	29.4	32.53
10^{-5}	32.46	31.46	29.1	32.57
Negative control	N/A	N/A	N/A	N/A
Negative control	N/A	N/A	N/A	N/A
Sloope, A	3.39	3.32	3.32	3.31
R^2	1.000	1.000	1.000	1.000
Effectiveness, E	97	100	100	100

N o t e. N/A means not available.

Successful testing of the proposed kit in qPCR with four devices of both Russian and foreign manufacturers allows us to recommend this system for diagnostic and screening in research laboratories.

Thus, a kit has been developed for qPCR detection of *SecY* gene fragment of *Candidatus* Phytoplasma solani, the causal agent of the black wood of grapevine. Analysis of field samples (leaves, roots and fragments of grapevine) which were presumably infected by *Candidatus* Phytoplasma solani with the proposed kit revealed DNA of this pathogen in all samples. qPCR data were confirmed by DNA sequencing. The developed qPCR system has a high sensitivity and specificity and is suitable for epiphytotic studies and evaluation of varietal resistance to this pathogen.

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