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GENE RESOURCES OF PERENNIAL WILD CEREALS INVOLVED IN BREEDING TO IMPROVE WHEAT CROP (review)

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Abstract

The reduction of wheat genetic diversity is an urgent problem in modern wheat breeding, which is primarily due to the limited number of varieties had been used in wheat pedigree. As a result of the depletion of the genetic pool of wheat, its resistance to phytopathogens has dropped, that generally reduces the stability of the agrophytocenosis. One of the ways to expand the genetic diversity of wheat is the transfer of genes of economically valuable traits from closely related genera and species, classified into three genetic pools: primary (varieties of hard and bread wheat), secondary (*Triticum* and *Aegilops* species), tertiary (most distant *Triticeae* species). The paper presents a review of success in gene transfer of economically valuable traits into the wheat genome from wheat's wild perennial relatives of the tertiary genetic pool: *Thinopyrum*, *Dasypyrum*, *Pseudoroegneria*, *Elymus*, and *Agropyron*. Representatives of these species have different levels of ploidy (di-, tetra, hexa- and even decaploids) and combine the genomes J (= E), St, W, Y, X, V, H, P, as well as their variants. Various levels of transfer of hereditary material into the wheat genome are considered, i.e. amphidiploids, addition and substitution lines, lines with translocations and small introgressions. Special attention is paid to amphidiploids, namely wheat-wheatgrass hybrids (PPG) combining the wheat genome and a whole or a part of the wheatgrass genome. The wheat-wheatgrass hybrids are considered both as an independent objects of cultivation and as a "breeding bridge", that is, an intermediate step in the transfer of genes from wheatgrass to wheat. The transfer of large chromatin fragments carrying the target gene is often associated with the additional transfer of undesirable genes which reduce the amount and impair the quality of the final wheat products. Therefore, introgressive lines of wheat are considered the most valuable forms, having a small chromatin insertion of an alien genome carrying a useful gene. Since the genomes of the tertiary genetic pool members are the most distant from the wheat genomes, an important problem considered in the review is the production of introgressions by recombination of homeologous chromosomes. The transfer of useful genes in wheat genome from its wild relatives is illustrated by examples, that consider the introgression of genes for resistance to fungal diseases (leaf and stem rust, powdery mildew, Fusarium blight, Septoria blight), viruses (yellow dwarfism streak mosaic), mite colonization, tolerance to drought, salinity and pre-harvest sprouting, storage proteins (glutenins) and perennial lifestyle of the plant. It is noted that wild relatives can serve as donors not only of genes responsible for resistance to stress factors, but also increase yields by increasing fertility, the number of spikelets and other elements of the yield structure, as well as improving the quality of the final product due to new variants of storage proteins. Special attention is paid to the development and use of molecular and molecular cytogenic markers which allow breeders to transfer target genes or regions of chromatin, as well as to monitor their introgression into the wheat genome in segregating populations. At the same time, in practical selection, different types of markers can be successfully used, i.e. those designed for the whole chromosome or its shoulder, linked to the chromatin region carrying the target gene, as well as the marker developed directly to the nucleotide sequence of the gene itself. Whole genome sequencing and genome editing technologies is noted to play in future a significant role in introduction of genetic material of wild relatives into wheat to improve its breeding programs.

Keywords: wheat, genes, wide hybridization, *Thinopyrum*, *Dasypyrum*, *Pseudoroegneria*, *Elymus, Agropyron*, wheatgrass, wheat-wheatgrass hybrids

Hexaploid wheat as a species arose after the second allopolyploidization nearly 8-10 thousand years ago. Its domestication occurred in the Fertile Crescent area, in the territory of modern south-eastern Turkey. Then, wheat followed the ways of human migration both to the West, Europe, and the East, Asia and finally to America and Australia. As a result, local varieties adapted to local conditions of cultivation appeared [1]. Traditional wheat varieties (landraces) have always been characterized by genetic inhomogeneity and heterogeneity. Due to it, variety populations have higher ecological sustainability and plasticity and are receptive to fluctuations in weather and climatic conditions [2]. Such variety populations of homozygous lines often change the population structure over time but provide resistance to biotic and abiotic stresses [3]. Since the 1970s, i.e., with the beginning of the active stage of the Green Revolution, and to the present time the active substitution of local varieties with commercial genetically homogeneous varieties suitable for high-intensity agricultural technologies has happened, resulting in the loss of biological diversity and a decrease in allelic variation [4]. The term "genetic erosion" [5, 6] was proposed in the early 1970s to describe the effects of these human activities, which reduce the genetic diversity of species until they are lost. Later, this term was adapted in the Russian scientific community both in the literal translation [7-11] and in the formulation "gene pool erosion" [12-15].

A reduction in genetic diversity is a global problem for most plants under cultivation, but in relation to wheat as the world most consumed crop (the area planted as of 2017 is 218 million hectares, the harvest is 772 million tons, 15% of all calories consumed by the world population), these processes are most wide-spread — 65-84% relative to the wild ancestral forms [16, 17]. The depletion of genetic diversity is primarily due to the widespread occurrence of similar varieties with overlapping pedigrees, the selection of which led to yield mainly. The situation is aggravated by the global climate change, urbanization, plowing of new lands, fires, and military actions; these factors lead to the loss of local varieties, as well as the reduction of the natural area of wild relatives of wheat, which could also be used to increase its genetic diversity [18]. As a consequence, more aggressive races of pathogens appear, epiphytoty outbreaks are recorded, the resilience of agroecosystems reduces, the dependence of agriculture on chemicals is growing [19].

In Russia, the analysis of gliadin-coding loci in wheat showed the beginning of genetic erosion in the Krasnodar Territory, Rostov Region and Nonblack Soil Zone and the prevalence of alleles of the Bezostaya 1 and Mironovskaya 808 varieties [20]. The comparison of the genealogical profiles of varieties in spring soft wheat in the Lower Volga Region revealed the degree of similarity higher than among the half-sibs, and in winter wheat in the Central and Volga-Vyatka Regions — at the level of full and half-sibs [21].

It is important to note that in recent years, the positive trend of the increase in the biodiversity of wheat varieties has been observed. Orabi et al. [22] found that the greatest decrease in genetic diversity among European wheat varieties occurred in the 1960s and 1980s; Martynov et al. [21] note that if in the 1970s, the pedigrees of the varieties included one or two landraces, the modern ones include 9-10 of them. However, the character of apparent diversity has changed. Modern breeding has resulted in an unbalanced predominance of wheat germ plasmas from southeastern, southern Europe and the Mediterranean, while genetic resources from East Europe and Asia are least used [1]. Over the past 70 years, the genetic diversity of Russian varieties has increased, however,

due to foreign breeding material. This process is accompanied by the loss of the original Russian material (soft wheat from 35 up to 50%, hard wheat up to 20%) and its substitution with the genetic resources from Europe, the United States and CIMMYT [21].

The main sources of increasing the genetic diversity of wheat are represented by three pools: primary (represented by local and traditional varieties and variety populations – landraces); secondary (other *Triticum* species) and tertiary (other genera — *Secale*, *Aegilops*, *Thinopyrum*, etc.). In the 1970s, a group of varieties with the genetic diversity differing from that of traditional and local varieties appeared, which is associated with the introgressions of foreign genetic material in the genome of wheat from its relatives, primarily rye, with distant hybridization [1]. Nowadays, alleles from 50 species representing 13 genera are known to be transferred to the wheat genome [23]. Among the donors of genes of economically valuable traits in the tertiary genetic pool, a special place is occupied by perennial cereals, in particular, such well-known and well-reputed species as intermediate wheatgrass and pontic wheatgrass [1].

According to modern classification, in perennial wild relatives of wheat, the chromosomal set is formed by a different combination of several genomes in polyploid species. The P genome is represented in di-, tetra-, and hexaploid (including segmental) *Agropyron cristatum* and other wheatgrass forms [24]. An important role is played by the *Pseudoroegneria* species with the St genome, which served, apparently, as the female parent in the polyploid *Thinopyrum* and *Elymus* species [25, 26] and thus represent one of the central genomes in perennial cereal species [27-29]. The members of the *Thinopyrum* genus carry the E gene, also denoted J; the range of ploidy of this genus vary from the diploids *Th. bessarabicum* (J^b) and *Th. elongatum* (J^e) up to the hexaploid forms of *Th. intermedium* ($J^rJ^{vs}St$) and decaploid *Th. ponticum* ($JJJJ^sJ^s$). The matter of the genomic composition of intermediate wheatgrass and pontic wheatgrass is still open and is studied by many researchers with the use of molecular cytogenetic approaches [29-31]. The listed *Thinopyrum* species are widely used in the breeding improvement of soft wheat as donors of economically valuable traits. The gene V is represented in only two species – in annual cereal *Dasypyrum villosum* ($2n = 14$, VV) and perennial cereal *D. breviaristatum*; the latter has both diploid and tetraploid forms. Whether the tetraploid form of *D. breviaristatum* is allo- or autotetraploid VVV^bV^b remains a subject to discuss [32, 33].

As a result of crossing wheat and perennial cereals, the following types of breeding material can be obtained: amphiploids, the hereditary material of which includes the complete genome of wheat and full (in the case of a diploid species) or partial (of a polyploid species) genome of a wild cereal; augmented lines (they have the complete genome of wheat combined with a pair of augmented chromosomes of wild cereal); substituted lines (their chromosome set is formed by the complete genome of wheat, with the exception of one pair of chromosomes, substituted on the chromosome of wild cereal); translocated lines (lines of wheat, one or more chromosomes of which carry translocations); introgressive lines (one or a few chromosomes carry introgressions – small inserts of chromatin of wild cereal in the wheat chromosomes).

A big problem in the transfer of useful genes in the genome of wheat is the so-called "genetic garbage": in the chromatin area of a wild relative embedded in the chromosome of wheat, in addition to the target gene, genes that degrade the final product quality may be situated. Unnecessary chromatin fragments are removed by chromosomal engineering with the use of spontaneous translocations, radiation exposure, tissue culture, and stimulated recombination. Spontaneous translocations, as a rule, centric ones (Robertsonian, occurring as a

result of disruption and fusion of chromosomes on the centromere), as well as exposure to radiation, lead to the formation of non-centric translocations. Stimulated translocations result from the removal from the wheat genome of the *Ph* gene on the chromosome 5BL, which prohibits homo- and homeologous conjugation: it is possible to use nullisomics at 5B or mutants *ph1b* of soft and *ph1c* of hard wheat, which have a deletion of the *Ph*-locus and allele Ph^1 *Aegilops speltoides*. The task of the breeder is to select plants with the target gene among the plants in which the dose of the rest of the chromatin was reduced; in the case of *ph*-mutants, it is necessary to restore the *Ph* allele in wheat after reducing the dose of introgression [34]. If the trait is supported by the group of genes located in different loci of the same chromosome or distributed between chromosomes, it is a hard task to pass it to wheat through separate introgressions; in this case, amphidiploids and augmented (or substituted) forms would show more severe manifestation of the symptom compared to the introgressive forms [35].

Work with wild relatives begins with the exact identification of the species belonging of the sample at the molecular-cytogenetic and molecular levels [36, 37]. An important role is played by the study of genes and allelic diversity in the genomes of wild relatives. This makes it possible to find candidate genes for economically valuable traits, assess the genetic diversity of populations, and develop molecular markers of those genes that can be transferred to the genome of soft and hard wheat [38-42]. Identification of molecular and cytogenetic markers specific to individual chromosomes or their sites plays a significant role, as introgressive forms are often selected not for a specific gene, but for a chromatin site associated (linked) with the target gene [43, 44].

Tall wheatgrass *Th. elongatum* (Host) D.R. Dewey is presented by diploid ($E = J^e$, $2n = 2\times = 14$) and tetraploid (E_1E_2, $2n = 4\times = 28$) forms. Augmented and substituted forms of soft wheat at the chromosome 7E and hard wheat at the chromosome 1E, resistant to Fusarium head blight, were created [45-48]. Disomically substituted lines of soft wheat 1E(1A), 1E(1D) and 6E(6D) showed resistance to Septoria blight, the lines 1E(1B), 2E(2B), 2E(2D) and 3E(3B) – to the yellow dwarf virus of cereals [49]. The salinization resistance genes at the chromosome 3E responsible for removing sodium ions from the cell were introgressed to the distal section of the soft wheat chromosome 3A by stimulated homological recombination [50]. The soft wheat line augmented with the chromosome 4E showed the best capacity for regrowth after harvesting in comparison with other augmented lines [51]. Li et al. [52] obtained substituted, augmented, and translocated lines, most of which showed resistance to wheat striped rust, on the basis of partial amphidiploid Trititrigia 8801 ($2n = 6\times = 42$, ABE), created with the participation of a tetraploid form of *Th. elongatum*.

Bessarabian wheatgrass *Th. bessarabicum* (Savul. & Rayss) Á. Löve (J^b, $2n = 2\times = 14$) is used to improve the resistance of soft wheat to adverse environmental factors. Bessarabian wheatgrass is also interesting as a source of the genes of resistance to salinization and eelworm *Meloidogyne chitwoodi* [53]. With the help of stimulated homologous recombination in the absence of the *Ph1* gene, translocated salt-tolerant forms with translocation of T5AS.5JL were obtained [54]. Low-molecular-weight glutenins have a certain value, which can affect the baking quality of soft wheat grains [55, 56]. In addition, a series of translocated forms of soft wheat with *Th. bessarabicum* chromosome segments was obtained [57, 58]. Grewal et al. [59] developed 1150 molecular SNP (single nucleotide polymorphism) markers for all seven chromosomes of Bessarabian wheatgrass, which can be used in marker-assisted selection (MAS) on the basis of series of recombinant wheat lines [59].

Virgate wheatgrass *Th. junceum* (L.) Á. Löve is a hexaploid ($2n = 6\times= 42$)

with the alleged genomic constitution $E^bE^bE^e$ (or JJE). On the basis of the partial octoploid amphidiploid, a series of wheat lines augmented by *Th. junceum* chromosomes was created [24]. The line AJDAj3 with the augmented chromosome of *Th. junceum* of the second homeologous group showed resistance to Fusarium head blight [60]; based on the salt-resistant line AJDAj5, augmented by the first homeologous group of *Th. junceum*, with the Ph^I gene from *Aegilops speltoides*, the recombinant lines of wheat that retained resistance to salinization were created [61]. The relative of virgate wheatgrass, wiry wheatgrass *Th. junceiforme* (Á. Löve & D. Löve) Á. Löve is a tetraploid ($2n = 4\times = 28$), combining the genomes J_1J_2 (J_1 is close to *Th. elongatum* or *Th. bessarabicum*) or JE (E^bE^e) [24, 62]. Based on it with the use of *T. turgidum* subsp. *dicoccon* (BA), a complete amphidiploid resistant to abiotic (drought, flooding) and biotic (Fusarium head blight and striped mosaic virus) stress factors was created [62].

Intermediate wheatgrass *Th. intermedium* (Host) Barkworth & D. R. Dewey [syn. *A. intermedium* (Host) Beauvoir, *Elytrigia intermedia* (Host) Nevski] ($2n = 6\times = 42$) is a wild cereal widely used in the breeding of soft wheat (due to the high combining ability with it) as a unique donor of resistance to biotic and abiotic stressors [63]. Various breeding lines of intermediate wheatgrass are tested for baking quality of the grain; its cultivation and selection is carried out [64-66]. Many wheat-wheatgrass hybrids (WWHs) with high protein content, resistant to yellow dwarf virus and wheat streak mosaic, powdery mildew, yellow leaf, and stem rust [67-70] have been created. In addition to wheat chromosomes, each WWH has its own unique set of wheatgrass chromosomes associated with certain economically valuable traits [71, 72]. WWH forms created by Tsitsin and his students have good baking qualities of grain, resistance to leaf rust, drought and salinization, germination at the root, are capable of regrowth and long-term lifestyle, are characterized by great biological diversity [73-77]. Many traits were subsequently successfully transferred to the genome of wheat directly from wheatgrass or by octaploid amphidiploids to disomically augmented, replaced and translocated lines of soft wheat having resistance to diseases and new protein subunits [78-80]. It is important to note that even disomically substituted forms may eventually become commercially successful varieties; an example of this are the varieties Tulaikovskaya and Belyanka and their derivatives in which resistance to brown rust is provided by the replacement of the chromosome 6D with the chromosome of intermediate wheatgrass 6J of different origin [81, 82]. It is shown that genes of economically useful traits are localized at different chromosomes of wheatgrass, which can be successfully introgressed into the genome of soft wheat through the exchange of sites between chromosomes [83]. Gene transfer from intermediate wheatgrass most often occurs in stages. For example, the resistance gene *Wsm3* was identified on the long arm of the wheatgrass chromosome 7SL in the disomically augmented line of soft wheat DtA7S#3 [84], on the basis of which a Robertsonian translocation in the wheat chromosome T7BS.7S#3L was obtained [85]; using *ph1b*-stimulated recombination, the line of T7BS 7BL-7S#3L with a smaller dose of wheatgrass chromatin that retained the gene *Wsm3* has been created [86]. Similarly, a translocation of the *Bdv2* gene was obtained: on the basis of amphidiploid TAF36, the augmented line with the wheatgrass chromosome 7S, resistant to the yellow dwarf virus of barley was created; by means of *ph*-mutation and tissue culture, a series of translocated lines carrying the target gene at a lower dose of the rest wheatgrass chromatin was obtained [87]. On the basis of the octoploid wheat-wheatgrass amphidiploid Zhong 5, the line Z4 with two non-Robertsonian translocations T3DS-3AS.3AL-7J^sS and T3AL-

$7J^sS.7J^sL$, among which the second carries the gene for resistance to yellow rust, was obtained [88]. By now, the genes of resistance to fungal rust diseases *Lr38*, *Sr44, Yr50*, *YrL693* [78, 84, 89], powdery mildew *Pm40* [90], *Pm43* [91], Fusarium wilt [92], viral diseases *Wsm1, Wsm3, Bdv2*, *Bdv3, Bdv4* [86, 93-96], and cornel aphid [97] were transferred from intermediate wheatgrass in different chromosomes in the form of small introgressions. Most often, resistance genes from the intermediate wheatgrass subgenomes J^r and J^{vs} are introgressed in chromosomes of the D-subgenome of wheat, more rarely in the subgenome A and very rarely in the subgenome B, which is probably due to the high degree of homology between the wheatgrass subgenomes J^r and J^{vs}, on the one hand, and the subgenome of wheat B — on the other hand [91].

Pontic wheatgrass *Th. ponticum* (Podp.) Z.-W. Liu & R.-C. Wang ($JJJJ^sJ^s$ or $E^eE^bE^xStSt$, $2n = 10\times = 70$) has a set of valuable characteristics, high resistance to fungal and bacterial diseases, high productivity, a powerful fibrous root system, strong development, etc., making this wheatgrass species very promising for crossing with wheat [67]. Pontic wheatgrass relatively easily crosses with soft wheat, which formed the basis for the creation of a series of WWHs, the genome of which combines chromosomes of both intermediate wheatgrass and pontic wheatgrass [71, 74]. WWHs can grow after harvesting and after wintering, are resistant to fungal and viral diseases and can be grown in the future as an independent culture [98, 99]. The genetic material of pontic wheatgrass is often involved in obtaining introgressive lines of wheat through "breeding bridges" in the form of WWHs, augmented and replaced lines. An example of the transfer of the leaf rust resistance gene *Lr19* from the pontic wheatgrass chromosome 7E to the soft wheat chromosome 7D can be called a classic one. The first step was to create the augmented line 7el1(7D) Agrus; then through γ-radiation, the line T4 (Agatha) with translocation of wheatgrass chromatin in the wheat chromosome 7D was obtained. Due to γ-irradiation and *ph1c*-stimulated homologous recombination, a series of lines with different doses of wheatgrass chromatin on the chromosomes 7D and 7B of soft wheat and 7B of hard wheat was created and it was found that this translocation, in addition to *Lr19*, carries the genes of resistance to striped rust *Sr25*, genes of the yellow pigment in the endosperm *Yp* (one of the possible candidates is the gene *Psy1*), as well as *Sd1* and *Sd2*, aggravating fertility and leading to a shift in the decomposition for translocation [100]. In addition to 7el1, a fragment of chromatin 7el2L carrying genes of resistance to fusariosis, which allows staking different resistance genes of pontic wheatgrass, was transferred in wheat [101-103]. During the introgression of the gene *Lr24*, in a similar manner, first a substituted line $3J^s$(3D) TAP 67 was obtained, and on its basis — a translocation at the chromosome 3D; then a chromatin fragment, in addition to the gene *Lr24*, also carried *Sr24* [104]. The effective gene *Sr26* (determines resistance including stem rust race Ug99), was transferred under the scheme of partial amphidiploid ($2n = 56$)—the substituted line 6Ag(6A)—translocation 6AgL-6AL; since this translocation reduced the yield by 15%, its dose was reduced from 90% to 30%, which allowed increasing the yield [105]; at the chromosome 6Ag, also a new resistance gene *SrB* was identified [106]. Pontic wheatgrass genes for resistance to leaf and stem rust *Lr19*, *Lr24*, *Lr29, Sr24*, *Sr25*, *Sr26*, *Sr43* [84, 107-110], colonization by the mite *Eriophyes tulipae Cmc2* [84], an unknown dominant gene for short stem [111], the gene of yellow pigment in the endosperm [112] and anthocyanin staining of the aleurone layer [113] were transferred to wheat chromosomes. Similarly to intermediate wheatgrass, in pontic wheatgrass, a large part of the chromatin introgressions with valuable genes occurs to the chromosomes of the subgenome D of soft wheat, which may be due to its proximity to the subgenomes of pontic wheat-

grass [111].

In breeding practice, the genus *Pseudoroegneria* is used as a donor of salt resistance and drought resistance. Hybrids between *P. spicata* (Pursh) Á. Löve (St, $2n = 2\times = 14$) and different types of wild ruttishness and *Secale montanum* were obtained [114]; new subunits of low-molecular-weight glutenins were identified [115]. The development of molecular markers of chromosomes in the species *Pseudoroegneria* is important, as the St-genome is represented in many polyploid species of perennial wild relatives of wheat, including species important in terms of breeding, such as intermediate wheatgrass, pontic wheatgrass, and in wild ruttishness species [24].

Perennial cereal *D. breviaristatum* (Lindb. F.) Frederiksen ($2n = 4\times = 28$, V^bV^b or VV^b) is represented by the diploid (Vb, $2n = 2\times = 14$) and tetraploid (V^bV^b or VV^b, $2n = 2\times = 42$) forms. On the basis of amphiploids obtained by hybridization of wheat and *D. breviaristatum*, the augmented lines carrying genes of resistance to striped, stem rust, powdery mildew were created [116, 117]. With the help of molecular markers, substitution of $2V^b$(2D) was revealed in lines of soft wheat resistant to stripe rust [118], on the basis of which the introgressive stable form with longer spikes was received [119]. The introgressions of the segments of the $1V^b$ chromosome allowed creating forms of wheat with new high molecular glutenins [119, 120].

The closest relative of *D. breviaristatum* [syn. *Haynaldia villosa*] is annual *Dasypyrum villosum* (L.) Borbás (V, $2n = 2\times = 14$), widely used as a donor of resistance to viral diseases in the augmented and substituted translocated lines [121, 122]. A major role in the involvement of genetic material of *Dasypyrum* in the breeding of soft wheat is played by molecular markers of not genes, but chromosomes and their arms, which allows marking and mapping the segments on the chromosome associated with certain symptoms [43, 44]. Due to the transfer of *D. villosum* chromatin in the genome of wheat, it was found that the chromosome 1V carries the genes of resistance to kernel smut and eyespot, as well as genes that improve grain quality [123-125]; at 2V, the genes that increase yield and genes of resistance to powdery mildew *Pm62* and eyespot [125-127] were discovered; at 3V, the genes of resistance to take-all disease (pathogen *Gaeumannomyces graminis*) and eyespot and striped rust [125, 128, 129] were identified; at 4V, the genes of resistance to eyespot *Pch3*, the virus of spindle streak mosaic of wheat *Wss1* [130-132] were localized; at the chromosome 5V, the genes of resistance to powdery mildew *Pm55* [133] were localized; the 6V chromosome carries the genes of resistance to powdery mildew [134], leaf rust *Lr6V*#4 [124], striped rust *SrHv6* [135] and bent-grass leaf-gall eelworm *CreV* [136].

The members of the genus *Agropyron* originally grew in the steppes of European Russia and south-east Siberia and may have been cultivated in the Volga Region to the east of Saratov. This genus is represented by 10 to 15 species, among which *A. cristatum* and *A. fragile* are introduced and grown in North America, and five species grow in China. The most characteristic representative of this genus is the tetraploid form of *A. cristatum*, growing in Central Europe and Central Asia, in Central Asia and Siberia, China and Mongolia, along with rarer diploid forms; hexaploids are found in Turkey, Iran, and Kazakhstan [24]. Based on augmented and substituted lines, applying gametocidal chromosomes, radiation exposure, and other methods, breeders transferred into chromosomes of wheat individual segments that carry genes for resistance to powdery mildew and leaf rust (chromosome 2P) [137, 138]; genes that increase productive tillering capacity and the number of grains per spike, and genes of resistance to leaf rust and powdery mildew (chromosome 6P) [139-142]; genes that increase drought hardiness and thousand-kernel weight (chromosome 7P) [143]. Thus, in

addition to stability genes, crested wheatgrass carries genes and QTL (quantitative trait loci), which positively affect the elements of the crop structure.

The *Elymus* genus is represented by more than 200 exclusively polyploid species combining the genomes St, H, Y, P, and W [24, 35]. In the progeny from crosses and backcrosses of allohexaploid apomictic species *E. rectisetus* with soft wheat, the disomically augmented line with the 1Y chromosome, which is characterized by moderate resistance to Helminthosporium and Septoria diseases [144, 145], was obtained, and the complement of the chromosomes of the 2nd and 5th homeologous groups provided good resistance to Fusarium head blight [60, 146]. *E. tsukushiensis*, which became a donor of the *Fhb6* gene (chromosome 1Ets#1S), transferred to the wheat chromosome 1AS [147], and *E. repens*, the chromatin of which was used to obtain different introgressive Fusarium-resistant lines of wheat [148, 149], also serve as sources of resistance to Fusarium. *E. trachycaulis* was the donor of the resistance gene of soft wheat to leaf rust *Lr55* [150]. Wheat – wild ruttishness hybrids based on *E. farctus* have great perspectives [151].

The use of molecular and cytogenetic markers allows introgressing targeted genes in the wheat genome, thereby greatly facilitating the work of breeders [152, 153]. Genome-wide sequencing data, which is currently being actively developed, and genomic editing technologies will undoubtedly improve the efficiency of the use of genetic resources of wild species [154-156].

Therefore, the successful use of the genetic potential of wild perennial wheat relatives makes it possible to expand the genetic diversity of wheat, significantly impoverished as a result of the limited use of the same varieties in pedigrees. The list of species of perennial wild relatives and useful genes transferred to the genome of wheat is certainly not limited to those listed in this review. This analysis showed that the general trend is the transfer and characterization of genes of wild relatives, which not only increase stability but also positively affect the elements of the structure of the crop and the quality of the final product, that is, clearly improve, and not just worsen these characteristics. Molecular and cytogenetic markers, methods of whole-genomic sequencing and genomic editing technologies will become effective tools for breeders. It is necessary to use all available resources to expand the wheat genetic base, involving in the selection both old varieties and populations of the *Triticum* and *Aegilops* species and new species and genera of *Triticeae* perennial cereals.

REFERENCES

1. Balfourier F., Bouchet S., Robert S., De Oliveira R., Rimbert H., Kitt J., Choulet F., International Wheat Genome Sequencing Consortium, BreedWheat Consortium, Paux E. Worldwide phylogeography and history of wheat genetic diversity. *Science Advances*, 2019, 5(5): EAAV0536 (doi: 10.1126/sciadv.aav0536).
2. Jaradat A.A. Phenotypic divergence in the meta-population of the Hourani durum wheat landrace. *J. Food Agric. Env.*, 2006, 4(3): 186-191 (doi: 10.1234/4.2006.942).
3. Jaradat A.A. Wheat landraces: a mini review. *Emir. J. Food Agric.*, 2013, 25(1): 20-29 (doi: 10.9755/ejfa.v25i1.15376).
4. Girma E. Genetic erosion of wheat (*Triticum* spp.): concept, research results and challenges. *Journal of Natural Sciences Research*, 2017, 7(23): 72-81.
5. Miller J. Genetic erosion: crop plants threatened by government neglect. Science New Series, 1973, 182(4118): 1231-1233 (doi: 10.1126/science.182.4118.1231).
6. Day P.R. Genetic variability of crops. *Annual Review of Phytopathology*, 1973, 11(1): 293-312 (doi: 10.1146/annurev.py.11.090173.001453).
7. Dzyubenko N.I. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Geneticheskie resursy kul'turnykh rastenii, problemy mobilizatsii, inventarizatsii, sokhraneniya i izucheniya genofonda vazhneishikh sel'skokhozyaistvennykh kul'tur dlya resheniya prioritetnykh zadach selektsii»* [Proc. Int. Conf. «Genetic resources of cultivated plants, problems of mobilization, inventory, preservation and study of gene pool for breeding priorities»]. St. Petersburg, 2001: 24-

26 (in Russ.).
8. Martynov S.P., Dobrotvorskaya T.V. Geneticheskaya eroziya v sortakh myagkoi pshenitsy, realizovannykh v Rossii. *Materialy konferentsii «Genetika v XXI veke: sovremennoe sostoyanie i perspektivy razvitiya»* [Proc. Conf. «Genetics in the 21st century: current state and development prospects»]. Moscow, 2004: 75 (in Russ.).
9. Pukhal'skii V.A. *Vestnik VOGiS,* 2005, 9(3): 306-316 (in Russ.).
10. Glazko V.I., Glazko T.T. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2010, 3: 101-114 (in Russ.).
11. Tarantul V.Z. *Tolkovyi slovar' po molekulyarnoi i kletochnoi biotekhnologii.* Tom 1 [Explanatory dictionary of molecular and cell biotechnology. Vol. 1]. Moscow, 2015 (in Russ.).
12. Glazko V.I. Genetic unit and sustainable development of agroecosystem. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2007, 6: 9-15 (in Russ.).
13. Goncharov N.P., Shumnyi V.K. *Vestnik VOGiS*, 2008, 12(4): 509-523 (in Russ.).
14. Gorbunov Yu., Soadatova R., Kazantseva E. *Genofond rastenii Krasnoi knigi Rossiiskoi Federatsii, sokhranyaemyi v kollektsiyakh botanicheskikh sadov i dendrariev* [The gene pool of plants of the Russian Federation Red Book, preserved in collections of botanical gardens and arboretums]. Moscow, 2012 (in Russ.).
15. Shamanin V.P., Pototskaya I.V., Trushchenko A.Yu., Chursin A.S., Kuz'mina S.P., Krotova L.A. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2012, 5(91): 13-16 (in Russ.).
16. *FAOSTAT*. Available http://www.fao.org/faostat/en/#data. Accessed 30.01.2019.
17. Smith S., Bubeck D., Nelson B., Stanek J., Gerke J. Genetic diversity and modern plant breeding. In: *Genetic diversity and erosion in plants. Indicators and prevention*. V. 1. M.R. Ahuja, S. Mohan Jain (eds.). Springer International Publishing, Switzerland, 2015: 55-88.
18. Govindaraj M., Vetriventhan M., Srinivasan M. Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. *Genetics Research International*, 2015, 2015: Article ID 431487 (doi: 10.1155/2015/431487).
19. Ablova I.B., Bespalova L.A., Kolesnikov F.A., Nabokov G.D., Kovtunenko V.Ya., Filobok V.A., Davoyan R.O., Khudokormova Zh.N., Mokhova L.M., Levchenko Yu.G., Tarkhov A.S. *Zernovoe khozyaistvo Rossii*, 2016, 5: 1-7 (in Russ.).
20. Novoselskaya-Dragovich A.Yu., Fisenko A.V., Imasheva A.G., Pukhalskiy V.A. Comparative analysis of the genetic diversity dynamics at gliadin loci in the winter common wheat *Triticum aestivum* L. cultivars developed in Serbia and Italy over 40 years of scientific breeding. *Russian Journal of Genetics*, 2007, 43(11): 1236-1242 (doi: 10.1134/S1022795407110051).
21. Martynov S.P., Dobrotvorskaya T.V. *Trudy po prikladnoi botanike, genetike i selektsii*, 2012, 169: 193-209 (in Russ.).
22. Orabi J., Jahoor A., Backes G. Changes in allelic frequency over time in European bread wheat (*Triticum aestivum* L.) varieties revealed using DArT and SSR markers. *Euphytica*, 2014, 197(3): 447-462 (doi: 10.1007/s10681-014-1080-x).
23. Wulff B.B., Moscou M.J. Strategies for transferring resistance into wheat: from wide crosses to GM cassettes. *Front. Plant Sci.*, 2014, 5: 692 (doi: 10.3389/fpls.2014.00692).
24. Wang R.R.C. *Agropyron* and *Psathyrostachys*. In: *Wild crop relatives: genomic and breeding resources. V. 1.* C. Kole (ed.). Springer Berlin Heidelberg, 2011: 77-108 (doi: 10.1007/978-3-642-14228-4_2).
25. Zhang C., Fan X., Yu H.Q., Zhang L., Wang X.L., Zhou Y.H. Different maternal genome donor to *Kengyilia* species inferred from chloroplast *trnL-F* sequences. *Biologia Plantarum*, 2009, 53(4): 759-763 (doi: 10.1007/s10535-009-0139-3).
26. Mahelka V., Kopecky D., Pastova L. On the genome constitution and evolution of intermediate wheatgrass (*Thinopyrum intermedium*: *Poaceae*, *Triticeae*). *BMC Evolutionary Biology*, 2011, 11(1): 127 (doi: 10.1186/1471-2148-11-127).
27. Wang Q., Xiang J., Gao A., Yang X., Liu W., Li X., Li L. Analysis of chromosomal structural polymorphisms in the St, P and Y genomes of *Triticeae* (*Poaceae*). *Genome*, 2010, 53: 241-249 (doi: 10.1139/g09-098).
28. Mason-Gamer R.J. Phylogeny of a genomically diverse group of *Elymus* (*Poaceae*) allopolyploids reveals multiple levels of reticulation. *PLoS ONE*. 2013, 8: e78449 (doi: 10.1371/journal.pone.0078449).
29. Wang R.R.-C., Larson S.R., Jensen K.B., Bushman S., DeHaan L., Wang S., Yan X. Genome evolution of intermediate wheatgrass as revealed by EST-SSR markers developed from its three progenitor diploid species. *Genome*, 2015, 58: 63-70 (doi: 10.1139/gen-2014-0186).
30. Chen Q., Conner R.L., Laroche A., Thomas J.B. Genome analysis of *Thinopyrum intermedium* and *Thinopyrum ponticum* using genomic in situ hybridization. *Genome*, 1998, 41(4): 580-586 (doi: 10.1139/g98-055).
31. Divashuk M.G., Khuat T.M., Kroupin P.Y., Kirov I.V., Romanov D.V., Kiseleva A.V., Khrustaleva L.I., Alexeev D.G., Zelenin A.S., Klimushina M.V., Razumova O.V., Karlov G.I. Variation in copy number of Ty3/Gypsy centromeric retrotransposons in the genomes of *Thinopyrum intermedium* and its diploid progenitors. *PLoS ONE*, 2016, 11(4): e0154241 (doi: 10.1371/journal.pone.0154241).

32. Baum B., Edwards T., Johnson D. What does the nr5S DNA multigene family tell us about the genomic relationship between *Dasypyrum breviaristatum* and *D. villosum* (*Triticeae*: *Poaceae*)? *Mol. Genet. Genomics*, 2014, 289: 553-565 (doi: 10.1007/s00438-014-0825-5).
33. Gradzielewska A., Tyrka M., Leśniowska-Nowak J., Nazaruk J. Genetic relationships among representatives of *Dasypyrum*, *Secale* and *Triticum* species revealed with RAPD and ISSR markers. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 2014, 42(2): 420-430 (doi: 10.15835/nbha.42.2.9662).
34. Zhang P., Dundas I.S., Xu S.S., Friebe B., McIntosh R.A., Raupp W.J. Chromosome engineering techniques for targeted introgression of rust resistance from wild wheat relatives. In: *Wheat rust diseases. Methods and protocols, methods in molecular biology, Vol. 1659*. S. Periyannan (ed.). Springer Science+Business Media LLC, 2017: 163-172 (doi: 10.1007/978-1-4939-7249-4_14).
35. Ceoloni C., Kuzmanovic L., Forte P., Virili M. E., Bitti A. Wheat-perennial *Triticeae* introgressions: major achievements and prospects. In: *Alien introgression in wheat: cytogenetics, molecular biology, and genomic*. M. Molnár-Láng, C. Ceoloni, J. Doležel (eds.). Springer International Publishing Switzerland, 2015: 273-314 (doi: 10.1007/978-3-319-23494-6_11).
36. Khuat T.M.L., Divashuk M.G., Kroupin P.Yu., Nguyen Ph.A., Kiseleva A.V., Karlov G.I. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2015, 2: 29-35 (in Russ.).
37. Alexandrov O.S., Divashuk M.G., Karlov G.I. Development of the St/J and V genome specific molecular marker based on 5S rDNA polymorphism in *Thinopyrum bessarabicum*, *Pseudoroegneria spicata*, and *Dasypyrum villosum*. *Moscow University Biological Sciences Bulletin*, 2018, 73(1): 18-23 (doi: 10.3103/S0096392518010017).
38. Kocheshkova A.A., Divashuk M.G., Krupin P.Yu., Karlov G.I. *Vestnik Bashkirskogo universiteta*, 2013, 18(3): 736-738 (in Russ.).
39. Pochtovyi A.A., Karlov G.I., Divashuk M.G. *Vestnik Bashkirskogo universiteta*, 2013, 18(3): 745-747 (in Russ.).
40. Klimushina M.V. *Sravnitel'nyi molekulyarno-geneticheskii analiz genov Wx u razlichnykh vidov triby pshenitsevykh. Avtoreferat kandidatskoi dissertatsii* [Comparative molecular genetic analysis of *Wx* genes in various species of the wheat tribe. PhD Thesis]. Moscow, 2013 (in Russ.).
41. Kocheshkova A.A., Divashuk M.G., Kroupin P.Y., Karlov G.I. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2014, 5: 5-12 (in Russ.).
42. Pochtovyy A.A., Kroupin P.Yu., Divashuk M.G., Kocheshkova A.A., Sokolov P.A., Karlov G.I. Clonning of *Dreb1* gene in wheat wild relatives and development of a DNA marker for its monitoring in wheat background. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2018, 53(3): 499-510 (doi: 10.15389/agrobiology.2018.3.499eng).
43. Kuznetsova V.M. *Materialy XIX Vserossiiskoi konferentsii molodykh uchenykh «Biotekhnologiya v rastenievodstve, zhivotnovodstve i sel'skokhozyaistvennoi mikrobiologii» (15-16 aprelya 2019 goda, g. Moskva*) [Proc. XIX Russian Conf. of young scientists «Biotechnologies in plant growing, livestock and agricultural microbiology»]. Moscow, 2019: 37-38 (in Russ.).
44. Sokolov P.A., Krupin P.Yu., Divashuk M.G., Karlov G.I. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2017, 4: 147-157 (in Russ.).
45. Shen X., Ohm H. Fusarium head blight resistance derived from *Lophopyrum elongatum* chromosome 7E and its augmentation with *Fhb1* in wheat. *Plant Breeding*, 2006, 125: 424-429 (doi: 10.1111/j.1439-0523.2006.01274.x).
46. Jauhar P.P., Peterson T.S., Xu S.S. Cytogenetic and molecular characterization of a durum alien disomic addition line with enhanced tolerance to Fusarium head blight. *Genome*, 2009, 52: 467-483 (doi 10.1139/g09-014).
47. Jauhar P.P., Peterson T.S. Cytological and molecular characterization of homoeologous group-1 chromosomes in hybrid derivatives of a durum disomic alien addition line. *Plant Genome*, 2011, 4: 102-109 (doi: 10.3835/plantgenome2011.01.0002).
48. Liu H., Dai Y., Chi D., Huang S., Li H., Duan Y., Cao W., Gao Y., Fedak G., Chen J. Production and molecular cytogenetic characterization of a durum wheat-*Thinopyrum elongatum* 7E disomic addition line with resistance to Fusarium Head Blight. *Cytogenet. Genome Res.*, 2017, 153(3): 165-173 (doi: 10.1159/000486382).
49. Anderson J.M., Bucholtz D.L., Sardesai N., Santini J.B., Gyulai G., Williams C.E., Stephen B., Goodwin S.B. Potential new genes for resistance to Mycosphaerella graminicola identified in *Triticum aestivum-Lophopyrum elongatum* disomic substitution lines. *Euphytica*, 2010, 172: 251-262 (doi: 10.1007/s10681-009-0061-y).
50. Mullan D., Mirzaghaderi G., Walker E., Colmer T., Francki M. Development of wheat—*Lophopyrum elongatum* recombinant lines for enhanced sodium 'exclusion' during salinity stress. *Theor. Appl. Genet.*, 2009, 119(7): 1313-1323 (doi: 10.1007/s00122-009-1136-9).
51. Lammer D., Cai X., Arterburn M., Chatelain J., Murray T., Jones S. A single chromosome addition from *Thinopyrum elongatum* confers a polycarpic, perennial habit to annual wheat. *J. Exp. Bot.*, 2004, 55: 1715-1720 (doi: 10.1093/jxb/erh209).
52. Li D., Long D., Li T., Wu Y., Wang Y., Zeng J., Xu L., Fan X., Sha L., Zhang H., Zhou Y., Kang H. Cytogenetics and stripe rust resistance of wheat—*Thinopyrum elongatum* hybrid deriva-

tives. *Molecular Cytogenetics*, 2018, 11: 16 (doi: 10.1186/s13039-018-0366-4).
53. Jensen K.B., Griffin G.D. Resistance of diploid *Triticeae* species and accessions to the Columbia root-knot nematode, *Meloidogyne chitwoodi*. *J. Nematol.*, 1994, 26(4S): 635-639.
54. King I., Purdie K., Rezanoor H., Koebner R., Miller T., Reader S., Nicholson P. Characterization of *Thinopyrum bessarabicum* chromosome segments in wheat using random amplified polymorphic DNAs (RAPIDs) and genomic in situ hybridization. *Theor. Appl. Genet.*, 1993, 86: 895-900 (doi: 10.1007/BF00211038).
55. Luo Z., Chen F., Feng D., Xia G. LMW-GS genes in *Agropyron elongatum* and their potential value in wheat breeding. *Theor. Appl. Genet.*, 2005, 111: 272-280 (doi: 10.1007/s00122-005-2021-9).
56. Gao X., Liu S.W., Sun Q., Xia G.M. High frequency of HMW-GS sequence variation through somatic hybridization between *Agropyron elongatum* and common wheat. *Planta*, 2010, 231: 245-250 (doi: 10.1007/s00425-009-1040-1).
57. Qi Z., Du P., Qian B., Zhuang L., Chen H., Chen T., Shen J., Guo J., Feng Y., Pei Z. Characterization of a wheat-*Thinopyrum bessarabicum* (T2JS-2BS.2BL) translocation line. *Theor. Appl. Genet.*, 2010, 121: 589-597 (doi: 10.1007/s00122-010-1332-7).
58. Patokar C., Sepsi A., Schwarzacher T., Kishii M., Heslop-Harrison J. Molecular cytogenetic characterization of novel wheat-*Thinopyrum bessarabicum* recombinant lines carrying intercalary translocations. *Chromosoma*, 2015, 125(1): 163-172 (doi: 10.1007/s00412-015-0537-6).
59. Grewal S., Yang C., Edwards S., Scholefield D., Ashling S., Burridge A., King I., King J. Characterisation of *Thinopyrum bessarabicum* chromosomes through genome-wide introgressions into wheat. *Theor. Appl. Genet.*, 2017, 131(2): 389-406 (doi: 10.1007/s00122-017-3009-y).
60. McArthur R., Zhu X., Oliver R., Klindworth D., Xu S., Stack R., Wang R., Cai X. Homoeology of *Thinopyrum junceum* and *Elymus rectisetus* chromosomes to wheat and disease resistance conferred by the *Thinopyrum* and *Elymus* chromosomes in wheat. *Chromosome Res.*, 2012, 20(6): 699-715 (doi: 10.1007/s10577-012-9307-y).
61. Wang R., Li X., Hu Z., Zhang J., Larson S., Zhang X., Grieve C., Shannon M. Development of salinity tolerant wheat recombinant lines from a wheat disomic addition line carrying a *Thinopyrum junceum* chromosome. *Int. J. Plant Sci.*, 2003, 164(1): 25-33 (doi: 10.1086/344556).
62. Li W., Zhang Q., Wang S., Langham M., Singh D., Bowden R., Xu S. Development and characterization of wheat—sea wheatgrass (*Thinopyrum junceiforme*) amphiploids for biotic stress resistance and abiotic stress tolerance. *Theor. Appl. Genet.*, 2018, 132(1): 163-175 (doi: 10.1007/s00122-018-3205-4).
63. Li H., Wang X. *Thinopyrum ponticum* and *Th. intermedium:* the promising source of resistance to fungal and viral diseases of wheat. *J. Genet. Genomics*, 2009, 36(9): 557-565 (doi: 10.1016/S1673-8527(08)60147-2).
64. Rahardjo C., Gajadeera C., Simsek S., Annor G., Schoenfuss T., Marti A., Ismail B. Chemical characterization, functionality, and baking quality of intermediate wheatgrass (*Thinopyrum intermedium*). *J. Cereal Sci.*, 2018, 83: 266-274 (doi: 10.1016/j.jcs.2018.09.002).
65. Banjade J. *Effects of dough conditioners on rheology and bread quality of intermediate wheatgrass. M.S. thesis.* University of Minnesota, 2018.
66. Jungers J.M., Frahm C.S., Tautges N.E., Ehlke N.J., Wells M.S., Wyse D.L., Sheaffer C.C. Growth, development, and biomass partitioning of the perennial grain crop *Thinopyrum intermedium*. *Ann. Appl. Biol.*, 2018, 172(3): 346-354 (doi: 10.1111/aab.12425).
67. Tsitsin N.V. *Mnogoletnyaya pshenitsa* [Perennial wheat]. Moscow, 1978 (in Russ.).
68. Cauderon Y., Saigne B., Dauge M. The resistance to wheat rusts of *Agropyron intermedium* and its use in wheat improvement. *Proc. 4th International Wheat Genet Symposium*. L.M.S. Sears, E.R. Sears (eds.). Columbia, Mo., 1973: 401-407
69. Georgieva M., Kruppa K., Tyankova N., Molnar Lang M. Molecular cytogenetic identification of a novel hexaploid wheat—*Thinopyrum intermedium* partial amphiploid with high protein content. *Turk. J. Biol.*, 2016, 40: 554-560 (doi: 10.3906/biy-1503-30).
70. Cui L., Ren Y., Murray T., Yan W., Guo Q., Niu Y., Sun Y., Li H. Development of perennial wheat through hybridization between wheat and wheatgrasses: a review. *Engineering*, 2018, 4(4): 507-513 (doi: 10.1016/j.eng.2018.07.003).
71. Kroupin P., Divashuk M., Belov V., Glukhova L., Aleksandrov O., Karlov G. Comparative molecular cytogenetic characterization of partial wheat-wheatgrass hybrids. *Rus. J. Genet.*, 2011, 47(4): 432-437 (doi: 10.1134/s1022795411040077).
72. Trifonova A.A., Boris K.V., Dedova L.V., Mel'nik V.A., Ivanova L.P., Kuz'mina N.P., Zavgorodnii S.V., Upelniek V.P. *Vavilovskii zhurnal genetiki i selektsii*, 2018, 22(6): 648-653 (doi: 10.18699/VJ18.406) (in Russ.).
73. Divashuk M.G., Krupin P.Yu., Bazhenov M.S., Klimushina M.V., Belov V.I., Semenova E.V., Karlov G.I. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2012, 5: 29-37 (in Russ.).
74. Belov V.I., Ivanova L.P., Zavgorodnii S.V., Upelniek V.P. *Byulleten' Glavnogo botanicheskogo sada*, 2013, 4(199): 49-55 (in Russ.).
75. Krupin P.Yu., Divashuk M.G., Belov V.I., Zhemchuzhina A.I., Kovalenko E.D.,

Upelniek V.P., Karlov G.I. Investigation of intermediary wheat-agropyron hybrids on resistance to leaf rust. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2013, 48(1): 68-73 (doi: 10.15389/agrobiology.2013.1.68eng).
76. Krupin P.Yu., Divashuk M.G., Bazhenov M.S., Gritsenko L.A., Tarakanov I.G., Upelniek V.P., Belov V.I., Pochtovyi A.A., Starikova E.V., Kkhuat Tkhi Mai L., Klimushina M.V., Davydova A.N., Karlov G.I. Salt tolerance polymorfism in seadlings of wheat-wheatgrass hybrids. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2013, 48(5): 44-53 (doi: 10.15389/agrobiology.2013.5.44rus) (in Russ.).
77. Kocheshkova A.A., Kroupin P.Y., Bazhenov M.S., Karlov G.I., Pochtovyy A.A., Divashuk M.G., Upelniek V.P., Belov V.I. Pre-harvest sprouting resistance and haplotype variation of *ThVp*-1 gene in the collection of wheat-wheatgrass hybrids. *PLoS ONE*, 2017, 12(11): e0188049 (doi: 10.1371/journal.pone.0188049).
78. Liu J., Chang Z., Zhang X., Yang Z., Li X., Jia J., Zhan H., Guo H., Wang J. Putative *Thinopyrum intermedium*-derived stripe rust resistance gene *Yr50* maps on wheat chromosome arm 4BL. *Theor. Appl. Genet.*, 2013, 126(1): 265-274 (doi: 10.1007/s00122-012-1979-3).
79. Zhan H., Zhang X., Li G., Pan Z., Hu J., Li X., Qiao L., Jia J., Guo H., Chang Z., Yang Z. Molecular characterization of a new wheat-*Thinopyrum intermedium* translocation line with resistance to powdery mildew and stripe rust. *Int. J. Mol. Sci.*, 2015, 16(1): 2162-2173 (doi: 10.3390/ijms1601216).
80. Wang Y., Wang H. Characterization of three novel wheat-*Thinopyrum intermedium* addition lines with novel storage protein subunits and resistance to both powdery mildew and stripe rust. *J. Genet. Genomics*, 2016 43(1): 45-48 (doi: 10.1016/j.jgg.2015.10.004).
81. Salina E.A., Adonina I.G., Stasyuk A.I., Leonova I.N., Badaeva E.D., Shishkina A.A., Kroupin P.Y., Divashuk M.G., Starikova E.V., Khuat T.M.L., Karlov G.I., Syukov V.V. A *Thinopyrum intermedium* chromosome in bread wheat cultivars as a source of genes conferring resistance to fungal diseases. *Euphytica*, 2015, 204(1): 91-101 (doi: 10.1007/s10681-014-1344-5).
82. Sibikeev S.N., Druzhin A.E., Badaeva E.D., Shishkina A.A., Dragovich A.Y., Gultyaeva E.I., Kroupin P.Y., Karlov G.I., Khuat T.M., Divashuk M.G. Comparative analysis of *Agropyron intermedium* (Host) Beauv 6Agi and 6Agi2 chromosomes in bread wheat cultivars and lines with wheat—wheatgrass substitutions. *Russ. J. Genet.*, 2017, 53(3): 314-324 (doi: 10.1134/s1022795417030115).
83. Davoyan R.O., Bebyakina I.V., Davoyan E.R., Zinchenco A.N., Zubanova Y.S., Mikov D.S. Introgression of common wheat lines with genetic material of *Agropyron glaucum*. *Russian Journal of Genetics: Applied Research*, 2016, 6(1): 54-61 (doi: 10.1134/s2079059716010056).
84. Friebe B., Jiang J.M., Raupp W.J., McIntosh R.A., Gill B.S. Characterization of wheat alien translocations conferring resistance to diseases and pests: current status. *Euphytica*, 1996, 91(1): 59-87 (doi: 10.1007/bf00035277).
85. Liu W., Seifers D.L., Qi L.L., Friebe B., Gill B.S. A compensating wheat-*Thinopyrum intermedium* Robertsonian translocation conferring resistance to wheat streak mosaic virus and *Triticum* mosaic virus. *Crop Sci.*, 2011, 51(6): 2382-2390 (doi: 10.2135/cropsci2011.03.0118).
86. Danilova T.V., Zhang G., Liu W., Friebe B., Gill B.S. Homoeologous recombination-based transfer and molecular cytogenetic mapping of a wheat streak mosaic virus and *Triticum* mosaic virus resistance gene *Wsm3* from *Thinopyrum intermedium* to wheat. *Theor. Appl. Genet.*, 2017, 130(3): 549-556 (doi: 10.1007/s00122-016-2834-8).
87. Banks P., Larkin P., Bariana H., Lagudah E., Appels R., Waterhouse P., Brettell R., Chen X., Xu H., Xin Z., Qian Y., Zhou X., Cheng Z., Zhou G. The use of cell culture for subchromosomal introgressions of barley yellow dwarf virus resistance from *Thinopyrum intermedium* to wheat. *Genome*, 1995, 38(2): 395-405 (doi: 10.1139/g95-051).
88. Lang T., La S., Li B., Yu Z., Chen Q., Li J., Yang E., Li G., Yang Z. Precise identification of wheat—*Thinopyrum intermedium* translocation chromosomes carrying resistance to wheat stripe rust in line Z4 and its derived progenies. *Genome*, 2018, 61(3): 177-185 (doi: 10.1139/gen-2017-0229).
89. Huang Q., Li X., Chen W.Q., Xiang Z.P., Zhong S.F., Chang Z.J., Zhang M., Zhang H.Y., Tan F.Q., Ren Z.L., Luo P.G. Genetic mapping of a putative *Thinopyrum intermedium*-derived stripe rust resistance gene on wheat chromosome 1B. *Theor. Appl. Genet.*, 2014, 127(4): 843-853 (doi: 10.1007/s00122-014-2261-7).
90. Luo P.G., Luo H.Y., Chang Z.J., Zhang H.Y., Zhang M., Ren Z.L. Characterization and chromosomal location of *Pm40* in common wheat: a new gene for resistance to powdery mildew derived from *Elytrigia intermedium*. *Theor. Appl. Genet.*, 2009, 118(6): 1059-1064 (doi: 10.1007/s00122-009-0962-0).
91. He R., Chang Z., Yang Z., Yuan Z., Zhan H., Zhan X., Liu J. Inheritance and mapping of powdery mildew resistance gene *Pm43* introgressed from *Thinopyrum intermedium* into wheat. *Theor. Appl. Genet.*, 2009, 118(6): 1173-1180 (doi: 10.1007/s00122-009-0971-z).
92. Fedak G., Han F. Characterization of derivatives from wheat—*Thinopyrum* wide crosses. *Cytogenet. Genome Res.*, 2005, 109(1-3): 350-359 (doi: 10.1159/000082420).

93. Zhang Z.Y., Xu J.S., Xu Q.J., Larkin P., Xin Z.Y. Development of novel PCR markers linked to the BYDV resistance gene *Bdv2* useful in wheat for marker assisted selection. *Theor. Appl. Genet.*, 2004, 109(2): 433-439 (doi: 10.1007/s00122-004-1649-1).
94. Ayala-Navarrete L., Tourton E., Mechanicos A.A., Larkin P.J. Comparison of *Thinopyrum intermedium* derivatives carrying barley yellow dwarf virus resistance in wheat. *Genome*, 2009, 52(6): 537-546 (doi: 10.1139/g09-028).
95. Zhang Z.Y., Lin Z.S., Xin Z.Y. Research progress in BYDV resistance genes derived from wheat and its wild relatives. *J. Genet. Genomics*, 2009, 36(9): 567-573 (doi: 10.1016/s1673-8527(08)60148-4).
96. Friebe B., Qi L.L., Wilson D.L., Chang Z.J., Seifers D.L., Martin T.J., Fritz A.K., Gill B.S. Wheat—*Thinopyrum intermedium* recombinants resistant to wheat streak mosaic virus and *Triticum* mosaic virus. *Crop Sci.*, 2009, 49(4): 1221-1226 (doi: 10.2135/cropsci2008.09.0513).
97. Friebe B., Mukai Y., Dhaliwal H.S., Martin T.J., Gill B.S. Identification of alien chromatin specifying resistance to wheat streak mosaic and greenbug in wheat germplasm by C-banding and in situ hybridization. *Theor. Appl. Genet.*, 1991, 81(3): 381-389 (doi: 10.1007/bf00228680).
98. Larkin P.J., Newell M.T., Hayes R.C., Aktar J., Norton M.R., Moroni S.J., Wade L.J. Progress in developing perennial wheats for grain and grazing. *Crop and Pasture Science*, 2014, 65(11): 1147-1164 (doi: 10.1071/CP13330).
99. Lloyd S. *Perennial wheat. Independent Project in Biology*. 2015. Available http://stud.epsilon.slu.se/7778/. No date.
100. Kuzmanovic L., Gennaro A., Benedettelli S., Dodd I.C., Quarrie S.A., Ceoloni C. Structural-functional dissection and characterization of yield-contributing traits originating from a group 7 chromosome of the wheatgrass species *Thinopyrum ponticum* after transfer into durum wheat. *J. Exp. Bot.,* 2014, 65(2): 509-525 (doi: 10.1093/jxb/ert393).
101. Shen X., Ohm H. Molecular mapping of *Thinopyrum*-derived Fusarium head blight resistance in common wheat. *Mol. Breeding*, 2007, 20(2): 131-140 (doi: 10.1007/s11032-007-9079-9).
102. Forte P., Virili M.E., Kuzmanovi L., Moscetti I., Gennaro A., D'Ovidio R., Ceoloni C. A novel assembly of *Thinopyrum ponticum* genes into the durum wheat genome: pyramiding Fusarium head blight resistance onto recombinant lines previously engineered for other beneficial traits from the same alien species. *Mol. Breeding*, 2014, 34(4): 1701-1716 (doi: 10.1007/s11032-014-0175-3).
103. Singh M., Mallick N., Chand S., Kumari P., Sharma J., Sivasamy M., Jayaprakash P., Prabhu K., Jha S., Vinod Marker-assisted pyramiding of *Thinopyrum*-derived leaf rust resistance genes *Lr19* and *Lr24* in bread wheat variety HD2733. *J. Genet.*, 2017, 96(6): 951-957 (doi: 10.1007/s12041-017-0859-7).
104. Li H., Chen Q., Conner R.L., Guo B., Zhang Y., Graf R.J., Laroche A., Jia X., Liu G., Chu C. Molecular characterization of a wheat—*Thinopyrum ponticum* partial amphiploid and its derivatives for resistance to leaf rust. *Genome*, 2003, 46(5): 906-913 (doi: 10.1139/g03-053).
105. Dundas I., Zhang P., Verlin D., Graner A., Shepherd K. Chromosome engineering and physical mapping of the *Thinopyrum ponticum* translocation in wheat carrying the rust resistance gene *Sr26*. *Crop Sci.*, 2015, 55(2): 648-657 (doi: 10.2135/cropsci2014.08.0590).
106. Mago R., Zhang P., Xia X., Zhang J., Hoxha S., Lagudah E., Graner A., Dundas I. Transfer of stem rust resistance gene *SrB* from *Thinopyrum ponticum* into wheat and development of a closely linked PCR-based marker. *Theor. Appl. Genet.*, 2019, 132(2): 371-382 (doi: 10.1007/s00122-018-3224-1).
107. McIntosh R.A., Dyck P.L., Green G.J. Inheritance of leaf rust and stem rust resistances in wheat cultivars Agent and Agatha. *Aust. J. Agric. Res.*, 1977, 28(1): 37-45 (doi: 10.1071/ar9770037).
108. Gupta S.K., Charpe A., Prabhu K.V., Haque Q.M.R. Identification and validation of molecular markers linked to the leaf rust resistance gene *Lr19* in wheat. *Theor. Appl. Genet.*, 2006, 113(6): 1027-1036 (doi: 10.1007/s00122-006-0362-7).
109. Friebe B., Jiang J., Knott D.R., Gill B.S. Compensation indices of radiation-induced wheat-*Agropyron elongatum* translocations conferring resistance to leaf rust and stem rust. *Crop Sci.*, 1994, 34(2): 400-404 (doi: 10.2135/cropsci1994.0011183x003400020018x).
110. Kim N.-S., Armstrong K., Knott D.R. Molecular detection of *Lophopyrum* chromatin in wheat-*Lophopyrum* recombinants and their use in physical mapping of chromosome 7D. *Theor. Appl. Genet.*, 1993, 85(5): 561-567 (doi: 10.1007/bf00220914).
111. Chen G., Zheng Q., Bao Y., Liu S., Wang H., Li X. Molecular cytogenetic identification of a novel dwarf wheat line with introgressed *Thinopyrum ponticum* chromatin. *J. Biosci.*, 2012, 37(1): 149-155 (doi: 10.1007/s12038-011-9175-1).
112. Pozniak C., Knox R., Clarke F., Clarke J. Identification of QTL and association of a phytoene synthase gene with endosperm color in durum wheat. *Theor. Appl. Genet.*, 2007, 114: 525-537 (doi: 10.1007/s00122-006-0453-5).
113. Liu L., Luo Q., Li H., Li B., Li Z., Zheng Q. Physical mapping of the blue-grained gene from *Thinopyrum ponticum* chromosome 4Ag and development of blue-grain-related molecular markers and a FISH probe based on SLAF-seq technology. *Theor. Appl. Genet.*, 2018, 131(11): 2359-

2370 (doi: 10.1007/s00122-018-3158-7).
114. Wang R. Diploid perennial intergeneric hybrids in the tribe *Triticeae*. III. Hybrids among *Secale montanum*, *Pseudoroegneria spicata*, and *Agropyron mongolicum*. *Genome*, 1987, 29(1): 80-84 (doi: 10.1139/g87-014).
115. Qin L., Liang Y., Yang D., Xia G., Liu S. Characterisation of low molecular weight glutenin subunit genes from *Pseudoroegneria spicata* and *Pd. strigosa*. *J. Appl. Genet.*, 2015, 56(1): 27-35 (doi: 10.1007/s13353-014-0229-6).
116. Yang Z.J., Zhang T., Liu C., Li G.R., Zhou J.P., Zhang Y., Ren Z.L. Identification of wheat—*Dasypyrum breviaristatum* addition lines with stripe rust resistance using C-banding and genomic in situ hybridization. In: *The 11th International wheat genetics symposium proceedings*. R. Appels, R. Eastwood, E. Lagudah, P. Langridge, M. Mackay, L. McIntyre, P. Sharp (eds.). Sydney University Press, Sydney, 2008: 1-2.
117. Liu C., Qi L., Liu W., Zhao W., Wilson J., Friebe B., Gill B. Development of a set of compensating *Triticum aestivum-Dasypyrum villosum* Robertsonian translocation lines. *Genome*, 2011, 54(10): 836-844 (doi: 10.1139/g11-051).
118. Li G.-R., Zhao J.-M., Li D.-H., Yang E.-N., Huang Y-.F., Liu C., Yang Z.-J. A novel wheat—*Dasypyrum breviaristatum* substitution line with stripe rust resistance. *Cytogenet. Genome Res.*, 2014, 143(4): 280-287 (doi: 10.1159/000366051).
119. Wang H., Yu Z., Li B., Lang T., Li G., Yang Z. Characterization of new wheat—*Dasypyrum breviaristatum* introgression lines with superior gene(s) for spike length and stripe rust resistance. *Cytogenet. Genome Res.*, 2018, 156: 117-125 (doi: 10.1159/000493562).
120. Wang H., Zhang H., Li B., Yu Z., Li G., Zhang J., Yang Z. Molecular cytogenetic characterization of new wheat—*Dasypyrum breviaristatum* introgression lines for improving grain quality of wheat. *Front. Plant Sci.*, 2018, 9: 365 (doi: 10.3389/fpls.2018.00365).
121. Gradzielewska A. The genus *Dasypyrum*—part 2. *Dasypyrum villosum* —a wild species used in wheat improvement. *Euphytica*, 2006, 152(3): 441-454 (doi: 10.1007/s10681-006-9245-x).
122. De Pace C., Vaccino P., Cionini P.G., Pasquini M., Bizzarri M., Qualset C.O. *Dasypyrum*. In: *Wild crop relatives: genomic and breeding resources. Cereals*. C. Kole (ed.). Springer, Berlin, 2011: 185-292 (doi: 10.1007/978-3-642-14228-4).
123. Uslu E., Miller T.E., Rezanoor N.H., Nicholson P. Resistance of *Dasyryrum villosum* to the cereal eyespot pathogens *Tapesia yallundae* and *Tapesia acuformis*. *Euphytica*, 1998, 103: 203-209 (doi: 10.1023/A:1018340018838).
124. Bizzarri M., Pasquini M., Matere A., Sereni L., Vida G., Sepsi A., Molnar-Lang M., De Pace C. *Dasypyrum villosum* 6V chromosome as source of adult plant resistance to *Puccinia triticina* in wheat. *Proc. the 53rd Italian society of agricultural genetics annual congress*. Torino, Italy, 2009: 16-19.
125. Zhao W., Qi L., Gao X., Zhang G., Dong J., Chen Q., Friebe B., Gill B. Development and characterization of two new *Triticum aestivum-Dasypyrum villosum* Robertsonian translocation lines T1DS.1V#3L and T1DL.1V#3S and their effect on grain quality. *Euphytica*, 2010, 175(3): 343-350 (doi: 10.1007/ s10681-010-0177-0).
126. Zhang R.Q., Hou F., Feng Y.G., Zhang W., Zhang M.Y., Chen P.D. Characterization of a *Triticum aestivum-Dasypyrum villosum* T2VS.2DL translocation line expressing a longer spike and more kernels traits. *Theor. Appl. Genet.*, 2015, 128(12): 2415-2425 (doi: 10.1007/s00122-015-2596-8).
127. Zhang R., Fan Y., Kong L., Wang Z., Wu J., Xing L., Cao A., Feng Y. *Pm62*, an adult-plant powdery mildew resistance gene introgressed from *Dasypyrum villosum* chromosome arm 2VL into wheat. *Theor. Appl. Genet.*, 2018, 131(12): 2613-2620 (doi: 10.1007/s00122-018-3176-5).
128. Huang D.H., Lin Z.S., Chen X., Zhang Z.Y., Chen C.C., Cheng S.H., Xin Z. Molecular characterization of a *Triticum durum-Haynaldia villosa* amphiploid and its derivatives for resistance to *Gaeumannomyces graminis* var. *tritici*. *Agricultural Sciences in China*, 2017, 6(5): 513-521 (doi: 10.1016/s1671-2927(07)60077-7).
129. Zhang J., Jiang Y., Wang Y., Guo Y., Long H., Deng G., Chen Q., Xuanet P. Molecular markers and cytogenetics to characterize a wheat-*Dasypyrum villosum* 3V (3D) substitution line conferring resistance to stripe rust. *PLoS ONE*, 2018, 13(8): e0202033 (doi: 10.1371/journal.pone.0202033).
130. Yildirim A., Jones S.S., Murray T.D. Mapping a gene conferring resistance to *Pseudocercosporella herpotrichoides* on chromosome 4V of *Dasypyrum villosum* in a wheat background. *Genome*, 1998, 41(1): 1-6 (doi: 10.1139/g97-092).
131. Yildirim A., Jones S.S., Murray T.D., Line R.F. Evaluation of *Dasypyrum villosum* populations for resistance to cereal eyespot and stripe rust pathogens. *Plant Dis.*, 2000; 84(1): 40-44 (doi: 10.1094/PDIS.2000.84.1.40).
132. Zhang Q., Li Q., Wang X., Wang H., Lang S., Wang Y, Wang S., Chen P., Liu D. Development and characterization of a *Triticum aestivum-Haynaldia villosa* translocation line T4VS.4DL conferring resistance to wheat spindle streak mosaic virus. *Euphytica*, 2005, 145(3): 317-332 (doi: 10.1007/s10681-005-1743-8).
133. Zhang R.Q., Sun B.X., Chen J., Cao A.Z., Xing L.P., Feng Y.G., Lan C., Chen P. *Pm55*, a

developmental-stage and tissue specific powdery mildew resistance gene introgressed from *Dasypyrum villosum* into common wheat. *Theor. Appl. Genet.*, 2016, 129(1): 1975-1984 (doi: 10.1007/s00122-016-2753-8).
134. Chen P.D., Qi L.L., Zhou B., Zhang S.Z., Liu D.J. Development and molecular cytogenetic analysis of wheat *Haynaldia villosa* 6VS/6AL translocation lines specifying resistance to powdery mildew. *Theor. Appl. Genet.*, 1995, 91(6-7): 1125-1128 (doi: 10.1007/BF00223930).
135. Pumphrey M., Jin Y., Rouse M., Qi L.L., Friebe B., Gill B.S. Resistance to stem rust race TTKS in wheat relative *Haynaldia villosa*. *Proc. the 11th international wheat genetics symposium*. R. Appels, E. Lagudah, P. Langridge, M. Mackay (eds.). University Press, Sydney, Australia, 2008: 151.
136. Zhang R.Q., Feng Y.G., Li H.F., Yuan H.X., Dai J.L., Cao A.Z., Xing L., Li H. Cereal cyst nematode resistance gene *CreV*, effective against *Heterodera filipjevi*, transferred from chromosome 6VL of *Dasypyrum villosum*, to bread wheat. *Mol. Breeding*, 2016, 36(9): 122 (doi: 10.1007/s11032-016-0549-9).
137. Li H., Jiang B., Wang J., Lu Y., Zhang J., Pan C., Yang X., Li X., Liu W., Li L. Mapping of novel powdery mildew resistance gene(s) from *Agropyron cristatum* chromosome 2P. *Theor. Appl. Genet.*, 2016, 130(1): 109-121 (doi: 10.1007/s00122-016-2797-9).
138. Jiang B., Liu T., Li H., Han H., Li L., Zhang J., Yang X., Zhou S., Li X., Liu W. Physical mapping of a novel locus conferring leaf rust resistance on the long arm of *Agropyron cristatum* chromosome 2P. *Front. Plant Sci.*, 2018, 9: 817 (doi: 10.3389/fpls.2018.00817).
139. Luan Y., Wang X., Liu W., Li C., Zhang J., Gao A., Wang Y., Yang X., Li L. Production and identification of wheat—*Agropyron cristatum* 6P translocation lines. *Planta*, 2010, 232(2): 501-510 (doi: 10.1007/s00425-010-1187-9).
140. Ye X., Lu Y., Liu W., Chen G., Han H., Zhang J., Yang X., Li X., Gao A., Li L. The effects of chromosome 6P on fertile tiller number of wheat as revealed in wheat—*Agropyron cristatum* chromosome 5A/6P translocation lines. *Theor. Appl. Genet.*, 2015, 128(5): 797-811 (doi: 10.1007/s00122-015-2466-4).
141. Song L., Lu Y., Zhang J., Pan C., Yang X., Li X., Liu W., Li L. Cytological and molecular analysis of wheat—*Agropyron cristatum* translocation lines with 6P chromosome fragments conferring superior agronomic traits in common wheat. *Genome*, 2016, 59(10): 840-850 (doi: 10.1139/gen-2016-0065).
142. Ma H., Zhang J., Zhang J., Zhou S., Han H., Liu W., Yang X., Li X., Li L. Development of P genome-specific SNPs and their application in tracing *Agropyron cristatum* introgressions in common wheat. *The Crop Journal*, 2018, 7(2): 151-162 (doi: 10.1016/j.cj.2018.07.003).
143. Lu M., Lu Y., Li H., Pan C., Guo Y., Zhang J., Yang X., Li X., Liu W., Li L. Transferring desirable genes from *Agropyron cristatum* 7P chromosome into common wheat. *PLoS ONE*, 2016, 11(7): e0159577 (doi: 10.1371/journal.pone.0159577).
144. Liu Z.W., Wang R.R.C., Carman J.G. Hybrids and backcross progenies between wheat (*Triticum aestivum* L.) and apomictic Australian wheatgrass [*Elymus rectisetus* (Nees in Lehm.) A. Löve and Connor]: karyotypic and genomic analyses. *Theor. Appl. Genet.*, 1994, 89(5): 599-605 (doi: 10.1007/bf00222454).
145. Oliver R.E., Cai X., Wang R.C., Xu S.S., Friesen T.L. Resistance to tan spot and *Stagonospora nodorum* blotch in wheat-alien species derivatives. *Plant Dis.*, 2008, 92(1): 150-157 (doi: 10.1094/PDIS-92-1-0150).
146. Dou Q.W., Lei Y.T., Li X.M., Mott I.W., Wang R.R.C. Characterization of alien grass chromosomes in backcross derivatives of *Triticum aestivum-Elymus rectisetus* hybrids by using molecular markers and multi-color FISH/GISH. *Genome*, 2012, 55: 337-347 (doi: 10.1139/g2012-018).
147. Cainong J., Bockus W., Feng Y., Chen P., Qi L., Sehgal S., Danilova T., Koo D., Friebe B., Gill B. Chromosome engineering, mapping, and transferring of resistance to Fusarium head blight disease from *Elymus tsukushiensis* into wheat. *Theor. Appl. Genet.*, 2015, 128(6): 1019-1027 (doi: 10.1007/s00122-015-2485-1).
148. Zeng J., Cao W., Hucl P., Yang Y., Xue A., Chi D., Fedak G. Molecular cytogenetic analysis of wheat-*Elymus repens* introgression lines with resistance to Fusarium head blight. *Genome*, 2013, 56(1): 75-82 (doi: 10.1139/gen-2012-0130).
149. Fedak G., Cao W., Wolfe D., Chi D., Xue A. Molecular characterization of Fusarium resistance from *Elymus repens* introgressed into bread wheat. *Cytology and Genetics*, 2017, 51(2): 130-133 (doi: 10.3103/s0095452717020025).
150. Friebe B., Wilson D.L., Raupp W.J., Gill B.S., Brown-Guedira G.L. Notice of release of KS04WGRC45 leaf rust-resistant hard white winter wheat germplasm. *Annu. Wheat Newsl.*, 2005, 51: 188-189.
151. Loshakova P.O., Fisenko A.V., Kalmykova L.P., Kuznetsova N.L., Upelniek V.P. *Dostizheniya nauki i tekhniki APK*, 2018: 32(9): 28-31 (in Russ.).
152. Wilkinson M.D., King R., Grimaldi R. Sequence diversity and identification of novel puroindoline and grain softness protein alleles in *Elymus*, *Agropyron* and related species. *Diversity*, 2018, 10(4): 114 (doi: 10.3390/d10040114).

153. Yu Z., Wang H., Xu Y., Li Y., Lang T., Yang Z., Li G. Characterization of chromosomal rearrangement in new wheat-*Thinopyrum intermedium* addition lines carrying *Thinopyrum*-specific grain hardness genes. *Agronomy*, 2019, 9(1): 18 (doi: 10.3390/agronomy9010018).
154. Kolchanov N.A., Kochetov A.V., Salina E.A., Pershina L.A. Khlestkina E.K., Shumny V. K. Status and prospects of marker-assisted and genomic plant breeding. *Herald of the Russian Academy of Sciences*, 2017, 87(2): 125-131 (doi: 10.1134/s1019331617020113).
155. Baral K., Coulman B., Biligetu B., Fu Y.-B. Genotyping-by-sequencing enhances genetic diversity analysis of crested wheatgrass [*Agropyron cristatum* (L.) Gaertn.]. *Int. J. Mol. Sci.*, 2018, 19(9): 2587 (doi: 10.3390/ijms19092587).
156. Liu L., Luo Q., Teng W., Li B., Li H., Li Y., Li Z., Zheng Q. Development of *Thinopyrum ponticum*-specific molecular markers and FISH probes based on SLAF-seq technology. *Planta*, 2018, 247(5): 1099-1108 (doi: 10.1007/s00425-018-2845-6).

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RISKS OF POLLEN-MEDIATED GENE FLOW FROM GENETICALLY MODIFIED MAIZE DURING CO-CULTIVATION WITH USUAL MAIZE VARIETIES (review)

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Abstract

Since 1985, active development of agricultural biotechnology has been associated with genetically modified (GM) plants. After the production of GM maize in the second half of the 1990s, the area of its crops has increased over 100-fold. Therefore, the GM maize spreeding and cross-pollination have become more practically relevant. Almost one third of the total area of all GM plants is occupied by GM maize. The Russian Federal Law No. 358 of 03.07.2016 prohibits the commercial use of GM plants in agriculture but allows their cultivation and testing for research purposes. This necessitates assessing and developing criteria, currently absent in Russia, for the safe co-cultivation of non-GM and GM varieties. This review analyzes the factors influencing pollen dispersion: wind (speed and direction), humidity (rain), physiology (viability), the pollen amount, the character of the landscape, the size, shape and orientation of the recipient fields, and the synchrony of flowering of the pollen donor and recipient. Early studies of gene flow in cross-pollination were reviewed Y. Devos et al., (2005) and O. Sanvido et al. (2008). In particular, the distance between GM and traditional maize recommended in the EU countries, with the same threshold for GM content in food, varies considerably (from 25 to 600 m) (Y. Devos et al., 2009; L. Riesgo et al., 2010). In addition to the distance between crops and the synchronicity of flowering, the frequency of cross-pollination depends on the field size and orientation (M. Langhof et al., 2010). Estimates of the cross-pollination frequency and the pollen counts at different distances from the GM donor allowed the researchers to recommend isolation distances of 10 to 200 m. If the isolation distance cannot be ensured, the recipient and/or donor field should be bordered by a barrier to pollen. In the recipient field, the outer rows of maize plants can be the barrier. After a 10-20 m maize barrier, almost none of the analyzed samples contains more than 0.9 % of GM material. For recipient fields of less than 1 ha in area and/or low-depth fields, an isolation distance of at least 50 m should be recommended, especially in the wind rose direction. Data on spreading GM maize with pollen in Europe, South America, Africa, and Asia provide recommendations for safe co-cultivation of non-GM and GM maize varieties and lines. The cytoplasmic male sterility (CMS) approach for GM- and non-GM maize co-cultivation was developed. The genetic control of CMS (N-, S-, C-types and CRISPR-mediated approach) and the CMS application history are discussed. For CMS hybrids, the isolation distances between GM and traditional maize crops may be significantly reduced (up to 10 m) without violation of the European requirements of a 0.9 % marking threshold. However, GM-maize with CMS is not used for practical cultivation. Russia has yet to develop its own measures and recommendations for the joint cultivation of GM and traditional maize.

Keywords: genetically modified corn, gene flow, pollen, CMS, GM crop co-cultivation, GMO regulations

Derivation of varieties by genetic engineering (GE) methods and their practical application have been actively developing as areas of agricultural biotechnology since 1985. Large-scale industrial production of genetically modified (GM) plants, particularly maize, has begun in 1996. The area of cultivation of agricultural GM crops since the beginning of their use has increased by more than 100 times – from 1.7 million hectares in 1996 to 185 million hectares in 2016, which amounted to about 12% of the world's acreage [1]. In 2017, GM varieties with herbicide resistance (either alone or in combination with insect pest resistance) were grown globally on a total of 166.4 million hectares [2].

Corn (*Zea mays* L.) is one of the most common crops, the production of which in the last 10 years in the world has grown from 600 to 1000 million tons. It adapts well to high temperatures, which in the context of climate warming creates the potential to expand areas of cultivation, including in Russia, where, according to the Federal State Statistics Service, almost 9 times more corn was grown in 2018 than in 1995 (http://www.gks.ru/free_doc/new_site/business/sx/val_1.xls). Among GM crops, maize is the second largest in the world after soybeans in terms of the crop area and the first in terms of harvest. In 2015, GM corn accounted for 53.6 million hectares or almost a third of the total crop area (185 million hectares) in the world [3]. In other words, there is a danger of gene transfer from GM corn to non-GM corn when they are grown together.

According to the Convention on Biological Diversity, 1993 (United Nations 1992; came into force in 1993), each participating country must develop a strategy and program for the preservation and use of its biological resources with their guaranteed and safe reproduction [4]. In particular, it is necessary to establish and approve ways and methods of regulating, managing and controlling the risks associated with the creation, use and distribution of GM varieties, as well as to develop methods for assessing possible risks in the cultivation of GM varieties for biodiversity preservation [5].

Russia is one of the countries that banned the commercial cultivation of GM crops (Federal Law dated July 5, 1996 No. 86-FZ “About State Regulation of Genetic Engineering Activity”, Federal Law dated July 3, 2016 No. 358-FZ “On Amendments Being Made to Particular Legislative Acts of the Russian Federation to Improve State Regulation of Genetic Engineering Activity”). Federal Law No. 86-FZ (1996) initially did not provide for the registration of GM varieties and the output of products based on them; but 10 years later, 15 GM crops were studied and allowed in Russia: 8 lines of corn, 3 lines of soybean, 1 line of sugar beet, 1 line of rice and 2 varieties of potatoes (Letter of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare “On Improving the Supervision of Food Products Containing GMO and GMM” dated August 20, 2008 No. 01/9044-8-32). Effective since 2018, Federal Law No. 358 also prohibits the commercial use of GM plants in agriculture but permits their cultivation and testing for scientific and research purposes. Consequently, a real necessity to develop criteria for the joint safe cultivation of untransformed and GM crops, including maize, taking into account the tasks of biodiversity preservation, appeared in Russia. This development includes the analysis of the best practices of experimental evaluation of potential risks in the cultivation of GM plants. This problem has many different aspects, such as considering the effects of herbicides and insecticides on non-target plant and insect species during cultivation of GM crops [6], but in this review, the material will be focused on evaluating the risks of distribution of GM corn with pollen in co-

cultivation of untransformed and GM maize varieties, which has not been studied in Russia.

Expansion of GM corn pollen in field conditions. Maize is a monoecious wind-pollinated species; plants reach a height of 3 m (sometimes up to 6-7 m), which increases the risk of spreading pollen of GM varieties carried by the wind for tens and hundreds of meters [7, 8]. Pollen transfer is influenced by many factors: wind (speed and direction), rain (humidity), physiology (viability), pollen quantity, landscape character, size, shape and orientation of the recipient field, the synchronicity of flowering of the pollen donor and recipient [9].

Pollen viability. Pollen viability (ability to germinate) is an important condition for cross-pollination. After leaving the anthers, corn pollen is viable for 1-24 hours [10, 11]. In the moderate climate of Europe (France), pollen viability is maintained up to 24 h [11]. Depending on atmospheric conditions in Iowa (USA), pollen germination decreased by 50% after 1-4 h [12]. In many years of research in the arid conditions of Mexico (area of Nayarit), a decrease in the pollen viability outside by 80% for 1 h and 100% for 2 hours was noted; in the driest conditions, 100% of pollen grains became unviable within 1 h [12].

Wind. Air flows during pollen expansion can lift pollen high into the atmosphere and distribute it over considerable distances. Pollen viability decreases with altitude, but at higher altitudes, lower air temperatures favor the maintenance of pollen viability. The main horizontal flow of pollen in corn was observed at an altitude of 6.5 m, and it was the same at a distance of 3-10 m from the source [13]. A small amount of pollen was recorded at a distance of 800-1000 m from the source, while the settling rate of pollen ranged within 0.0002-0.001 grains/($m^2 \cdot s$) [13, 14]. On average, the frequency of cross-pollination (% of grains) was the same at a distance of 28 m down the wind and at a distance of 10 m against the wind [15].

Wind speed is the main variable that determines the amount of pollen dispersed after the release of pollen grains from anthers. The time and distance of the pollen grain fall on the snout of the recipient plant depend on gravity, on the one hand, and turbulence and airflow, on the other [16]. Corn pollen grains in comparison with pollen of other wind-pollinated species are relatively large (average diameter 90 microns) and heavy (0.25 rg), so corn pollen has a high settling rate [14, 17]. About 95-99% of the pollen is set at a distance of about 30 m from the source. At a distance of more than 30-50 m, its amount is significantly reduced, but the clear distance beyond which it is not detected is unknown [13, 14, 17]. The relative density of pollen in experiments in Massachusetts (USA) fell to about 2% at a distance of 60 m from the edge of crops and remained within 0.50-0.75% at a distance of 500 m from the donor culture [18]. In another paper, it was reported on the settling rate of pollen downwind at a distance of 10 m from the source — 10-100 grains/($m^2 \cdot s$), 800 and 1000 m — 0.001-0.0002 grain/($m^2 \cdot s$) [13]. In a study carried out in Mexico, no correlation between wind speed and cross-section percentages was observed, meaning that the role of wind speed may not be relevant to cross-section frequency [8].

Humidity, rain. In the air stream, pollen can be captured by water droplets and/or fall on wet flowers, where it bursts and dies. As a rule, corn pollen falls out of anthers in dry conditions, mainly from morning to noon [13, 14]. When it rains, pollen release is delayed because anthers do not crack in wet conditions [13, 14]. However, the published data to quantify the impact of rain on anthers opening and maize pollen flow is not still available.

Field dimensions of the GM pollen source and the recipient field. A cloud of pollen of recipient plants over the field of the recipient competes with donor pollen. The larger the field, the greater the mass of the recipient's pollen. Field

tests have shown that for a donor of pollen with a given size, the cross-pollination frequency decreases as the size of the recipient field increases [14, 19, 20]. This indicator, expressed as a percentage of GM plants, decreased from 1.8% to 0.8% when the size of the pollen recipient field increased from 0.25 to 1 ha [19]. The results of research with fodder corn in Germany, when the donor's fields of GM corn were surrounded by an isogenic non-GM variety, confirmed these results [14]. However, later in Germany, it was found that the size of the donor field 0.3-23 ha did not affect the frequency of cross-pollination [21]. In experiments in Spain, the size of the donor field of corn influenced the number of GM grains found in the recipient field, but to a lesser degree than the size of the recipient field [22].

Shape and orientation of pollen donor and recipient fields. The study of the influence of the shape of the fields of recipients and pollen donors showed that the volume of their cross-pollination can be easily reduced by changing the square shape of the recipient field to a rectangular of the same area and placing it accordingly [14, 23]. For example, if a 5-hectare recipient field had a long side facing the pollen source, the cross-pollination frequency was 10.7%, if the short side – only 3.4% [24].

Synchrony of the donor and recipient flowering. Synchronization of pollen dispersion and snouts release is crucial for determining the survival rate of corn pollen [14, 25]. The better the synchronicity between pollen donor flowering and the snouts release of the recipient plant, the higher the probability of cross-pollination [11, 14, 25, 26]. Different sowing times can lead to a difference in flowering time, limiting cross-pollination. In Spain, the difference in sowing time on average of 1 week reduced the frequency of cross-pollination in the first row of recipient fields by 50%, 3 weeks – by 75% [20, 27]. In Mediterranean European countries, this approach can be applied without crop losses [20, 27].

Barriers to Pollen. Plants around the source or recipient of pollen can act as barriers. Thus, the presence of several rows of taller plants on the outside of the donor field reduces the frequency of crosses, i.e., the effect is similar to increasing the distance to the recipient plants. In addition, barriers introduce competing pollen if they are plants of the same species and/or physically impede pollen transfer through the air by affecting flows and filtration [18, 28]. A barrier of corn and trees reduced cross-pollination (immediately behind the barrier) by 50% more efficiently than the sown earth [14]. A barrier of tall sorghum plants reduced the safe distance of the joint cultivation of GM and non-GM corn from 35 to 20 m with a threshold value of the frequency of cross-pollination below 0.9% [29].

Landscape influence. The highly diverse nature of the landscapes of the European Union (EU) suggests that measures to ensure the coexistence of GM and non-GM plants should be adapted to regional conditions under the SIGMEA program for the sustainable introduction of GM crops into European agriculture. In 2010, four landscape-specific maize growing zones in Alsace and Aragon (France) were compared and regional landscapes were classified by risk degree for different threshold values of 0.1; 0.4, and 0.9% [30].

Risk analysis of the spread of GM corn varieties with pollen in different countries. *Europe. EU Countries.* Directive 2001/18/EC dated March 12, 2001 on the deliberate release of genetically modified organisms into the environment is in force in the European Union and was substantially amended in 2015. Directive 2015/412/EC established the right of EU member states to restrict or prohibit the cultivation of GMO approved in the EU in their territories. The prohibition can be imposed not only for scientific but also for political, socio-economic reasons. Permission to grow a GM crop can be ob-

tained only after an individual assessment of the potential danger of each GMO to human and animal health.

A study in 2000-2003 in 15 counties of England showed a quick reduction in the rate of cross-pollination in corn at a distance of the first 20 m from the donor culture; however, marker genes of GM varieties of fodder and sweet corn were detected by PCR at distances of 80 and 200 m [31, 32]. SCIMAC (Supply Chain Initiative on Modified Agricultural Crops, UK), an organization established to regulate the use of GM plants in England in the production of agricultural products, recommended a separating distance of 80 m for the joint cultivation of GM and non-GM corn varieties [31].

Due to the inclusion of 17 GM corn varieties in the EU catalogue of crop varieties on September 8, 2004, an increase in the commercial acreage of transgenic maize was expected in some countries [14] and thresholds (0.9%) were set for allowing the accidental and technically unavoidable presence of GM material in non-GM products. To reduce pollen dispersion, the EU has adopted a regulation that establishes 200 m as the minimum isolation between the pollen source and the recipient field (with the use of physical barriers – 100 m) [14]. The distances originally proposed by EU member states for isolation of traditional and GM corn ranged from 15 to 800 m [33].

An empirical one-dimensional model of gene flow mediated by corn pollen allowed establishing the practice of growing GM and traditional plants while maintaining the threshold value of 0.9% for GM grain in non-GM grain [34]. Based on the model describing the decrease in the rate of germination of pollen grains in the atmosphere, it can be assumed that the decrease in the settling rate is accompanied by a decrease in the viability of pollen [35]. A simple dispersion model is proposed to illustrate the possible effects of changes in the deposition rate and germination rate on pollen propagation and cross-pollination of corn. The results show that modern pollen propagation models that do not take into account these changes overestimate cross-pollination rates [35]. An approach to modelling pollen-mediated gene flow from multiple sources is proposed [8]. It is based on generalized linear mixed models that quantify variability across years and locations to determine which isolation methods will effectively meet the 0.9% threshold. Data for the new model were obtained from a database of experiments conducted in 2000-2010 in Canada, France, Germany, Italy, the Netherlands, Poland, Romania, Slovakia, and the United States. According to this model, for 1 ha of a non-GM field, surrounded from all four sides by GM fields, 12 m of border rows in combination with a 12-meter band out of crop are enough to ensure a threshold of 0.9% (at the 95% significance level) established by the EU [9]. Based on a comparison of four methods for assessing the presence of GM corn in the fields of non-GM recipients with mathematical processing of the results, a more reliable method for recording GM crosses over the entire depth of the field was proposed [36].

Cultivation of GM corn in Europe until 2005 was limited to Spain (about 58 thousand hectares in 2004). Since 2005, GM corn varieties have been cultivated in the Czech Republic, Slovakia, France, Germany, and Portugal [36]. However, since 2008, France has prohibited the cultivation of the GM corn variety MON810 resistant to insect pests [33]. Germany, after further research and public debate, also prohibited the use of the MON810 on its territory [37]. GM corn crops in the Czech Republic and Slovakia have only declined since 2008 until a total prohibition in 2017 [2]. In Portugal and Spain to 2017, the area under GM corn decreased by 10% relative to 2016 (https://www.infogm.org/6391-europe-GMO-drop-of-transgenic-crops?lang=fr).

Italy. In Italy (Lombardy), experiments were conducted to study the ef-

fect of different flowering periods of the GM donor and non-GM recipient of corn on the number of GM plants in the recipient's crops. A slight or complete absence of pollen flow reduction was found with the difference in flowering time between the source and the recipient up to 3 days. For the 4-5 days period, the flow of pollen decreased by 25%, for 6 days — by 50%, for more than 7 days — by 100%. The cross-pollination threshold (0.9%) was reached at a buffer zone of 25 m, with corn plants being a more effective barrier rather than fallow (out of crops) lands [38].

Romania. In 2008-2009, the distribution of GM corn pollen of the MON810 variety was studied in the South (Călărași district), East (Brăila district) and West (Timiș district) of Romania to assess potential risk [42]. A plot sized 300×250 m planted with transgenic plants was surrounded by crops of non-transgenic seeds. The average distance at which the content of GM grains was less than 0.9% in all four directions (north, south, east, west) was 20 m in 2008, and 25 m in 2009 [39]. On this basis, for the spatial isolation of commercial GM varieties, the authors considered a distance of 35 m sufficient. In 2016, Romania stopped growing GM corn [2].

The Netherlands. In 2006-2007, field tests of the effect of the two insulating distances (25 and 250 m) specified in the Dutch Coexistence Committee (Holland) resolution were conducted in three regions of the Netherlands [40]. A field with the genetically modified donor (DKC3421YG, a modified version of the MON810 variety, 1 ha) was surrounded in four directions by fields (0.25 ha) with traditional corn (variety DKC3420), located at a distance of 25 and 250 m. PCR analysis showed the presence of 0.080-0.084% GM grains for 25 m distance and 0.005-0.007% for 250 m.

It is important to note that the European Union has adopted a stricter regulatory framework for GM plants compared to other countries [41], despite the fact that Europe grows less GM corn than on all other continents except Australia, although, according to Italian scientists who analyzed 1,783 publications for the period from 2002 to 2012, no direct threat from the use of GM plants was revealed [42]. Directive 2015/412/EC of the European Parliament and the Council dated March 11, 2015, as already noted, allows the EU member states to determine independently the amount and rules of cultivation of GM plants [43]. By October 2015, 19 of the 28 EU countries had applied to abandon the cultivation of GM corn MON810 [44]; by 2017, this variety was grown only by Portugal and Spain [45].

Russia. In 2012, the Russian Federation approved the Comprehensive Program for the Development of Biotechnology (Including Agricultural Biotechnology) for the Period up to 2020 [46]. In 2013, Russia ranked 12th in the world in corn production [47]. As per the Federal State Statistics Service, in 2016, the yield of corn grain was almost 9 times higher than in 1995 (respectively 15.0 million tons against 1.7 million tons), but over the next 2 years it decreased, reaching 13 million tons in 2017, and 11 million tons in 2018 (http://www.gks.ru/free_doc/new_site/business/sx/val_1.xls). At the same time, the acreage for corn in the period from 1995 to 2018 in Russia, according to the Federal State Statistics Service, increased by nearly 4 times (from 643 thousand hectares to 2452 thousand ha) (http://www.gks.ru/free_doc/new_site/business/sx/posev_pl1.xls).

Currently, GM corn in Russia is not officially grown, although in 2008, 8 lines of GM corn of American origin were ready for mass production (Letter of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare "On Improving the Supervision of Food Products Containing GMO and GMM" dated August 20, 2008 No. 01/9044-8-32).

For comparison, the area of the world's biotechnological crops increased

from 1.7 million hectares in 1996 to 189.8 million hectares in 2017 (by more than 100 times) [48]. In 2016, the total world income of farmers from using GM corn amounted to 2.1 billion USD, the total return for the years 1996-2016 was 13.1 billion USD, among which 4.5 billion USD (34%) was due to increased yield, the rest – due to the reduction of production costs [49].

The review by Zhuchenko [50] presents an analysis of the risks associated with the creation, cultivation, and use of GM plants in agriculture. The limitations of genetic engineering, as well as possible evolutionary, biological and environmental consequences of the widespread use of genetically modified organisms, are considered. It is important to note that some of the limitations and dangers of genetic engineering mentioned in the review (in particular, the lack of site-directed gene integration methods, or insertion of introduced genes into a specific host DNA site) have already been overcome by modern CRISPR/Cas technologies. For modeling the microevolution of a population, in which the GM species are presented, Zhivotovsky [51] considered two scenarios of the behavior of mixed populations depending on the adaptation potential of GM plants. According to one scenario, GM plants that carry useful adaptations (for example, heat and salt resistance) may gain an advantage over other populations for a short period of time and displace them, but then die due to their own narrow adaptation with a significant and varied change in conditions ("Trojan horse hypothesis"). Similar conclusions were given earlier by other authors [52]. However, experimental risk assessment of GM corn distribution in Russia has not been carried out; criteria and recommendations for its safe cultivation together with untransformed varieties in Russia are absent.

North America. Mexico. Mexico is considered the center of origin and biodiversity of corn, the domestication of which began in its territory more than 8 thousand years ago [53]. In Mexico, more than 9 thousand species have been created and stored [54], including the populations of subspecies of corn (*Zea Mexicana, Z. parviglumis*) and teosinte plant species that participated in the breeding of modern corn in Mexico [55].

As early as 2001, GM corn imported from the United States was found in local corn populations in Northern Mexico (Oaxaca state) [56], although no GM corn was detected in a later 2-year study of 125 Oaxaca fields using PCR [57]. However, no more than 10 plants (corn ears) from each field were analyzed and combined samples (from 300 to 810 and even 5,630) were used [57]. The authors also do not indicate at what distance the studied fields were from the fields where GM corn varieties were tested in Mexico in 1998. Experimental testing of the ability of donor corn pollen to spread and pollinate recipient plants in Mexico showed the highest frequency of crossing near the pollen source – 12.9% at a distance of 1 m. This value fell sharply to 4.6; 2.7; 1.4; 1.0; 0.9; 0.5 and 0.5% as the distance increased, respectively, to 2, 4, 8, 12, 16, 20, and 25 m. At a distance of more than 20 m, the crossing frequency at all points was below 1% [8]. It should be noted that in Mexico, unlike many other countries, the additional factor of increased risk of uncontrolled spread of GM corn is active: during cultivation, small-scale farmers traditionally exchange grain for planting and sow mixed populations of corn (the so-called creolization) [58]. The observed distribution of GM corn in Mexico under the described conditions showed that in sowing 5 hectares of GM corn in each of the 13 regions, in 10 years, GM plants will be present in 87.85% of all crops [55].

To protect the genetic resources of corn in Mexico, the GM Biosafety act was drafted in 2008, which provides for the establishment of isolation zones for areas that are considered centers of corn origin; the protection of species for which Mexico is considered a center of origin and genetic diversity; the use

of isolation distances (250 m) for experimental field studies with transgenic corn, with additional restrictions in regions identified as centers of origin [8]. Since 1996, Mexico has approved 170 biotech crops for food, feed, and cultivation, including 75 GM corn lines [2]. In 2017, Mexico approved five GM corn lines for production.

The USA. The marked gap between American and European approaches to GM crops is observed. The USA has 1.4 times more arable land than the EU, with almost 600 times more land allocated to GM crops [45]. This huge difference reflects the different attitudes towards genetically modified organisms (GMOs) in Europe and the United States. In Europe and America, regulation is based on fundamentally different models of risk assessment inherent in GMOs. These approaches are called process-based (principle-based) and product-based [59]. In the first case, the risk character is recognized for the process of genetic modification, in the second – for its result, the object (GMO or derivative products) [60]. Legislation of all countries can be divided according to this criterion into two types. The first includes regulation in the EU, the second – in the United States and Canada. Most of the countries that follow the process-oriented approach participate in the Cartagena Protocol on Biosafety (2000), while the countries implementing the product-oriented approach largely do not participate. This causes significant differences in the requirements for GM crops but does not directly affect the possibility of their cultivation. The process-oriented approach is adopted in Brazil and India, which are among the leaders in the use of biotechnology in agriculture [61].

In the United States since 1986, the Coordinated Framework for Regulation of Biotechnology, updated in 1991, is active. It serves as a guideline for federal oversight and regulation of GM foods, including GM crops, food products, and GMO use. In implementing the regulation, U.S. regulatory authorities should rely solely on the risks inherent in the final product, without regard to biotechnological processes of its manufacture [62]. The USA has no federal legislation on GMOs in the United States and the United States is not a party to the Cartagena Protocol on Biosafety and the Convention on Biological Diversity [2].

The first commercial herbicide-resistant GM corn appeared in the U.S. market in 1998. By 2009, genetically modified corn in the United States accounted for 85% [63]. According to the evaluation of American researchers, the yield of GM corn from 1996 to 2010 in the central corn belt of the United States only slightly increased, unlike GM soybeans. However, whether this was due to the use of GM corn or other factors is not analyzed in detail [64]. The US legislation on GM plants is the most liberal, although in the US, there is a theoretical danger of crossing corn with eastern gamagrass *Tripsacum dactyloides* L., which is widespread in the eastern and northern part of the United States [65]. Eastern gamagrass belongs to the same genus of the family *Poaceae* as corn *Zea mays* L., and grows naturally in the same region of the United States where corn is commercially produced. To study the gene flow from corn to gamagrass, experimental crosses were made between transgenic corn used as a male parent and gamagrass as a female parent to assess the possibility of interspecific hybridization [66]. No evidence of gene flow from transgenic corn to gamagrass was observed in nature, although the two species grew in close proximity for many years and had ample opportunities for interbreeding [66].

South America. Colombia. The Colombian Agricultural Institute (Instituto Colombiano Agropecuario, ICA) in 2010 established a distance requirement of 500 m between local and transgenic corn fields [67]. In 2015, Colombia reviewed 60 randomly selected plots of corn in the valley of San Juan planted in groups at distances of 2.2-4 km in the area of 15×8 km. The

areas of ordinary corn varied from 8 to 114 hectares, GM corn – from 95 to 125 hectares. The effects of the distribution of GM corn with pollen were analyzed by three independent methods (two enzyme immunoassay and PCR). The results showed the presence of transgenic sequences in leaves (more than 88% of plots) and seeds (12% of plots) of non-GM corn [68].

Uruguay. Uruguay's government regulations stipulate that when GM corn is grown, at least 10 percent of the area should be occupied by non-GM corn as a biodiversity safety zone. Government resolutions (2003-2004) set a minimum distance of 250 m between GM and non-GM corn [16]. In 2010, Uruguay studied cases and determined the frequency of cross-pollination between commercial GM and non-GM varieties. The technique included detection of GM varieties by sandwich-ELISA and PCR methods. The proportion of transgenic seedlings in the offspring of non-GM crops was 0.56; 0.83 and 0.13% at three sampling points at a distance of 40, 100 and 330 m from GM crops, respectively [16].

Argentina. Argentina is the third country in the world in terms of the cultivation volume of GM crops [69]. In 2010, their GM crop acreage here accounted for 21% of the world's biotech crops (99% for soybeans, 83% for corn, 94% for cotton) [70]. In 2016, this value was already 97% for corn, almost 100% for soybeans and 95% for cotton [3].

Data on environmental and agrotechnical risks obtained in EU countries based on assessments and the need to have certain isolation zones are perceived in Argentina as an obstacle to the realization of national interests [52], as the use of GM crops in Brazil and Argentina has resulted in a higher average income for farmers. The combined economic impact of 24.8 billion USD for Brazil and 21.1 billion USD for Argentina was achieved mainly by reducing the cost of selective herbicide and achieving higher yields [3].

Brazil. Since the first GM crop was grown in Brazil in 1998, their share in crops has increased to 88% for corn, 96% for soybeans and 78% for cotton crops [3]. Brazil requires a buffer distance of 100 m from the edge of the GM corn field to the beginning of the non-GM corn field. Alternatively, a 20-meter buffer consisting of at least 10 rows of non-GM corn along the edge of the GM corn field can be used. Buffer zones were established by the Brazilian National Technical Biosafety Commission on the basis of gene flow studies, as well as taking into account national legislation, which set a threshold of 1% for GM crops [71].

Africa. Compared to other African countries, the Republic of South Africa has adopted GM crop biotechnology from the very beginning. The first field tests of GM crops were conducted in South Africa in 1989; the first commercial production of GM corn was approved in 1997 [46]. The main legislation in South Africa concerning GMOs, including their limited use, trial release, commercial release, and import and export, is the GMOs Act 1997 (Genetically Modified Organisms Act No. 15 of 1997, Statutes of the Republic of South Africa – Agriculture) [46]. South Africa is currently the eighth producer of GM crops in the world. The study of the level of cross-pollination between GM and non-GM corn in South Africa showed that <0.1-1.0% of pollen reached 45 m distance, < 0.01-0.1% of pollen moved to a 145 m distance, and <0.01-0.001% of pollen to a 473 m distance [72].

Asia. China and Japan. At a threshold of 0.9%, the insulation distance in China was estimated to be 50 and 25 m on a two-stage model [73]. Japanese food labeling rules for GM products are not particularly strict. New varieties of GM crops are grown on isolated farmland. Once the safety of such varieties is proven, they are allowed to grow on traditional farms. In Japan, how-

ever, the introduction of GM crops for food production has met strong public opposition. More than 70% of Japanese consumers surveyed are against growing GM varieties and eating them [74].

The use of corn varieties with male sterility to limit the spread of corn with pollen. One of the approaches for the joint safe cultivation of GM and non-GM corn is the use of plants with cytoplasmic male sterility (CMS), in which pollen is defective (functionally defective) or is formed in small quantities. CMS is inherited through the maternal line. At first, three types of CMS were known: T-type (Texas), S-type (USDA) and C-type (Charrua type) [75, 76]. In the 1990s, the corn mitochondrial gene *T-urf13* was found to encode a small protein URF-13 (13 kDa) expressed in mitochondrial crystals, which is responsible for the T-type of corn CMS and also increases susceptibility to fungal pathogens [76, 77]. The mitochondrial genome of the male-sterile corn S-cytoplasm contains a repeating R-region of DNA having two open reading frames [76]. The restoration of the fertility of S-type CMS is mainly controlled by the nuclear restorer *Rf3*, which is located in the 2nd chromosome [78]. For CMS of C-type, *Rf4*, located in the 8th chromosome, is a dominant gene restoring fertility [79].

T-type CMS was widely presented in corn hybrids in North America in the 1950s and 1960s, mainly to limit seed self-reproduction by farmers [76]. In addition, for the introduction of CMS corn to agricultural practices, it was necessary to ensure comparable yields. However, weather conditions in the early 1970s contributed to the development of leaf blight in the varieties of corn with T-type CMS, which led to a loss of up to 15% of the crop in the states of the "corn belt" of the United States (more than 6 billion USD at the current rate). The varieties without T-type CMS were immune to infection, which limited the use of CMS in practice [80]. Due to the susceptibility of T-type CMS to the race of the fungus *Bipolaris maydis* (formerly known as *Helminthosporium maydis*, race T), other cytoplasm groups providing corn CMS were looked for. In the United States, S-type CMS (often referred to as USDA-type) was found in the offspring of the variety Teopod (Iowa) [76]. C-type CMS, identified in the Brazilian corn of the variety Charrua in 1970, was used in the creation of hybrids, but C-type CMS was sensitive to the race of *B. maydis*, found only in China so far [76].

To assess whether the CMS of corn hybrids reliably reduces pollen-mediated gene flow from GM plants [81], two-year field experiments with three corn hybrids with CMS were conducted in three regions of Germany. The characteristics of panicles, pollen viability, and cross-pollination frequency were investigated. CMS stability depended on the genotype, weather conditions of the year and location. One CMS corn hybrid showed high CMS-C stability and very low cross-pollination rate (<1%). The other two hybrids with the CMS types S and T were characterized by high variability and formed fertile panicles with small or large amounts of pollen, respectively. In all corn hybrids with CMS, cross-pollination was by 84-99% lower than in a conventional fully fertile variety. It was found that when using corn hybrids with CMS, the isolation distances between adjacent GM and non-GM fields can be greatly reduced (up to 10 m) while maintaining the set threshold of 0.9% [81].

If the corn hybrid with CMS and the pollinator plant provide another genetic background, yield can be increased significantly. The so-called Plus-Hybrid-Effect combines the potential benefits of CMS and the Xenia effect (increased female fertility associated with resource redistribution or greater seed viability) [81-83]. In the review by Wan et al. [84], the main achievements in the identification and characterization of CMS genes in corn, Arabidopsis, and rice

are summarized. In particular, corn to date is characterized by 17 CMS genes, 13 of which are orthologs of CSM genes of rice and/or Arabidopsis.

In 1998, DuPont (USA) developed the GM lines DP-32138-1 (commercial name 32138 SPT maintainer), PH-ØØØ676-7, PH-ØØØ678-9, and PH-ØØØ68Ø-2, and Bayer Crop Science (USA) introduced the GM line ACS-ZMØØ1-9 (commercial name InVigor™ Maize) and ACS-ZMØØ5-4 (commercial name InVigor™ Maize), approved for commercialization by ISAAA (GM Approval Database, http://www.isaaa.org/gmap-provaldatabase/default.asp). The line DP-32138-1 (commercial cultivation in the USA in 2011 and in Brazil in 2015) has the transferred genes *ms45* (restores fertility due to the normalization of development of the cell wall of microspores), *zm-aa1* (its expression in the immature pollen hydrolyzes starch and makes the pollen sterile) and the marker gene *dsRed2*, encoding red fluorescent protein for easy selection. The lines PH-ØØØ678-9, PH-ØØØ676-7, and PH-ØØØ68Ø-2 (commercial cultivation in the USA in 1998) have the transferred *dam* gene, which provides CMS, preventing the formation of functional anthers and pollen, and the *pat* gene for resistance to phosphinothricin. The lines ACS-ZMØØ1-9 and ACS-ZMØØ5-4 have the transferred *barnase* gene (causes male sterility; fused with a tapetum-specific promoter TA29 and activated in tissue during the formation of pollen, the product has ribonuclease activity, inhibiting the synthesis of RNA in the tapetum cells of the anther), the gene *bla* (neutralizes β-lactam antibiotics, such as ampicillin) and the *bar* gene (in its presence, the enzyme phosphinothricin-N-acetyltransferase is produced, which acetylates herbicide phosphinothricin on the amino group, making it non-toxic to plants).

In 2016, DuPont Pioneer (USA) developed a system for the production of hybrid seeds using male sterility to produce hybrids of corn and other cross-pollinating crops [85]. The main element of this system is the so-called Seed Production Technology (SPT), which includes several stages. First, using the vector bearing the SPT design (contains the gene for male fertility of wild type *Ms45* to restore fertility, the mortality gen of pollen *ZmAA* that violates its normal development, and the gene of the fluorescent marker *DsRed2*, which facilitates the sorting of seeds), the method of agrobacterium transformation of immature embryos of corn is used to receive an SPT transgenic corn maintainer line. When the male sterile line (with a mutant gene *ms45/ms45*) is pollinated with pollen of the line that contains the SPT design, almost 100% of the resulting seeds have the genotype *ms45/ms45* and can be used as female lines with CMS for crossing and produce hybrid seeds. A Multi-Control Sterility (MCS) hybrid seed production system has been developed to limit the rate of transgenic transfer through pollen lines containing the SPT design. The transgenic line is obtained with the agrobacterium transformation of mutants at the genes *ms7* or *ms30* using a vector containing the gene of male fertility (*ZmMs*) to restore fertility, two genes for fertility disturbance of pollen, which are able to inhibit the formation or function of transgenic pollen, the fluorescent marker (*DsRed2*) and the gene of resistance to herbicides (*bar*) to prevent contamination of the seeds [86]. In lines containing these structures, the rate of transmission of the transgene is reduced significantly.

In 2014, Monsanto (USA) developed the Roundup Hybridization System (RHS) for the production of hybrid seeds [87]. The RHS mechanism is based on obtaining plants resistant to this herbicide through the introduction of the *CP4-EPSP* gene from *Agrobacterium* sp. CP4 that encodes 5-enolpyruvyl-shikimate-3-phosphate-synthase insensitive to glyphosate, with the subsequent selective decrease in the expression of this gene in male reproductive tissues. As a result, when exposed to glyphosate, defective pollen (glyphosate-mediated male sterility) is

formed with virtually no negative effects on other organs [87].

Despite the fact that the three-gene hybrid system on the basis of CSM was used in the commercial production of hybrid seeds of GM corn, its widespread use was prevented by serious problems [84]. Most of the developed CMS strategies have not been successfully applied due to the lack of a cost-effective, environmentally friendly and genetically stable strategy for large-scale reproduction of parent lines with CMS [84]. The necessary data was not found in the available literature on the large-scale sale and cultivation of GM corn seeds with CMS, although in 2017 the report on the commercial cultivation of GM rape with CMS was published [2].

Since 2016, CRISPR/Cas9 technology has been used to create corn lines with CMS. Xie et al. [88] studied the *ms33* gene of corn, encoding glycerol-3-phosphate acyltransferase (GPAT), a key enzyme in the synthesis of glycerolipid. The form obtained using CRISPR/Cas9 technology turned out to be a full-fledged mutant with CMS without anthers and mature pollen grains. Loss of *ZmMs33* gene expression in anthers violates the metabolism and development of the tapetum, which causes damage of anther cuticle and blocks the formation of pollen. In 2018, the vector CRISPR/Cas9 for editing the *MS8* gene, encoding β-1,3-galactosyltransferase that affects the meiotic stage of anther development, was developed [89]. The authors obtained eight transgenic lines (T_0). The results of sequencing showed that genes *MS8* in these lines of the T_0 generation are not mutated. However, mutations have been identified in the gene *MS8* among the descendants of the transgenic lines H17 to F_1 (obtained by crossing with the inbred line Zong31) and F_2 (reproduced by self-pollination of F_1). Mutations in the *MS8* gene and CMS can be consistently inherited in generations and passed on to other elite inbred lines to produce hybrid products [89]. With the help of the CRISPR/Cas9 technology, corn mutants at the *MS45* gene required for pollen development were also produced [90]. Plants of the generation T_0 containing two-allelic mutations in the *MS45* gene were with CMS. CMS lines created using CRISPR/Cas9 technology have not yet been tested in practical production. Thus, CMS has been used for more than half a century to limit the crossing of the donor and recipient of corn pollen, but this approach has not yet been widely used to create GM corn for various reasons. Perhaps, attempts to create corn varieties with CMS using CRISPR/Cas9 technology will be more successful.

Early studies of gene flow in cross-pollination are discussed in summarizing papers [14, 91]. However, differences in approaches, analytical methods, and experimental schemes impede comparison of results and complicate the identification of measures to limit cross-pollination in the field conditions. In particular, the distance between GM and non-GM corn recommended in EU countries at the same threshold of GM content in food varies significantly (from 25 to 600 m) [92, 93]. In addition to the distance between crops and the synchronicity of flowering, the frequency of cross-pollination depends on the size and orientation of the fields [94]. Most studies of the distribution of GM varieties were based on a single source of donor pollen, often with a smaller or equal field size relative to the recipient [34, 91]. Later, models based on multiple pollen sources were created, based on numerous data of field experiments [95]. The established requirements to the minimum distance and flowering time when grown transgenic and non-transgenic corn discriminate farmers, but the additional costs of these requirements (up to 300 Euro/ha) severely limit the economic benefits of the growers of GM corn in Europe [92, 96].

Thus, since the second half of the 1990s, the area under GM corn has increased by more than 100 times; therefore, the issues of its distribution and cross-pollination have become more relevant in practical terms. Based on cross-

pollination and pollen counting data at different distances from the source, a range of isolation distances from 10 to 50 m is recommended. If the proper isolation distance cannot be ensured, the area of the recipient and/or donor may be bordered by a pollen barrier. In the recipient field, the outer rows of corn can be considered the pollen barrier. After the 10-20 m wide corn pollen barrier, almost no analyzed sample contains more than 0.9% of GM material. For recipient areas of less than 1 ha and/or shallow depth, an isolation distance of at least 50 m may be recommended, especially in the direction of the wind pattern. When using corn hybrids with CMS, isolation distances between adjacent GM and non-GM corn fields can be significantly reduced (to 10 m) while maintaining compliance with European requirements (marking threshold 0.9%). However, for practical mass cultivation of GM corn and corn modified with the CRISPR/Cas9 technology with CMS is not yet used. Russia has yet to develop its measures and recommendations for the joint cultivation of GM and non-GM corn.

REFERENCES

1. Pellegrino E., Bedini S., Nuti M., Ercoli L. Impact of genetically engineered maize on agronomic, environmental and toxicological traits: a meta-analysis of 21 years of field data. *Scientific Reports*, 2018, 8(3113): 1-12 (doi: 10.1038/s41598-018-21284-2).
2. *ISAAA. Global Status of Commercialized Biotech/GM Crops: 2017. Biotech Crop Adoption Surges as Economic Benefits Accumulate in 22 years. ISAAA Brief No. 53.* ISAAA, Ithaca, NY, 2017.
3. *ISAAA. Global Status of Commercialized Biotech/GM Crops: 2016. ISAAA Brief No. 52.* ISAAA, Ithaca, NY, 2016.
4. Golikov A.G., Stepanova N.G., Krasovskii O.A., Skryabin K.G. *Biotekhnologiya*, 1997, 1: 53-58 (in Russ.).
5. Chesnokov Yu.V. *Vavilovskii zhurnal genetiki i selektsii*, 2011, 15(4): 818-827 (in Russ.).
6. Romeis J., Naranjo S.E., Meissle M., Shelton A.M. Genetically engineered crops help support conservation biological control. *Biological Control*, 2019, 130: 136-154 (doi: 10.1016/j.biocontrol.2018.10.001).
7. Bannert M., Stamp P. Cross-pollination of maize at long distance. *European Journal of Agronomy*, 2007, 27: 44-51 (doi: 10.1016/j.eja.2007.01.002).
8. Baltazar B., Castro Espinoza L., Espinoza Banda A., de la Fuente Martнnez J.M., Garzyn Tiznado J.A., González García J., Gutiérrez M.A., Guzmán Rodríguez J.L., Heredia Díaz O., Horak M.J., Madueco Martínez J.I., Schapaugh A.W., Stojšin D., Uribe Montes H.R., Zavala García F. Pollen-mediated gene flow in maize: implications for isolation requirements and coexistence in Mexico, the center of origin of maize. *PloS ONE*, 2015, 10: e0131549 (doi: 10.1371/journal.pone.0131549).
9. Marceau A., Gustafson D.I., Brants I.O., Leprince F., Foueillassar X., Riesgo L., Areale F.-J., Sowaf S., Kraicg J., Badeah E.M. Updated empirical model of genetically modified maize grain production practices to achieve European Union labeling thresholds. *Crop Science*, 2013, 53: 1712-1721 (doi: 10.2135/cropsci2012.04.0224).
10. Luna S., Figueroa J., Baltazar B., Gomez R., Townsend R., Schoper J.B. Maize pollen longevity and distance isolation requirements for effective pollen control. *Crop Science*, 2001, 41: 1551-1557 (doi:m10.2135/cropsci2001.4151551x).
11. Angevin F., Klein E., Choimet C., Meynard J., de Rouw A., Sohbi Y. Modélisation des effets des systиmes de culture et du climat sur les pollinisations croisйes chez le maпs. Isolement des collectes et maotrise des dissйminations au champ. In: *Rapport du groupe 3 du programme de recherche: pertinence йconomique et faisabilitй d'une filiure sans utilisation d'OGM, INRAFNSEA.* J.-M. Meynard, M. Le Bail (eds.). Thiverval-Grignon, France, 2001: 21-36.
12. Aylor D. Survival of maize (*Zea mays*) pollen exposed in the atmosphere. *Agricultural and Forest Meteorology*, 2004, 123: 125-133 (doi: 10.1016/j.agrformet.2003.12.007).
13. Jarosz N., Loubet B., Durand B., Foueillassar X., Huber L. Variations in maize pollen emission and deposition in relation to microclimate. *Environmental Science & Technology*, 2005, 39: 4377-4384 (doi: 10.1021/es0494252).
14. Devos Y., Reheul D., De Schrijver A. The co-existence between transgenic and non-transgenic maize in the European Union: a focus on pollen flow and cross-fertilization. *Environmental Biosafety Research*, 2005, 4(2): 71-87 (doi: 10.1051/ebr:2005013).
15. Ma B.L., Subedi K.D., Reid L.M. Extent of cross-fertilization in maize by pollen from neighboring transgenic hybrids. *Crop Science*, 2004, 44: 1273-1282 (doi: 10.2135/cropsci2004.1273).
16. Galeano P., Debat C.M., Ruibal F., Fraguas L.F., Galván G.A. Cross-fertilization between

genetically modified and non-genetically modified maize crops in Uruguay. *Environmental Biosafety Research*, 2010, 9(3): 147-154 (doi: 10.1051/ebr/2011100).
17. Aylor D., Schultes N., Shields E. An aerobiological framework for assessing cross-pollination in maize. *Agricultural and Forest Meteorology*, 2003, 119: 111-129 (doi: 10.1016/S0168-1923(03)00159-X).
18. Emberlin J., Adams-Groom B., Tidmarsh J. A report on the dispersal of maize pollen. In: *Report commissioned by and available from the Soil Association National Pollen Research Unit*. Bristol House, Bristol, UK, 1999: 40-56.
19. Mele E. Spanish study shows that coexistence is possible. *Agricultural Biotechnology International Conference*, 2004, 3: 2.
20. Ortega Molina J. *Results of the studies into the coexistence of genetically modified and conventional maize. COPA-COGECA colloquy on the co-existence and thresholds of adventitious presence on GMOs in conventional seeds, 2004*. Available http://www.copa-cogeca.be/pdf/9.pdf. No date.
21. Weber W.E., Bringezu T., Broer I., Eder J., Holz F. Coexistence between GM and non-GM maize crops — tested in 2004 at the field scale level (Erprobungsanbau 2004). *Journal of Agronomy and Crop Science*, 2007, 193: 79-92 (doi:10.1111/j.1439-037X.2006.00245.x).
22. Palaudelmas M., Mele E., Monfort A., Serra J., Salvia J., Messeguer J. Assessment of the influence of field size on maize gene flow using SSR analysis. *Transgenic Research*, 2012, 21(3): 471-483 (doi: 10.1007/s11248-011-9549-z).
23. Ingram J. The separation distances required to ensure cross-pollination is below specified limits in non-seed crops of sugar beet, maize and oilseed rape. *Plant Varieties and Seeds*, 2000, 13(3): 181-199.
24. Novotny E., Perdang J. Report on a model for pollen transport by wind. In: *Report for the Chardon LL hearing*. London, 2002.
25. Westgate M., Lizaso J., Batchelor W. Quantitative relationship between pollen-shed density and grain yield in maize. *Crop Science*, 2003, 43: 934-942 (doi: 10.2135/cropsci2003.9340).
26. Bock A.-K., Lheureux K., Libeau-Dulos M., Nilsagard H., Rodriguez-Cerezo E. *Scenarios for co-existence of genetically modified, conventional and organic crops in European agriculture*. IPTS-JRC, Seville, Spain, 2002.
27. Brookes G., Barfoot P., Melé E., Messeguer J., Bénétrix F., Bloc D., Foueillassar X., Fabié A., Poeydomenge C. *Genetically modified maize: pollen movement and crop coexistence*. PG Economics Ltd., Dorchester, UK, 2004.
28. Du M., Kawashima S., Matsuo K., Yonemura S., Inoue S. Simulation of the effect of a cornfield on wind and pollen deposition. In: *International Congress on Modelling and Simulation*. F. Ghassemi, P. Whetton, R. Little, M. Littleboy (eds.). Australian National University, 2001: 899-903.
29. Liu Y., Chen F., Guan X., Li J. High crop barrier reduces gene flow from transgenic to conventional maize in large fields. *European Journal of Agronomy*, 2015, 71: 135-140 (doi: 10.1016/j.eja.2015.09.005).
30. Le Bail M., Lecroart B., Gauffreteau A., Angevin F., Messean A. Effect of the structural variables of landscapes on the risks of spatial dissemination between GM and non-GM maize. *European Journal of Agronomy*, 2010, 33: 12-23 (doi: 10.1016/j.eja.2010.02.002).
31. Henry C., Morgan D., Weekes R., Daniels R., Boffey C. *Farm scale evaluations of GM crops: monitoring gene flow from GM crops to non-GM equivalent crops in the vicinity: Part I: Forage maize*. DEFRA report EPG, 2003.
32. Weekes R., Allnutt T., Boffey C., Morgan S., Bilton M., Daniels R., Henry C. A study of crop-to-crop gene flow using farm scale sites of fodder maize (*Zea may*s L.) in the UK. *Transgenic Research*, 2007, 16(2): 203-211 (doi: 10.1007/s11248-006-9036-0).
33. Devos Y., Demont M., Sanvido O. Coexistence in the EU — return of the moratorium on GM crops? *Nature Biotechnology*, 2008, 26(11): 1223-1225 (doi: 10.1038/nbt1108-1223).
34. Gustafson D.I., Brants I.O., Horak M.J., Remund K.M., Rosenbaum E.W., Soteres J.K. Empirical modeling of genetically-modified maize grain production practices to achieve European Union labeling thresholds. *Crop Science*, 2006, 46: 2133-2140 (doi: 10.2135/cropsci2006.01.0060).
35. Chamecki M., Gleicher S.C., Dufault N.S., Isard S.A. Diurnal variation in settling velocity of pollen released from maize and consequences for atmospheric dispersion and cross-pollination. *Agricultural and Forest Meteorology*, 2011, 151: 1055-1065 (doi: 10.1016/j.agrformet.2011.03.009).
36. Makowski D., Bancal R., Bensadoun A., Monod H., Messéan A. Sampling strategies for evaluating the rate of adventitious transgene presence in non-genetically modified crop fields. *Risk Analysis*, 2017, 37(9): 1693-1705 (doi: 10.1111/risa.12745).
37. Bohn T., Primicerio R., Traavik T. The German ban on GM maize MON810: scientifically justified or unjustified? *Environmental Sciences Europe*, 2012, 24(22): 1-7 (doi: 10.1186/2190-4715-24-22).
38. Della Porta G., Ederle D., Bucchini L., Prandi M., Verderio A., Pozzi C. Maize pollen mediated gene flow in the Po valley (Italy): Source—recipient distance and effect of flowering time. *European Journal of Agronomy*, 2008, 28: 255-265 (doi: 10.1016/j.eja.2007.07.009).
39. Popescu S., Leprince F., Ioja-Boldura O., Marutescu A., Sabau I., Marcela Badea E. Quantification of pollen mediated gene flow in maize. *Romanian Biotechnological Letters*, 2010, 15: 5351-5360.
40. Van de Wiel C.C.M., Groeneveld R.M.W., Dolstra O., Kok E.J., Scholtens I.M.J., Thissen J.,

Smulders M.J.M., Lotz L.A.P. Pollen-mediated gene flow in maize tested for coexistence of GM and non-GM crops in the Netherlands: Effect of isolation distances between fields. *Njas-Wageningen Journal of Life Sciences*, 2009, 56: 405-423 (doi: 10.1016/S1573-5214(09)80007-9).
41. Jaffe G. Regulating transgenic crops: a comparative analysis of different regulatory processes. *Transgenic Research*, 2004, 13: 5-19 (doi: 10.1023/B:TRAG.0000017198.80801.fb).
42. Nicolia A., Manzo A., Veronesi F., Rosellini D. An overview of the last 10 years of genetically engineered crop safety research. *Critical Review Biotechnology*, 2014, 34(1): 77-88 (doi:10.3109/07388551.2013.823595).
43. Sirsi E. Coexistence: a new perspective, a new field. *Agriculture and Agricultural Science Procedia*, 2016, 8: 449-454 (doi: 10.1016/j.aaspro.2016.02.042).
44. Ujj O. European and American views on genetically modified foods. *The New Atlantis*, 2016, 49: 77-92.
45. *EC (European Commission). 2015b. Fact sheet: Questions and answers on EU's policies on GMOs.* Available http://europa.eu/rapid/press-release_MEMO-15-4778_en.htm. Accessed 30.11.2015.
46. *Restrictions on Genetically Modified Organisms.* Washington: Law Library of Congress, Global Legal Research Center, 2014. Available https://www.loc.gov/law/help/restrictions-on-gmos/index.php. No date.
47. Deppermann A., Balkovic J., Bundle S.-C., Di Fulvio F., Havlík P., Leclиre D., Lesiv M., Prishchepov A., Schepaschenko D. Increasing crop production in Russia and Ukraine— regional and global impacts from intensification and recultivation. *Environmental Research Letters*, 2018, 13: 025008 (doi: 10.1088/1748-9326/aaa4a4).
48. Clive J. *Global status of commercialized biotech/gm crops: 2014. ISAAA Brief No. 49.* ISAAA, Ithaca, NY, 2014.
49. Brookes G., Barfoot P. Farm income and production impacts of using GM crop technology 1996-2016. *GM Crops & Food*, 2018, 9: 59-89 (doi: 10.1080/21645698.2018.1464866).
50. Zhuchenko A.A. Role of genetic engeneering in adaptive system of plant breeding (myths and reality). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2003, 1: 3-33.
51. Zhivotovskii L.A. V sbornike: *GMO — skrytaya ugroza Rossii* /Pod redaktsiei I.V. Starikova [In: GMO — the hidden threat to Russia. I.V. Starikov (ed.)]. Moscow, 2004: 94-104 (in Russ.).
52. Muir W.M., Howard R.D. Possible ecological risks of transgenic organism release when transgenes affect mating success: Sexual selection and the Trojan gene hypothesis. *Proc. Natl. Acad. Sci. USA*, 1999, 96 (24): 13853−13856 (doi: 10.1073/pnas.96.24.13853).
53. Piperno D.R., Flannery K.V. The earliest archaeological maize (*Zea mays* L.) from highland Mexico: new accelerator mass spectrometry dates and their implications. *Proc. Natl. Acad. Sci. USA*, 2001, 98: 2101-2103 (doi: 10.1073/pnas.98.4.2101).
54. Cárdenas F. Latin American maize germplasm regeneration and conservation. In: *Proc. Workshop CIMMYT, Mexico, June 4-6, 1996.* S. Taba (ed.). CIMMYT, Mexico, 1997: 74.
55. Serratos-Hernandez J.-A., Islas-Gutierrez F., Buendia-Rjdrigueez E., Berthaud J. Gene flow scenarios with transgenic maize in Mexico. *Environmental Biosafety Research*, 2004. 3: 149-157 (doi: 10.1051/ebr:2004013).
56. Quist D., Chapela I.H. Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. *Nature*, 2001, 41: 541-543 (doi: 10.1038/35107068).
57. Ortiz-Garcıa S., Ezcurra E., Schoel B., Acevedo F., Soberon J., Snow A.A. Absence of detectable transgenes in local landraces of maize in Oaxaca, Mexico (2003-2004). *Proc. Natl. Acad. Sci. USA*, 2005, 102(35): 12338-12343 (doi: 10.1073/ pnas.0503356102).
58. Bellon M.R., Berthaud J. Transgenic maize and the evolution of landrace diversity in Mexico. The importance of farmers' behavior. *Plant Physiology*, 2004, 134: 883-888 (doi: 10.1104/pp.103.038331).
59. McHughen A. A critical assessment of regulatory triggers for products of biotechnology: Product vs. process. *GM Crops & Food*, 2016, 7: 125-158 (doi: 10.1080/21645698.2016.1228516).
60. Ramessar K., Capell T., Twyman R.M., Quemada H., Christou P. Trace and traceability — a call for regulatory harmony. *Natural Biotechnology*, 2008, 26: 975-978 (doi: 10.1038/nbt0908-975).
61. Tetsuya I., Motoko A. A future scenario of the global regulatory landscape regarding genome-edited crops. *GM Crops & Food*, 2017, 8(1): 44-56 (doi: 10.1080/21645698.2016.1261787).
62. Baram M. Governance of GM crop and food safety in the United States. In: *Governing risk in GM agriculture.* M. Baram, M. Bourrier (eds.). Cambridge University Press. 2011: 15-55.
63. Nabradi A., Popp J. Economics of GM crop cultivation. *Applied Studies in Agribusiness and Commerce*, 2011, 05: 7-19 (doi: 10.19041/Apstract/2011/3-4/1)
64. Xu Z., Hennessy D.A., Sardana K., Moschini G.C. The realized yield eject of genetically engineered crops: U.S. maize and soybean. *Crop Science*, 2013, 53: 735-745 (doi: 10.2135/cropsci2012.06.0399).
65. Eastham K., Sweet J. Genetically modified organisms (GMOs): the significance of gene flow through pollen transfer. In: *European Environment Agency. Environmental issue report.* D. Gee (ed.). Copenhagen, 2002, 28: 38-42.
66. Lee M.S., Anderson E.K., Stojšin D., McPherson M.A., Baltazar B., Horak M.J., de la Fuente J.M., Wu K., Crowley J.H., Rayburn A.L., Lee D.K. Assessment of the potential for

gene flow from transgenic maize (*Zea mays* L.) to eastern gamagrass (*Tripsacum dactyloides* L.). *Transgenic Research*, 2017, 26(4): 501-514 (doi: 10.1007/s11248-017-0020-7).
67. Instituto Colombiano Agropecuario. *Por medio de la cual se implementa el plan de manejo, bioseguridad y seguimiento para siembras controladas de maíz genéticamente modificado. Resoluciyn No. 2894.* Bogota, 2010.
68. Chaparro-Giraldo A., Blanco M.J.T., López-Pazos S.A. Evidence of gene flow between transgenic and non-transgenic maize in Colombia. *Agronomна Colombiana*, 2015, 33(3): 297-304 (doi: 10.15446/agron.colomb.v33n3.51501).
69. Vicién C., Trigo E. The Argentinian GMO biosafety system: an evolving perspective. In: *Genetically modified organisms in developing countries risk analysis and governance*. A. Adendle, E. Jane Morris, J. Denis (eds.). Cambridge University Press, Cambridge, UK, 2017: 247-257 (doi: 10.1017/9781316585269.022).
70. Burachik M. Experience from use of GMOs in Argentinian agriculture, economy and environment. *New Biotechnology*, 2010, 27(5): 588-592 (doi: 10.1016/j.nbt.2010.05.011).
71. Coelho M.V.S. Coexistence in Brazil. In: *The coexistence of genetically modified, organic and conventional foods.* N. Kalaitzandonakes, P. Phillips, J. Wesseler, S. Smyth (eds.). Natural Resource Management and Policy, Springer, NY, USA, 2016, 49: 87-94 (doi: 10.1007/978-1-4939-3727-1_8).
72. Viljoen C., Chetty L. A case study of GM maize gene flow in South Africa. *Environmental Sciences Europe*, 2011, 23: 1-8 (doi: 10.1186/2190-4715-23-8).
73. Wang C.Y., Kuo B.J., Hsu Y.H., Yiu T.J., Lin W.S. Using the two-step model based on the field border consideration to evaluate pollen-mediated gene flow (PMGF) model and the isolation distance of GM maize in Potzu city of Chiayi county. *Crop, Environment & Bioinformatics*, 2013, 10: 172-189 (doi: 10.1371/journal.pone.0131549).
74. Smyth S.J. Genetically modified crops, regulatory delays, and international trade. *Food Energy Security*, 2017, 6: 78-86 (doi: 10.1002/fes3.100).
75. Bückmann H., Capellades G., Hamouzová K., Holec J., Soukup J., Messeguer J., Melé E., Nadal A., Guillen X.P., Pla M., Serra J., Thiele K., Schiemann J. Cytoplasmic male sterility as a biological confinement tool for maize coexistence: Optimization of pollinator spatial arrangement. *Plant, Soil and Environment*, 2017, 63: 145-151 (doi: 10.17221/761/2016-PSE).
76. Bruns H.A. Southern corn leaf blight: a story worth retelling. *Agronomy Journal.* 2017, 109: 1218-1224 (doi: 10.2134/agronj2017.01.0006).
77. Schnable P.S., Wise R.P. The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends in Plant Science*, 1998, 3: 175-180 (doi: 10.1016/S1360-1385(98)01235-7).
78. Su A., Song W., Xing J., Zhao Y., Zhang R., Li C., Duan M., Luo M., Shi Z., Zhao J. identification of genes potentially associated with the fertility instability of S-type cytoplasmic male sterility in maize via bulked segregant RNA-seq. *PLoS ONE*, 2016, 11(9): e0163489 (doi: 10.1371/journal.pone.0163489).
79. Liu Y., Wei G., Xia Y., Liu X., Tang J., Lu Y., Lan H., Zhang S., Li C., Cao M. Comparative transcriptome analysis reveals that tricarboxylic acid cycle-related genes are associated with maize CMS-C fertility restoration. *BMC Plant Biology*, 2018, 18(1): 190 (doi: 10.1186/s12870-018-1409-z).
80. Herman R.A., Zhuang M., Storer N.P., Cnudde F., Delaney B. Risk-only assessment of genetically engineered crops is risky. *Trends in Plant Science*, 2019, 24(1): 58-68 (doi: 10.1016/j.tplants.2018.10.001).
81. Bückmann H., Hüsken A., Schiemann J. Applicability of cytoplasmic male sterility (CMS) as a reliable biological confinement method for the cultivation of genetically modified maize in Germany. *Journal of Agricultural Science and Technology,* 2013, A3: 385-403.
82. Bückmann H., Thiele K., Schiemann J., Husken A. Influence of air temperature on the stability of cytoplasmic male sterility (CMS) in maize (*Zea mays* L.). *AgBioForum*, 2014, 2: 205-212.
83. Bückmann H., Thiele K., Schiemann J. CMS maize: a tool to reduce the distance between GM and non-GM maize. *EuroChoices*, 2016, 15(1): 31-35 (doi: 10.1111/1746-692X.12116).
84. Wan X., Wu S., Li Z., Dong Z., An X., Ma B., Tian Y., Li J. Maize genic male-sterility genes and their applications in hybrid breeding: progress and perspectives. *Molecular Plant*, 2019, 12(3): 321-342 (doi: 10.1016/j.molp.2019.01.014).
85. Wu Y., Fox T.W., Trimnell M.R., Wang L., Xu R.J., Cigan A.M., Huffman G.A., Garnaat C.W., Hershey H., Albertsen M.C. Development of a novel recessive genetic male sterility system for hybrid seed production in maize and other cross pollinating crops. *Plant Biotechnology Journal*, 2016, 14: 1046-1054 (doi: 10.1111/pbi.12477).
86. Zhang D., Wu S., An X., Xie K., Dong Z., Zhou Y., Xu L., Fang W., Liu S., Liu S., Zhu T., Li J., Rao L., Zhao J., Wan X. Construction of a multicontrol sterility system for a maize male-sterile line and hybrid seed production based on the ZmMs7 gene encoding a PHD-finger transcription factor. *Plant Biotechnology Journal*, 2018, 16: 459-471 (doi: 10.1111/pbi.12786).
87. Feng P.C., Qi Y., Chiu T., Stoecker M.A., Schuster C.L., Johnson S.C., Fonseca A.E., Huang J. Improving hybrid seed production in corn with glyphosate-mediated male sterility.

Pest Management Science, 2014, 70: 212-218 (doi: 10.1002/ps.3526).
88. Xie K., Wu S., Li Z., Zhou Y., Zhang D., Dong Z., An X., Zhu T., Zhang S., Liu S., Li J., Wan X. Map-based cloning and characterization of *Zea mays* male sterility33 (*ZmMs33*) gene, encoding a glycerol-3-phosphate acyltransferase. *Theoretical and Applied Genetics*, 2018, 131: 1363 (doi: 10.1007/s00122-018-3083-9).
89. Chen R., Xu Q., Liu Y., Zhang J., Ren D., Wang G., Liu Y. Generation of transgene-free maize male sterile lines using the CRISPR/Cas9 system. *Frontiers in Plant Science*, 2018, 9: 1180 (doi: 10.3389/fpls.2018.01180).
90. Svitashev S., Schwartz C., Lenderts B., Young J.K., Mark C.A. Genome editing in maize directed by CRISPR—Cas9 ribonucleoprotein complexes. *Nature Communications,* 2016, 7: 13274 (doi: 10.1038/ncomms13274).
91. Sanvido O., Widmer F., Winzeler M., Streit B., Szerencsits E., Bigler F. Definition and feasibility of isolation distances for transgenic maize cultivation. *Transgenic Research*, 2008, 17: 317-335 (doi: 10.1007/s11248-007-9103-1).
92. Devos Y., Demont M., Dillen K., Reheul D., Kaiser M., Sanvido O. Coexistence of genetically modified (GM) and non-GM crops in the European Union (review). *Agronomy for Sustainable Development*, 2009, 29: 11-30 (doi: 10.1051/agro:2008051).
93. Riesgo L., Areal F.J., Sanvido O., Rodriguez-Cerezo E. Distances needed to limit cross-fertilization between GM and conventional maize in Europe. *Nature Biotechnology*, 2010, 28: 780-782 (doi: 10.1038/nbt0810-780).
94. Langhof M., Hommel B., Hüsken A., Njontie C., Schiemann J., Wehling P., Wilhelm R., Rühl G. Coexistence in maize: isolation distance in dependence on conventional maize field depth and separate edge harvest. *Crop Science*, 2010, 50: 1496-1508 (doi: 10.2135/cropsci2009.11.0641).
95. Marceau A., Saint-Jean S., Loubet B., Foueillassar X., Huber L. Biophysical characteristics of maize pollen: Variability during emission and consequences on cross-pollination risks. *Field Crops Research*, 2012, 127: 51-63 (doi: 10.1016/j.fcr.2011.11.006).
96. Venus T.J., Dillen K., Punt M.J., Wesseler J.H. The costs of coexistence measures for genetically modified maize in Germany. *Journal of Agricultural Economics*, 2017, 68: 407-426 (doi:10.1111/1477-9552.12178).

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PLANT CELL WALL IN SYMBIOTIC INTERACTIONS. PECTINS (review)

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Abstract

Since plant cells, unlike animals, are immobile and limited by rigid cell walls, often the properties of the plant extracellular matrix play a crucial role in the plant development. The extracellular matrix, in particular the cell walls, are involved in the molecular dialogue between partners during the interaction of plants and microorganisms during the formation of legume-rhizobial symbiosis (N.J. Brewin, 2004; M.K. Rich et al., 2014). Legume-rhizobial symbiosis is a convenient model for studying changes in the composition of the plant cell wall caused by interactions with bacteria. Colonization of host cells with nodule bacteria, rhizobia, involves the sequential reorganization of the plant-microbial interface. The bacterial components of the symbiotic interface include various surface polysaccharides (A.V. Tsyganova et al., 2012). Plant components include the cell wall, the extracellular matrix and the plasma membrane. In this review, we have summarized the data demonstrating the involvement of pectins, the polysaccharides of the cell wall matrix, in the legume-rhizobial symbiosis (K.H. Caffall et al., 2009; M.A. Atmodjo et al., 2013; C.T. Anderson, 2015). The greatest progress has been made in the study of homogalacturonan, for which highly specific monoclonal antibodies have been obtained (J.P. Knox et al., 1990; Y. Verhertbruggen et al., 2009). The level of methyl-esterification of homogalacturonan determines its function in nodules. It was shown that low methyl-esterified homogalacturonan is involved in increasing the rigidity of the cell walls and walls of infection threads (K.A. VandenBosch et al., 1989; A.L. Rae et al., 1992) that is especially manifested in ineffective interaction with rhizobia (K.A. Ivanova et al., 2015) and during the action of abiotic factors (M. Redondo-Nieto et al., 2003, 2007; M. Sujkowska-Rybkowska et al., 2015). High methyl-esterified homogalacturonan is observed in the cell walls at all stages of nodule development (A.L. Rae et al., 1992; A.V. Tsyganova et al., 2019). The absence of well characterized antibodies complicates the study of rhamnogalacturonan-II (M.A. O'Neill et al., 2004). However, it was shown that in nodules rhamnogalacturonan-II is present in the cell wall at the border with the plasma membrane, in undifferentiated symbiosomes, and also in the matrix of infection threads (M. Redondo-Nieto et al., 2003, 2007; M. Reguera et al., 2010). Probably, rhamnogalacturonan-II in combination with boron and arabinogalactan-protein extensins promotes movement of rhizobia in the matrix of infection threads (M. Reguera et al., 2010). Only recently, we conducted the first studies aimed at identifying the role of rhamnogalacturonan-I in the development of nodules (A.V. Tsyganova et al., 2019). It has been shown that rhamnogalacturonan-I is present in the cell wall of the meristem cells, vascular bundles and in the walls of the infection threads. However, its precise function remains unknown, although it was suggested that rhamnogalacturonan-I is involved in the perception of rhizobia as pathogens during ineffective symbiosis (A.V. Tsyganova et al., 2019). Thus, to date, it has been shown that all types of pectins are involved in the development of a symbiotic nodule. It is important to note that plant plays a central role in the remodelling of the cell wall during symbiotic interaction and the construction of the plant-microbe interface.

Keywords: legume-rhizobial symbiosis, plant-microbe interface, cell wall, infection thread, homogalacturonan, rhamnogalacturonans

Interaction with microorganisms, both pathogenic and symbiotic, is intrinsic to any naturally growing plant. Each plant cell is surrounded by a network of cellulose microfibrils, i.e. a cell wall that is almost impenetrable to mi-

croorganisms [1-3]. Cell wall, as an exterior boundary of plant body, mediates many interactions with the environment, including interactions with biotic factors [4-6]. In the process of evolution microorganisms had developed two strategies to overcome a barrier created by a wall cell and to penetrate inside a cell. The first is lysis of a plant cell wall used by many pathogens. The second is reorganization of a cell wall at microorganism penetration point. This strategy is also used by rhizobia when penetrating into the root. As a result of local inhibition of cellulose synthesis and modification of intracellular matrix, rhizobia may, in fact, create subcellular entry point through the cellulose network with minimal damage of a host cell and manifestation of stress and defence reactions [7-9]. At intracellular growth of infection thread, its wall is built like a phragmoplast [7, 9, 10]. At incompatible interactions between rhizobia and legumes, the intracellular matrix is modified and becomes more resistant to invasion due to fusion of secondary metabolites, such as suberin [11], or through cross-linking of cell wall proteins by hydrogen peroxide [12].

Cell walls and plasma membranes are involved in the molecule exchange between partners during the interaction of plants and microorganisms, thereat symbiotic interactions arrive at full functionality due to development of an extensive contact surface between the host and microsymbiont – symbiotic interface [7, 8]. Cell walls mainly consist of polysaccharides, which could be grouped into three main categories: cellulose, pectins and hemicelluloses [1, 3]. In addition to polysaccharides, most plant cell walls contain structural proteins, such as extensins and arabinogalactan-glycoproteins.

For the first time, the role of cell wall polysaccharides in molecular dialogue between partners during the interaction of plants and rhizobia was shown in studies of research group from John Innes Centre [10, 13, 14].

In present review we discuss participation of a broad class of cell wall polysaccharides – pectins in legume-rhizobial symbiosis.

Pectin is an important cell wall component in dicotyledon plants, which, possibly, is one of the most complex macromolecules in nature. It consists of 17 different monosaccharide based on which various polysaccharides forming a network are created [15]. Pectins belong to a family of polysaccharides rich in galacturonic acid, including homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and substituted galacturonan, rhamnogalacturonan-II (RG-II); several plant cell walls also contain additional substituted galacturonans, known as apiogalacturonan and xylogalacturonan [16-18]. Pectin polysaccharides have numerous functions in plants: promoting cell adhesion, for instance, by dimerization of RG-II and cross-linking of HG; influencing on formation of secondary walls in fiber and wood tissue; creating reservoir of oligosaccharide signal molecules, which are important for growth, development, and defence reactions in plants; ensuring ionic transport and water regime as hydrating polymers, and influencing on seed germination [17, 19, 20]. As a result of many years of research, monoclonal antibodies detecting various pectin polymers in a plant cell wall have been obtained. Microscopic analysis of cell walls labeled by specific antibodies had shown that pectin contributes significantly to heterogeneity of plant cell walls in various tissues and at different stages of ontogenesis [15, 16, 21-23].

Cell wall homogalacturonans at formation of symbiotic nodules. HG is about 65% of pectin representing a linear backbone of (1-4)-linked α-D-galacturonic acids, provided that HG is methylated in part and is acetylated to a lesser extent [17, 18]. During cellular differentiation, HG is synthesized at plant Golgi apparatus and is secreted in form of polymers with high degree of methylation [16, 24]. Degree of HG methyl-esterification and acetylation is different in various plants; it is regulated during development by pectin methylesterases, inhibitors of pectin methylesterases, as well as by subtilisin-like

serine proteases and by at least one ubiquitin E3 ligase [25]. Activity of pectin degrading enzymes directly depends on presence of methyl esters [26, 27]. Pectin de-esterification in a cell wall by pectin methylesterase results in one of two opposite scenarios [28, 29]: blocks of de-esterificated residues of galacturonic acid may be cross-linked by Ca^{2+} ions in form of «egg-boxes» to harden cell wall [5, 16, 25, 30] or may be macerated by pectin degrading enzymes (for instance, polygalacturonases or pectate lyases), to soften cell wall [4, 31-33].

Pectin methylesterase participates in differentiation of uninfected cells in nodules formed at secondary roots of tropic legumes, such as *Sesbania rostrata* Bremek. and Oberm, with *Azorhizobium caulinodans* [26]. In *Medicago truncatula* Gaertn., *MtPER* gene out of eight differentially expressed pectin methylesterase genes relates to symbiotic and functions at early stages of interaction with *Sinorhizobium meliloti* [34].

Electron microscopical studies had shown that rhizobia penetrate into small degraded area of plant cell wall. Known role of enzymes degrading the cell wall during the infection process allows bacteria to penetrate inside the plants [4, 33]. *HrpW* gene referring to type III secretion system, which product had shown enzymatic activity of pectate lyase, was found in *Rhizobium etli* involved in symbiosis with *Phaseolus vulgaris* L. [35]. One of the first evidences of participation of plant enzymes degrading a cell wall during the infection process was obtained when studying the polygalacturonase gene of *M. sativa* L. (*MsPG3*) specifically expressed during the symbiosis with *S. meliloti* [36]. Pectate lyase (LjNPL), which is induced by Nod-factors and, evidently, participates in penetration of rhizobia into infection threads was found in *Lotus japonicus* (Regel) K. Larsen [37].

Immune-chemical method is often used for detailed localization of HG in plant tissues, at that partial or high methyl-esterified HG is detected by JIM7 and LM20 antibodies, whereas low methyl-esterified or de-esterified – by JIM5 and LM19 antibodies [38, 39]. Immunogold microscopy had shown different localization of pectin epitopes on cell walls [29]. De-esterified HG detected by JIM5 antibody is located on internal surface of primary cell walls adjacent to plasma membrane, in the middle lamella and mostly on the exterior surface of cell walls adjacent to intercellular spaces. For instance, high methyl-esterified HG detected by JIM7 antibody is distributed evenly throughout the cell wall [38, 40].

HG localization is studied in detail in various legumes inoculated by rhizobial symbiontes. Thus, high content of de-esterified HG was found in symbiotic nodules of pea (*Pisum sativum* L.) in primary cell walls of infected cells and in infection thread walls in nodules [14]. However, JIM5 label in older nonproliferating cells was mainly limited by the middle lamella. Upon studying the indeterminate root nodules of *Vicia hirsuta* (L.) Gray and *P. sativum* induced by *R. leguminosarum*, it was shown that cellulose, xyloglucan and HG with various degree of methyl-esterification were present in walls of infection thread [10]. Determinate nodules of *P. vulgaris* had very narrow infection threads with little or no matrix material and more densely fibrillar wall, which was largely labeled by JIM5 antibody detecting the de-esterified HG [10]. In symbiotic nodules of *P. sativum* and *M. truncatula* localization of high methyl-esterified HG detectable by JIM7 antibody is appropriate to isodiametrically growing cells filled by developing bacteroids [41].

HG localization and distribution was analyzed when other components of legume-rhizobial interface were studied. Immune-cytochemical study of ENOD2 nodulin proline-rich extensin in nodules of *P. sativum* and *Glycine max* (L.) Merr. had shown that de-esterified HG is localized in intercellular spaces, where ENOD2 is accumulated [42]. Positively-charged lysine and histidine residues of ENOD2 could facilitate its interaction with negatively-charged lateral

chains of pectins of cell walls, thus, promoting pectin gelling on the boundary of cell wall and intercellular space [42]. Vesicular transport components, in particular VAMP721d and VAMP721e, play an important role in formation of symbiotic interface in *M. truncatula* nodules [43]. Large bacterial clusters embedded into the matrix of methyl-esterified and de-esterified HG surrounded by membrane were found in *G. max* nodules with partially silenced *GmVAMP721d* gene. Besides, GmVAMP721d is partially colocalized with pectate lyase [44]. Therefore, biological role of VAMP721d could be explained by its participation in delivery of pectin modifying enzymes to release site of rhizobia into the host cell cytoplasm. Moreover, abnormal endocytosis of de-esterified HG is observed in young infected cells of such nodules [44].

Immune-chemical study of boron-deficit *P. sativum* plants had shown that, apart from control nodules, de-esterified HG detected by JIM5 antibody was evenly localized along the entire thickness of a cell wall and even in cytoplasm [45, 46]. At that, distribution of high methyl-esterified HG detected by JIM7 antibody remained unchanged. For investigation of possible participation of pectins in inhibition of aluminum-based growth, distribution of HG with different degree of methyl-esterification in apoplast of *P. sativum* nodules was studied [47]. When treated by aluminum, number of epitope labels of de-esterified HG and HG with association of pectic chains through calcium ions in thick infection thread walls had been increased. In contrast, epitope of high methyl-esterified HG was evenly distributed along all cell walls in nodules, and its quantity had been decreased during treatment. These results denote the specific role of HG de-esterification during thickening of a cell wall and inhibition of growth [47].

The study of ineffective legume-rhizobial interaction with the use of symbiotic mutants of *P. sativum* had shown that abundant deposition of de-esterified HG marked by JIM5 antibody in single or double mutants carrying mutation in *PsSym33* gene was observed in walls of infection threads [11]. De-esterified HG in RisFixV (*Pssym42*) mutant was unevenly distributed in callose-impregnated walls of infection threads and in cell walls of infected cells. The other specific feature of RisFixV (*Pssym42*) was presence of JIM5 label around the degrading bacteroids, which points out the incapsulation of the ineffective bacteroids by de-esterified HG [11]. In pea mutants, without any abnormalities in development of infection threads, the SGEFix$^-$-3 (*Pssym26*) and Sprint-2Fix$^-$ (*Pssym31*), distribution of de-esterified HG did not differ from that in the wild type, and quantity of HG was insignificant [41].

The same pattern of de-esterified HG distribution was observed in nodules of *M. truncatula* ineffective symbiotic mutants *ipd3*, *dnf1-1* and *efd-1* [41]. Herewith, quantity of deposited de-esterified HG was lower in all *M. truncatula* mutant genotypes than in wild type. However, in *ipd3* mutant nodules content of de-esterified HG had increased with age. It is interesting to note that doubling of the quantity of de-esterified HG labels was also observed in *M. truncatula* mutant *dnf1-1*, which is characterized by undifferentiated bacteroids [41].

Cell wall rhamnogalacturonan II at formation of symbiotic nodules. RG-II comprises ~ 10% of pectin and is the most complicated, as well as the most structurally conservative pectin polysaccharide. It is known, that RG-II molecules are interrelated forming RG-II dimers that is covalently cross-linked by a borate diester [17, 18]. Complex and conservative RG-II with 12 various sugars and over 20 types of links has the critical function in growth and development of plants, provided that even insignificant changes in RG-II structure may negatively influence on the growth of plants (to result in the dwarf phenotype) [28]. There is a growing body of evidences that boron and calcium are synergetic at formation of the primary pectin network of cell walls and that

change in properties of such network influence on plant growth [48-50]. Cell wall pectins, in particular RG-II, contain over 60% of the total quantity of boron in a cell. Numerous studies had shown that boron is a necessary microelement at development of legume-rhizobial symbiosis [48, 50-54].

Since currently existing RG-II antibodies are not characterized in detail, it restrains their use at determination of RG-II localization in a cell wall [49]. However, results obtained with the use of such antibodies suggest that RG-II is distributed all along the primary wall and is especially abundant near the plasma membrane (as compared to the middle lamella, where it lacks) [55]. After alkaline treatment of several, but not all tissues, quantity of labels in primary walls is increased, i.e. it could be assumed that epitopes detected by RG-II antibodies are masked due to esterification [49].

While studying the nodule formation in control plants *P. sativum,* localization of RG-II was found in the interface between the plasma membrane and cell wall [46]. Unlike the control nodules, RG-II label in boron-deficit plants located evenly along the thickness of cell wall and even in cytoplasm; thereat addition of Ca^{2+} had not restored the initial localization of RG-II in full [46]. It was shown later that RG-II in nodules of control plants *P. sativum* had also localized at peribacteroid membrane of undifferentiated bacteroids, however gradually disappeared later during the bacteroid maturation [45]. Symbiosomes in boron-deficit nodules had differentiated in aberrational manner and no RG-II was present on peribacteroid membrane due to the abnormal transport of vesicles with pectin to cell membranes [45]. RG-II in combination with boron and arabinogalactan-protein extensins in control plants was observed in infection thread matrix separated from the rhizobia cells by exopolysaccharide capsule [56]. RG-II in combination with arabinogalactan-protein extensins in boron-deficit plants was closely related to rhizobia surface in the lumen of infection threads [56]. Besides, rhizobia colonies grown without boron in free-living culture had weakly formed the capsule.

RG-II distribution in *G. max* plants with partially silenced *GmVAMP721d* gene was unchanged as compared to control nodules [44].

Cell wall rhamnogalacturonan I at formation of symbiotic nodules. RG-I comprises 20-35% of total pectin cell wall. RG-I has a disaccharide repeat backbone of (1,2)-linked residues of α-L-rhamnose and (1,4)-linked residues of α-D-galacturonic acid. Depending on a cell type and development stage, up to 20-80% of rhamnose residues relates to side (mainly linear, but also branched) arabinan, galactan, arabinogalactan or fucosyl chains [17, 18]. RG-I, provided all differences in its structure, usually forms a part of thin primary cell walls, where it plays a role of some "glue" for connecting with neighboring cells [57]. Herewith, RG-I specific for tissue and development stage is yet characterized for a single cell type, the plant fibre forming so called gelatin-like type of cell walls [57, 58]. RG-I is the most structurally heterogenic pectin, and prevalence and composition of side chains of RG-I significantly varies depending on the cell type, development stage and type of plant [39]. RG-I backbone plays an important role in integrity and functioning of cell wall since its degradation caused by hydrolase expression results in morphological changes [33]. Herewith, part of RG-I molecules interacts with cellulose microfibrils, and RG-I with galactan side chains with average polymerization degree is capable of self-linking due to galactose chains [57]. Studying of RG-I due to cell functions is focused on the role of such polysaccharides in ensuring of mechanic properties of a cell wall, such as its hardness and elasticity [23, 57, 59]. However, main contribution of galactan-rich RG-I to mechanical hardness of a cell wall is unknown.

Immunogold study had shown that distribution of RG-I in tissues of

leaves and roots is limited by the middle lamella, whereas 80-90% of labeling relates to expanded areas of middle lamella at intercellular junctions. At cell expansion, RG-I is present in cisterns and vesicles of Golgi apparatus, which confirms that Golgi apparatus serves the main synthesis point of non-cellulose polysaccharides of a cell wall. Besides, it was found that RG-I is practically absent in forming cell plate at cell division [60, 61].

Functions of side RG-I chains are still unclear in general. Arabinan and galactan side chains of RG-I are characterized by clear and mutually exclusive localization; generally, arabinan usually prevails in younger cells, whilst galactan share is usually higher during cell expansion [19, 21, 23, 62, 63]. Arabinan replacement by galactan in epidermal cells denotes the switch-over to differentiation of such cells and makes cell walls less flexible and more resistant to degradation. It is assumed that complementary pattern of epitope of galactan side chains of RG-I may reflect the need for maintenance of elastic properties in tissue with high density of sieve elements [64].

RG-I (1,5)-α-L arabinan side chains in *Arabidopsis* are abundant in cell wall of pollen tube. They may play an important role in prevention of formation of HG-polymers and participate in cell adhesion [65]. At early stages of rhizobial infection processes the polar growth of pollen tubes and infection threads may have similar mechanisms and regulators [34]. However, the role of RG-I in development of legume-rhizobial symbiosis was not studied thus far. We have for the first time studied distribution of RG-I in legume-rhizobial nodules in *P. sativum* and *M. truncatula* [41, 66]. For this end, LM5 antibodies detecting (1→4)-β-galactan side chain of RG-I were used. Fluorescent label was registered in meristem cell walls and vascular bundles in nodules of both species, as well as in endoderm, which consists of cells serving an "oxygen barrier", as well as in three-cell junctions in pea nodules. Besides, LM5 antibody had labeled walls of infection threads in *P. sativum* nodules, but label was absent in *M. truncatula* nodules. At the same time, LM5 antibody label was absent in walls of some infection threads in pea mutant SGEFix$^-$-2 (*Pssym33-3*), and mutant SGEFix$^-$-3 (*Pssym26*) had abnormal accumulation of epitope of galactan side chain of RG-I in cell wall of uninfected cells in senescence zone of nodule [41].

Recently discovered ability of *Pectobacterium atrosepticum* to form bacterial emboli in vessels of primary xylem [67] gives insight into the possible role of RG-I at development of ineffective nodules. Thus, while studying of *P. sativum* mutant SGEFix$^-$-2 (*Pssym33-3*) we have found that cell wall material containing high methyl-esterified HG and linear (1→4)-β-galactan side chain of RG-I was accumulated around tonoplast in infected cells of nodules [66]. Besides, such material formed certain outgrowths and drops in the lumen of the vacuole. Electron-dense matrix with LM5 antibody label, in which rhizobia was embedded, was observed in some infection threads. These phenotypic features of the mutation in *PsSym33* gene resemble of formation of bacterial emboli at invasion of *P. atrosepticum* [67]. Previously, intensive defence reactions – suberinization, deposition of de-esterified HG in walls of infection threads and increase of peroxidase gene expression were described in *Pssym33-3* mutant [11]. Deposition of cell wall material around the tonoplast and formation of pectin gel in matrix of infection threads is another manifestation of defence reaction in host plant and perception of rhizobia as a pathogen.

Thus, pectin which is one of the main components of cell wall plays an important role in establishment and functioning of symbiotic interface in legume nodules. Although recently the role of all three main pectin types: homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II has been analyzed, our knowledge about the functions of each pectin type are unequal. The most

studied is homogalacturonan, which is explained by existence of high specific monoclonal antibodies to this polysaccharide. Function of homogalacturonan in nodules is determined by its methylation level. It was found that low methyl-esterified homogalacturonan participates in rigidity increase of cell walls and walls of infection threads, especially at ineffective interaction with rhizobia and under influence of abiotic stresses. High methyl-esterified homogalacturonan participates at all developmental stages of nodule. Studying of rhamnogalacturonan-II is challenged due to absence of characterized antibodies. However, it is reported that in nodules rhamnogalacturonan-II is present in the cell wall at the border with the plasma membrane, in undifferentiated symbiosomes, and also in the matrix of infection threads. It is assumed that rhamnogalacturonan-II in combination with boron and arabinogalactan-protein extensins facilitates movement of rhizobia in infection threads. With regard to rhamnogalacturonan-I, which role in development of nodules remained unstudied until recently, we had shown that it is present in meristem cell walls, vascular bundles and infection thread walls. However, the exact function of rhamnogalacturonan-I is not yet clear. Therefore, although participation of all pectin types in development of symbiotic nodules it now known, identification of their specific functions needs further research. In particular, analysis of rhamnogalacturonan-II in ineffective mutant nodules with abnormalities in development of infection thread may confirm the hypothesis of its participation in growth of infection thread and movement of bacteria along it. Studying of rhamnogalacturonan-I and its side chains with the use of various antibodies is of great interest to confirm species-specificity of the content of infection thread walls.

REFERENCES

1. Cosgrove D.J. Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.*, 2005, 6(11): 850-861 (doi: 10.1038/nrm1746).
2. Malinovsky F.G., Fangel J.U., Willats W.G.T. The role of the cell wall in plant immunity. *Front. Plant. Sci.*, 2014, 5: 178 (doi: 10.3389/fpls.2014.00178).
3. Keegstra K. Plant cell walls. *Plant Physiol.*, 2010, 154(2): 483-486 (doi: 10.1104/pp.110.161240).
4. Lionetti V., Cervone F., Bellincampi D. Methyl esterification of pectin plays a role during plant—pathogen interactions and affects plant resistance to diseases. *J. Plant Physiol.*, 2012, 169(16): 1623-1630 (doi: 10.1016/j.jplph.2012.05.006).
5. Palin R., Geitmann A. The role of pectin in plant morphogenesis. *Biosystems*, 2012, 109(3): 397-402 (doi: 10.1016/j.biosystems.2012.04.006).
6. Bellincampi D., Cervone F., Lionetti V. Plant cell wall dynamics and wall-related susceptibility in plant—pathogen interactions. *Front. Plant. Sci.*, 2014, 5: 228 (doi: 10.3389/fpls.2014.00228).
7. Brewin N.J. Plant cell wall remodelling in the Rhizobium—legume symbiosis. *Crit. Rev. Plant Sci.*, 2004, 23(4): 293-316 (doi: 10.1080/07352680490480734).
8. Rich M.K., Schorderet M., Reinhardt D. The role of the cell wall compartment in mutualistic symbioses of plants. *Front. Plant. Sci.*, 2014, 5: 238 (doi: 10.3389/fpls.2014.00238).
9. Parniske M. Intracellular accommodation of microbes by plants: a common developmental program for symbiosis and disease? *Curr. Opin. Plant Biol.*, 2000, 3(4): 320-328 (doi: 10.1016/S1369-5266(00)00088-1).
10. Rae A.L., Bonfante-Fasolo P., Brewin N.J. Structure and growth of infection threads in the legume symbiosis with *Rhizobium leguminosarum*. *Plant J.*, 1992, 2(3): 385-395 (doi: 10.1111/j.1365-313X.1992.00385.x).
11. Ivanova K.A., Tsyganova A.V., Brewin N.J., Tikhonovich I.A., Tsyganov V.E. Induction of host defences by *Rhizobium* during ineffective nodulation of pea (*Pisum sativum* L.) carrying symbiotically defective mutations *sym40* (*PsEFD*), *sym33* (*PsIPD3/PsCYCLOPS*) and *sym42*. *Protoplasma*, 2015, 252(6): 1505-1517 (doi: 10.1007/s00709-015-0780-y).
12. Tsyganova A.V., Tsyganov V.E., Borisov A.Yu., Tikhonovich I.A., Brevin N.D. *Ekologicheskaya genetika*, 2009, 7(3): 3-9 (in Russ.).
13. Bradley D.J., Wood E.A., Larkins A.P., Galfre G., Butcher G.W., Brewin N.J. Isolation of monoclonal antibodies reacting with peribacteriod membranes and other components of pea root nodules containing *Rhizobium leguminosarum*. *Planta*, 1988, 173(2): 149-160 (doi: 10.1007/bf00403006).
14. VandenBosch K.A., Bradley D.J., Knox J.P., Perotto S., Butcher G.W., Brewin N.J. Common

components of the infection thread matrix and the intercellular space identified by immunocytochemical analysis of pea nodules and uninfected roots. *EMBO J.*, 1989, 8(2): 335-341 (doi: 10.1002/j.1460-2075.1989.tb03382.x).
15. Vincken J.-P., Schols H.A., Oomen R.J., McCann M.C., Ulvskov P., Voragen A.G., Visser R.G. If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiol.*, 2003, 132(4): 1781-1789 (doi: 10.1104/pp.103.022350).
16. Anderson C.T. We be jammin': an update on pectin biosynthesis, trafficking and dynamics. *J. Exp. Bot.*, 2015, 67(2): 495-502 (doi: 10.1093/jxb/erv501).
17. Atmodjo M.A., Hao Z., Mohnen D. Evolving views of pectin biosynthesis. *Annu. Rev. Plant Biol.*, 2013, 64(1): 747-779 (doi: 10.1146/annurev-arplant-042811-105534).
18. Caffall K.H., Mohnen D. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.*, 2009, 344(14): 1879-1900 (doi: 10.1016/j.carres.2009.05.021).
19. Saffer A.M. Expanding roles for pectins in plant development. *J. Integr. Plant. Biol.*, 2018, 60(10): 910-923 (doi: 10.1111/jipb.12662).
20. Larskaya I.A., Gorshkova T.A. Plant oligosaccharides — outsiders among elicitors? *Biochemistry (Moscow)*, 2015, 80(7): 881-900 (doi: 10.1134/s0006297915070081).
21. Willats W.G., Steele-King C.G., Marcus S.E., Knox J.P. Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. *Plant J.*, 1999, 20(6): 619-628 (doi: 10.1046/j.1365-313X.1999.00629.x).
22. Peaucelle A., Louvet R., Johansen J.N., Höfte H., Laufs P., Pelloux J., Mouille G. *Arabidopsis* phyllotaxis is controlled by the methyl-esterification status of cell-wall pectins. *Curr. Biol.*, 2008, 18(24): 1943-1948 (doi: 10.1016/j.cub.2008.10.065).
23. McCartney L., Ormerod A.P., Gidley M.J., Knox J.P. Temporal and spatial regulation of pectic (1→4)-β-D-galactan in cell walls of developing pea cotyledons: implications for mechanical properties. *Plant J.*, 2000, 22(2): 105-113 (doi: 10.1046/j.1365-313x.2000.00719.x).
24. Mohnen D. Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.*, 2008, 11(3): 266-277 (doi: 10.1016/j.pbi.2008.03.006).
25. Wolf S., Mouille G., Pelloux J. Homogalacturonan methyl-esterification and plant development. *Mol. Plant*, 2009, 2(5): 851-860 (doi: 10.1093/mp/ssp066).
26. Lievens S., Goormachtig S., Herman S., Holsters M. Patterns of pectin methylesterase transcripts in developing stem nodules of *Sesbania rostrata*. *Mol. Plant-Microbe Interact.*, 2002, 15(2): 164-168 (doi: 10.1094/MPMI.2002.15.2.164).
27. Prade R.A., Zhan D., Ayoubi P., Mort A.J. Pectins, pectinases and plant-microbe interactions. *Biotechnol. Genet. Eng. Rev.*, 1999, 16(1): 361-392 (doi: 10.1080/02648725.1999.10647984).
28. Levesque-Tremblay G., Pelloux J., Braybrook S.A., Müller K. Tuning of pectin methylesterification: consequences for cell wall biomechanics and development. *Planta*, 2015, 242(4): 791-811 (doi: 10.1007/s00425-015-2358-5).
29. Wolf S., Greiner S. Growth control by cell wall pectins. *Protoplasma*, 2012, 249(2): 169-175 (doi: 10.1007/s00709-011-0371-5).
30. Pelloux J., Rustérucci C., Mellerowicz E.J. New insights into pectin methylesterase structure and function. *Trends Plant Sci.*, 2007, 12(6): 267-277 (doi: 10.1016/j.tplants.2007.04.001).
31. Lionetti V., Fabri E., De Caroli M., Hansen A.R., Willats W.G.T., Piro G., Bellincampi D. Three pectin methylesterase inhibitors protect cell wall integrity for *Arabidopsis* immunity to *Botrytis*. *Plant Physiol.*, 2017, 173(3): 1844-1863 (doi: 10.1104/pp.16.01185).
32. Peaucelle A., Braybrook S.A., Le Guillou L., Bron E., Kuhlemeier C., Höfte H. Pectin-induced changes in cell wall mechanics underlie organ initiation in Arabidopsis. *Curr. Biol.*, 2011, 21(20): 1720-1726 (doi: 10.1016/j.cub.2011.08.057).
33. Pogorelko G., Lionetti V., Bellincampi D., Zabotina O. Cell wall integrity: Targeted post-synthetic modifications to reveal its role in plant growth and defense against pathogens. *Plant Signal Behav.*, 2013, 8(9): e25435 (doi: 10.4161/psb.25435).
34. Rodríguez-Llorente I.D., Pérez-Hormaeche J., Mounadi K.E., Dary M., Caviedes M.A., Cosson V., Kondorosi A., Ratet P., Palomares A.J. From pollen tubes to infection threads: recruitment of *Medicago* floral pectic genes for symbiosis. *Plant J.*, 2004, 39(4): 587-598 (doi: 10.1111/j.1365-313X.2004.02155.x).
35. Fauvart M., Verstraeten N., Dombrecht B., Venmans R., Beullens S., Heusdens C., Michiels J. *Rhizobium etli* HrpW is a pectin-degrading enzyme and differs from phytopathogenic homologues in enzymically crucial tryptophan and glycine residues. *Microbiology*, 2009, 155(9): 3045-3054 (doi: 10.1099/mic.0.027599-0).
36. Mucoz J.A., Coronado C., Pérez-Hormaeche J., Kondorosi A., Ratet P., Palomares A.J. *MsPG3*, a *Medicago sativa* polygalacturonase gene expressed during the alfalfa—*Rhizobium meliloti* interaction. *PNAS USA*, 1998, 95(16): 9687-9692 (doi: 10.1073/pnas.95.16.9687).
37. Xie F., Murray J.D., Kim J., Heckmann A.B., Edwards A., Oldroyd G.E.D., Downie J.A. Legume pectate lyase required for root infection by rhizobia. *PNAS USA*, 2012, 109(2): 633-638 (doi: 10.1073/pnas.1113992109).
38. Knox J.P., Linstead P.J., King J., Cooper C., Roberts K. Pectin esterification is spatially

regulated both within cell walls and between developing tissues of root apices. *Planta*, 1990, 181(4): 512-521 (doi: 10.1007/bf00193004).
39. Verhertbruggen Y., Marcus S.E., Haeger A., Ordaz-Ortiz J.J., Knox J.P. An extended set of monoclonal antibodies to pectic homogalacturonan. *Carbohydr. Res.*, 2009, 344(14): 1858-1862 (doi: 10.1016/j.carres.2008.11.010).
40. Guillemin F., Guillon F., Bonnin E., Devaux M.-F., Chevalier T., Knox J.P., Liners F., Thibault J.-F. Distribution of pectic epitopes in cell walls of the sugar beet root. *Planta*, 2005, 222(2): 355-371 (doi: 10.1007/s00425-005-1535-3).
41. Tsyganova A.V., Seliverstova E.V., Ivanova K.A., Brewin N.J., Tsyganov V.E. Comparative analysis of remodelling of the plant—microbe interface in *Pisum sativum* and *Medicago truncatula* symbiotic nodules. *Protoplasma*, 2019, 256(4): 983-996 (doi: 10.1007/s00709-019-01355-5).
42. Sherrier D.J., Taylor G.S., Silverstein K.A.T., Gonzales M.B., VandenBosch K.A. Accumulation of extracellular proteins bearing unique proline-rich motifs in intercellular spaces of the legume nodule parenchyma. *Protoplasma*, 2005, 225(1): 43-55 (doi: 10.1007/s00709-005-0090-x).
43. Ivanov S., Fedorova E.E., Limpens E., De Mita S., Genre A., Bonfante P., Bisseling T. *Rhizobium*—legume symbiosis shares an exocytotic pathway required for arbuscule formation. *PNAS USA*, 2012, 109(21): 8316-8321 (doi: 10.1073/pnas.1200407109).
44. Gavrin A., Chiasson D., Ovchinnikova E., Kaiser B.N., Bisseling T., Fedorova E.E. VAMP721a and VAMP721d are important for pectin dynamics and release of bacteria in soybean nodules. *New Phytol.*, 2016, 210(3): 1011-1021 (doi: 10.1111/nph.13837).
45. Redondo-Nieto M., Pulido L., Reguera M., Bonilla I., Bolaños L. Developmentally regulated membrane glycoproteins sharing antigenicity with rhamnogalacturonan II are not detected in nodulated boron deficient *Pisum sativum*. *Plant Cell Environ.*, 2007, 30(11): 1436-1443 (doi: 10.1111/j.1365-3040.2007.01721.x).
46. Redondo-Nieto M., Wilmot A.R., El-Hamdaoui A., Bonilla I., Bolaños L. Relationship between boron and calcium in the N_2-fixing legume—rhizobia symbiosis. *Plant Cell Environ.*, 2003, 26(11): 1905-1915 (doi: 10.1046/j.1365-3040.2003.01107.x).
47. Sujkowska-Rybkowska M., Borucki W. Pectins esterification in the apoplast of aluminum-treated pea root nodules. *J. Plant Physiol.*, 2015, 184: 1-7 (doi: 10.1016/j.jplph.2015.05.011).
48. Carpena R.O., Esteban E., Sarro M.J., Pecalosa J., Gárate A.N., Lucena J.J., Zornoza P. Boron and calcium distribution in nitrogen-fixing pea plants. *Plant Sci.*, 2000, 151(2): 163-170 (doi: 10.1016/S0168-9452(99)00210-1).
49. O'Neill M.A., Ishii T., Albersheim P., Darvill A.G. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol.*, 2004, 55(1): 109-139 (doi: 10.1146/annurev.arplant.55.031903.141750).
50. Redondo-Nieto M., Maunoury N., Mergaert P., Kondorosi E., Bonilla I., Bolaños L. Boron and calcium induce major changes in gene expression during legume nodule organogenesis. Does boron have a role in signalling? *New Phytol.*, 2012, 195(1): 14-19 (doi: 10.1111/j.1469-8137.2012.04176.x).
51. Bolaños L., Brewin N.J., Bonilla I. Effects of Boron on *Rhizobium*-legume cell-surface interactions and nodule development. *Plant Physiol.*, 1996, 110(4): 1249-1256 (doi: 10.1104/pp.110.4.1249).
52. Bolaños L., Cebrián A., Redondo-Nieto M., Rivilla R., Bonilla I. Lectin-like glycoprotein PsNLEC-1 is not correctly glycosylated and targeted in boron-deficient pea nodules. *Mol. Plant-Microbe Interact.*, 2001, 14(5): 663-670 (doi: 10.1094/MPMI.2001.14.5.663).
53. Bolaños L., Esteban E., de Lorenzo C., Fernandez-Pascual M., de Felipe M.R., Garate A., Bonilla I. Essentiality of boron for symbiotic dinitrogen fixation in pea (*Pisum sativum*) rhizobium nodules. *Plant Physiol.*, 1994, 104(1): 85-90 (doi: 10.1104/pp.104.1.85).
54. Bonilla I., Mergold-Villasenor C., Campos M.E., Sanchez N., Perez H., Lopez L., Castrejon L., Sanchez F., Cassab G.I. The aberrant cell walls of boron-deficient bean root nodules have no covalently bound hydroxyproline/proline-rich proteins. *Plant Physiol.*, 1997, 115(4): 1329-1340 (doi: 10.1104/pp.115.4.1329).
55. Matoh T., Takasaki M., Takabe K., Kobayashi M. Immunocytochemistry of rhamnogalacturonan II in cell walls of higher plants. *Plant Cell Physiol.*, 1998, 39(5): 483-491 (doi: 10.1093/oxfordjournals.pcp.a029395).
56. Reguera M., Abreu I., Brewin N.J., Bonilla I., Bolaños L. Borate promotes the formation of a complex between legume AGP-extensin and rhamnogalacturonan II and enhances production of *Rhizobium* capsular polysaccharide during infection thread development in *Pisum sativum* symbiotic root nodules. *Plant Cell Environ.*, 2010, 33(12): 2112-2120 (doi: 10.1111/j.1365-3040.2010.02209.x).
57. Gorshkova T.A., Kozlova L.V., Mikshina P.V. *Biokhimiya*, 2013, 78(7): 1068-1088 (in Russ.).
58. Mikshina P.V., Petrova A.A., Faizullin D.A., Zuev Yu.F., Gorshkova T.A. *Biokhimiya*, 2015, 80(7): 1088-1098 (in Russ.).
59. Lee K.J.D., Cornuault V., Manfield I.W., Ralet M.-C., Knox P.J. Multi-scale spatial heterogeneity of pectic rhamnogalacturonan I (RG-I) structural features in tobacco seed endosperm cell walls. *Plant J.*, 2013, 75(6): 1018-1027 (doi: 10.1111/tpj.12263).

60. Jones L., Seymour G.B., Knox J.P. Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1→4)-β-D-galactan. *Plant Physiol.*, 1997, 113(4): 1405-1412 (doi: 10.1104/pp.113.4.1405).
61. Moore P.J., Staehelin L.A. Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta*, 1988, 174(4): 433-445 (doi: 10.1007/bf00634471).
62. Corral-Martínez P., García-Fortea E., Bernard S., Driouich A., Seguí-Simarro J.M. Ultrastructural immunolocalization of arabinogalactan protein, pectin and hemicellulose epitopes through anther development in *Brassica napus*. *Plant Cell Physiol.*, 2016, 57(10): 2161-2174 (doi: 10.1093/pcp/pcw133).
63. Liu J., Hou J., Chen H., Pei K., Li Y., He X.-Q. Dynamic changes of pectin epitopes in cell walls during the development of the procambium–cambium continuum in poplar. *Int. J. Mol. Sci.*, 2017, 18(8): 1716 (doi: 10.3390/ijms18081716).
64. Torode T.A., O'Neill R., Marcus S.E., Cornuault V., Pose S., Lauder R.P., Kračun S.K., Rydahl M.G., Andersen M.C., Willats W.G. Branched pectic galactan in phloem-sieve-element cell walls: implications for cell mechanics. *Plant Physiol.*, 2018, 176(2): 1547-1558 (doi: 10.1104/pp.17.01568).
65. Lehner A., Dardelle F., Soret-Morvan O., Lerouge P., Driouich A., Mollet J.-C. Pectins in the cell wall of *Arabidopsis thaliana* pollen tube and pistil. *Plant Signal Behav.*, 2010, 5(10): 1282-1285 (doi: 10.4161/psb.5.10.13040).
66. Tsyganova A.V., Seliverstova E.V., Brewin N.J., Tsyganov V.E. Bacterial release is accompanied by ectopic accumulation of cell wall material around the vacuole in nodules of *Pisum sativum sym33-3* allele encoding transcription factor PsCYCLOPS/PsIPD3. *Protoplasma*, 2019 (doi: 10.1007/s00709-019-01383-1) [Epub ahead of print].
67. Gorshkov V.Y., Daminova A.G., Mikshina P.V., Petrova O.E., Ageeva M.V., Salnikov V.V., Gorshkova T.A., Gogolev Y.V. Pathogen-induced conditioning of the primary xylem vessels — a prerequisite for the formation of bacterial emboli by *Pectobacterium atrosepticum*. *Plant Biol.*, 2016, 18(4): 609-617 (doi: 10.1111/plb.12448).

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PHYSIOLOGICAL MECHANISMS AND GENETIC FACTORS OF THE TEA PLANT *Camellia sinensis* (L.) Kuntze RESPONSE TO DROUGHT (review)

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Abstract

The main constraint in the tea plants growth in the world is drought, which reduces the productivity of plantations by 15-45 % (R.M. Bhagat et al., 2010; R.D. Baruah et al., 2012). In this regard, physiological (M. Mukhopadhyay et al., 2014; T.K. Maritim et al., 2015) and molecular mechanisms (W.D. Wang et al, 2016; Y. Guo et al., 2017) drought tolerance of tea plants are a matter of great interest. The purpose of this review is to summarize the international experience of phenotyping and genotyping of tea drought response to create a comprehensive picture of the plant response to osmotic stress and to understand the reproducibility of response mechanisms in different climatic regions. During drought stress the main signaling role is played by abscisic, jasmonic and salicylic acids, as well as ethylene (S.C. Liu et al., 2016), the metabolic pathway of which includes cascades of physiological changes and involves response genes (T. Umezawa et al., 2010). It was reported that tea plants had increased expression of genes encoding cytokinin biosynthesis enzymes (trans-zeatin and cis-zeatin and isopentyniladine) under drought, and during recovery its expression decreased. It is assumed that an increase in cytokinin content may partially mitigate the negative effect of stress on photosynthetic apparatus and slow down leaf senescence induced by stress. An important adaptive response of tea plant to drought is an increase in the concentration of proline, glycine-betaine, mannitol and other osmolytes which neutralize reactive oxygen species, protect macromolecules from damage by free radicals, and maintain the osmotic potential of the cell (W.D. Wang et al., 2016). Under the drought in tea plant starch decomposes to glucose, and mannitol, trehalose, and sucrose contents increase. The accumulation of reactive oxygen species (ROS) directly correlates with the accumulation of glucose, to prevent the negative effects of stress. In addition, it has been shown that many genes involved in the metabolism and signaling of phytohormones, osmolytes, antioxidants and carbohydrates are also involved in tolerance to osmotic stress (S. Gupta et al., 2013; Y. Guo et al., 2017). Several families of transcription factors play a crucial role in the regulation of tea response to drought in tea. In particular, 39 *CsbHLH* genes were identified with increased expression in drought conditions (X. Cui et al., 2018). From the NAC family, the *CsNAC17* and *CsNAC30* genes have been identified that can be used in the breeding for drought tolerance of tea (Y.-X. Wang et al., 2016). From the WRKY family, the *CsWRKY2* gene has been identified which is involved in the mechanisms of protection from drought and can act as an activator or repressor of abscisic acid (ABA). From the DREB gene family, 29 *CsDREB* have been identified, which increase drought tolerance of tea through ABA-dependent and ABA-independent pathways and can act as a link between different biochemical networks in response to drought (M. Wang et al., 2017). From the HD-Zip family, *Cshdz* genes have been identified which are divided into 4 groups according to their functions, of which HD-Zip I and HD-Zip IV play the major role in drought response in tea (W. Shen et al., 2018). Of the HSP (HSF) family, 47 transcription factors were identified in tea, including 7 *CsHSP90*, 18 *CsHSP70*, and 22 *CsHSP* genes the expression of which increases resistance to oxidative stress, protection of photosystem II and stabilizes photosynthesis during drought (J. Chen et al., 2018). The transcription factors of the bZIP family also play

the important role in ABA-mediated drought response. From the Dof family, 29 transcription factors were revealed in tea plants and their increased expression was shown in the resistant cultivars under drought. The important role of CsDof-22 in ABA biosynthesis has been revealed (H. Li et al., 2016). An increased expression of the SBP family *CsSBP* genes in tea plants led to assumption of its participation in signaling pathways involving ABA, gibberellic acid, and methyl jasmonate (P. Wang et al., 2018). The genes of the CsLOX1, CsLOX6 and CsLOX7 family of lipoxygenases in tea can also play an important role in drought response (J. Zhu et al., 2018). In addition, miRNA play an important role in gene regulation at transcription and translation level in tea plants (Y. Guo et al., 2017). Despite the great progress in the functional genomics of tea plant further research is needed to identify the location of various genes in regulatory networks and their impact in drought tolerance.

Keywords: tea plant, *Camellia sinensis*, drought, phytohormones, osmolytes, antioxidant system, transcription factors

Tea *Camellia sinensis* (L.) Kuntze is the most important industrial crop cultivated in more than 50 countries of the world, including in the humid subtropical zone of Russia. This perennial plant, growing in one place for up to 70 years or more, periodically encounters prolonged drought (1-2 months) in the summer period, which leads to oxidative stress and the formation of mineral deficiency. According to various authors, these abiotic factors become one of the main reasons for the decrease in tea plantation yields (on average by 15-45%) [1-3] and even partial death of plants (up to 19%) [4]. The predicted global climate changes towards aridization [5] will further aggravate the existing problems of tea cultivation and actualize the tasks to increase its endurance to extreme environmental conditions [6-9]. In this regard, the physiological, biochemical, and molecular mechanisms of tea plant resistance to drought and the effectiveness of using various exogenous inducers are studied, and the selection search for more drought-tolerant varieties is performed [10]. Over the years of research in foreign countries, significant experimental material has been accumulated concerning the physiological, biochemical, and molecular mechanisms of the resistance of tea and other crops to drought [11-14], which can be used as a scientific basis for the development of selection research in Russia.

The purpose of this report is to summarize the international experience of phenotyping and genotyping of tea plants on the basis of resistance to stress factors in order to create a holistic picture of the plant's response to osmotic stress and to understand the reproducibility of response mechanisms in different climatic zones.

The most effective strategies for plant adaptation to drought, including tea, include the so-called mechanisms of stress avoidance by shortening the life cycle, growing season and flowering, which are aimed at reducing water loss by the organism [15, 16]. The most important morphological symptoms of plant adaptation are the characteristics of the root system (biomass, length, depth, and density of roots) [17-19], the ratio of root/shoot biomass, the number and size of leaves, the area and nature of the leaf surface, the ratio of leaf mass to surface, the structure of the photosynthetic apparatus, the structure and form of chloroplasts [20-24]. The main physiological mechanisms of avoidance are the reduction of water loss through stomatal control of transpiration [25-27], a decrease in the number and size of stomata [28], an increase in the viscosity of the cytoplasm, and maintenance of the osmotic potential of the cell. All these processes are based on cascades of biochemical reactions involving three groups of metabolites — phytohormones, osmolytes, and antioxidant components.

Phytohormones. According to modern research, among phytohormones, abscisic (ABA), salicylic and jasmonic acids, as well as ethylene, play an important role in responding to drought. A comparative study of two tea varieties, which are contrasting in terms of resistance, revealed an increase in the content of abscisic and salicylic acids 4 days after the onset of drought [25].

Other phytohormones, such as indoleacetic acid (IAA) and cytokinins, were involved in plant recovery after drought. In the case of the tea plant, the ABA-dependent response path to drought has been studied quite fully, although some links have not yet been identified. ABA-dependent signaling primarily affects constitutively expressed transcription factors, which then induce direct effector stress resistance genes [29]. A key regulatory link in ABA biosynthesis in both roots and leaves is probably catalyzed by 9-cis-epoxycarotenoid deoxygenase, which is an enzyme that converts the epoxycarotenoid precursor into xanthoxin in plastids. ABA initiates the formation of reactive oxygen species, which in a chain activate superoxide dismutase (SOD), which is the first line of defense against free radicals [12, 25]. The increasing activity of SOD leads to an increase in the amount of H_2O_2, activation of Ca^2 channels, and calcium-dependent protein kinases that regulate stomata closure [26, 30-32]. When recovering from drought, the amount of ABA decreases, as a result of which the calcium concentration in the cytosol supposedly reduces, the influx of potassium and anions into the stomata trailing cells decreases and, as a result, stomata open [26, 33-35].

Under the influence of drought, the expression of the jasmonic acid synthesis gene increases, which turns into a more active compound (–)-jasmonoyl-L-isoleucine (JA-Ile) [26]. Genes encoding the main components of ethylene signaling are also activated. After the onset of stress, the expression of the 1-aminocyclopropane-1-carboxylic acid (ACC) gene is increased, which indicates the accumulation of ethylene, and it is suppressed during the recovery period after drought [26].

Salicylic acid is a phenolic compound involved in the regulation of photosynthesis, nitrogen metabolism, proline, glycine-beta-in synthesis, an antioxidant defense system, and water potential under stress conditions [36]. The main regulator of salicylic acid metabolism is the *NPR1* gene, a decrease in the expression of which under conditions of drought led to a weakening of tea resistance to disease [26].

Indoleacetic acid (IAA) regulates many physiological processes. In a tea plant, the expression of genes associated with the synthesis of IAA decreased in response to drought [26]. However, to maintain the activity of physiological processes under these conditions, alternative routes for the synthesis of IAA from indole via tryptophan were activated.

It was reported that during a drought period, the expression of genes encoding cytokinin biosynthesis enzymes (trans- and cis-zeatin and isopentinyladenine) increased in a tea plant, and during the recovery after stress, it decreased. Apparently, an increase in the content of cytokinins can partially mitigate the negative effect of stress on photosynthetic activity and slow down accelerated leaf aging [36].

Osmolytes. Another strategy for adapting to drought is to increase the synthesis of osmolytes – substances with a low molecular weight dissolved in the cytosol, which are not toxic to the plant even in high concentrations [37-39]. Osmolytes increase the viscosity of the cytoplasm, absorb reactive oxygen species and protect macromolecules from free radicals, thereby maintaining the integrity of the membranes and the metabolic activity of tissues, which ensures the resumption of growth after improving the water regime [40-42]. These substances belong to three main classes: amino acids (glutamine, proline, glycine-betaine, carnitine), sugars (starch, di- and monosaccharides) and polyols (mannitol, sorbitol) [43]. Proline [44], which is synthesized from glutamate (with the participation of the pyrroline-5-carboxylate synthase enzyme) or ornithine [26, 43] is especially important among amino acids. The accumulation of proline is accompanied by the prevention of protein denaturation, the preservation of the struc-

ture and activity of enzymes, as well as the protection of membranes from ROS damage during moisture deficiency and high solar activity. The most studied components of osmotic regulation include glycine-betaine [45], which has a positive effect on enzyme stability and membrane integrity, acting as an osmoprotector and also indirectly participating in transduction signals [46]. In the paper by Maritim et al. [19], accumulation of proline and glycine-betaine under simulated water deficiency stress in the leaves of 8 varieties of tea was shown, which is more pronounced in drought-tolerant varieties. Based on this, it was concluded that proline can be used as a biochemical marker for screening genetic material for drought tolerance.

A significant contribution of carbohydrates to cell resistance under osmotic stress has also been shown [25, 26, 47]. So, under drought conditions in tea plants, starch was decomposed to glucose, and the synthesis of mannitol, trehalose, and sucrose increased. The accumulation of reactive oxygen species (ROS) was directly correlated with the accumulation of glucose, which is involved in the closure of stomata and increases the adaptability of the plant, prevents the decomposing of chlorophyll and transpiration of water under osmotic stress. In addition, soluble sugars play a dual function, as they are associated with both anabolism and catabolism of ROS, such as the oxidative pentose phosphate pathway and NADPH production [48-51]. Physiological and molecular genetic studies conducted by Liu et al. [25, 26] showed that the concentration of soluble sugars in the tea plant increased significantly as the drought intensified, and then rapidly decreased after rehydration. These results suggested that photoassimilated carbon was mainly used for the synthesis of osmolytes, and starch was mainly decomposed to glucose.

Antioxidant components. Antioxidant defense systems include both enzyme (SOD, catalase, peroxidase, ascorbate peroxidase, glutathione reductase) and non-enzymatic (cysteine, reduced glutathione, and ascorbic acid) components [27, 37, 52]. It has been established that in addition to catalase, various peroxidases and peroxiredoxins [25, 53, 46], several more enzymes are involved in the process of liquidation of hydrogen peroxide and free radicals: dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase [31, 54]. Transcriptomic studies by Liu et al. [26] have shown that under drought conditions in a tea plant, the expression of the gene of ribulose bisphosphate carboxylase (RuBisCO). the key enzyme of the Calvin cycle, decreased, indicating inhibition of carbon dioxide fixation. Moreover, the synthesis of regulatory enzymes of glycolysis of hexokinase under drought conditions did not stop. Researchers have suggested that the tea plant maintains ATP supply by maintaining glycolytic metabolism [26]. The role of β-carotene in protecting against oxidative stress and maintaining photochemical processes was identified [55, 56], which presumably involves the direct quenching of triplet chlorophyll, which prevents the formation of reactive oxygen species [37].

Other signaling paths. The group of molecular messengers that regulate the work of response genes is very numerous. Thus, aquaporins, the family of the main membrane (plasma and vacuolar) proteins, regulate the passive exchange of water through membranes [57]. Dehydrins and heat shock proteins (chaperones) stabilize the structure of other proteins and macromolecules, which prevents their denaturation under stress [58].

Protein kinases and protein phosphatases often act together to phosphorylate and dephosphorylate their targets. Protein kinases in a tea plant play a positive role, and protein phosphatases play a negative regulatory role in the response to drought, ensuring the maintenance of homeostasis and signal transduction in the tea plant. According to Wan et al. [59-62], all 29 calcium-dependent

protein kinases found in tea contain cis-elements of the multiple stress response in the promoter region of the gene.

Genetic factors. Among all the mechanisms, transcription factors are the main regulators of the plant's response to abiotic stress. Tea is supposed to have 12 families of transcription factors involved in response to drought: AP2/EREBP, bHLH, bZIP, HD-ZIP, HSF (HSP), MYB, NAC, WRKY, zinc-finger protein TFs, SCL, ARR, SPL [63-66].

One of the largest families of bHLH transcription factors (basic helix-loop-helix) is widespread in eukaryotes. These factors determine signal transmission and secondary metabolism of brassinosteroids, jasmonic acid, anthocyanin synthesis, modulation of plant growth and development, control of embryo development, branching of shoots, flower and fruit development. In addition, bHLHs are involved in ABA signaling and plant response to abiotic stresses. 39 *CsbHLH* genes were identified in tea, the expression of which is enhanced under drought conditions [67].

The NAC family of transcription factors (NAM-ATAF1/2-CUC) regulates the formation of the apical meristem, lateral roots, secondary cell wall, leaf aging, seed development, flavonoid biosynthesis. Many genes of the NAC family are involved in stress response and hormonal signaling. An analysis of these genes expression in tea plant during drought revealed the candidate genes *CsNAC17* and *CsNAC30*, which can be used in breeding tea for drought tolerance [68].

The WRKY transcription factor group is involved in responses to abiotic stress. The *CsWRKY2* gene was identified in tea, which is involved in protection against drought. Its greatest expression was observed in leaves, the smallest – in flowers and shoots. A high degree of expression of *CsWRKY2* was noted under the conditions of drought and cold. It has been shown that WRKY proteins can act as ABA activators or repressors [69].

One of the most extensive families of plant transcription factors is DREBs (dehydration-responsive element-binding proteins). Twenty-nine *CsDREB* genes were identified in tea; their localization in the cell nucleus was shown. The expression of these genes was enhanced by various abiotic stresses, including drought. Overexpression of *CsDREB* genes increases resistance to stress through both ABA-dependent and ABA-independent path. An analysis of the expression of *CsDREB* genes showed that they can act as a link between different response chains in the response of tea to stress [70].

The HD-Zip family of proteins (homeodomain-leucine zipper) is an important group of transcription factors, which is divided into four subgroups (HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV). Thirty-three transcription factors belonging to these subgroups were found in tea. Among them, HD-Zip I and HD-Zip IV are most involved in response to drought. HD-Zip I proteins mainly respond to external signals, such as extreme temperatures, drought, and other abiotic stresses, while regulating the processes of growth and adaptation of plants to environmental factors. The HD-Zip IV subgroup is involved in root formation, cell differentiation, trichome formation, and anthocyanin accumulation. The results of the analysis of tea *Cshdz* genes expression confirmed their participation in various stress responses, including drought [71].

Heat shock proteins (HSPs) play an important role in the growth and development of plants and protect cell structures under stress. Forty-seven *CsHSP* genes, including 7 *CsHSP90*, 18 *CsHSP70*, and 22 *CssHSP* genes, were identified in tea, the expression of which increases resistance to oxidative stress, protecting the photosystem II and supporting the photosynthetic apparatus [72].

The transcription factors of the bZIP family (basic region/leucine zipper)

are divided into 13 groups (AL and S). The most studied group A includes the so-called ABRE-binding factors (ABFs) responsible for the work of ABA. These and other bZIP family genes are involved in the response and mechanisms of tea plant resistance to drought, act as positive regulators of resistance to oxidative stress, and play a central role in biochemical cascades involving glucose and ABA [73].

The Dof family of transcription factors (DNA-binding with one finger) regulates the expression of genes involved in seed maturation and germination, flowering periods, accumulation of secondary metabolites, and also in protective processes. Twenty-nine transcriptional Dof factors were identified in tea and the participation of *CsDof-22* in ABA biosynthesis was shown. An increase in the expression of *CsDof* genes was observed in resistant tea varieties under stress [74].

SBPs (SQUAMOSA promoter binding protein) encode transcription factors, are involved in sporogenesis, shoot and leaf development, flowering, fertilization, fruit ripening, hormonal signaling, and responses to abiotic and biotic stresses in many plant species. Overexpression of these genes was observed in response to an increase in the content of jasmonic acid and led to an increase in the content of superoxide dismutase and peroxidase. Tea plant shows increased expression of *CsSBP* genes in the buds and leaves; these reactions can be associated with signaling pathways involving ABA, gibberellic acid, and methyl jasmonate [75].

Oxylypins are oxidized derivatives of fatty acids, including jasmonic acid, hydroxy-, oxo- or keto- fatty acids, volatile aldehydes, and are important signaling molecules in higher plants. Lipoxygenases (LOXs) are a family of iron-containing enzymes that catalyze the oxidation of polyunsaturated fatty acids, which initiates the biosynthesis of oxylipins. In a tea plant, the lipoxygenase family genes *CsLOX1*, *CsLOX6,* and *CsLOX7* are involved in response to stresses (cold, drought, biotic stress) via an ABA-independent pathway [76].

MicroRNAs regulate the expression of transcription factors at the translation stage. Sixty-two microRNAs were identified in tea; they are involved in the response to drought through the regulation of transcription and suppression of translation. It was found that microRNAs expression varies depending on the strength of the stress factor and manifests itself in the form of morphological, physiological, and biochemical changes [77].

Thus, many biochemical and genetic signals have been established and described for tea plants; they activate genetic matrices in response to osmotic stress, which leads to physiological and metabolic changes that ensure plant resistance. This process involves many genes involved in the metabolism and signaling of phytohormones, the metabolism of osmolytes, the regulation of antioxidant activity, and the regulation of stomatal apparatus functions. However, the role of all genetic factors in the response to drought still requires further study to fully understand their place in cascades of biochemical reactions. Therefore, despite significant progress in the functional genomics of plants, including tea, further studies are required to identify the place of various genes in regulatory networks and the response to drought.

REFERENCES

1. Bhagat R.M., Baruah R.D., Safigue S. Climate and tea [*Camellia sinensis* (L.) O. Kuntze] production with special reference to north eastern India: a review. *Journal of Environmental Research and Development*, 2010, 4(4): 1017-1028.
2. Baruah R.D., Bhagat R.M. Climate trends of Northeastern India: a longterm pragmatic analysis for tea production. *Two and a Bud*, 2012, 59(2): 46-49.
3. Malyukova L.S. *Plodovodstvo i yagodovodstvo Rossii*, 2014, 38(1): 255-261 (in Russ.).

4. Cheruiyot E.K., Mumera L.M., Ng'etich W.K., Hassanali A., Wachira F.N. High fertilizer rates increase susceptibility of tea to water stress. *Journal of Plant Nutrition*, 2009, 33(1): 115-129 (doi: 10.1080/01904160903392659).
5. Reynolds M.P., Ortiz R. Adapting crops to climate change: a summary. In: *Climate change and crop production*. M.P. Reynolds (ed.). CAB International, 2010: 1-8 (doi: 10.1079/9781845936334.0001).
6. Sinclair T.R. Challenges in breeding for yield increase for drought. *Trends in Plant Science*, 2011, 16(6): 289-293 (doi: 10.1016/j.tplants.2011.02.008).
7. Ashraf M. Inducing drought tolerance in plants: recent advances. *Biotechnology Advances*, 2010, 28(1): 169-183 (doi: 10.1016/j.biotechadv.2009.11.005).
8. Varshney R.K., Bansal K.C., Aggarwal P.K., Datta S.K., Craufurd P.Q. Agricultural biotechnology for crop improvement in a variable climate: hope or hype? *Trends in Plant Science*, 2011; 16(7): 363-371 (doi: 10.1016/j.tplants.2011.03.004).
9. Tsonev S., Todorovska E., Avramova V., Kolev S., Abu-Mhadi N., Christov N.K. Genomics assisted improvement of drought tolerance in maize: QTL approaches. *Biotechnology & Biotechnological Equipment*, 2009, 23(4): 1410-1413 (doi: 10.2478/V10133-009-0004-8).
10. Mukhopadhyay M., Mondal T.K. The physio-chemical responses of Camellia plants to abiotic stresses. *J. Plant Sci. Res.*, 2014, 1(1): 1-12.
11. Bernier J., Atlin G.N., Serraj R., Kumar A., Spaner D. Breeding upland rice for drought resistance. *Journal of the Science of Food and Agriculture*, 2008, 88(6): 927-939 (doi: 10.1002/jsfa.3153).
12. Fleury D., Jefferies S., Kuchel H., Langridge P. Genetic and genomic tools to improve drought tolerance in wheat. *Journal of Experimental Botany*, 2010, 61(12): 3211-3222 (doi: 10.1093/jxb/erq152).
13. Manavalan L.P., Guttikonda S.K., Tran L.S., Nguyen H.T. Physiological and molecular approaches to improve drought resistance in soybean. *Plant and Cell Physiology*, 2009, 50(7): 1260-1276 (doi: 10.1093/pcp/pcp082).
14. Yadav R.S., Sehgal D., Vadez V. Using genetic mapping and genomics approaches in understanding and improving drought tolerance in pearl millet. *Journal of Experimental Botany*, 2011, 62(2): 397-408 (doi: 10.1093/jxb/erq265).
15. Araus J.L., Slafer G.A., Reynolds M.P., Royo C. Plant breeding and water relations in C_3 cereals: what should we breed for? *Annals of Botany*, 2002, 89 (7): 925-940 (doi: 10.1093/aob/mcf049).
16. Pchikhachev E.K., Korzun B.V. *Subtropicheskie kul'tury*, 2010, 1(4): 219-220 (in Russ.).
17. Tuov M.T. *Nauchnye osnovy povysheniya kachestva i produktivnosti chainykh plantatsii Rossii. Doktorskaya dissertatsiya* [Scientific basis for improving quality and productivity of tea plantations in Russia. DSc Thesis]. Sochi, 1997 (in Russ.).
18. Das A., Das S., Mondal T.K. Identification of differentially expressed gene profiles in young roots of tea (*Camellia sinensis* (L.) O. Kuntze) subjected to drought stress using suppression subtractive hybridization. *Plant Mol. Biol. Rep.*, 2012, 30(5): 1088-1101 (doi: 10.1007/s11105-012-0422-x).
19. Maritim T.K., Kamunya S.M., Mireji P., Mwendia C.M., Muoki R.C., Cheruiyot E.K., Wachira F.N. Physiological and biochemical response of tea (*Camellia sinensis* (L.) O. Kuntze) to water-deficit stress. *The Journal of Horticultural Science and Biotechnology*, 2015, 90(4): 395-400 (doi: 10.1080/14620316.2015.11513200).
20. Jaleel C.A., Manivannan P., Wahid A., Farooq M., Somasundaram R., Panneerselvam R. Drought stress in plants: a review on morphological characteristics and pigments composition. *Int. J. Agric. Biol.*, 2009, 11(1): 100-105.
21. Waheed A., Hamid F.S., Shan A.H., Ahmad H., Khalid A., Abbasi F.M., Ahmad N., Aslam S., Sarwar S. Response of different tea (*Camellia sinensis* L.) clones against drought stress. *J. Mater. Environ. Sci.*, 2012, 3(2): 395-410.
22. Gvasaliya M.V. *Spontannye i indutsirovannye sorta i formy chaya (Samellia sinensis (L.) Kuntze) vo vlazhnykh subtropikakh Rossii i Abkhazii, perspektivy ikh razmnozheniya i sokhraneniya v kul'ture in vitro. Kandidatskaya dissertatsiya* [Spontaneous and induced varieties and forms of tea (*Camellia sinensis* (L.) Kuntze) in the humid subtropics of Russia and Abkhazia, the prospects for their reproduction and preservation in culture in vitro. PhD Thesis]. Krasnodar, 2015 (in Russ.).
23. Nyabundi K.W., Owuor P.O., Netondo G.W., Bore J.K. Genotype and environment interactions of yields and yield components of tea (*Camellia sinensis*) cultivars in Kenya. *American Journal of Plant Sciences*, 2016, 7(6): 855-869 (doi: 10.4236/ajps.2016.76081).
24. Vavilova L.V., Korzun B.V. *Novye tekhnologii*, 2016, 4: 114-120 (in Russ.).
25. Liu S.C., Yao M.Z., Ma C.L., Jin J.Q., Ma J.Q., Li C.F. Physiological changes and differential gene expression of tea plant under dehydration and rehydration conditions. *Scientia Horticulturae*, 2015, 184(5): 129-141 (doi: 10.1016/j.scienta.2014.12.036).
26. Liu S.C., Jin J.Q., Ma J.Q., Yao M.Z., Ma C.L., Li C.F., Ding Z.T., Chen L. Transcriptomic analysis of tea plant responding to drought stress and recovery. *PLoS ONE*, 2016, 11(1): e0147306 (doi: 10.1371/journal.pone.0147306).
27. Li X., Liu F. Drought stress memory and drought stress tolerance in plants: biochemical and

molecular basis. In: *Drought stress tolerance in plants.* M. Hossain, S. Wani, S. Bhattacharjee, D. Burritt, L.S. Tran (eds.). Springer, Cham, 2016, Vol. 1: 17-44 (doi: 10.1007/978-3-319-28899-4).
28. Daszkowska-Golec A.D., Szarejko I. Open or close the gate-stomata action under the control of phytohormones in drought stress conditions. *Front. Plant Sci.*, 2013, 4: 138 (doi: 10.3389/fpls.2013.00138).
29. Umezawa T., Nakashima K., Miyakawa T., Kuromori T., Tanokura M., Shinozaki K., Yamaguchi-Shinozaki K. Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant Cell Physiol.*, 2010, 51(11): 1821-1839 (doi: 10.1093/pcp/pcq156).
30. Miller G., Schlauch K., Tam R., Cortes D., Torres M.A., Shulaev V., Jeffery L. Dang, Mittler R. The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Science Signaling*, 2009, 2(84): 1-10 (doi: 10.1126/scisignal.2000448).
31. Upadhyaya H., Dutta B.K., Sahoo L., Panda S.K. Comparative effect of Ca, K, Mn and B on post-drought stress recovery in tea [*Camellia sinensis* (L.) O. Kuntze]. *American Journal of Plant Sciences*, 2012, 3(4): 443-460 (doi: 10.4236/ajps.2012.34054).
32. Meyer S., Mumm P., Imes D., Endler A., Weder B., Al-Rasheid K.A.S., Geiger D., Marten I., Martinoia E., Hedrich R. AtALMT12 represents an R-type anion channel required for stomatal movement in Arabidopsis guard cells. *The Plant Journal*, 2010, 63(6): 1054-1062 (doi: 10.1111/j.1365-313X.2010.04302.x).
33. Upadhyaya H., Panda S.K., Dutta B.K. CaCl2 improves post-drought recovery potential in *Camellia sinensis* (L) O. Kuntze. *Plant Cell Rep.*, 2011, 30(4): 495-503 (doi: 10.1007/s00299-010-0958-x).
34. Gao X.Y., Yang G.P., Xu Z.Q. Xu F.C. Effect of calcium on antioxidant enzymes of lipid peroxidation of soy-bean leaves under water stress. *J. South China Agric. Univ.*, 1999, 2: 58-62.
35. Bowler C., Fluhr B. The role of calcium and activated oxygen as signals for controlling cross-tolerance. *Trends Plant Sci.*, 2000, 5(6): 241-243 (doi: 10.1016/S1360-1385(00)01628-9).
36. Miura K., Tada Y. Regulation of water, salinity, and cold stress responses by salicylic acid. *Front. Plant Sci.*, 2014, 5: 4 (doi: 10.3389/fpls.2014.00004).
37. Defez R., Andreozzi A., Dickinson M., Charlton A., Tadini L., Pesaresi P., Bianco C. Improved drought stress response in alfalfa plants nodulated by an IAA over-producing *Rhizobium* strain. *Front Microbiol.*, 2017, 14(8): 2466 (doi: 10.3389/fmicb.2017.02466).
38. Farooq M., Wahid A., Kobayashi N., Fujita D., Basra S.M.A. Plant drought stress: effects, mechanisms and management. *Agron. Sustain. Dev.*, 2009, 29(1): 185-212 (doi: 10.1051/agro:2008021).
39. Damayanthi M.M.N., Mohotti A.J., Nissanka S.P. Comparison of tolerant ability of nature field grown tea (*Camellia sinensis* L.) cultivars exposed to a drought stress in Passara Area. *Tropical Agricultural Research*, 2010, 22(1): 66-75 (doi: 10.4038/tar.v22i1.2671).
40. Mukhopadhyay M., Ghosh P.D., Mondal T.K. Effect of boron deficiency on photosynthesis and antioxidant responses of young tea (*Camellia sinensis* (L.) O. Kuntze) plantlets. *Russ. J. Plant Physiol.*, 2013, 60(5): 633-639 (doi: 10.1134/S1021443713030096).
41. Pritula Z.V., Malyukova L.S. *Problemy agrokhimii i ekologii*, 2017, 3: 31-34 (in Russ.).
42. Tholakalabavi A., Zwiazek, J.J, Thorpe, T.A. Effect of mannitol and glucose-induced osmotic stress on growth, water relations, and solute composition of cell suspension cultures of poplar (*Populus deltoids* var. *Occidentalis*) in relation to anthocyanin accumulation. *In Vitro Cell Dev. Biol. — Plant*, 1994, 30(3): 164-170 (doi: 10.1007/BF02632208).
43. Serraj R., Sinclair T.R. Osmolyte accumulation: can it really help increase crop yield under drought conditions? *Plant, Cell Environ.*, 2002, 25(2): 333-341 (doi: 10.1046/j.1365-3040.2002.00754.x).
44. Guo Y., Zhao S., Zhu C.H., Chang X., Yue C.H., Wang Z.H., Lin Y., Lai Z.H. Identification of drought-responsive miRNAs and physiological characterization of tea plant (*Camellia sinensis* L.) under drought stress. *BMC Plant Biol.*, 2017, 17(1): 211 (doi: 10.1186/s12870-017-1172-6).
45. Szabados L., Savoure A. Proline: a multifunctional amino acid. *Trends Plant Sci.*, 2010, 15(2): 89-97 (doi: 10.1016/j.tplants.2009.11.009).
46. Farooq M., Basra S.M.A., Wahid A., Cheema Z.A., Cheema M.A., Khaliq A. Physiological role of exogenously applied glycinebetaine in improving drought tolerance of fine grain aromatic rice (*Oryza sativa* L.). *J. Agron. Crop Sci.*, 2008, 194(5): 325-333 (doi: 10.1111/j.1439-037X.2008.00323.x).
47. Upadhyaya H., Panda S.K. Abiotic stress responses in tea [*Camellia sinensis* (L.) O. Kuntze]: an overview. *Reviews in Agricultural Science*, 2013, 1: 1-10 (doi: 10.7831/ras.1.1).
48. Ciereszko I. Sucrose metabolism in plant tissues under stress conditions: key enzymes, localization and function In: *Compartmentation of responses to stresses in higher plants, true or false.* W. Maksymiec (ed.). Transworld Research Network, Trivandrum, 2009: 193-218 (ISBN: 978-81-7895-422-6).
49. ElSayed A.I., Rafudeen M.S., Golldack D. Physiological aspects of raffinose family oligosaccharides in plants: protection against abiotic stress. *Plant Biol*ogy, 2014, 16(1): 1-8 (doi: 10.1111/plb.12053).

50. Sami F., Yusuf M., Faizan M., Faraz A., Hayat S. Role of sugars under abiotic stress. *Plant Physiology and Biochemistry*, 2016, 109: 54-61 (doi: 10.1016/j.plaphy.2016.09.005).
51. Thalmann M., Santelia D. Starch as a determinant of plant fitness under abiotic stress. *New Phytologist*, 2017, 214(3): 943-951 (doi: 10.1111/nph.14491).
52. Kamanga R.M., Mbega E., Ndakidemi P. Drought tolerance mechanisms in plants: physiological responses associated with water deficit stress in *Solanum lycopersicum Adv. Crop. Sci. Tech.*, 2018, 6(3): 362 (doi: 10.4172/2329-8863.1000362).
53. Das A., Mukhopadhyay M., Sarkar B., Saha D., Mondal T.K. Influence of drought stress on cellular ultrastructure and antioxidant system in tea cultivars with different drought sensitivities. *J. Environ. Biol.*, 2015, 36(4): 875-882.
54. Belous O.G. *Aktivnost' katalazy v list'yakh chaya v zone vlazhnykh subtropikov Rossii.* LAP LAMBERT Academic Publishing, Saarbruchen, 2012.
55. Pedranzani H., Vigliocco A. Evaluation of jasmonic acid and salicylic acid levels in abiotic stress tolerance: Past and present. In: *Mechanisms behind phytohormonal signalling and crop abiotic stress tolerance. Chapter 15.* V.P. Singh, S. Singh, S.M. Prasad (eds.). Nova Science Publishers, 2017: 1-60.
56. Manivannan P., Jaleel C.A., Kishorekumar A., Sankar B., Somasundaram R., Sridharan R., Panneerselvam R. Drought stress induced changes in the biochemical parameters and photosynthetic pigments of cotton (*Gossypium hirsutum* L.). *Indian J. Appl. Pure Biol.*, 2007, 22: 369-372.
57. Pritula Z.V., Malyukova L.S., Velikii A.V. *Plodovodstvo i yagodovodstvo Rossii*, 2017, 51: 299-307 (in Russ.).
58. Tyerman S.D., Niemietz C.M., Bramley H. Plant aquaporins: multifunctional water and solute channels with expanding roles. *Plant, Cell & Environment*, 2002, 25(2): 173-194 (doi: 10.1046/j.0016-8025.2001.00791.x).
59. Wahid A., Gelani S., Ashraf M., Foolad M.R. Heat tolerance in plants: an overview. *Environmental and Experimental Botany*, 2007, 61(3): 199-223 (doi: 10.1016/j.envexpbot.2007.05.011).
60. Spalding E.P., Harper J.F. The ins and outs of cellular Ca^{2+} transport. *Current Opinion in Plant Biology*, 2011, 14(6): 715-720 (doi: 10.1016/j.pbi.2011.08.001).
61. Saruhashi M., Ghosh T.K., Arai K., Ishizaki Y., Hagiwara K., Komatsu K., Shiwa Y., Izumikawa T., Yoshikawa H., Umezawa T., Sakata Y., Takezawa D. Plant Raf-like kinase integrates abscisic acid and hyperosmotic stress signaling upstream of SNF1-related protein kinase 2. *Proceedings of the National Academy of Sciences*, 2015, 112(46): E6388-6396 (doi: 10.1073/pnas.1511238112).
62. Wan X., O'Quinn R.P., Pierce H.L., Joglekar A.P., Gall W.E., DeLuca J.G., Carroll C.W., Liu S.-T., Yen T. J., McEwen B.F., Stukenberg T., Desai A., Salmon E.D. Protein architecture of the human kinetochore microtubule attachment site. *Cell*, 2009, 137(4): 672-684 (doi: 10.1016/j.cell.2009.03.035).
63. Kim T.H., Böhmer M., Hu H.H., Nishimura N., Schroeder J.I. Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. *Annual Review of Plant Biology*, 2010, 61: 561-591 (doi: 10.1146/annurev-arplant-042809-112226).
64. Gupta S., Bharalee R., Bhorali P., Das S.K., Bhagawati P., Bandyopadhyay T., B. Gohain, Agarwal N., Ahmed P., Borchetia S., Kalita M.C., Handique A.K., Das S. Molecular analysis of drought tolerance in tea by cDNA-AFLP based transcript profiling. *Mol. Biotechnol.*, 2013, 53(3): 237-248 (doi: 10.1007/s12033-012-9517-8).
65. Gelmesa D., Dechassa N., Mohammed D., Gebre E., Monneveux P., Bündig C., Winkelmann T. In vitro screening of potato genotypes for osmotic stress tolerance. *Open Agriculture*, 2017, 2(1): 308-316 (doi: 10.1515/opag-2017-0035).
66. Wang W.D., Xin H.H., Wang M.L., Ma Q.P., Wang L., Kaleri N.A., Wang Y.H., Li X.H. Transcriptomic analysis reveals the molecular mechanisms of drought-stress-induced decreases in *Camellia sinensis* leaf quality. *Front Plant Sci.*, 2016, 7(795): 385-412 (doi: 10.3389/fpls.2016.00385).
67. Cui X., Wang Y.-X., Liu Z.-W., Wang W.-L., Li H., Zhuang J. Transcriptome-wide identification and expression profile analysisof the bHLH family genes in *Camellia sinensis. Functional & Integrative Genomics*, 2018, 18(5): 489-503 (doi: 10.1007/s10142-018-0608-x).
68. Wang Y.-X., Liu Z.-W., Wu Z.-J., Li H., Zhuang J. Transcriptome-wide identification and expression analysis of the NAC gene family in tea plant [*Camellia sinensis* (L.) O. Kuntze]. *PLoS ONE*, 2016, 11(11): e0166727 (doi: 10.1371/journal.pone.0166727).
69. Wang Y., Shu Z., Wang W., Jiang X., Li D., Pan J., Li X. CsWRKY2, a novel WRKY gene from *Camellia sinensis*, is involved in cold and drought stress responses. *Biologia Plantarum*, 2016, 60(3): 443-451 (doi: 10.1007/s10535-016-0618-2).
70. Wang M., Zhuang J., Zou Z., Li Q., Xin H., Li X. Overexpression of a *Camellia sinensis* DREB transcription factor gene (CsDREB) increases salt and drought tolerance in transgenic *Arabidopsis thaliana. J. Plant Biol.*, 2017, 60: 452-461 (doi: 10.1007/s12374-016-0547-9).
71. Shen W., Li H., Teng R., Wang Y., Wang W., Zhuang J. Genomic and transcriptomic analyses of HD-Zip family transcription factors and their responses to abiotic stress in tea plant (*Camellia sinensis*). *Genomics*, 2018 (In Press, Corrected Proof) (doi: 10.1016/j.ygeno.2018.07.009).

72. Chen J., Gao T., Wan S., Zhang Y., Yang J., Yu Y., Wang W. Genome-wide identification, classification and expression analysis of the HSP gene superfamily in tea plant (*Camellia sinensis*). *Int. J. Mol. Sci.*, 2018, 19(9): 2633 (doi: 10.3390/ijms19092633).
73. Wang L., Cao H., Qian W., Yao L., Hao X., Li N., Yang Y., Wang X. Identification of a novel bZIP transcription factor in *Camellia sinensis* as a negative regulator of freezing tolerance in transgenic arabidopsis. *Annals of Botany*, 2017, 119(7): 1195-1209 (doi: 10.1093/aob/mcx011).
74. Li H., Huang W., Liu Z.-W., Wang Y.-X., Zhuang J. Transcriptome-based analysis of Dof family transcription factors and their responses to abiotic stress in tea plant (*Camellia sinensis*). *Int J Genomics*, 2016: 5614142 (doi: 10.1155/2016/5614142).
75. Wang P., Chen D., Zheng Y., Jin S., Yang J., Ye N. Identification and expression analyses of SBP-box genes reveal their involvement in abiotic stress and hormone response in tea plant (*Camellia sinensis*). *Int. J. Mol. Sci.*, 2018, 19(11): 3404 (doi: 10.3390/ijms19113404).
76. Zhu J., Wang X., Guo L., Xu Q., Zhao S., Li F., Yan X., Liu S., Wei C. Characterization and alternative splicing profiles of the lipoxygenase gene family in tea plant (*Camellia sinensis*). *Plant and Cell Physiology*, 2018, 59(9): 1765-1781 (doi: 10.1093/pcp/pcy091).
77. Guo Y., Zhao S., Zhu C., Chang X., Yue C., Wang Z., Lin Y., Lai Z. Identification of drought-responsive miRNAs and physiological characterization of tea plant (*Camellia sinensis* L.) under drought stress. *BMC Plant Biology*, 2017, 17: 211 (doi: 10.1186/s12870-017-1172-6).

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QTL MAPPING OF ESTERASE ISOZYME FORMS IN *Brassica rapa* L. MATURE SEEDS

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Abstract

Since the 1960s, isoenzymes have been well known as one of the most common biochemical markers. Establishing the overall variability of the isoenzyme systems and identifying their genetic control retain their relevance, allowing researchers to reveal the fine mechanisms of the relationship of the organism with the environment and homeostasis and to develop effective biochemical markers for rapid assessment of genetically and selectively significant material. In this paper, the chromosome loci responsible for the activity of 13 different esterase forms of mature seeds in *Brassica rapa* L. were identified and mapped for the first time. The doubled haploid lines of two mapping populations, DH30 and DH38, were studied. All identified esterase isoforms were divided into three groups according to their electrophoretic mobility. The group of isoforms A1-A3 had a high molecular weight and low electrophoretic mobility. The group of B1-B7 isoforms exhibited an average molecular weight and an average electrophoretic mobility. The C1-C3 group consisted of isoforms having a low molecular weight and, consequently, the highest electrophoretic mobility. Each of the parental forms, as well as each of the studied lines of mapping populations, had its own unique electrophoretic spectrum of esterase isoforms. Based on the electrophoretic data obtained for both populations, a QTL analysis was carried out and chromosome loci were identified, determining the manifestation of each esterase isoform identified in the mapping lines of populations DH30 and DH38. The composite interval mapping approach, combined with a permutation test (1000 iteration) and a confidence level of $p < 0.05$, allowed us to identify and locate QTLs on chromosomes that determine the manifestation of all esterase isoforms identified by gel electrophoresis, with the exception of A1 isoform for the DH38 population and B7 isoform for the DH30 population. For these two isoforms, no QTL analysis results were obtained because of limitation in initial data on these isoforms in the corresponding mapping population. A total of 35 QTLs for esterase isoforms were mapped for DH30 mapping population and 39 QTLs for DH38 population. As a result of the QTL analysis, molecular markers genetically linked to the identified loci and the percentage of phenotypic variability determined by each of the identified QTLs were also identified. According to isoenzyme analysis, the heterozygosity of both populations in each H_l locus and total heterozygosity H_{total}, as well as $Var(H_l)$ heterozygosity dispersions for one locus and the variance of average heterozygosity within each population $Var(H_{total})$ were calculated. The identified heterozygosity was considered as the average portion of loci with two different alleles in one locus in one individual and could be defined as the observed heterozygosity characterizing the part of the genes for which the studied population is heterozygous. It was shown that in the studied populations of doubled QTL haploid lines, which determine the complex of esterases isoenzymes, are found mainly in the 2nd, 4th, 6th, and 9th linkage groups and form blocks of co-adapted genes and genomic co-adapted gene blocks, which emphasizes the importance of the contribution of

these loci in the ontogenesis and adaptability of plants *B. rapa*. In general, the carried out molecular genetic mapping and biochemical analysis of the studied biochemical traits of various manifestation of esterase isoforms in mature seeds of *B. rapa* revealed genetic determinants of the studied characters, as well as the genome distribution of mapped QTLs, which in the long term makes it possible to conduct effective molecular and genetic screening of collection accessions and breeding material of the *B. rapa* species according to these biochemical characters when performing genetic and selection investigations in this species.

Keywords: *Brassica rapa* L., biochemical analysis, esterase isoforms, mature seeds, QTL mapping, population heterozygosity

Since the 1960s, isoenzymes have been well known as one of the most common biochemical markers suitable for establishing genetic variability in both natural and artificial populations of plants [1-3] and animals [4-6]. Establishing the overall variability of the isoenzyme systems and identifying their genetic control retain their relevance, allowing researchers not only to reveal the fine mechanisms of the relationship of the organism with the environment and homeostasis during growth and development in both normal and extremal conditions [7] but also to develop effective biochemical markers for rapid assessment of genetically and selectively significant material [8].

Esterases (ES 3.1.1) combine a large number of different carboxyl ether hydrolases. Some of them have very broad substrate specificity and can hydrolyze both endogenous and exogenous ether bonds of different structures [9]. The fact that the enzymes showing esteratic activity can also hydrolyze non-ether bonds raises questions about the evolutionary adaptive physiological and ecological role of these esterases. Thus, the possibility of inhibiting α and β-esterases in plants has been described for aryl esterases in young cassava leaves and evaluated as a marker of pathogenesis after infection with bacteria [10]. Inhibition tests showed that organophosphates (insecticides) inhibit some plant esterases and activate the other ones in *Aspidosperma polyneuron* [11]. Consequently, plant esterases can serve as biochemical indicators used to detect insecticide residues and determine their toxicity in the control of environmental pollution. In general, the esterase system of organisms is characterized by the presence of a large number of isoenzymes and significant individual and population variability. In animals, most esterase enzymes are non-specific and exhibit overlapping substrate specificity [3]. In some cases, these isoenzymes are relatively specific, such as cholinesterases and carbonyl anhydrases.

The functional characteristics of plant esterases have not yet been sufficiently studied, although in plants esterases seem to represent one of the most studied groups of isoenzymes. As early as the late 1960s and early 1970s, esterases were studied in corn [1, 12, 13], barley [2], potatoes [14], cotton [15, 16], oats [17, 18], sugar beet [19], wheat [20, 21] and many other species. The most detailed genetic control of esterases is described in wheat [21-25]. In addition, the "alien"-wheat genealogy of chromosomes was defined [4, 20] and "alien"-wheat hybrids were identified [26, 27], and the variability of esterases of hexaploid genotypes was revealed [28]. All these papers were aimed at establishing not so much the physiological as the genetic component of such a biochemical system as higher plant esterases. The absence of epistatic interactions and the codominant nature of the inheritance of esterase isoforms make them a convenient tool for the rapid and effective study of the processes of biochemical adaptation to changing environmental conditions. This type of biochemical markers is convenient for solving practical problems of selection as a means of accelerating and simplifying the selection process of breeding material.

In the previous studies, a polymorphism in the isozyme profile of esterases isolated from mature seeds in samples of the varietal and line-breeding material of

hexaploid wheat (*Triticum aestivum* L.) was identified. The average heterozygosity (*H*) of the samples at 10 identified loci encoding isoforms, coding esterases, was 0.924 [29]. The most promising parental forms differing in phenotypic traits of breeding interest and the spectra of esterase isoforms – the varieties Zlata, Mera, and the homozygous lines AFI91, AFI177, ITMI7, ITMI44, ITMI83 and ITMI115 were identified and the possibility of determining the polymorphism of esterases in the hybrid generations was defined. Another study [30] shows a wide range of diversity of electrophoretic profiles of isoenzymes of mature seeds (eight isoforms with molecular masses from 37.7 to 57.6 kD) in 25 samples of radish (*Raphanus sativus* L.), which were divided into 13 electrophoretic enzyme types that differ from each other by the presence or absence of certain areas. The most common was the electrophoretic enzyme type of Gr. 1, spread among 24% of the estimated samples. The enzyme type of Gr. 5 was characterized by a maximum number of zones – 8, the enzyme types of Gr. 3 and Gr. 12 – the smallest (4 zones). The seventh and eighth zones of esterases (M_r = 39.7 kD and M_r = 37.7 kD, respectively) were monomorphic; the remaining six zones were polymorphic. The frequency of each zone for different enzyme types ranged from 6.58 to 17.11%. The obtained results allowed selecting promising source material for breeding.

However, to date, no studies have been carried out for the lines of doubled haploids on the esterase isoenzyme spectrum. The advantage of this approach is that, for example, molecular genetic maps of the mapping populations of *Brassica rapa* L. DH30 and DH38 are saturated with SSR and AFLP molecular markers with a mapping interval of 2.4-2.6 cm. The denser the markers are, the more accurate the map is. AFLP markers are currently relatively rare but still used in conjunction with other types of markers. At the same time, in the absence of other known markers of esterase isoforms, in the future for routine screening of breeding or other genetic material, it is possible to convert AFLP markers located in peaks detected by QTL into SCAR or CAPS markers. All this allows taking the issue of the reliability and reproducibility of the results when using markers obtained using certain genetic material off the table, as this question arises very often, if not always [29-31]. Different positions of loci of quantitative traits of breeding interest in the mentioned populations DH30 and DH38 have been noted in many studies, which is quite natural since the populations are different genetically [31]. However, this is even more interesting because for mass collection or selection screening the most reliable markers of loci are those in close positions in both populations.

In the present study, the isoenzyme analysis of *Brassica rapa* L. doubled haploid lines was conducted for the first time, which allowed not only identifying the localization of the loci determining the manifestation of the analyzed isoforms of esterases in the linkage groups on the chromosomes but also finding a statistical relationship between the identified QTLs (quantitative trait loci) of these isozymes and the observed heterozygosity for each locus.

The aim of the paper was the mapping of QTLs that determine the expression of different esterase isoforms of mature seeds of doubled haploid lines of *Brassica rapa* L.

Techniques. The esteratic profiles of the seeds were analyzed for 80 lines of the mapping populations of *Brassica rapa* L. (3 parent forms, 50 DH38 descending lines, 27 DH30 descending lines). The details of obtaining and using the DH30 and DH38 populations for QTL mapping of morphological and biochemical traits in *B. rapa* were described in the previous paper [31].

Mature seeds were ground thoroughly in a porcelain mortar, 100 mg of the obtained flour was placed in test tubes of the Eppendorf type, 1 ml of hexane was added, the mixture was thoroughly stirred and left for degreasing overnight in a refrigerator at 4 °C, then centrifuged 10 min at 15000 rpm, the super-

natant fluid was decanted and the sample was left under a draught to dry in air. Enzymes were extracted for 14-18 hours from fat-free and dried samples with 0.05 M Tris-HCl buffer (pH 8.3) in the presence of 2-mercaptoethanol (2 μl/ml) and glycerol (10%), at the flour to buffer ratio of 1:4 and a temperature in the range from 4 to 8 °C. The samples were centrifuged for 10 min, the supernatant liquid was collected and samples were frozen at –20 °C to prevent inactivation of the isoenzymes during storage. Before entering to the electrophoresis chamber, the samples were defrosted at room temperature. Isoenzymes were separated by native vertical electrophoresis in PAAG [32]; the concentration of separating and concentrating gels was 11 and 5%, respectively. Electrophoresis was carried out in a Mini-PROTEAN Tetra Cell chamber (Bio-Rad Laboratories, Inc., USA). Prestained Protein Ladder (Thermo Scientific, USA) was used as molecular mass markers. Protein (20 μg) was added to each pocket of the concentrating gel. The protein concentration in enzyme preparations was determined by the dye-binding method according to Bradford [33], with bovine serum albumin (Promega Corporation, USA) as a standard. Electrophoresis was performed in the cold (10-15 °C), at 10 V/cm for 2.5 h. After separation, the gel was treated with a reagent for nonspecific esterase [34]. This gel was stained in freshly prepared dye solution consisting of 100 mg α-naphthyl acetate and 120 mg β-naphthyl acetate (Sigma-Aldrich Chemie GmbH, Switzerland), dissolved in 10 ml of 70% ethanol, 500 mg of Fast Blue RR (Sigma-Aldrich Co., USA); 4 ml of propanol and 60 ml of 0.1 M phosphate buffer (pH 6.0). After the appearance of brownish-violet bands, the excess dye was removed with 10% acetic acid. The received zymograms were scanned (Epson Expression 10000XL, GE Healthcare, USA). Evaluation of each sample (R_f value of all bands in the track, calculation of molecular weight based on standards, calculation of the relative amount of each zone in the track) was carried out using Phoretix 1D Advanced software (TotalLab, Ltd., UK).

For mapping of the identified QTLs, MAPQTL 6.0 software (Kyazma B.V., Netherlands) was used [35] to detect for each esterase isoform in each population the presence and location of candidate QTL in the linkage group (at 5 cM mapping interval), to estimate LOD (logarithm of odds) values (p = 0.05) and the degree of phenotypic expression of the trait explained by the corresponding QTL (% Expl.). LOD significance was assessed in the permutation test (1000 iterations) [36].

The trait-marker correlation coefficients were calculated as a statistically significant association of the marker locus with the QTL conferring the trait (for p = 0.05 significance level) based on empiric variants for each trait-marker pair [37]. Using data on mapping of the detected QTL, the maps were graphically constructed with MapChart 2.2 software (https://www.wur.nl/en/Research-Results/Research-Institutes/plant-research/biometris/Software-Service.htm) [38]. Statistical data processing was carried out by multivariate analysis methods [35].

The degree of the observed heterozygosity H for each locus was determined, as well as its average value for several loci (total heterozygosity H_{total}). The population heterozygosity at each locus H_l and the total heterozygosity H_{total} were calculated according to the description [39, 40] by the following formulas:

$$H_l = 2n(1 - \sum_k x_k^2)/2n - 1,$$
$$H_{total} = \sum_{l=1}^{r} H_l/r,$$

where l is the sequence number of the locus, n is the population size, x_k is the estimated frequency of the k-th allele of the l-th locus, r is the total number of loci.

The variance of heterozygosity $Var(H_l)$ at one locus and the variance of the average heterozygosity within populations $Var(H_{total})$ were found [41]:

$$Var(H_l) = H_l(1 - H_l)/n,$$
$$Var(H_{total}) = \frac{1}{nr^2}\sum_l H_l(1 - H_l) + \frac{1}{nr^2}\sum_l \sum_{l \neq \hat{l}} (H_{l\hat{l}} - H_l H_{\hat{l}}).$$

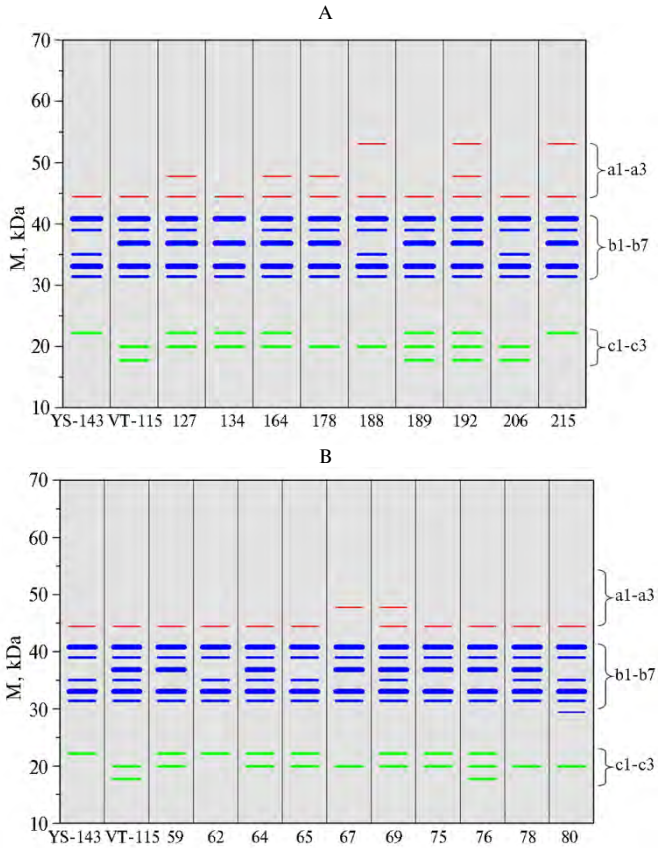

Fig. 1. The diagrams of electropherograms of esterase isoforms in the mapping populations DH30 (A) **and DH38** (B) of ***Brassica rapa* L.**: YS-143 — male parent, VT-115, PC-175 — female parents; the figures at the bottom — line numbers of mapping populations; A1-A3, B1-B7 and C1-C3 — groups of esterase isoforms of respectively large, medium and small molecular weight; M —of molecular weight marker (Prestained Protein Ladder, Thermo Scientific, USA).

Results. Electrophoretic analysis of isozymes of esterases of mature seeds for lines of doubled haploids from two mapping populations of *B. rapa* and their three parental forms revealed 13 isoforms of esteratic enzymes, which were divided into three groups by electrophoretic mobility (Fig. 1). The group of isoforms A1-A3 had large molecular weight and low electrophoretic mobility, the group B1-B7 showed medium molecular weight and mobility, the isoforms of the group C1-C3 had a small molecular weight and the highest electrophoretic mobility. Note, the intensity of the zones A1-A3 and C1-C3 varied relatively little, while B1-B7 varied significantly. In addition, in contrast to groups A1-A3 and C1-C3, including three isoforms, the group B1-B7 was represented by seven esterase isoforms.

Each of the parent forms, as well as each of the studied lines of mapping populations, had its unique electrophoretic spectrum of esterase isoforms. The group C1-C3 consisted of isoforms inherent in both the male and each of the female samples. This group of esterases exhibited a codominant type of inheritance. The group B1-B7 from the parent forms consisted of six electrophoretic zones, five of which were identical, and the area B3 absent among the male plant YS-143 was presented in both female forms, the VT-115 and PC-175, showing the dominant type of inheritance. In the case of the DH30 population, the area V4 was found in the male form YS-143 and was absent in the female form VT-115, also showing the dominant type of inheritance. In the line 97 of the mapping DH30 population, as well as the lines 80, 123, 127 and 136 of the mapping population DH38, the B7 isoform was found that was absent in all three parental forms. The appearance of this isoform in these five lines seems to be due to the transgressive nature of genetic rearrangements or changes in the genome of these lines that occurred during their creation and selection in culture in vitro, that is, due to somaclonal variability. It is also possible that this is one

of the sinapine esterases identified in *Brassicaceae* previously [42]. The same can be said for the ratio of the A1 and A2 isoforms found in a number of lines in both DH30 and DH38 populations. It should be noted that the A3 isoform is presented in all three of the parental forms and is absent in the lines 28 and 67 of the DH30 population, and the lines 1, 4, 67, 90, 124, and 134 of the DH38 population. The presence of the isoforms A1 and A2 in different lines of both populations varied. Their presence in a particular line seems to be due to the same reasons as the appearance of the isoform B7.

On the basis of electropherograms for both populations, QTL analysis was conducted and the loci on the chromosomes that determine the appearance of each of the esterase isoforms detected from lines of the mapping populations DH38 and DH30 were identified (Table 1, see the journal website http://www.agrobiology.ru).

The approach of composite interval QTL mapping used in this study in combination with the permutation test (1000 iterations) at the significance level $p < 0.05$ allowed identifying and localizing on chromosomes the loci (QTLs) that determine the appearance of all isoforms of esterases identified by means of gel-electrophoresis (see Fig. 1) except for A1 in the DH38 population and V7 in DH30. For these two isoforms, the QTL analysis results were not obtained due to the small amount of relevant baseline data for each mapping population.

The results of QTL mapping revealed molecular markers genetically linked to the identified loci, which in the future can be used for molecular genetic analysis of *B. rapa* samples, and the localization of the detected QTLs in the linkage groups was identified (Fig. 2, see at the journal website http://www.agrobiology.ru). So, in a population of DH30 for the A1 isoforms, seven loci on the chromosomes that determine its manifestation were identified. The maximum LOD values were found for the 1st (3.70) and 5th (3.76) linkage groups. For A2 in the DH30 population, three, and, in the DH38 population, six loci determining the manifestation of this isoform were found. It should be noted that the location of the QTL of the isoform A2 on the 4th and 9th chromosomes coincided in both populations, indicating the evolutionary consolidation of this trait, which apparently had adaptive significance. The maximum LOD (of 2.09) in the population DH30 was calculated for the 9th chromosome, and for DH38, the obtained LOD values ranged from 2.28 to 4.88 for different linkage groups. In contrast, the value of LOD for A3 isoforms was relatively low, 1.29 for a single QTL in the population DH30 and 1.30-1.79 for QTLs detected in DH38; however, the proportion of phenotypic variability, which determined the QTLs identified for the isoforms A3, accounted for 23.4% of the population DH30 and 14.6-19.5% in the case of DH38.

The isoform B1 is also characterized by low LOD value for both populations, i.e. 1.36-1.53 and 1.33-1.75 if the amount of phenotypic variation is 20.2-25.4 and 13.3-17.1% for DH30 and DH38, respectively. In general, three QTLs from the population DH30 and two from DH38 were mapped. QTLs of the isoform B3 were not numerous and had low LOD values. Thus, for DH30, they were 2.00 and 1.37-2.53, respectively, for DH38 — 4.00 and 1.32-1.78. However, for B3, as for A2, the location of the QTL mapped in the 6th linkage group coincided, indicating the evolutionarily adaptive nature of this isoform. The number of QTLs underlying the expression of the isoform B2 was small as well, i.e. four in the population DH30 and two for DH38. However, the LOD value (1.36-3.36 and 1.93-2.47 for DH30 and DH38, respectively) and the proportion of controlled phenotypic variability (23.9-48.0 and 19.9-24.8%, respectively) were higher than for B3. Almost the same results were obtained for the isoform B4. Two QTLs were identified for it in each of the mapping populations. The

value of LOD and the percentage of phenotypic variation ranged between 1.48-1.55 and 46.1-47.8% for DH30, 1.33-1.49 and 20.3 and 22.4% for DH38. The isoform B5, though it had a low LOD (1.32-1.52 and 1.93-2.07 for DH30 and DH38 respectively) with a small percentage of phenotypic variation (22.3-25.3 and 18.3-19.5%), however, as well as B3, had a QTL, localized in both populations at the 6th linkage group in similar positions, which confirms the conclusion about the evolutionary adaptive nature of inheritance of these isoforms of esterases of *B. rapa* seeds. The population DH30 had five QTL isoforms B6 at the 3rd, 6th, 7th, and 8th chromosomes; DH38 had three QTLs in the 8th, 9th and 10th linkage groups. LOD scores were relatively small, 1.31-1.84 and 1.31-1.48 respectively; the proportion of phenotypic variation was 23.9-29.7% and 11.9-14.9% for DH30 and DH38. It is interesting to note that for the DH30 population, the QTL identified in the 6th linkage group was located in the same place as the QTL of the isoform B5, which indicates the importance of this chromosomal locus in *B. rapa*. The B7 isoform was identified and mapped only in the DH38 population. In total, five QTLs were localized, which were located on the 1st, 2nd, 7th and 9th chromosomes. LOD values ranged from 1.53 to 2.13; the proportion of phenotypic variability due to the identified QTL was from 82.8 to 91.4%.

For C1, only one QTL was found in each of the mapping populations. The LOD values (1.39 and 1.32 for DH30 and DH38) and the percentage of phenotypic variability due to the corresponding QTL (32.9 and 18.9%) were relatively small. C2 QTLs were identified in DH30 in the 2nd, 4th and 5th linkage groups (1.46-2.20 and 31.2-43.0%), in DH38 – in the 3rd and 8th groups (1.29-2.31 and 18.2-30.7%). The C3 isoform had three mapped QTLs in the population DH30 and four QTLs – in DH38. The LOD values of the identified QTLs varied from 1.50-2.77 in DH30 and 1.51-2.51 in DH38, and the proportion of the phenotypic variability in DH38 and DH30 was 68.5-88.1 and 43.3-65.0%, respectively. QTLs for the C3 isoform mapped on the 2nd chromosome (as in the case of A2, B3, and B5) were identified in both mapping populations. This fact confirms that the specified locus of the 2nd chromosome has evolutionary significance and its structure was fixed in the process of ontogenetic and phylogenetic adaptation in the *B. rapa* species. In all probability, the stability of the typical morphological and biochemical features of the plant is associated with such adaptively significant chromosome loci and the increased adaptive ability to adverse environmental factors as well [43-45].

2. Heterozygosity and its variance in *Brassica rapa* L. mapping lines of DH38 and DH30 populations based on data of isozyme analysis of esterases

Indicator	Isozymes												
	A1	A2	A3	B1	B2	B3	B4	B5	B6	B7	C1	C2	C3
Population DH30													
H_l	0.352	0.616	0.139	0	0.139	0.475	0.491	0	0	0.073	0.475	0.391	0.391
$Var(H_l)$	0.008	0.009	0.004	0	0.004	0.009	0.009	0	0	0.002	0.009	0.009	0.009
Average heterozygosity H_{total} = 0.272; dispersion $Var(H_{ototal})$ = 0.007													
Population DH38													
H_l	0	0.213	0.213	0.078	0.149	0.424	0.476	0	0	0.149	0.465	0.465	0.368
$Var(H_l)$	0	0.003	0.003	0.001	0.002	0.005	0.005	0	0	0.002	0.005	0.005	0.005
Average heterozygosity H_{total} = 0.231; dispersion $Var(H_{ototal})$ = 0.003													

According to isozyme analysis, the heterozygosity of populations for each locus H_l and total heterozygosity H_{total}, as well as the variance of heterozygosity $Var(H_l)$ at one locus and the variance of mean heterozygosity within each of the *Var* populations (H_{total}) was calculated (Table 2).

Since the dispersion of the average heterozygosity should take into account covariations between heterozygosity at different loci (l and $\hat{l}$), which is due

to their dependence on the frequency of double heterozygotes $H_{l\hat{l}}$ at these loci, the formula proposed by Weir [41] was used. The revealed heterozygosity was considered as the average portion of loci with two different alleles in one locus in one individual and could be defined as the observed heterozygosity characterizing a part of genes on which the studied population is heterozygous. The used formulas [39-41] make it possible to calculate any polynomial in a set of variables distributed multinominally, which, in turn, allows considering the revealed heterozygosity as a measure of information polymorphism widely used in the preparation and implementation of genetic selection programs. The formation of any breeding significant properties depends on the genotype of the individual [46, 47]. At the same time, information polymorphism is nothing but a reflection of phenotypic manifestations of genetic determinants (genes, chromosome loci) distributed in the genome, determining the studied features [48].

To summarize, the QTLs determining biochemical traits, i.e. the spectra of esterase isoforms of mature seeds in mapping populations of *B. rapa* doubled haploids, were mapped by us for the first time and the molecular markers genetically linked to the QTLs of these traits were identified, which allows efficient molecular genetic screening of sample collections and breeding material. Mechanisms that control the biosynthesis and accumulation of biologically active substances such as isoenzymes that determine the biochemical characteristics of morphotypes occurs as a result of evolutionary-adaptive formation and inheritance. Modification of these mechanisms can lead to a sharp change in the number and set of isoenzymes in various organs, in particular, in seeds, which, in turn, in *B. rapa* affects the morphological and adaptive qualitative characteristics of the species. The study of genetic determinants of esterase isoforms in the future can allow controlling the process of genetic variability on these adaptively significant features by combining different genetic derivatives from different parental forms in one organism. Analysis of genetic variability is very important for understanding the genetics of quantitative traits that play a key role in breeding programs to improve economically significant properties in *B. rapa*. As a result, it would be extremely important to determine the functions of genes that affect their expression, molecular genetic mechanisms of expression and, finally, characterize the molecular variability of genomes inside and outside the species. It is interesting to note that similar data were obtained earlier for wheat [29] and radish [30], which also revealed polymorphism between samples of promising breeding material in the spectra of isoenzyme esterases forms of mature seeds. In addition, these results echo those described in the analysis of the esterase-lipase gene family in *Arabidopsis thaliana* [49] and *Olea europaea* [50]. The data obtained on these species (both in this study and earlier), in the future, are supposed to allow the identification of the genetic-evolutionary and phylogenetic relationships of the structure and functioning of genomes in mono- and dicotyledonous plants.

Note should be made of the revealed coincidence of the positions of loci that control the isoenzyme profiles of esterases and other biochemical features studied earlier, such as the content of free amino acids, phenolic compounds, carotenoids, and chlorophylls. These loci were localized in the lower part of the A03 chromosome where the gene for the transition to flowering *BrFLC5* is situated, as well as in the middle and in the lower part of the A06 linkage group [31].

Thus, molecular genetic mapping and analysis of the spectra of esterase isoforms in mature *Brassica rapa* seeds revealed genetic determinants of these traits. The distribution of mapped QTLs in the genome has been identified. It was shown that in the studied populations of doubled haploid lines, QTLs determining

a set of esterase isoenzymes are located mainly in the 2nd, 4th, 6th and 9th linkage groups and form the blocks of co-adapted genes and genomic co-adapted blocks of genes, underscoring the importance of the contribution of these loci in the ontogeny and adaptiveness of *B. rapa* plants. In general, the coincidence of localization of the detected QTLs supports the observed biochemical correlations (e.g., the location of esterase isoforms on the electrophoretogram), and the blocks of genes combined into a genome areas in the linkage groups A02, A03, A04, A06 and A09, and the QTLs identified in the linkage groups reflect the genetic correlations between such characteristics as spectra of esterase isoforms and other biochemical properties of plants.

REFERENCES

1. Schwartz D., Fuehsman C., McGrath H.K. Allelic isozymes of the pH 7.5 esterases in maize. *Genetics*, 1965, 52: 1265.
2. Solari R.M., Favret E.A. Polymorphism in endosperm proteins of barley and its genetic control. In: *Barley genetics II*. R. Nilan (ed.). Washington University Press, Pulman WA, 1971: 23-31.
3. Artemova N.V. Chromosomal control of the isoenzymes of alcohol dehydrogenase, esterase and amylase in different rye varieties. *Genetika*, 1982, 18: 661-667.
4. Coates P.M., Mestriner M.A., Hopkinson D.A. A preliminary genetic interpretation of the esterase isozymes of human tissues. *Ann. Hum. Genet.*, 1975, 39: 1-20 (doi: 10.1111/j.1469-1809.1975.tb00103.x).
5. Lith H.A., Bieman M., Zutphen B.F.M. Purification and molecular properties of rabbit liver esterase ES-1A. *Eur. J. Biochem.*, 1989, 184: 545-551 (doi: 10.1111/j.1432-1033.1989.tb15048.x).
6. Deimilling O., Wassmer B. Genetic characterization of esterase-28 (ES-28) of the house mouse. *Biochem. Genet.*, 1991, 29: 55-64 (doi: 10.1007/BF00578240).
7. Ivachenko L.E. *Uspekhi sovremennogo estestvoznaniya*, 2010, 9: 96-98 (in Russ.).
8. Chesnokov Yu.V. *Molekulyarno-geneticheskie markery i ikh ispol'zovanie v predselektsionnykh issledovaniyakh* [Molecular genetic markers and their use in preselection studies]. St. Petersburg, 2013 (in Russ.).
9. Walker C.H., Macknees M.I. Esterases: problems of identification and classification. *Biochem. Pharmacol.*, 1983, 32: 3265-3269.
10. Pereira A.J., Lapenta A.S., Vidigal-Filho P.S., Machado M.F.P.S. Differential esterase expression in leaves of *Manihot esculenta* Crantz infected by *Xanthomonas axonopodis* pv. *manihotis*. *Biochem. Genet.*, 2001, 39: 289-296 (doi: 10.1023/A:1012251228986).
11. Carvalho V.M., Marques R.M., Lapenta A.S., Machado M.F. Functional classification of esterases from leaves of *Aspidosperma polyneuron* M. Arg. (*Apocynaceae*). *Genet. Mol. Biol.*, 2003, 26(2): 195-198 (doi: 10.1590/S1415-47572003000200013).
12. MacDonald T., Brewbaker J. Isoenzyme polymorphism in flowering plants. VIII. Genetic control and dimeric nature of transaminase hybrid maize isoenzymes. *J. Hered.*, 1972, 63: 11-14 (doi: 10.1093/oxfordjournals.jhered.a108209).
13. Scandalios J.G. Genetic control of multiple molecular forms of enzymes in plants. *Biochem. Genet.*, 1969, 3: 37-79 (doi: 10.1007/BF00485973).
14. Desborough S., Peloquin J. Esterases isozymes from *Solanum* tubers. *Phytochemistry*, 1967, 6: 1398-1402 (doi: 10.1016/S0031-9422(00)86051-3).
15. Cherry J.P., Katterman F.R.H., Endrizzi J.E. Comparative studies of seed proteins of species of *Gossypium* by gel electrophoresis. *Evolution*, 1970, 24: 231-447 (doi: 10.2307/2406817).
16. Cherry J.P. A comparative study of seed proteins of allopolyploids of *Gossypium* by gel electrophoresis. *Canadian Journal of Genetics and Cytology*, 1971, 13: 155-158 (doi: 10.1139/g71-024).
17. Marshall D.R., Allard R.W. Isozyme polymorphism in natural populations of *Avena fatua* and *A. barbata*. *Heredity*, 1970, 25: 273-282 (doi: 10.1038/hdy.1970.38).
18. Murray B.E., Craig I.L., Rajhathy T. A protein electrophoretic study of three amphidiploids and eight species of *Avena*. *Canadian Journal of Genetics and Cytology*, 1970, 12: 651-665 (doi: 10.1139/g70-085).
19. Lerch B., Wolf G. Isolation of phosphodiesterase from sugar beet leaves. *Biochimica et Biophysica Acta (BBA) — Enzymology*, 1972, 258: 200-218 (doi: 10.1016/0005-2744(72)90979-5).
20. Barber H.N., Driscoll C.J., Vickery R.S. Enzymic markers for wheat and rye chromosomes. In: *Proceedings III International Wheat Genetics Symposium*. Canberra, 1968: 116-122.
21. May C.E., Vickery R.S., Driscoll C.S. Gene control in hexaploid wheat. In: *Proceedings 4th International Wheat Genetics Symposium*. Columbia, 1973: 843-849.
22. Nakai Y. Isozyme variations in *Aegilops* and *Triticum*. 2. Esterase and acid phosphatase isozymes studied by the gel isoelectrofocusing method. *Seiken Ziho*, 1973, 24: 45-73 (doi:

10.1038/hdy.1977.102).
23. Nakai Y. Isozyme variations in *Aegilops* and *Triticum*. 3. Chromosomal basis of the esterase isozyme production in different organs of Chinese spring wheat. *Bot. Mag. Tokyo*, 1976, 89(3): 219-234 (doi: 10.1007/BF02488344).
24. Cubadda R., Bozzini A., Quattrucci E. Genetic control of esterases in common wheat. *Theor. Appl. Genet.*, 1975, 45: 290-293 (doi: 10.1007/BF00276681).
25. Jaaska V. Electrophoretic survey of seedling esterases in wheats in relation to their phylogeny. *Theor. Appl. Genet.*, 1980, 56: 273-284 (doi: 10.1007/BF00282570).
26. Fedak G. Increased homoeologous chromosome pairing in *Hordeum vulgare* × *Triticum aestivum* hybrids. *Nature*, 1977, 266: 529-530 (doi: 10.1007/BF00264329).
27. Fejer O., Belea A. Cytology and isoenzymes of some *Triticum* auto- and amphidiploids. *Wheat Information Service,* 1978, 47/48: 41-44.
28. Alexandrescu N., Saulescu N. Esterase variability in kernels of *Triticum aestivum* cultivars. *Revue roumaine de biochimie*, 1982, 19: 93-100.
29. Rudakova A.S., Rudakov S.V., Davydova N.V., Mirskaya G.V., Zhuravleva E.V., Chesnokov Yu.V. Isozymic analysis of esterases in mature seeds of hexaploid soft wheat (Triticum aestivum L.). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(3): 327-334 (doi: 10.15389/agrobiology.2016.3.327rus).
30. Rudakova A.S., Rudakov S.V., Artem'eva A.M., Kurina A.B., Kocherina N.V., Chesnokov Yu.V. Izuchenie polimorfizma esteraznogo sostava zrelykh semyan obraztsov redisa (*Raphanus sativus* L.) kollektsii VIR. *Ovoshchi Rossii*, 2017, 5: 3-8 (doi: 10.18619/2072-9146-2017-5-3-8) (in Russ.).
31. Artem'eva A.M., Solov'eva A.E., Kocherina N.V., Berensen F.A., Rudneva E.N., Chesnokov Yu.V. *Fiziologiya rastenii*, 2016, 63: 275-289 (doi: 10.7868/S0015330316020044) (in Russ.).
32. Davis B.J. Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the NY Academy of Science*, 1964, 121: 404-427 (doi: 10.1111/j.1749-6632.1964.tb14213.x).
33. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, 1976, 72: 248-254 (doi: 10.1016/0003-2697(76)90527-3).
34. Meon S. Protein, esterase and peroxidase patterns of phytophtora isolates from Cocoa in Malaysia. *Journal of Islamic Academy of Sciences*, 1988, 1(2): 154-158.
35. Van Ooijen J.W. *MapQTL 6. Software for the mapping of quantitative trait loci in experimental populations of diploid species.* Wageningen, Netherlands, 2009.
36. Kocherina N.V., Artemyeva A.M., Chesnokov Yu.V. Use of LOD-score technology in mapping quantitative trait loci in plants. *Russian Agricultural Sciences*, 2011, 37: 201-204 (doi: 10.3103/S1068367411030098).
37. Ronald P.S., Penner G.A., Brown P.D., Brule-Babel A. Identification of RAPD markers for percent hull in oat. *Genome*, 1997, 40: 873-878 (doi: 10.1139/g97-813).
38. Voorrips R.E. MapChart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.*, 2002, 93: 77-78 (doi: 10.1093/jhered/93.1.77).
39. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 1978, 89(3): 583-590.
40. Lefèvre F., Charrie A. Isozyme diversity within African *Manihot* germplasm. *Euphytica*, 1992, 66: 73-80 (doi: 10.1007/BF00023510).
41. Veir B. *Analiz geneticheskikh dannykh* [Genetic data analysis]. Moscow, 1995: 132-135 (in Russ.).
42. Clauss K., Baumert A., Nimtz M., Milkowski C., Strack D. Role of a GDSL lipase-like protein as sinapine esterase in Brassicaceae. *Plant J.*, 2008, 53(5): 802-813 (doi: 10.1111/j.1365-313X.2007.03374.x).
43. De Souza A., Hull P.A., Gille S., Pauly M. Identification and functional characterization of the distinct plant pectin esterases PAE8 and PAE9 and their deletion mutants. *Planta*, 2014, 240(5): 1123-1138 (doi: 10.1007/s00425-014-2139-6).
44. Biely P. Microbial carbohydrate esterases deacetylating plant polysaccharides. *Biotechnol. Adv.*, 2012, 30(6): 1575-1588 (doi: 10.1016/j.biotechadv.2012.04.010).
45. Gao Z., Qian Q., Liu X., Yan M., Feng Q., Dong G., Liu J. *Dwarf 88*, a novel putative esterase gene affecting architecture of rice plant. *Plant Molecular Biology*, 2009, 71(3): 265-276 (doi: 10.1007/s11103-009-9522-x).
46. Sandukhadze B.I., Zhuravleva E.V., Kochetygov G.V. *Ozimaya pshenitsa Nechernozem'ya v reshenii prodovol'stvennoi bezopasnosti Rossiiskoi Federatsii* [Winter wheat of Nonchernozem zone in addressing the food security of the Russian Federation]. Moscow, 2011 (in Russ.).
47. Casacuberta J.M., Jackson S., Panaud O., Purugganan M., Wendel J. Evolution of plant phenotypes, from genomes to traits. *G3: Genes, Genomes, Genetics*, 2016, 6(4): 775-778 (doi: 10.1534/g3.115.025502).
48. Yang W., Guo Z., Huang C., Duan L., Chen G., Jiang N., Fang W., Feng H., Xie W.,

Lian X., Wang G., Luo Q., Zhang Q., Liu Q., Xiong L. Combining high-throughput phenotyping and genome-wide association studies to reveal natural genetic variation in rice. *Nature Communications*, 2014, 5: 5087 (doi: 10.1038/ncomms6087).

49. Lai C.P., Huang L.M., Chen L.O., Chan M.T., Shaw J.F. Genome-wide analysis of GDSL-type esterases/lipases in Arabidopsis. *Plant Mol. Biol.*, 2017, 95(1-2):181-197 (doi: 10.1007/s11103-017-0648-y).
50. Rejyn J.D., Zienkiewicz A., Rodríguez-García M.I., Castro A.J. Profiling and functional classification of esterases in olive (*Olea europaea*) pollen during germination. *Ann. Bot.*, 2012, 110(5): 1035-1045 (doi: 10.1093/aob/mcs174).

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PRODUCTIVITY OF LEGUME-RHIZOBIAL COMPLEX UNDER THE INFLUENCE OF GROWTH-STIMULATING MICROORGANISMS

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Abstract

The lack of a general transition to environmentally oriented (environmentally friendly) agriculture is primarily due to the fact that use of biologicals shows unstable effects. An impact of the introduced microorganisms on the native soil microbiota, in particular, on natural microbial-plant relations, remains little studied. The purpose of this work was to study the effect of growth-stimulating strains of *Paenibacillus ehimensis* IB 739, *Pseudomonas koreensis* IB-4 and *Ps. chlororaphis* IB-51 on the legume plant—an aboriginal microbial community ecosystem. Seeds of pea (*Pisum sativum* L.) variety Chishminsky 95, white lupine (*Lupinus albus* L.) variety Dega, chickpea (*Cicer arietinum* L.) variety Zavolzhsky, alfalfa changeable (*Medicago* × *varia Martyn*) variety Galia, white melilot (*Melilotus albus* Medik.) variety Chermasan were treated with liquid cultures of rhizosphere bacteria strains. Sterile tap water was the control, and well-known microbiological fertilizer Azotovit® based on *Azotobacter chroococcum* B-9029 strain was the standard. Seed germination percentage, plant size, the number of root nodules, root rot lesions, nitrogen accumulation in the soil and nitrogen assimilation by plants, and abundance of inoculants in the soil served as estimates of the effect of seed inoculation during 45-day pot experiment carried out under room temperature and natural lighting. Plants, along with the soil samples for counting inoculants, were collected on day 18 and day 45, the samples for assessing nitrogen accumulation were collected on day 45. The obtained data indicate the stimulating effect of strains *P. ehimensis* IB 739, *Ps. koreensis* IB-4, and *Ps. chlororaphis* IB-51 on the formation and function of various legume-rhizobial communities. Treatment with plant growth-promoting (PGP) microorganisms improved seed germination, plant growth and development. In seed treating with *Ps. chlororaphis* IB-51, the root length predominantly increased, whereas *Ps. koreensis* IB-4 strain stimulated the development of the aerial parts. The *Ps. chlororaphis* IB-51 and *Ps. koreensis* IB-4 were most effective on pea, lupine and chickpea plants with no growth stimulation on alfalfa and melilot plants The inoculation of seeds with tested bacterial strains was found to suppress of the development of microscopic fungi in the rhizosphere, as a result, the root rot on peas decreased from 66.7 % to 25.3-43.8 %, on lupine from 35.9 % to 20.3-25.0 %. The inoculants showed no inhibitory effect on rhizobia, on the contrary, nodulation became more abundant. After seed bacterization of fodder crops with *P. ehimensis* IB 739, the number of nodules on the roots increased 1.9-2.6 times. *P. ehimensis* IB 739 proved to be the most active in providing accumulation of nitrogen in plants and in the soil. The nitrogen concentration in the treated pea plants was 8.5 % vs. 3.9 % in the control, in lupine — 8.6 % vs. 5.0 % in the control. To summarize, the growth-promoting properties of a strain do not guarantee its favorable effect on the productivity of leguminous plants. Strains having similar characteristics can significantly differ in their effectiveness on the same legume crops.

Keywords: PGPB, *Pseudomonas, Paenibacillus,* leguminous plants, growth-promoting activity, nodule formation, nitrogen fixation, plant-microorganism interaction

To date, researchers know of a number of direct [1-3] and mediated [4, 5] positive bacterial effects on plants. It has been discovered that the same plant growth-promoting bacterium, PGPB, can have a variety of phyto-positive traits [6, 7]. Unfortunately, how introduced microbial species may affect the aboriginal soil microbiota remains to be seen, in particular, the effects of biologicals on the native microbe-plant symbiosis [8]. The efficiency of a biological preparation has been proven to depend not only on its functionality but also on whether the biological control agents it contains manage to fill a niche in the existing microbial community [9-11].

The legume-rhizobial community is a well-known example of a balanced phytomicrobiome. Nodule bacteria are naturally symbiotic with legumes and provide the latter with extra nitrogen. However, only some Rhizobium strains are able to boost crop productivity by synthesizing phytohormones [12, 13] and controlling phytopathogens [14, 15]. In this regard, it seems important to analyze how introducing microorganisms with useful traits might potentiate the legume ecosystem.

The biological activity of the plant growth-promoting bacteria *Paenibacillus ehimensis* IB 739 (VKM B-2680D), *Pseudomonas koreensis* IB-4 (VKM B-2830D), and *Ps. chlororaphis* IB-51 is very complex, as all the three strains antagonize phytopathogens while also being capable of producing phytohormones and improving the plant nitrogen intake. The ability of the strains *P. ehimensis* IB 739 and *Ps. koreensis* IB-4 to fix nitrogen is comparable to the nitrogenase activity of bacteria from the genus *Azotobacter* [16]. All of these strains produce cytokinins, albeit in negligible amounts; only *Ps. chlororaphis* IB-51 can produce auxins. Notably, *P. ehimensis* IB 739 and *Ps. koreensis* IB-4 also produce exopolysaccharides (EPS) [17, 18]. This is a positive trait for plant inoculation, as the presence of EPS in a bacterial preparation improves the viability of microbial cells on the seed surface.

As noted above, whether the useful traits of this or that microbe are practical depends on whether these bacteria survive in the rhizosphere of the host plant [19, 20]. Experimentation on spring wheat and cucumbers has revealed that *P. ehimensis* IB 739, *Ps. koreensis* IB-4, and *Ps. chlororaphis* IB-51 are able to successfully colonize the rhizosphere; the microbial population remains high throughout the growing season [21, 22].

This paper is the first to present a comprehensive comparison of the effects that introducing Rhizobacteria strains, each of which has a unique combination of growth-promoting traits, has on perennial and non-perennial legumes; it shows that even if a strain is able to promote the growth of legumes, it might still fail to boost productivity.

The goal hereof is to analyze how the growth-promoting strains *Paenibacillus ehimensis* IB 739, *Pseudomonas koreensis* IB-4, and *Ps. chlororaphis* IB-51 affect a plant's ecosystem, i.e. the aboriginal microbial community.

Techniques. The experimental tests carried out in 2017 used clayey-illuvial chernozem: 4.2% total humus, 0.5% total nitrogen, 5.6 mg of labile phosphorus per 100 g of soil, $pH_{wat.}$ 6.3). To optimize the air and water parameters, each pot contained drainage (a 2-centimeter high layer) and air-dry non-sterile soil (1,300 g); the content of each pot was moisturized to 60% of its total water capacity. Phosphorus was quantified by Kirsanov's method; acidity was tested by potentiometry [23].

Seeds of pea (*Pisum sativum* L.) variety Chishminsky 95, white lupine (*Lupinus albus* L.) variety Dega, chickpea (*Cicer arietinum* L.) variety Zavolzhsky, alfalfa changeable (*Medicago* × *varia* Martyn) variety Galia, and white melilot (*Melilotus albus* Medik.) variety Chermasan were sterilized by soaking in 1%

potassium permanganate solution over 30 minutes, then rinsed 5 times with plenty of sterile tap water, then submerged in water for 24 hours for swelling, after which they were placed on moistened filter paper to grow over 24 hours at room temperature; the research team thus rated their germination in laboratory conditions.

Seeds were treated with a liquid culture of bacterial strains, the microbial titer of which was known (10^8 to 10^9 CFU/ml). *P. ehimensis* IB 739 was cultured in Medium I [24], *Ps. koreensis* IB-4 and *Ps. chlororaphis* IB-51 were cultured in King's B medium [25]. Seeds were inoculated at 10^5 cells per seed for larger seeds (pea, lupine, and chickpea) or at 10^3 cell per seed for smaller seeds (alfalfa, melilot). Pea, lupine, chickpea (5 seedlings each), alfalfa, and melilot (10 seedlings each) were planted in pots. Planting depth varied from 1 to 3 cm, although plants whose seed lobes would not surface (pea and chickpea) were planted deeper. For control, seeds were treated with sterile tap water. For additional monitoring of how the concentration of nitrogen in the soil was changed, some pots contained soil and but no plants. For reference, the team used Azotovit®, a well-known microbiological fertilizer based on *Azotobacter chroococcum* B-9029 (registered by Industrial Innovations LLC, Russia, http://azotovit.ru). Azototovit producers recommend it as a universal multipurpose fertilizer that can boost plant nitrogen intake, suppress phytopathogenic microflora, and promote the growth of the vegetative system (leaves, stems, and inflorescences), thus improving the yield.

Experiments were carried out at room temperature in natural light over 45 days. Plants were sampled on Days 18 and 45, the soil was sampled to analyze some microbial populations on Days 18 and 45; nitrogen was only quantified for Day 45 samples. Plants were extracted with soil and had their roots carefully washed; then the team measured the size and quantity of newly sprouted nodules. Total Kjeldahl nitrogen, TKN, was measured for dried plant samples. Root rot was analyzed and reported per the guidelines in [26]. The microbial population of the rhizosphere was quantified by inoculating serially diluted soil suspension onto agarized nutrient media: MPA (microorganisms using organic nitrogen), Ashby's medium (aerobic free-living nitrogen-fixing bacteria and oligonitrophiles), and Czapek-Dox medium mixed with lactic acid (micromycetes) [27].

Data was processed statistically in Microsoft Excel. For processing, the researchers calculated the means (M) and standard errors of the mean (±SEM). Significance was tested by Student's *t*-test with a threshold $p \leq 0.05$.

Results. Soil analysis showed the availability of high-solubility phosphates to legumes was average in the case of neutral pH. Such soil is hospitable to active forms of spontaneous nodule-bacteria strains.

Table 1 summarizes the data to visualize the positive effects of *P. ehimensis* IB 739, *Ps. koreensis* IB-4, and *Ps. chlororaphis* IB-51 on plants [16, 17, 21, 22]. The legumes experimented upon differ in habitat. Apparently, while *Rhizobium leguminosarum* (a pea microsymbiont) are ubiquitous, chickpea and lupine might lack the microsymbionts they need if planted in soil that has never previously hosted such plants.

For non-perennial legumes (pea, lupine, and chickpea), the laboratory germination rate was 92.5% to 98.0%. Forage grasses (alfalfa and melilot) had a lower germination rate of 77.0% and 82.0%, respectively. In this experiment, *Ps. koreensis* IB-4, *P. ehimensis* IB 739 cultures, and Azotovit® had positive effects on legume germination. Interestingly, alfalfa was the most susceptive species.

The germination rate peaked in seeds treated with a liquid *Ps. koreensis*

IB-4, culture, which resulted in a 15-20% increase in germination against the controls. *Ps. chlororaphis* IB-51 had very little to no effect (on pea and chickpea). These results are not consistent with data on the phytohormonal activity of strains, see Table 1. Thus, *Ps. chlororaphis* IB-51 far exceeds *Ps. koreensis* IB-4 in the synthesis of plant growth regulators; however, it did not have any positive effect on germination rates.

Indole-3-acetic acid (IAA) can be inhibitive at higher concentrations [28], with varying the amount of inoculated bacterial producers being the easiest way to regulate phytohormone concentration in the rhizosphere [29-31]. Earlier, Loginov found that a higher titer of *Ps. chlororaphis* IB-51 had negative effects on the germination rates in some vegetables [18]. Apparently, a similar effect was observed in this experiment, too. It has been hypothesized that if the strain-synthesized phytohormones are in a complex with exopolysaccharides, as is the case for *P. ehimensis* IB 739 and *Ps. koreensis* IB-4, the gradual dissociation of this complex mitigates and prolongs the phytohormonal effects on plants [32].

Auxins are believed to mainly stimulate root growth (lengthening and branching [31]) while also positively affecting the leaves. In turn, cytokinins mainly activate shoot growth rather than root growth; in fact, they might even inhibit the latter [33]. In this experiment, seed groups exposed to *Ps. chlororaphis* IB-51 (which produces auxins and cytokinins) mainly tended to grow longer roots, see Figure 1A, and more leaves. Growth promotion of the aerial part was mainly observed for seeds inoculated with *Ps. koreensis* IB-4 (a cytokinin producer) and Azotovit®, see Figure 1B. However, it would be undue to say that different strains affect different parts of their symbiotic plants. Thus, *P. ehimensis* IB 739 (a cytokinin producer) promoted the growth of shoots in pea, alfalfa, and melilot; and the growth of roots in lupine and chickpea.

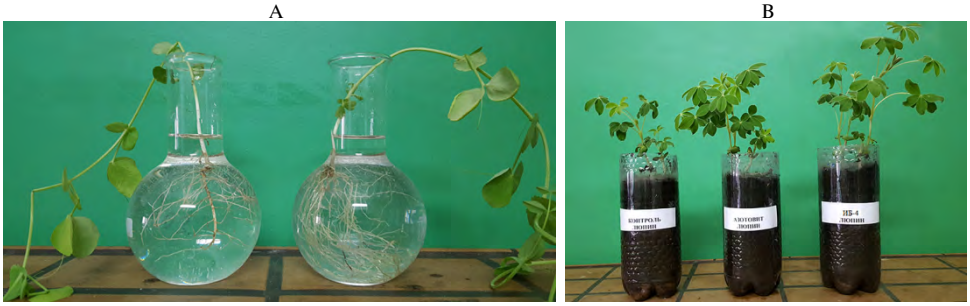

Fig. 1. Growth of roots and shoots in legumes under inoculation with PGPB strains: (A) pea (*Pisum sativum* L.), control to the left, *Pseudomonas chlororaphis* IB-51 treatment to the right; (B) white lupine (*Lupinus albus* L.), left to right: Azototovit® treatment, *Ps. koreensis* IB-4 treatment.

Ps. koreensis IB-4 and *Ps. chlororaphis* IB-51 affected pea, lupine, and chickpea the most; they had either limited or zero effect on alfalfa and melilot. Perhaps, this was due to toxins present in alfalfa and melilot seed husks, in particular, alkaloids [34], while the Pseudomonas strains under analysis were less resistant to microbicides than *Paenibacillus* or *Azotobacter*.

First nodules emerged on ~ Day 10 after sprouting. However, chickpea roots did not develop nodules throughout the experiment, which was logical. Chickpea is not a priority crop in the Republic of Bashkortostan; soil might lack the nodule bacteria specific to this species. Initially, pea roots developed the largest number of nodules, perhaps due to the fast growth of this species, see Table 2. However, melilot took lead by the end of the experiment in nodules/plant. Nodules would emerge on the taproot and on the secondary/side roots (mainly in the root hair zone), as well as on the stem. Treatment with a liquid *P. ehimensis* IB 739, *Ps. chlororaphis* IB-51 culture or Azotovit® resulted

1. Known positive effects of the analyzed plant growth-promoting bacteria (M±SEM) [16, 17, 21, 22]

Strain	Phytohormone synthesis, ng/ml LC		Ability to fix atmospheric nitrogen		Phytopathogen-antagonistic mechanisms		
	cytokinins	IAA	growth in Ashby's medium	nitrogenase activity, μg $N_2 \cdot ml^{-1} \cdot h^{-1}$	antibiotics	proteases	chitinases
Paenibacillus ehimensis IB 739	190.3±25.3	–	Moderate	0.67±0.021	+	+	+
Pseudomonas koreensis IB-4	119.0±17.3	40.4±4.7	Abundant	0.68±0.030	+	+	–
Ps. chlororaphis IB-51	205.4±42.1	878.1±93.2	Moderate	0.08±0.002	+	+	–

N o t e. LC stands for liquid culture, IAA stands for indole-3-acetic acid. Dash means that enzymes and metabolites were not detected..

2. Morphophysiological indicators of legumes as affected by treating seeds with growth-promoting strains at different post-inoculation times (M±SEM, pot test, 2017)

Test group	Day 18				Day 45			
	length of the aerial part, cm	taproot length, cm	number of leaves	number of nodules per plant	length of the aerial part, cm	taproot length, cm	number of leaves	number of nodules per plant
P e a (*Pisum sativum* L.), cv. Chishmishinskii 95								
Control (water)	32.3±1.7	11.0±0.8	6.3±0.7	2.1±0.9	73.7±5.8	16.2±0.9	12.7±1.3	9.0±2.5
Paenibacillus ehimensis IB 739	38.5±2.1*	11.3±0.8	7.0±0.5	3.7±1.2	77.6±4.1	15.7±1.5	14.5±2.1	12.7±4.4
Pseudomonas koreensis IB-4	38.0±2.2*	10.8±0.6	6.7±0.6	1.8±0.3	85.5±4.7*	18.2±0.9*	15.4±1.1*	20.4±3.8*
Ps. chlororaphis IB-51	36.3±2.8	12.7±0.7*	5.7±0.5	14.0±2.4*	76.3±6.3	18.4±0.8*	15.0±0.8*	20.3±5.1*
Azotovit®	38.7±2.5*	7.6±0.6*	5.5±0.3	6.8±3.9	84.0±4.2*	14.0±1.1*	14.0±2.5	10.4±1.7
W h i t e l u p i n e (*Lupinus albus* L.). cv. Dega								
Control (water)	14.7±0.9	8.3±0.6	4.0±0.5	0.3±0.1	29.1±2.5	14.5±2.1	10.5±1.4	10.2±2.0
Paenibacillus ehimensis IB 739	16.2±1.4	8.8±0.5	4.0±0.2	6.3±0.5*	30.0±3.2	18.3±1.5*	11.3±2.9	10.7±1.8
Pseudomonas koreensis IB-4	18.5±1.1*	8.8±0.5	5.0±0.6	–	34.3±2.6*	14.6±1.8	11.7±2.5	8.3±1.7
Ps. chlororaphis IB-51	16.8±1.3	10.0±0.8*	5.0±0.5	0.5±0.1	36.0±3.8*	18.7±1.5*	13.3±1.2*	17.0±2.5*
Azotovit®	17.7±1.1*	8.3±0.6	4.0±0.4	–	35.7±3.9*	15.3±2.8	11.3±1.9	16.0±1.9*
C h i c k p e a (*Cicer arietinum* L.), cv. Zavolzhskii								
Control (water)	28.5±1.9	12.7±1.1	9.4±0.6	–	38.1±3.0	17.0±1.8	16.0±2.8	–
Paenibacillus ehimensis IB 739	31.0±2.1	17.5±1.0*	10.3±0.7	–	40.0±3.7	20.3±2.5	15.3±2.1	–
Pseudomonas koreensis IB-4	27.0±1.5	15.3±0.8*	9.7±0.6	–	44.0±2.5*	22.5±3.1*	14.7±1.9	–
Ps. chlororaphis IB-51	31.3±1.8	13.8±0.8	10.7±0.9	–	44.0±2.7*	21.7±2.7*	17.0±2.9	–
Azotovit®	30.3±2.3	11.2±0.6	9.0±0.5	–	45.7±3.1*	16.3±2.5	16.0±2.9	–
A l f a l f a (*Medicago* × *varia* Martyn), cv. Galiya								
Control (water)	9.3±0.8	5.2±0.7	3.1±0.3	1.9±0.5	18.9±2.2	15.0±1.9	7.2±1.3	7.2±2.1
Paenibacillus ehimensis IB 739	9.8±0.7	4.1±0.5	3.0±0.3	1.8±0.3	23.1±1.7*	15.1±2.1	10.7±1.9*	18.4±4.9*

Table 2 continued

Pseudomonas koreensis IB-4	9.5±1.0	5.9±0.5	3.3±0.3	3.8±1.5	17.8±2.7	14.2±2.5	8.1±1.8	11.1±3.0
Ps. chlororaphis IB-51	8.8±0.7	4.7±0.4	2.8±0.4	1.8±0.2	18.1±2.1	14.8±2.5	8.0±1.9	10.6±1.9
Azotovit®	10.4±0.9	6.0±0.5	3.1±0.3	2.4±0.5	19.1±2.9	14.9±2.7	9.3±0.6*	25.2±6.7*
White melilot (*Melilotus albus* Medik.), cv. Chermasan								
Control (water)	7.2±0.6	5.0±0.4	2.9±0.3	1.5±0.6	14.0±0.9	14.3±1.1	5.6±0.7	12.5±2.6
P. ehimensis IB 739	8.5±0.5*	5.5±0.4	3.3±0.3	1.3±0.3	15.6±1.8	15.4±2.5	6.4±1.1	23.9±3.8*
Pseudomonas koreensis IB-4	8.3±0.6	5.6±0.5	2.7±0.2	3.0±1.0	14.0±1.3	12.3±2.0	6.0±1.0	13.6±1.8
Ps. chlororaphis IB-51	6.7±0.5	4.6±0.4	3.1±0.4	2.1±0.5	16.2±1.9	15.1±1.9	7.0±0.5*	16.6±2.9
Azotovit®	6.6±0.6	6.4±0.3*	2.8±0.3	0.6±0.3	20.6±3.1*	16.3±0.7*	7.7±0.9*	37.1±7.8*

Note. Dash means no nodules emerged.
* Difference from the control is statistically significant at $p \leq 0.05$.

3. Different microbial populations (CFU/g of abs. dry soil) **of the rhizosphere of legumes as affected by treating seeds with growth-promoting strains at different post-inoculation times** (*M*±SEM, pot test, 2017)

Test group	Pea (*Pisum sativum* L.)		Lupine (*Lupinus albus* L.)		Chickpea (*Cicer arietinum* L.)		Alfalfa (*Medicago* × *varia* Martyn)		White melilot (*Melilotus albus* Medik.)	
	Day 18	Day 45	Day 18	Day 45	Day 18	Day 45	Day 18	Day 45	Day 18	Day 45
Microorganisms using organic nitrogen, $\times 10^7$										
Control (water)	5.4±0.7	4.4±0.8	3.5±0.6	5.8±0.5	5.3±0.9	3.9±0.3	2.7±0.8	0.9±0.5	1.0±0.3	1.9±0.5
Paenibacillus ehimensis IB 739	6.3±0.8	1.0±0.1*	2.4±0.6	0.8±0.2*	6.4±0.7	1.2±0.2*	1.4±0.6	2.0±0.8	0.3±0.1*	2.5±0.5
Pseudomonas koreensis IB-4	4.3±0.6	1.7±0.2*	4.3±0.4	3.1±0.3*	3.3±1.3	1.3±0.2*	0.6±0.1*	2.0±0.7	0.5±0.2	3.1±0.9
Ps. chlororaphis IB-51	5.4±0.3	6.4±1.3	3.2±0.4	1.9±0.2*	4.2±0.7	0.7±0.1*	0.7±0.2*	2.0±0.6	1.0±0.2	2.9±0.6
Azotovit®	4.1±0.7	2.3±0.3*	3.2±0.5	1.5±0.2*	6.7±0.9	1.4±0.3*	1.1±0.4*	1.9±0.6	1.2±0.3	1.6±0.3
Aerobic free-living nitrogen-fixing bacteria and oligonitrophyles, $\times 10^7$										
Control (water)	1.2±0.3	2.0±0.4	1.6±0.2	1.2±0.4	2.8±0.6	1.1±0.2	1.2±0.5	1.7±0.3	0.8±0.2	1.3±0.5
P. ehimensis IB 739	2.1±0.6	2.4±0.2	1.6±0.2	1.3±0.3	3.6±0.5	1.1±0.1	1.5±0.3	1.5±0.3	0.9±0.2	2.1±0.5
Pseudomonas koreensis IB-4	1.6±0.3	1.5±0.3	2.0±0.3	0.8±0.2	3.0±0.4	1.2±0.1	0.5±0.2	1.7±0.5	0.9±0.2	2.5±0.8
Ps. chlororaphis IB-51	1.1±0.3	1.5±0.4	1.7±0.3	2.1±0.6	1.6±0.8	0.8±0.2	0.9±0.2	1.8±0.3	0.8±0.1	1.5±0.4
Azotovit®	0.8±0.2	1.1±0.6	1.9±0.3	1.2±0.3	2.2±0.4	1.2±0.2	1.4±0.3	1.8±0.2	0.5±0.1	1.9±0.5
Micromycetes, $\times 10^4$										
Control (water)	8.6±0.8	4.2±0.3	35.0±3.6	3.4±0.4	13.3±1.1	1.4±0.4	11.3±1.0	2.5±0.5	9.8±0.9	2.9±0.5
P. ehimensis IB 739	4.1±0.5*	1.8±0.2*	4.5±0.4*	3.0±0.3	3.3±0.3*	2.2±0.5	4.9±0.5*	3.9±1.0	1.6±0.3*	3.2±0.6
Pseudomonas koreensis IB-4	3.2±0.4*	1.5±0.1*	2.0±0.2*	2.6±0.5	2.1±0.2*	1.8±0.3	0.8±0.1*	2.3±0.5	0.5±0.1*	3.9±0.6
Ps. chlororaphis IB-51	4.7±0.6*	5.0±0.5	2.8±0.3*	1.1±0.2*	4.9±0.5*	1.4±0.2	2.3±0.3*	3.6±0.7	1.6±0.2*	2.2±0.4
Azotovit®	8.1±0.5	1.5±0.2*	3.4±0.4*	3.1±0.3	7.3±0.6*	2.3±0.5	1.7±0.2*	1.3±0.8	4.7±0.5*	2.6±0.4

* Difference from the control is statistically significant at $p \leq 0.05$.

in the emergence of larger pink nodules, mainly clustered around the taproot.

Bacterial inoculation mainly had positive effects on nodulation; however, plants of different genera responded differently to introducing PGPB into the microbial community of their rhizosphere. Pea plants treated with *Ps. chlororaphis* IB-51 and lupine treated with *P. ehimensis* IB 739 had several times higher nodulation than their respective controls ($p \leq 0.05$) as early as on Week 3. In alfalfa and melilot, there was no significant difference in nodulation between the controls and the experimental groups after two weeks of growth; by the end of the experiment, plants treated with *P. ehimensis* IB 739 and Azotovit® had 1.9 to 3.5 times more nodules ($p \leq 0.05$). Pseudomonas strains did not induce statistically significant differences in forages compared to the controls. In general, *P. ehimensis* IB 739 and Azotovit® promoted the nodulation of lupine, alfalfa, and pea; *Ps. chlororaphis* IB-51 was active in lupine and pea rhizosphere; *Ps. koreensis* IB-4 only affected pea.

No correlation was found between the number of nodules and the growth of roots. Roots were the longest in specimens treated with *P. ehimensis* IB 739 and *Ps. chlororaphis* IB-51, while *Ps. koreensis* IB-4 and Azotovit® did not have any significant effect on these indicators. The well-developed root system of lupines treated with *P. ehimensis* IB 739 had fewer nodules than their Azotovit®-treated counterparts.

Analysis of nitrogen fixation is for preliminary evaluations only, as the experiment only lasted until flowering.

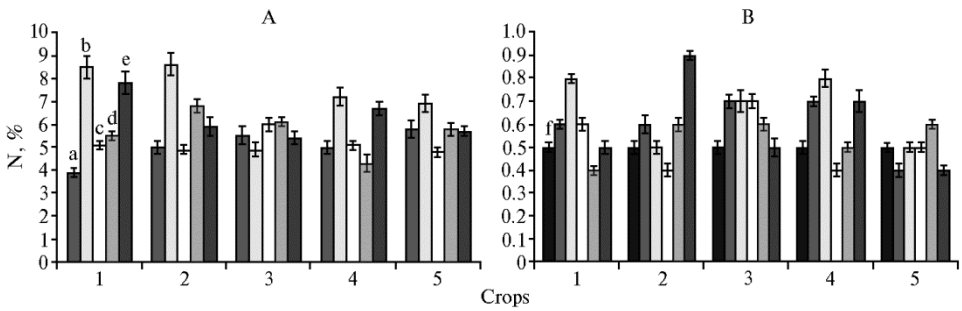

Fig. 2. Nitrogen content of pea (*Pisum sativum* L.) cv. Chishminskii 95 (1)**, white lupine (*Lupinus albus* L.) cv. Dega** (2)**, chickpea (*Cicer arietinum* L.) cv. Zavolzhskii** (3)**, alfalfa changeable (*Medicago × varia* Martyn) cv. Galiya** (4)**, white melilot (*Melilotus albus* Medik.) cv. Chermasan** (5) (A) **and soil after growing the plants** (B) **from seeds treated with PGPB strains:** a — controls, b — *Paenibacillus ehimensis* IB 739, c — *Pseudomonas koreensis* IB-4, d — *Ps. chlororaphis* IB-51, e — Azotovit®, f — plantless soil (pot test, 2017).

Nitrogen content of roots and biomass peaked in plants inoculated with *P. ehimensis* IB 739 and treated with Azotovit®, see Figure 2A. In pea, the indicator was 7.8% to 8.5% against 3.9% in the controls; in alfalfa, 6.7% to 7.2% against 5.0% ($p \leq 0.05$). After the plants were removed, the nitrogen content of soil was found to have been boosted from the initial value (0.5%) for pea, chickpea, and alfalfa treated with *P. ehimensis* IB 739, lupine treated with Azotovit®, as well as in the soil where chickpea had been exposed to *Ps. koreensis* IB-4 or not inoculated at all ($p \leq 0.05$), see Figure 2B. Apparently, *P. ehimensis* IB 739 was the greatest nitrogen fixation booster.

Notably, these data are not consistent with the nodulation readings. For instance, chickpea did not develop any nodules at all. Therefore, it only fixed nitrogen due to the non-Rhizobia nitrogen-fixing bacteria present in the rhizosphere of the host plant.

In *P. ehimensis* IB 739 experiments, the nitrogen reserves in soil were depleted while plants accumulated the element. At the same time, lupine treated

with Azotovit® did not consume nitrogen despite boosted nitrogen concentrations in the soil.

Analysis of these data did not identify any correlation between the nitrogenase/nodulating activity of the strains and the intensity of fixing nitrogen. Despite *P. ehimensis* IB 739 and *Ps. koreensis* IB-4 being equally able to fix atmospheric nitrogen, see Table 1, only *P. ehimensis* IB 739 boosted the nitrogen concentrations in soil and in peas. Seed treatment with a liquid *Ps. chlororaphis* IB-51 culture boosted nodulation of pea and lupine due to auxin synthesis, but neither the plants nor the soil accumulated nitrogen.

Microbiological assay of legume rhizosphere showed the microbial population near large-seed plants peaked early, then dropped. On the contrary, the microbial population of the rhizosphere was growing towards the end of the experiment in forages, see Table 3. This was assumingly due to the difference in lifecycles and nutrient accumulation strategies of perennials and non-perennials. By the end of the experiment, legumes had completed their active growth phase, which directly correlated with the displacement of nitrogen and other nutrients from vegetative to reproductive organs. However, perennials still continued to grow actively and to accumulate nutrients in their roots, which provided nutrition to the microbial community.

Notably, the rhizospheric population of heterotrophs was significantly larger in non-inoculated plants than in their inoculated counterparts ($p \leq 0.05$) by the end of the experiment. This can be interpreted as indirect evidence of introduced strains affecting the aboriginal bacterial community. The population of nitrogen-fixing microorganisms in the rhizosphere was 10^7 CFU/g of soil throughout the experiment for controls and experimental plants alike. Perennials tended to increase the nitrogen-fixing population towards the end of the experiment.

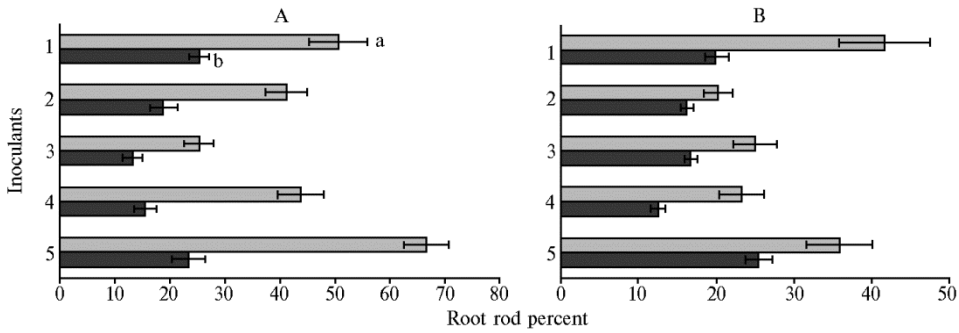

Fig. 3. Root rot infection in pea (*Pisum sativum* L.) cv. Chishminskii 95 (A) **and white lupine (*Lupinus albus* L.) cv. Dega** (B) **on Days 18** (a) **and 45** (b) **after inoculating the seeds with PGPB strains:** 1 — Azotovit®, 2 — *Pseudomonas chlororaphis* IB-51, 3 — *Ps. koreensis* IB-4, 4 — *Paenibacillus ehimensis* IB 739, 5 — controls (pot test, 2017).

Bacterial inoculation, as well as treatment with Azotovit®, suppressed the growth of microscopic fungi in the rhizosphere of 2-week plants. By the end of the experiment, the difference in micromycete population between the controls and the experimental groups was not significant. However, the strains continued to inhibit root rot throughout the experiment with an effectiveness of 33.7% to 62.1% for pea, 30.4% to 50.9% for white lupine, see Figure 3. Root rot morbidity was the highest in plants treated with Azotovit®.

Thus, the key finding is that introducing PGPB will not necessarily have positive effects on plants. Other researchers reported that bacterial treatment effectiveness could vary for different varieties of the same species [35, 36]. This is consistent with the reports of Yefimov et al. [37], who state that legumes are ex-

tremely selective with respect to introduced bacterial strain and may respond differently to biologicals.

Auxin-like substances are known to simulate nodulation [38]. At the same time, low IAA concentration stimulates nodulation while higher concentrations inhibit it [39]. This study failed to identify any correlation between the nodulation and the bacterial growth promotion, whether its performance or nature (cytokinin- or auxin-based). However, since the strains did not inhibit nodulation, it can be assumed that they did not suppress, or compete against, the aboriginal soil Rhizobia.

Speaking of the role the free-living nitrogen-fixing bacteria have to play in plant nitrogen intake, the experiment proves that greater biological nitrogen concentrations in the soil will not necessarily result in a greater accumulation thereof in plant roots and biomass [40]. One of the reasons why could be that the atmospheric nitrogen fixed by diazotrophs may remain unavailable to plants, as it localizes in microbial biomass [41].

Thus, inoculating legume seeds with *Paenibacillus* and *Pseudomonas*, both of which combine such positive effects of phytohormone production, nitrogenase activity, and antibiotic synthesis, did improve the germination rates, shoot and root growth, nitrogen intake, nodulation, and root-rot morbidity. Different legumes responded differently to PGPB introduction. In general, the experiments suggest *Paenibacillus ehimensis* IB 739 is good for alfalfa and white melilot, *Pseudomonas koreensis* IB-4 and *Pseudomonas chlororaphis* IB-51 are good for pea and white lupine.

REFERENCES

1. Sziderics A.H., Rasche F., Trognitz F., Sessitsch A., Wilhelm E. Bacterial endophytes contribute to abiotic stress adaptation in pepper plants (*Capsicum annuum* L.). *Canadian Journal of Microbiology*, 2007, 53(11): 1195-1202 (doi: 10.1139/W07-082).
2. Joo G.J., Kim Y.M., Kim J.T., Rhee I.K., Kim J.H., Lee I.J. Gibberellins-producing rhizobacteria increase endogenous gibberellins content and promote growth of red peppers. *Journal of Microbiology*, 2005, 43(6): 510-515.
3. Yang J., Kloepper J.W., Ryu C.M. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science*, 2009, 14(1): 1-4 (doi: 10.1016/j.tplants.2008.10.004).
4. Forchetti G., Masciarelli O., Alemano S., Alvares D., Abdala G. Endophytic bacteria in sunflower (*Helianthus annuus* L.): isolation, characterization, and production of jasmonates and abscisic acid in culture medium. *Applied Microbiology and Biotechnology*, 2007, 76(5): 1145-1152 (doi: 10.1007/s00253-007-1077-7).
5. Francis I., Holsters M., Vereecke D. The Gram-positive side of plant-microbe interactions. *Environmental Microbiology*, 2010, 12(1): 1-12 (doi: 10.1111/j.1462-2920.2009.01989.x).
6. van Loon L.C. Plant responses to plant growth-promoting rhizobacteria. *European Journal of Plant Pathology*, 2007, 119(3): 243-254 (doi: 10.1007/s10658-007-9165-1).
7. Berg G. Plant—microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology*, 2009, 84(1): 11-18 (doi: 10.1007/s00253-009-2092-7).
8. Raaijmakers J.M., Paulitz T.C., Steinberg C., Alabouvette C., Möenne-Loccoz Y. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*, 2009, 321(1-2): 341-361 (doi: 10.1007/s11104-008-9568-6).
9. Chandrashekhara, Niranjanranj S., Deepak S.A., Amrutesh K.N., Shetty N.P., Shetty H.S. Endophytic bacteria from different plant origin enhance growth and induce downy mildew resistance in pearl millet. *Asian Journal of Plant Pathology*, 2007, 1(1): 1-11 (doi: 10.3923/ajppaj.2007.1.11).
10. Walters D.R., Fountaine J.M. Practical application of induced resistance to plant diseases: an appraisal of effectiveness under field conditions. *The Journal of Agricultural Science*, 2009, 147(5): 523-535 (doi: 10.1017/S0021859609008806).
11. Spaepen S., Vanderleyden J., Remans R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiology Reviews*, 2007, 31(4): 425-448 (doi: 10.1111/j.1574-6976.2007.00072.x).
12. Gouws L.M., Botes E., Wiese A.J., Trenkamp S., Torres-Jerez I., Tang Y., Hills P.N., Usadel B., Lloyd J.R., Fernie A.R., Kossmann J., van der Merwe M. The plant growth promot-

ing substance, lumichrome, mimics starch, and ethylene-associated symbiotic responses in lotus and tomato roots. *Front. Plant Sci.*, 2012, 3: 120 (doi: 10.3389/fpls.2012.00120).
13. Khan W., Prithiviraj B., Smith D.L. Nod factor [Nod Bj V ($C_{18:1}$, MeFuc)] and lumichrome enhance photosynthesis and growth of corn and soybean. *Journal of Plant Physiology*, 2008, 165(13): 1342-1351 (doi: 10.1016/j.jplph.2007.11.001).
14. Hemissi I., Mabrouk Y., Abdi N., Bouraoui M., Saidi M., Sifi B. Effects of some *Rhizobium* strains on chickpea growth and biological control of *Rhizoctonia solani*. *African Journal of Microbiology Research*, 2011, 24(5): 4080-4090 (doi: 10.5897/AJMR10.903).
15. Akhtar M.S., Shakeel U., Siddiqui Z.A. Biocontrol of *Fusarium* wilt by *Bacillus pumilis*, *Pseudomonas alcaligenes* and *Rhizobium* sp. on lentil. *Turk. J. Biol.*, 2010, 34: 1-7 (doi: 10.3906/biy-0809-12).
16. Loginov O.N., Chetverikov S.P., Davletshin T.K., Kuzina E.V. *Shtamm azotfiksiruyushchikh bakterii Pseudomonas sp. dlya polucheniya biopreparata protiv zabolevanii pshenitsy, vyzyvaemykh fitopatogennymi gribami, i povysheniya urozhainosti. Patent RF № 2529958.* Zayavl. 20.08.2013. Opubl. 10.10.2014. Byul. № 28 [The strain of nitrogen-fixing bacteria *Pseudomonas* sp. for obtaining a biological product against wheat diseases caused by pathogenic fungi, and increasing yields. Patent RF № 2529958. Claims 20.08.2013. Publ. 10.10.2014. Bul. № 28] (in Russ.).
17. Rafikova G.F., Korshunova T.Yu., Minnebaev L.F., Chetverikov S.P., Loginov O.N. *Mikrobiologiya*, 2016, 85(3): 317-326 (doi: 10.7868/S0026365616030137) (in Russ.).
18. Khudaigulov G.G., Loginov O.N., Melent'ev A.I. *Izvestiya Samarskogo nauchnogo tsentra Rossiiskoi akademii nauk*, 2011, 5: 214-217 (in Russ.).
19. Boronin A.M. *Sorosovskii obrazovatel'nyi zhurnal*, 1998, 10: 25-31 (in Russ.).
20. Ivanchina N.V., Garipova S.R. *Agrokhimiya*, 2012, 7: 87-95 (in Russ.).
21. Melent'ev A.I. *Aerobnye sporoobrazuyushchie bakterii Bacillus Cohn v agroekosistemakh* [Aerobic spore-forming bacteria *Bacillus* Cohn in agroecosystems]. Moscow, 2007 (in Russ.).
22. Loginov O.N. *Bakterii Pseudomonas i Azotobacter kak ob"ekty sel'skokhozyaistvennoi biotekhnologii* [Bacteria *Pseudomonas* and *Azotobacter* as objects of agricultural biotechnology]. Moscow, 2005 (in Russ.).
23. Spirina V.Z., Solov'eva T.P. *Agrokhimicheskie metody issledovaniya pochv, rastenii i udobrenii* [Agrochemical methods for studying soils, plants and fertilizers]. Tomsk, 2014 (in Russ.).
24. Kuz'mina L.Yu. *Issledovanie vzaimodeistvii bakterii-antagonistov s rasteniyami i fitopatogennymi gribami. Avtoreferat kandidatskoi dissertatsii* [Study of the interactions of antagonist bacteria with plants and phytopathogenic fungi. PhD Thesis]. Kazan', 1998 (in Russ.).
25. Smirnov V.V., Kiprianova E.A. *Bakterii roda Pseudomonas* [*Pseudomonas* sp. bacteria]. Kiev, 1990 (in Russ.).
26. *Metodicheskie ukazaniya po gosudarstvennym ispytaniyam fungitsidov, antibiotikov i protravitelei semyan sel'skokhozyaistvennykh kul'tur* [Guidelines for State trials of fungicides, antibiotics and seed treaters of agricultural crops]. Moscow, 1985 (in Russ.).
27. Dzerzhinskaya I.S. *Pitatel'nye sredy dlya vydeleniya i kul'tivirovaniya mikroorganizmov* [Nutrient media for isolation and cultivation of microorganisms]. Astrakhan', 2008 (in Russ.).
28. Long H.H., Furuya N., Kurose D., Takeshita M., Takanami Y. Isolation of endophytic bacteria from *Solanum* sp. and their antibacterial activity against plant pathogenic bacteria. *Journal of the Faculty of Agriculture, Kyushu University*, 2003, 48(1-2): 21-28.
29. Persello-Cartieaux F., David P., Sarrobert C., Thibaud M.C., Achouak W., Robaglia C., Nussaume L. Utilization of mutants to analyze the interaction between *Arabidopsis thaliana* and its naturally root-associated *Pseudomonas*. *Planta*, 2001, 212(2): 190-198 (doi: 10.1007/s004250000384).
30. Compant S., Reiter B., Sessitch A., Nowak J., Clement C., Barka E.A. Endophytic colonization of *Vitis vinifera* L. by a plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Applied Microbiology and Biotechnology*, 2005, 71(4): 1685-1693 (doi: 10.1128/AEM.71.4.1685-1693.2005).
31. Idris E.E.S., Iglesias D.J., Talon M., Borriss R. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol. Plant-Microbe Interact.*, 2007, 20(6): 619-626 (doi: 10.1094/MPMI-20-6-0619).
32. Veselov S.Yu., Ivanova T.N., Simonyan M.V., Melent'ev A.I. *Prikladnaya biokhimiya i mikrobiologiya*, 1998, 34(2): 175-179 (in Russ.).
33. Kudoyarova G.R., Kurdish I.K., Melent'ev A.I. *Izvestiya UNTS RAN*, 2011, 3-4: 5-16 (in Russ.).
34. Joy S.G., George P. Phytochemical analysis of alfalfa (*Medicago sativa*) seed extract by soxhlet extraction using different solvents. *American Journal of Advanced Drug Delivery*, 2014, 2(2): 145-152.
35. Garipova S.R., Garifullina D.V., Markova O.V., Ivanchina N.V., Khairullin R.M. *Agrokhimiya*, 2010, 11: 50-58 (in Russ.).
36. Kumari S., Khanna V. Effect of antagonistic rhizobacteria inoculated with *Mesorhizobium ciceri* on control of fusarium wilt in chickpea (*Cicer arietinum* L.). *African Journal of Microbiology Research*, 2014, 8(12): 1255-1265 (doi: 10.5897/AJMR2013.6481).
37. Efimov V.N., Vorobeikov G.A., Patil A.B., Miryugina T.A., Bredikhin V.N., Khoty-

anovich A.V. *Agrokhimiya*, 1996, 1: 10-15 (in Russ.).
38. Ferguson B.J., Mathesius U. Phytohormone regulation of legume-rhizobia interactions. *Journal of Chemical Ecology*, 2014, 40(7): 770-790 (doi: 10.1007/s10886-014-0472-7).
39. van Noorden G.E., Ross J.J., Reid J.B., Rolfe B.G., Mathesius U. Defective long-distance auxin transport regulation in the *Medicago truncatula* super numeric nodules mutant. *Plant Physiology*, 2006, 140(4): 1494-1506 (doi: 10.1104/pp.105.075879).
40. Spehn E.M., Scherer-Lorenzen M., Schmid B., Hector A., Caldeira M.C., Dimitrakopoulos P.G., Finn J.A., Jumpponen A., O'Donnovan G., Pereira J.S., Schulze E.D., Troumbis A.Y., Kцrner C. The role of legumes as a component of biodiversity in a cross-European study of grassland biomass nitrogen. *Oikos*, 2002, 98: 205-218 (doi: 10.1034/j.1600-0706.2002.980203.x).
41. Shabaev V.P. *Pochvovedenie*, 2010, 4: 471-476 (in Russ.).

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POLYFUNCTIONAL PROPERTIES OF THE *Bacillus thuringiensis* var. *thuringiensis* INDUSTRIAL STRAIN 800/15

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Abstract

Crop losses caused by pests can reach 40-50 % and even more. Application of biological methods for regulation of the harmful species is promising and providing ecological safety. Biological preparations based on the living cultures of microorganisms and their metabolites meet these requirements. Currently, the crystal-forming bacterium *Bacillus thuringiensis* is considered to be the most important species for production of biological insecticides, since this bacterium exhibits high specificity in relation to the target pathogens, safety for humans and the environment. At ARRIAM, the biological preparation based on the *Bacillus thuringiensis* var. *thuringiensis* (*Bt*H_1) 800/15 strain was developed. The strain was isolated from larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say.) in the Leningrad region, studied for culture-morphological, biochemical and serological properties and identified according to the classification of De Barjac and Bonnefoi. Sequencing of the genes encoding 16S RNA and B-subunit of the DNA-gyrase (GyrB) confirmed that the isolated strain 800/15 belongs to *Bacillus thuringiensis* var. *thuringiensis*. The BtH_1 800/15 strain was deposited in the Russian Collection of Agricultural Microorganisms (RCAM) under the registration number 611 (Patent of the Russian Federation RU 2514211 C1 of 27.04.2014). This paper is the first to report that the BtH1 800/15-based biologicals increases the germination of seeds, the height of seedlings and the root length of various crops, and also revealed the inhibitory activity against phytopathogenic fungi. The goal of this study was to investigate whether the biological preparation based on the BtH_1 800/15 strain has multifunctional properties including entomocidal activity against mass insect pests of crops, growth-stimulating effect on economically significant plant species and antifungal activity against phytopathogenic fungi. The preparation based on the BtH_1 800/15 strain is a liquid that is easily diluted with water to the required concentration and contains the components of the cultural medium, spores and entomocidal exo- and endotoxins. The initial values of the biological activity of the preparation were as follows: titer was 3.5×10^9 CFU/ml, exotoxin content for the *Musca domestica* Linn. larvae in LC_{50} was 3.1 μl/g of feed, entomocidal activity for the larvae of the Colorado beetle *Leptinotarsa decemlineata* Say. in LC_{50} was 0,28 %. The paper presents the data of field trials of the effectiveness of the preparation carried out on different agricultural crops in the period of 2014-2017 in the Leningrad Region, Krasnodar and Primorsky Krai against phytophagous insects, the Colorado beetle (*L. decemlineata* Say.), the 28-spotted potato ladybird (*Henosepilachna vigintioctomaculata* Motsch.), the diamondback moth (*Plutella xylostella* L.), the cabbage white and the small white (*Pieris brassica* L., *P. rapae* L.), cabbage moth (*Barathra brassicae* L.), gooseberry sawfly (*Pteronidea ribesii* Scop.), red spider mite (*Tetranichus urtica* Koch.) and whitefly (*Trialeurodes vaporariorum* West.). Field tests demonstrated the effectiveness of this biological preparation against

harmful phytophagous insects (66.7-100 %). The laboratory tests revealed that the preparation did not exhibit phytotoxicity, moreover, it showed a growth-stimulating effect on the seed germination (up to 32 %), as well as the height of seedlings and root length (up to 52 %). The efficacy of the preparation against phytopathogenous fungi did not exceed 54 % and was inferior to the preparation based on the BtH_{10} strain 56. The combined use of the biological preparation based on the BtH_1 800/15 strain with the chemical insecticide Decis Extra, CE (emulsion concentrate) on potato against *H. vigintioctomaculata* Motsch. was very efficient (100 %) even if the application rates were reduced 2 and 3 times, respectively. This combination of biological and chemical insecticides is economically valuable and can be successfully used in potato fields when they are pest-infected, with the predominance of larvae of older ages and imago, which allows a significant reduction of the pesticide load. Overall, data obtained show that the biological preparation based on the *Bacillus thuringiensis* var. *thuringiensis* strain 800/15 has multifunctional properties, including entomocidal, antifungal and growth-stimulating activities, and is also promising for joint use with chemical insecticides.

Keywords: *Bacillus thuringiensis*, phytophagous insects, phytopathogens, polyfunctional properties, entomocidal, antifungal and growth-stimulating activity

The world market for chemical pesticides has recently entered a slowdown, while the market for biologicals is on the rise. The two markets are expected to equate by 2050 [1]. Biologicals are state-of-the-art alternatives to conventional chemical pesticides. They are eco-friendly; safe for humans, warm-blooded animals, and beneficial organisms; highly selective; effective against specific pests [2, 3].

Crops are increasingly protected by means of biologicals based on the genus *Bacillus*, which is pathogenic to phytophagous insects [4-6], phytopathogens [7-9], and plant pathogenic nematodes [10]. Biologicals based on various subspecies of *B. thuringiensis* Berliner (Bt) take the lead [11-13]. Insecticidal efforts at farms mainly used biologicals based on three pathogenic Bt variants. Pathovar A comprises Bt subspecies (var. *thuringiensis*), whose endotoxin crystals have greatest effects on *Lepidoptera*, Pathovar B comprises Bt subspecies (var. *israelensis*) that affect *Diptera* larvae, and Pathovar C comprises Bt subspecies (var. *tenebrionis*, var. *darmstadiensis*) that affect *Coleoptera* [4]. There has been discovered Pathovar F (for *fungi*) that affects plant pathogenic fungi [14].

The insecticidal traits of Bt arise from the synthesis of various toxins [15-17], the most important family of which is Cry, which comprises crystal delta endotoxins [18, 19]. In an insect's intestines, the protein crystal transforms into a protoxin, which further becomes a true toxin when exposed to serine proteases. The true toxin causes pathological processes and septicemia, a condition that enables microorganisms to colonize the pest's circulatory system, killing it. The thermostable water-soluble nucleotide β-exotoxin is released by bacteria into the environment; this very important substance has a metatoxic effect, as it tampers with the host insect's growth and metamorphosis [4, 20].

Bt-based biologicals have polyenzymatic traits; they have been discovered to produce a variety of hydrolases, which is seemingly what kills pests (insects and fungi) [21, 22]. Bt antifungal effects are also associated with the synthesis of other lytic enzymes, in particular, proteases and chitinases, which destroy the cell walls of phytopathogenic fungi [23, 24]. Recently, *Bacillus* bacteria have been discovered to synthesize lipopeptide antibiotics that have an antagonistic effect [25-27].

One aspect to the multifunctionality of *Bacillus* bacteria is that they can promote plant growth [28-30]. They colonize soil rhizosphere and rhizoplane, where they produce physiological agents that may activate plant resilience genes [31]. R&D of Bt-based biologicals requires finding and selecting active strains from natural sources (soil, affected insects, infected tissue and organs of plants); strains are selectively engineered to be easy to produce, to have strong broad-spectrum effects [32].

The All-Russian Research Institute of Agricultural Microbiology (ARRI-

AM) has developed a liquid broad-spectrum biological against mass plant pests, which is based on *B. thuringiensis* var. *thuringiensis* 800/15 (BtH_1 800/15) (Russian Patent No. 2514211 dd. April 27, 2014) [33]; it can be easily diluted in water to the required concentration and easily disseminated using standard sprayers.

This paper presents the first study of the plant growth promotion effects of the BtH_1 800/15-based biological, as well as its antifungal effects against phytopathogenic fungi. The biological has been found to improve germination rate by 6% to 21%, the seedling height by 4% to 52%, and the root length by 12% to 52% in a variety of crops. Its inhibitory efficacy against phytopathogens is up to 54%.

The goal hereof was to study the multifunctionality of a biological based on the strain *Bacillus thuringiensis* var. *thuringiensis* 800/15.

Techniques. Bacillus thuringiensis var. *thuringiensis* 800/15 (BtH_1 800/15) was isolated from dead Colorado beetle larvae collected in the Leningrad Region; the team studied the cultural, morphological, biochemical, and serological traits of the strain to classify it per De Barjac and Bonnefoi [34-36].

Gene sequences encoding 16S rRNA and B-subunits (*gyrB*) were employed to define the isolate by molecular phylogenetics. The strain was cultured in TY medium for 24 hours, after which 10^8 bacterial cells were moved to TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated for 10 minutes at 95 °C. The resulting DNA was used to amplify the sequences encoding 16S rRNA (27f 5′-GTTTGATCMTGGCTCAG-3′ primers), 1492R (5′-TACGGYTACCTTGTTACGACTT-3') (37), *gyrB* (gyrB_F 5′-CTTGAAGGACTAGARGCAGT-3′, gyrB_Rf 5′-CCTTCACGAACATCYTCACC-3′) [38] by PCR. The reaction mixture had a volume of 20 rl, which comprised 1 rl of bacterial DNA, 0.5 rl of each primer, and 10 rl of Fermentas-DreamTaq green PCR master mix (Thermo Fisher Scientific, USA). PCR was carried out on a T100tm Thermal Cycler (Bio-Rad, USA) using the following procedure: 15 minutes at 95 °C (for initial denaturation); 30 s at 94 °C (denaturation), 30 s at 55 °C (annealing), 1 min at 72 °C (elongation) (31 cycles); 10 minutes at 72 °C (final elongation). Sanger sequencing was performed at the Collective Lab for Genomic Technologies, Proteomics, and Cell Biology, ARRIAM [39]. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the closest strains in terms of 16S rRNA and *gyrB* sequences.

BtH_1 800/15 was deposited in the Russian Collection of Agricultural Microorganisms (RCAM) as a spore microorganism under the registration number 611. BtH_1 800/15 was cultured in dense nutrient media (meat-peptone agar, MPA, and fish agar, FA) at 28-30 °C until the spore generation and crystal endotoxin synthesis were complete. Microscopy was performed on Day 7 [40] using black aniline dye. Strain productivity was measured in yeast-polysaccharide media by submerged cultivation in Erlenmeyer flasks on an aerated rocker at 220 rpm and 28 °C over 72 hours.

ARRIAM's Ecos branch based in Kolpino, St. Petersburg, used this strain to produce a liquid biological. Cellular population, entomocidal activity (LC_{50} for the larvae of *Leptinotarsa decemlineata* Say from a natural population), and the exotoxin content (LC_{50} for domestic fly *Musca domestica* Linn.) were determined per [32].

The antifungal effects of Bt against various plant pathogenic fungi were determined by the agar block method in Petri dishes [41]. The biological based on BtH_1 800/15 was compared against a reference based on BtH_{10} 56. For tests, the team used the plant pathogenic fungi *Botrytis cinerea* Pers. (strain C-5), *Pythium* spp. (strain C-2), *Bipolaris sorokiniana* (Sacc.) Shoemaker (strain C-20), *Fusarium avenaceum* (Fr.) Sacc. (strain C-8), *F. solani* App. et Wr. (strain C-15), *Verticillium dahlia* Kleb. (strain 27). Liquid Bt biological (5% concentration) was

injected in a molten Czapek medium cooled down to 40 °C. Blocks cut out of a 10-day culture of the fungi were placed on the congealed agar. For control, the team used a medium that did not contain the biological. Fungal colony diameters were measured 5 days later. The inhibitory efficacy of Bt was calculated by the Abbot's formula [42]:

$$X = \frac{Dc - Dt}{Dc} \times 100,$$

where X is the degree of inhibiting fungal colony growth, %; D_c and D_t are the diameters of fungal colonies (control and test, respectively), cm.

The phytoregulatory effects of the BtH_1 800/15-based biological were assessed by laboratory germination of seeds in rolls of filter paper at 26 °C and a humidity of 86%. Tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), marrow (*Cucurbita pepo* var. *giromontina*), beet (*Beta vulgaris*), pumpkin (*Cucurbita pepo*), and cabbage (*Brassica oleracea*) seeds were soaked for 3 hours in a liquid culture of BtH_1 800/15, as well as in a nutrient medium for control. Germination was rated in 5 days; seedling and root length was measured in 3 weeks [43].

Field tests were carried out at farms in the Leningrad Region, Krasnodar Territory, and Primorye Territory in 2014-2017. Vegetating *Solanum tuberosum* plants were treated with a liquid biological suspension while the Colorado beetle larvae L_{1-2} were hatching. In the Krasnodar Territory, soy (*Glycine max*) was treated against red spider mite (*Tetranychus urticae* L.) in the field, while cucumbers were treated against red spider mite and whitefly (*Trialeurodes vaporariorum* West.) indoors. The biological was applied at 15 to 20 l/ha; the solution application rate was 200 to 400 l/ha.

In the Leningrad Region, white cabbage (*Brassica oleracea*) was treated against diamondback moth (*Plutella xylostella* L.), cabbage butterfly, and small white (*Pieris brassicae* L., *P. rapae* L.), cabbage moth (*Barathra brassicae* L.) (10-15 l/ha); gooseberry (*Ribes uva-crispa*) was treated against common gooseberry sawfly (*Pteronidea ribesii* Scop.) (20 l/ha). In the Primorye Territory, potato was treated against 28-spotted potato ladybird (*Henosepilachna vigintioctomaculata* Motsch.) with the tested biological, as well as with Decis Extra, CE (emulsion concentrate, Decis®, Bayer AG, Germany), both applied in recommended dosages (15 and 0.03 l/ha, respectively), also mixed in a tank with reduced dosage (7.5 l/ha and 0.01 l/ha).

The collected data was processed by analysis of variance at 95% CI [44]. For processing, the researchers calculated the means (*M*) and the standard errors of the mean (±SEM). Significance was tested by Student's *t*-test at a confidence interval of 95% ($p < 0.05$).

Results. Following the conventional approach [34-36], the strain 800/15 had earlier been described as *B. thuringiensis* var. *thuringiensis*. We confirmed its taxonomy by molecular systematics. Using BLAST, *Bacillus cereus* VLS-S-1, *Bacillus thuringiensis* HC2, *Bacillus toyonensis* PgBe301 were identified as strains of a nearly identical 16S rRNA sequence. Thus, the 16S rRNA locus is too conservative for accurate identification of the strain's taxonomy, but provides proving the strain to be a *Bacillus*.

To clarify the phylogenetic position of 800/15, wwe sequenced its gene *gyrB* that encodes the B-subunit of DNA gyrase. This locus is less conservative than 16S rDNA, which helps differentiate phylogenetically close groups. The *gyrB* sequence in 800/15 was found to be closer to that of *Bacillus thuringiensis* ATCC 10792 and *Bacillus thuringiensis* Bt 407, earlier classified as serotype BtH_1 [37]. Thus, molecular systematics confirmed that 800/15 was indeed *B. thuringiensis* var. *thuringiensis*.

The next step was to find whether the BtH_1 800/15-based biological was

multifunctional. To that end, its samples were first tested for activity. The titer was 3.5-3.6±0.2 billion CFU/ml, with the *Musca domestica* exotoxin concentration at LC_{50} being 3.1-3.2±0.1 rl/g of food and the entomocidal activity against *Leptinotarsa decemlineata* larvae at LC_{50} being in the range 0.26-0.28±0.02%.

In field tests, the biological killed 70% to 100% of insects, see Table 1. Its application improved the yield of potatoes (Leningrad Region, Krasnodar Territory) and cabbage (Leningrad Region) as compared to the controls (no data available).

1. Efficacy of the liquid *Bacillus thuringiensis* var. *thuringiensis* 800/15-based biological as tested on phytophages in different Russian regions over 2014-2017

Pest species	Geographic location	Crop	Biological efficacy, %
Leptinotarsa decemlineata Say.	Krasnodar Territory, Leningrad Region	Potato	95.9-98.8
Henosepilachna vigintioctomaculata Motsch.	Primorye Territory	Potato	66.7-70.0
Tetranichus urticae Koch.)		Cucumber	97.6-98.4
	Krasnodar Territory	Soybean	95.8-96.2
Trialeurodes vaporariorum West.	Krasnodar Territory	Cucumber	99.8-100.0
Plutella maculipennis Curt.	Leningrad Region	Cabbage	80.0-90.1
Pieris brassicae L.	Leningrad Region	Cabbage	94.7-95.6
Pieris rapae L.	Leningrad Region	Cabbage	84.5-96.7
Barathra brassicae L.	Leningrad Region	Cabbage	90.0-92.8
Pteronidear ribesii Scop.	Leningrad Region	Gooseberry	70.8-72.4

N o t e. Efficacies are averaged-based.

Laboratory tests showed the biological was not phytotoxic; on the contrary, it promoted plant growth, e.g. it increased the germination rates of: beet and cabbage by 6%, cucumbers by 10%, marrows by 21%, tomatoes by 11%, and pumpkins by 21%; beet seedlings gained 4% in height, cucumbers gained 8%, marrows gained 52%, cabbage gained 36%, and pumpkins gained 38%; root length rose by 12% in beet, 19% in cucumbers, 52% in marrows, 28% in tomatoes, 48% in cabbage, and 42% in pumpkins, see Table 2. The crops responded differently to BtH_1 800/15. Marrows, tomatoes, cabbage, and pumpkins were stimulated to a far greater extent than beet or cucumbers. Seedlings that sprouted from treated tomato, cabbage, pumpkin, and marrow seeds were 6.4±0.7 to 14±0.75 cm or 36% to 52% higher than controls (4.2±0.65 to 9.9±0.25 cm); the roots of treated cabbage, pumpkin, and marrow were 7.4±0.55; 15.9±0.75, or 22.5±0.9 cm longer than the controls (5.0±0.25; 11.2±0.45, or 14.8±0.75 cm (difference from the controls deemed significant at $p < 0.05$). The biological also proved to significantly boost the pumpkin, cabbage, and marrow root growth (by 42-52%).

The BtH_1 800/15-based biological had antifungal effects, albeit weaker than those of BtH_{10} 56, see Table 3. On average, the fungi *Botrytis cinerea* Pers., *Pythium* spp., *Bipolaris sorokiniana* and *Verticillium dahliae* were inhibited by BtH_1 800/15 by 51.15±1.75; 40.35±1.65; 21.55±1.45, and 18.45±0.45%, respectively. A further selection of BtH_1 800/15 for antifungal effects requires more specific criteria, which will be set forth by further research.

In the Primorye Territory, the BtH_1 800/15-based biological was compared against Decis Extra, CE for protection against 28-spotted potato ladybird. Tests showed mixing both agents in lower doses was as efficient as the chemical reference, as 100% of the pest was dead on the 10th post-treatment day. Thus, combining these agents is appropriate and cost-effective for fields inhabited by this pest, predominantly by older larvae and imago.

Laboratory tests showed that neither at sublethal dosages nor at higher-than-recommended concentrations in combination did Decis Extra hinder the viability of BtH_1.

2. *Bacillus thuringiensis* var. *thuringiensis* 800/15-based biological and its phytoregulatory effects on different crops

Crop	Seed germination, %			Seedling height, cm			Root length, cm		
	controls	post-treatment		controls	post-treatment		controls	post-treatment	
		total	percent of the control		total	percent of the control		total	percent of the control
Beet	92-96	99	106	4.2-5.0	4.8-5.1	104	3.5-4.0	3.8-4.7	112
Cucumbers	87-90	99	110	4.6-5.3	5.2-5.7	108	4.9-5.2	4.9-5.2	119
Marrows	77-80	91-96	120	9.7-10.2	11.4-15.7	142	14.0-15.9	14.0-15.9	152
Tomatoes	88-91	99	111	4.3-4.8	5.6-7.0	152	12.9-14.0	12.9-14.0	128
Cabbage	92-94	99	106	4.9-5.9	7.1-7.9	136	4.8-5.3	4.8-5.3	148
Pumpkin	77-80	91-98	120	7.4-8.3	10.4-11.3	138	10.7-11.8	10.7-11.8	142

3. Antifungal effects of the strains *Bacillus thuringiensis* var. *thuringiensis* (BtH_1) and *Bacillus thuringiensis* var. *darmstadiensis* (BtH_{10})

Вид фитопатогенного гриба	Ингибирование роста колоний гриба через 5 сут, %	
	BtH_1 800/15	BtH_{10} 56
Botrytis cinerea Pers (штамм С-5)	48,3-54,0	100
Pythium spp. (штамм С-2)	38,7-42,0	83,2-86,5
Bipolaris sorokiniana (Sacc) Shoemaker (штамм С-20)	20,1-23,0	74,1-75,3
Fusarium avenacrum (Fr.) (Sacc) (штамм С-8)	0	50,0-51,2
Fusarium solani App. et Wr. (штамм С-3)	0	25,2-26,0
Verticillum dahlia Kleb. (штамм 27)	18,0-18,9	50,7-52,0

Примечание. Ингибирующую активность препарата рассчитывали по формуле W.S. Abbot (см. раздел «Методика»).

Combining chemicals and biologicals subjects plants to less stress and is a cost-effective solution, as both agents can thus be applied in lesser quantities. The synergistic effects of *B. thuringiensis*-based biologicals in combination with chemicals have been noted in research literature. Thus, using *B. thuringiensis* var. *israelensis*-based bactoculicide in combination with dimilin, a chitin synthesis inhibitor, at a quarter to an eighth of the recommended dosage did kill up to 91.2% *Lycoriella solani* Winn. while boosting the yield by up to 58.6% [45]. Ivantsova evaluated *B. thuringiensis* var. *darmstadiensis*-based bacicol in combination with Fastac®, BASF SE, Germany, at low-toxic dosage; the agents amplified each other, and the combination was extremely efficient against mustard pests [46]. Bactoculicide has been proven effective against *Cricotopus sylvestris* Fabr. in combinations with Proponit®, Arysta LifeScience S.A.S., France, Basagran®, BASF SE, Germany, 2M-4X MCPA, Bayer AG, Germany, and Ordram®, Syngenta AG, Switzerland. The herbicides had no identifiable negative effects on the viability of *B. thuringiensis* var. *israelensis* [47].

Thus, the *Bacillus thuringiensis* var. *thuringiensis* 800/15-biological has entomocidal, plant growth-promoting, and antifungal effects. It is a promising solution for combating mass pests affecting a wide range of crops, from potatoes and cruciferous crops to berries. Use in combination with a chemical insecticide seems appropriate and cost-effective for fields populated with older larvae and imago of *Leptinotarsa decemlineata* Say. and *Henosepilachna vigintioctomaculata* Motsch.

REFERENCES

1. Olson S. An analysis of the biopesticide market now and where it is going. *Outlooks on Pest Management*, 2015, 26(5): 203-206 (doi: 10.1564/v26_oct_04).
2. Siegel J.P. The Mammalian Safety of *Bacillus thuringiensis*- Based Insecticides. *Journal of Invertebrate Pathology*, 2001, 77(1): 13-21 (doi: 10.1006/jipa.2000.5000).
3. Raymond B., Federici B.A. In deference of *Bacillus thuringiensis*, the safest and most success the microbial insecticide available to humanity — a response to EFSA. *FEMS Microbiology Ecology*, 2017, 93(7): fix084 (doi: 10.1093/femsec/fix084).
4. Kandybin N.V., Patyka T.I, Ermolova V.P., Patyka I.F. *Mikrobiokontrol' chislennosti nasekomykh i ego dominanta Bacillus thuringiensis.* St. Petersburg—Pushkin, 2009 (in Russ.).
5. Polanczyk R.A., Pires da Silva R.F., Fiuza L.M. Effectiveness of *Bacillus thuringiensis* against *Spodoptera frugipera* (*Lepidoptera*: *Noctuidae*). *Brazilian Journal of Microbiology*, 2000, 31(3): 165-167 (doi: 10.1590/S1517-83822000000300003).
6. Eswarapriya B., Gopalsamy B., Kameswari B., Meera R., Devi P. Insecticidal activity of *Bacillus thuringiensis* IBt-15 strain against *Plutella xylostella*. *International Journal of PharmTech Research*, 2010, 2(3): 2048-2053.
7. Seo D.J. Nguyen D.M.S., Song Y.S., Jung W.J. Induction of defense response against *Rhizoctonia solani* in cucumber plants by endophytic bacterium *Bacillus thuringiensis* GS1. *J. Microbiol. Biotechnol.*, 2012, 22(3): 407-415 (doi: 10.4014/jmb.1107.07027).
8. Martinez-Absalón S., Rojas-Solís D., Hernández-Leyn R., Prieto-Barajas C., Orozco-Mosqueda M., Peña-Cabriales J.J., Sakuda S., Valencia-Cantero E., Santoyo G. Potential use and mode of action of the new strain *Bacillus thuringiensis* UM96 for the biological control of the grey mould phytopathogen *Botrytis cinerea*. *Biocontrol Science and Technology*, 2014, 24(12):

1349-1362 (doi: 10.1080/09583157.2014.940846).
9. Heydari A., Pessaraki M. A review on biological control of fungal plant pathogens using microbial antagonists. *Journal of Biological Sciences*, 2010, 1(4): 273-290 (doi: 10.3923/jbs.2010.273.290).
10. Mohammed S.H., El Saedy M.A., Enan M.R., Nasser E.I., Ghareeb A., Salah A.M. Biocontrol efficiency of *Bacillus thuringiensis* toxins against root-knot nematode, *Meloidogyne incognita*. *J. Cell Mol. Biol.*, 2008, 7(1): 57-66.
11. Pane C., Villecco D., Campanile F., Zaccardelli M. Novel strains of *Bacillus* isolated from compost and compost-amended soils as biological control agents against soil-borne phytopathogenic fungi. *Biocontrol Science and Technology*, 2012, 22(12): 1373-1388 (doi: 10.1080/09583157.2012.729143).
12. Akram W., Mahboob A., Jave A.A. *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against *Fusarium* wilt. *European Journal of Microbiology and Immunology*, 2013, 3(4): 275-280 (doi: 10.1556/EuJMI.3.2013.4.7).
13. Tao A., Pang F., Huang S., Yu G., Li B., Wang T. Characterization of endophytic *Bacillus thuringiensis* strains isolated from wheat plants as biocontrol agents against wheat flag smut. *Biocontrol Science and Technology*, 2014, 24(8): 901-924 (doi: 10.1080/09583157.2014.904502).
14. Smirnov O.V. *Patotipy Bacillus thuringiensis i ekologicheskie osnovy ikh ispol'zovaniya v zashchite rastenii. Avtoreferat doktorskoi dissertatsii* [Pathotypes of *Bacillus thuringiensis* and the ecological basis of their use in plant protection. DSc Thesis]. Petersburg—Pushkin, 2000 (in Russ.).
15. Shrestha A., Sultana R., Chae J.-C., Kim K., Lee K.-J. *Bacillus thuringiensis* C25 which is rich in cell wall degrading enzymes efficiently controls lettuce drop caused by *Sclerotinia minor*. *Eur. J. Plant Pathol.*, 2015, 142(3): 577-589 (doi: 10.1007/s10658-015-0636-5).
16. Loseva O., Ibrahim M., Candas M., Koller C.N., Bauer L.S., Bulla Jr L.A. Changes in protease activity and Cry3Aa toxin binding in the Solorado potato beetle: implications for insect resistance to *Bacillus thuringiensis* toxins. *Insect Biochemistry and Molecular Biology*, 2002, 32(5): 567-577 (doi: 10.1016/S0965-1748(01)00137-0).
17. Zhong C.H., Ellar D.J., Bishop A., Johnson C., Lin S.S., Hart E.R. Characterization of *Bacillus thuringiensis* δ-endotoxin which is toxic to insects in three orders. *Journal of Invertebrate Pathology*, 2000, 76(2): 131-139 (doi: 10.1006/jipa.2000.4962).
18. Aronson A.I., Shai Y. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiology Letters*, 2001, 195(1): 1-8 (doi: 10.1111/j.1574-6968.2001.tb10489.x).
19. Knaak N., Rohr A., Fiuza L. In vitro effect of *Bacillus thuringiensis* strains and Cry proteins in phytopathogenic fungi of paddy rice-field. *Brazil. J. Microbiol.*, 2007, 38(3): 526-530 (doi: 10.1590/S1517-83822007000300027).
20. Shternshis M.V., Belyaev A.A., Tsvetkova V.P. Shpatova T.V., Lelyak A.A., Bakhvalov S.A. *Biopreparaty na osnove bakterii roda Bacillus dlya upravleniya zdorov'em rastenii* [*Bacillus*-based biological products for plant health management]. Novosibirsk, 2016 (in Russ.).
21. Wagner W., Möhrlen F., Schnetter W. Characterization of the proteolytic enzymes in the midgut of the European Cockchafer, *Melolontha melolontha* (*Coleoptera*: *Scarabaidae*). *Insect Biochemistry and Molecular Biology*, 2002, 32(7): 803-814 (doi: 10.1016/S0965-1748(01)00167-9).
22. Smirnov O.V., Grishechkina S.D. Polyfunctional activity of *Bacillus thuringiensis* Berliner. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2011, 3: 123-126 (in Russ.).
23. Reyes-Ramírez A., Escudero-Abarca B.I., Aguilar-Uscanga G., Hayward-Jones P.M., Barboza-Corona J.E. Antifungal activity of *Bacillus thuringiensis* chitinase and its potential for the biocontrol of phytopathogenic fungi in soybean seeds. *Journal of Food Science*, 2004, 69(5): M131-M134 (doi: 10.1111/j.1365-2621.2004.tb10721.x).
24. Xiao L., Xie C.-C., Cai J., Lin Z.-J., Chen Y.-H. Identification and characterization of chitinase producing *Bacillus* slowing significant antifungal activity. *Curr. Microbiol.*, 2009, 58(5): 528-533 (doi: 10.1007/s00284-009-9363-5).
25. Kim P.I., Bai H., Bai D., Chae H., Chung S., Kim Y., Park R., Chi Y.-T. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *Journal of Applied Microbiology*, 2004, 97(5): 942-949 (doi: 10.1111/j.1365-2672.2004.02356.x).
26. Yánez-Mendizábal V., Zeriouh H., Viñas I., Torres R., Usall J., de Vicente A., Pérez-García A., Teixidó N. Biological control of peach brown rot (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides. *Eur. J. Plant Pathol.*, 2011, 132(4): 609-619 (doi: 10.1007/s10658-011-9905-0).
27. Elkahoui S., Djébalin N., Karkouch I., Hadj Ibrahim A., Kalai L., Bachkouel S., Tabbene O., Limam F. Mass spectrometry identification of antifungal lipopeptides from Bacillus sp. BCLRB2 against *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. *Appl. Biochem. Microbiol.*, 2014, 50(2): 161-165 (doi: 10.1134/S0003683814020082).
28. Choudhary D.K., Johri B.N. Interactions of *Bacillus* spp. and plants — With special reference to induced systemic resistance (ISP). *Microbiological Research*, 2009, 164(5): 493-513 (doi: 10.1016/j.micres.2008.08.007).
29. Kumar P., Dubey R.C., Maheshwari D.K. *Bacillus* strain isolated from rhizosphere showed

plant growth promoting and antagonistic activity against phytopathogens. *Microbiological Research*, 2012, 167(8): 493-499 (doi: 10.1016/j.micres.2012.05.002).
30. Chebotar' V.K., Naumkina T.S., Borisov A.Yu. *Kompleksnoe mikrobnoe udobrenie «Bisolbimiks»* /Pod redaktsiei A.Yu. Borisova [Complex microbial fertilizer Bisolbimix. A.Yu. Borisov (ed.)]. St. Petersburg, 2015 (in Russ.).
31. Partida-Martínez L.P., Heil M. The microbe-free plant root: fact or artifact? *Frontiers in Plant Science*, 2011, 2(100): 1-16 (doi: 10.3389/fpls.2011.00100).
32. Grishechkina S.D., Ermolova V.P., Minina G.N., Safronova V.I., Bologova E.V. *Metodika. Kollektsiya shtammov bakterii-simbiontov vrednykh nasekomykh i gryzunov, prigodnykh dlya biokontrolya chislennosti vreditelei sel'skokhozyaistvennykh rastenii* [Collection of bacterial symbionts in harmful insects and rodents, suitable for biocontrol of agricultural plant pests]. St. Petersburg, 2014 (in Russ.).
33. Tikhonovich I.A., Ermolova V.P., Grishechkina S.D., Romanova T.A. *Shtamm bakterii Bacillus thuringiensis var. thuringiensis №800/15 v kachestve sredstva dlya polucheniya entomotsidnogo biopreparata*. Patent RU 2514211 S1. Zayavl. 10.10.2012. Opubl. 27.04.2014. Byul. № 12 [The strain of *Bacillus thuringiensis* var. *thuringiensis* №800/15 for entomocidal biopreparation. Patent RU 2514211 S1. Claims 10.10.2012. Publ. 27.04.2014. Bul. № 12] (in Russ.).
34. De Barjac H., Bonnefoi A. Essai de classification biochmique et seroloque de 24 coucher de *Bacillus* du type *B. thuringiensis. Entomophaga*, 1962, 7(1): 5-31 (doi: 10.1007/BF02375988).
35. De Barjac H., Bonnefoi A. A classification strains of *Bacillus thuringiensis Berliner* with a key to their differentiation. *Journal of Invertebrate Pathology*, 1968, 11(3): 335-347 (doi: 10.1016/0022-2011(68)90182-1).
36. De Barjac H., Bonnefoi A. Misse au point sur la classification des *Bacillus thuringiensis. Entomophaga*, 1973, 18(1): 5-17 (doi: 10.1007/BF02373009).
37. Lane D.J. 16S/23S rRNA sequencing. In: *Nucleic acid technics in bacterial systematics.* E. Stackebrandt, M. Goodfellow (eds.). John Wiley & Sons, Chichester, NY, 1991: 115-175.
38. Punina N.V., Zotov V.S., Parkhomenko A.L., Parkhomenko T.U., Topunov A.F. Genetic diversity of *Bacillus thuringiensis* from different geo-ecological regions of Ukraine by analyzing the 16S rRNA and *gyrB* genes and by AP-PCR and saAFLP. *Acta Naturae*, 2013, 5(1): 90-100.
39. Sanger F., Nicklen S., Coulson A.R. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 1977, 74(12): 5463-5467 (doi: 10.1073/pnas.74.12.5463).
40. Kol'chevskii A.G., Rybina L.M., Kolomiets V.Ya. *Vydelenie i otbor vysokovirulentnykh kul'tur Bacillus thuringiensis var. galleriae. Metodicheskie rekomendatsii* /Pod redaktsiei V.A. Pavlyushina [Isolation and selection of highly virulent cultures of *Bacillus thuringiensis* var. *galleriae*. Guidelines. V.A. Pavlyushin (ed.)]. Leningrad, 1987 (in Russ.).
41. *Metody eksperimental'noi mikologii* /Pod redaktsiei V.I. Bilai [Methods of experimental mycology. V.I. Bilai (ed.)]. Kiev, 1982 (in Russ.).
42. Abbot W.S. A method for computing the effectiveness of insecticide. *Journal of Economic Entomology*, 1925, 18(2): 265-267 (doi: 10.1093/jee/18.2.265a).
43. Benken A.A., Khatskevich L.K., Grishechkina S.D. *Uskorennyi metod otsenki bolezneustoichivosti sortoobraztsov yachmenya i paraziticheskikh svoistv vozbuditelya fuzarioznoi kornevoi gnili. Informatsionnyi listok* [A rapid assessment of disease resistance in barley varieties and parasitic activity of the Fusarium root rot causative agent]. St. Petersburg, 1983 (in Russ.).
44. Dospekhov B.A. *Metodika polevogo opyta* [Methods of field trials]. Moscow, 1985 (in Russ.).
45. Mokrousova E.P., Glazunova I.N., Kandybin N.V., Ermolova V.P. *Sovmestnoe primenenie ingibitorov sinteza khitina s mikrobiologicheskimi preparatami protiv vreditelei shampin'onov. Problemy entomologii v Rossii* [Combined use of chitin synthesis inhibitors with microbiological preparations against champignon pests. Problems of entomology in Russia. Vol. 2]. St. Petersburg, 1998, tom 2: 40-41 (in Russ.).
46. Ivantsova E.A. *Optimizatsiya fitosanitarnogo sostoyaniya agrobiotsenozov Nizhnego Povolzh'ya. Avtoreferat doktorskoi dissertatsii* [Optimization of the phytosanitary state of agrobiocenoses in the condition Lower Volga region. DSc Thesis]. Saratov, 2009 (in Russ.).
47. Stus' A.A., Ermolova V.P. Kandybin N.V. *Byull. VNIISKHM*, 1989, 52: 25-27 (in Russ.).

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ENTOMOPATHOGENIC FUNGUS *Beauveria bassiana* (Bals-Criv.) Vuill. AS A PROMISING AGENT FOR THE RASPBERRY CANE MIDGE *Resseliella theobaldi* (Barnes) BIOCONTROL

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Abstract

Red raspberry *Rubus idaeus* L. is a widespread crop including Eurasian continent. This berry crop is often damaged by the dangerous pest, the raspberry cane midge *Resselliella theobaldi* (Barnes). This insect pest commonly destroys 30-80 % of raspberry canes and decreases berry yield 5-6-fold. Chemical insecticides predominate in raspberry plant protection against the pest. However, the trend of recent years lays in replacement of chemicals for ecologically safe biological agents, especially for soft fruit plant protection. The entomopathogenic fungus *Beauveria bassiana* (Bals-Criv.) Vuill. is a well-known agent for biocontrol of *Diptera* insects, including the cane midge. In this paper for the first time we have used a Siberian isolate of *B. bassiana* as a biological agent for regulation of Siberian population of this dangerous pest of the red raspberry. The aim of the research was to demonstrate capability of Siberian *B. bassiana* strain to suppress the number of raspberry cane midge in the laboratory and in the field conditions for two red raspberry cultivars. *B. bassiana* strain IC-1480-25 was isolated from Colorado beetle dead larvae in Novosibirsk region. In the laboratory the influence of *B. bassiana* was studied by counting of adults flying from soil where larvae of last instar were treated with entomopathogenic fungus at 10^6-10^7 CFU/ml concentration. Laboratory test efficacy was more than 80 %. In 2015-2016 the impact of *B. bassiana* on *R. theobaldi* was studied in the field conditions (plantation of Siberian Garden, Novosibirsk region). Raspberry cultivars Altai Zorenka (cv. 1) and Yellow Giant (cv. 2) differed in the susceptibility to the insect were field tested. In order to provide a reliable occupancy of ecological niche by phytophagous insect and to obtain reliable results, the artificial cane splits were created before the strain application. The results of field test were in line with the laboratory experiments. The *B. bassiana* activity depended on weather conditions, particularly on humidity. In the more humid 2015, the contrast between two varieties in response to *B. bassiana* was more pronounced. The efficacy of treatment with 10^7 CFU/ml fungal suspension was less than 60 % for cv. 1 and 100 % (all larvae died) for cv. 2. The results of this research evidence that the suppression of raspberry cane midge by the potential biocontrol agent depends on berry cultivar, weather conditions and *B. bassiana* dose. Larvae number and their mortality as well as degree of bark splits occupancy is mainly determined by the differences in raspberry variety and their response to weather changes, and also by the impact of weather on insect population. These results confirm the data of Russian and foreign researchers that 10^7 CFU/ml concentration of *B. bassiana* provides a marked effect on *Diptera* pests.

Keywords: red raspberry, raspberry cane midge, fungus *Beauveria bassiana*, pest number regulation, biological efficacy

Red raspberry *Rubus idaeus* L. is a widespread crop [1, 2], including in Eurasia [3-6]. This berry crop is often damaged by a dangerous pest, the raspberry cane midge *Resseliella theobaldi* (Barnes) [7-10]. Females lay eggs in fis-

sures on raspberry canes, whether naturally occurring or resulting from damage, after which the hatched larvae destroy the stem tissue. The phytophage can destroy up to 30-80% of raspberry shoots, resulting in 5-6 times lower yield. The pest migrated to Western Siberia from the European regions of the former USSR on plantings; this happened in the first half of the 1970s at the latest. Here, the midge adapted successfully and formed a local population, further spreading across the region; it was not detected immediately since the imago is small, and the larvae are well-capable of hiding. In the Novosibirsk Region, the midge was first discovered in 1983; scientists further studied its harmfulness and researched into the opportunities to suppress its population [11].

Raspberries are conventionally protected against the midge by chemical insecticides [12-14] based on a variety of compounds [15, 16]. However, the use of chemicals causes an increasing concern [17] and a discussion of the need to minimize pesticide loads while keeping high product quality [18], which draws attention to eco-friendly biologicals, especially for the treatment of plantings [19, 20]. The feasibility of controlling the population of raspberry cane midge was first shown for the Russian biological based on *Bacillus thuringiensis* subsp. *israelensis*, which had been developed to control the populations of dipterans [20]. However, for the order *Diptera*, which includes raspberry cane midge, the most appropriate biological population control agent is the entomopathogenic fungus *Beauveria bassiana* (Bals-Criv.) Vuill. [21-24]. Earlier laboratory tests prove three strains of *Beauveria bassiana* isolated in different geographical locations to have a >90% efficacy against raspberry cane midge [25]. Since the midge affects the inner cortex of canes (primary cortex parenchyma and the non-corked periderm), the ability of *B. bassiana* to colonize the insides of plants is crucial [26-29].

This paper is the first to demonstrate the effectiveness of a local *B. bassiana* isolate as a bioagent for controlling the Siberian population of raspberry cane midge.

The goal hereof was to test the siberian strain of *beauveria bassiana* as a suppressor of raspberry cane midge larvae in laboratory and field tests on two red raspberry varieties.

Techniques. Experiments were carried out in 2015 and 2016. *B. bassiana* strain IC-1480-25 from the collection of Issledovatelsky Tsentr LLC was isolated from dead Colorado beetle larvae in the Novosibirsk Region. To culture the strain, the team prepared various nutrient media (pH 6.5), poured 500 ml of each in a 2,000-ml Erlenmeyer flask, closed the flasks with cotton and gauze plugs, and sterilized in an autoclave at 121 °C over 40 minutes. Post-sterilization pH was 6.3. Nutrient media were cooled to 25 °C and inoculated with a culture grown in potato glucose agar, and then incubated in a thermostatic shaker at 25 °C over 8 to 10 days.

To assess the effects of the bioagent on raspberry cane midge larvae, a suspension of fungal conidia (10^6 and 10^7 CFU/ml) was placed in 125-ml plastic cups, each pre-filled with 100 g of soil sampled from under raspberry plants. Experiments were run four times. For control, the soil in cups was treated with 25 ml of water. During the experiment, the soil was moisturized systematically with 10 ml of water per cup by manually spraying it on the surface [24]. After 14 to 20 days of room-temperature incubation, 20 ready-to-pupation third-instar *R. theobaldi* larvae were placed onto each sample; the larvae had been extracted with a brush from raspberry cane fissures of the same plantation where the soil had been sampled prior to the experiment. The cups were covered with nylon smeared with Polyfix entomological glue by ITs Khimtek LLC, Russia; this would cause mature insects born from the pupae to stick to the nylon surface; 20 days later, the team counted the imagoes that had left the soil.

Biological efficacy (*BE*, %) was calculated by Abbott's formula: $BE = (K_1 \cdot K_c) \cdot (K_0 \cdot K_2)^{-1} \cdot 100$, where K_0 is the number of living specimens before treatment (experiment), K_1 is the number of living specimens after treatment (experiment), Kc is the number of living specimens before treatment (control), K_2 is the number of living specimens after treatment (control).

In-field effects of *B. bassiana* on midge larvae were evaluated at experimental sites of the Siberian Garden, Novosibirsk Region, in 2015 and 2016 for two cultivars: Zorenka Altaya, which is relatively resilient to midge, and Zheltyi gigant, which is not. The fungal suspensions were applied at concentrations of 10^6 and 10^7 CFU/ml. For reference, the team used the Iskra-M chemical by Tekhnoexport, Russia, while untreated raspberry canes were evaluated as controls. Larvae populations per fissure and larvae mortality rates were counted to assess the efficacy of the bioagent.

To enable the phytophage to fill the niche more reliably for more significant results, fissures had been made in canes on purpose. To that end, a 10-cm cut was made in each cane at 30 to 50 cm above ground with a dissecting needle, which would attract midge females. A 1 to 2-mm epidermis strip was exfoliated from the fissure to make a pocket for imagoes to lay eggs; canes were treated with the fungal suspension on the same day. For treatment, the experimenters used Orion-Kwazar back-carried sprayers (Kwazar Corporation Sp. z o.o., Poland); the application rate was 500 to 1,000 l/ha. Five or 6 labeled canes were cut two weeks after treatment to count larvae populations per fissure as well as their mortality rates.

Statistical processing was done in ANOVA and Microsoft Excel 2010 with means (*M*) and standard errors of the mean (±SEM) calculated. Significance was assessed by Student's *t*-test at $p < 0.05$ [30].

Results. Laboratory experiments run to test *B. bassiana* effects on raspberry cane midge at two concentrations revealed a statistically significant ($p < 0.05$) reduction in the flown-out imago numbers against the controls. Thus, the biological efficacy of both concentrations (10^6 and 10^7 CFU/ml) was at least 80%, see Table 1.

1. Hatching of *Resseliella theobaldi* (Barnes) imago when exposed to the entomopathogenic fungus *Beauveria bassiana* (Bals-Criv.) Vuill. (lab test)

Group	Imago hatching		Biological efficacy, %
	average number per group (*M*±SEM)	against the original larvae population, %	
Comttol	14.3±0.7		
B. bassiana, ×10^6 CFU/ml	2.8±0.6	13.8	80.5
B. bassiana, ×10^7 CFU/ml	1.3±0.5	6.3	91.0

These results are consistent with the data on the laboratory performance of *Beauveria* (10^7 CFU/ml) isolated by Borisov [25] in three remote locations in Russia and Ukraine.

Laboratory test results were confirmed in field tests in 2015 and 2016, see Table 2. In 2015, treating Zorenka Altaya canes with *B. bassiana* at two concentrations resulted in a statistically significant drop in larvae populations ($p < 0.05$), with a maximum of 1 living larva per fissure. Biological efficacy thus reached 54.0% to 57.6%. The chemical Iskra M reduced the living population to 0.4 specimens per fissure with a 94% mortality rate and a 70.0% biological efficacy. Zheltyi gigant contained living larvae only if treated with *B. bassiana* at 10^6 CFU/ml. The higher concentration of 10^7 CFU/ml killed all the larvae. The biological efficacy of this concentration (10^7 CFU/ml) was high, although still below that of the chemical. Experiments were repeated in 2016; for Zorenka Altaya, the results were nearly the same. Zheltyi gigant did not demon-

strate as drastic difference between the two concentrations as it had done a year earlier, see Table 2. It seems that cultivars might differ in attractiveness for the pest, which affects the effects of *B. bassiana*. Fungal agents are known to act differently depending on weather, in particular on the humidity. The heavier rainfall in 2015 might have boosted the efficacy of the fungal agent for Zheltyi gigant plants.

2. *Resseliella theobaldi* (Barnes) specimens per cortex fissure when exposed to *Beauveria bassiana* (Bals-Criv.) Vuill. and to a chemical insecticide (*M*±SEM, SKhA Siberian Garden, Novosibirsk Region)

Group	Larvaes				Mortality, %		Biological efficacy, %	
	total number		alive					
	2015	2016	2015	2016	2015	2016	2015	2016 год
Cv. Zorenka Altaya								
Control	6.0±0.9	14.6±0.1	4.3±0.1	10.4±0.2	28.3	28.8		
B. bassiana, ×10^6 CFU/ml	5.1±0.7	5.2±0.2^{ab}	0.8±0.2^{a}	1.0±0.3^{ab}	84.3	80.8	54.0	52.0
B. bassiana, ×10^7 CFU/ml	5.7±0.7	5.5±0.2^{ab}	0.7±0.3^{a}	0.9±0.2^{ab}	87.7	83.6	57.6	54.9
Iskra-M, 0.2 % (st)	6.8±1.0	7.4±0.1^{ac}	0.4±0.2^{a}	0.1±0.1^{ac}	94.1	98.8	70.2	70.0
Cv. Zheltyi gigant								
Control	9.1±2.8	16.8±0.2	8.2±0.1	13.0±0.2	9.9	22.6		
B. bassiana, ×10^6 CFU/ml	11.9±3.3^{b}	3.4±0.4^{ab}	6.1±0.2^{a}	1.0±0.3^{a}	48.5	70.6	39.5	48.0
B. bassiana, ×10^7 CFU/ml	7.7±2.9	2.7±0.5^{ab}	0	0.7±0.3^{a}	100.0	74.1	77.1	51.5
Iskra-M, 0.2 % (st)	5.0±3.7	8.0±0.4^{ac}	0	0	100.0	100.0	90.1	77.4

Note. a — difference from control is statistically significant at $p < 0.05$, b — difference from standard (st) is statistically significant at $p < 0.05$, c — difference from the preparation of lower is statistically significant at $p < 0.05$.

The results are consistent with those of other studies [22, 24, 31-33], which prove that Beauveria must be applied at a minimum of 10^7 CFU/ml to be effective against *Diptera*. Notably, these papers only present laboratory tests. Thus, in vitro infestation of Mexican fruit fly *Anastrepha ludens* (Loew) larvae, pupae, and imagoes with Brazilian, Mexican, and Ecuadorian strains of *B. bassiana* killed only the mature insects in numbers [22]. In laboratory tests, contact and oral transmission of Beauveria (10^8 CFU/ml) to mature olive fruit fly *Bactrocera oleae* (Gmelin) killed 50% of the specimens [24]. Three *B. bassiana* strains were lab-tested on the pupae of onion fly *Delia antiqua* (Meigen) (*Diptera*: *Anthomyiidae*). At 10^7 CFU/ml, the fungus killed 35.4% to 52.5% of the insects. In vitro tests also demonstrated the effects of *B. bassiana* on the puparia and imago of *Ceratitis capitata* (Wiedemann) (*Diptera*: *Tephritidae*). *B. bassiana strain* Bb-1333 caused a >50% mortality [31]. Beauveria has been isolated from the same insect species in Brazil [32]. The authors have not found any publications on the effects of this fungal biocontrol agent on raspberry cane midge, whether in vivo or in vitro. *B. bassiana* is known for its ability to spread under the cortex [34], which likely contributes to its action against raspberry cane midge as shown by this study.

Thus, the paper presents lab and field test results to demonstrate that the population of raspberry cane midge *Resseliella theobaldi* can be controlled by the Siberian strain of *Beauveria bassiana*. It shows that the suppressive effects depend on the raspberry cultivar, the weather, and the concentration of *B. bassiana*, which must be at least 10^7 CFU/ml in suspension. The difference in larvae populations and mortality, as well as in their presence in cane cortex fissures, mainly depends on the cultivar, its reaction to weather, and the weather effects on raspberry cane midge population.

REFERENCES

1. Williams I.H. Effects of environment on *Rubus idaeus* L. V. Dormancy and flowering of the mature shoot. *Journal of Horticultural Science*, 1960, 35(3): 214-220 (doi: 10.1080/00221589.1960.11513985).
2. Kazakov I.V. *Malina i ezhevika* [Raspberry and blackberry]. Moscow, 2001 (in Russ.).

3. Kichina V.V. *Krupnoplodnye maliny Rossii* [Large raspberries of Russia]. Moscow, 2005 (in Russ.).
4. Jennings S.N., Brennan R.M. Improvement of raspberry cultivars in Scotland. *Acta Horticulturae*, 2002, 585: 179-183 (doi: 10.17660/ActaHortic.2002.585.27).
5. Sønsteby A., Heide O.M. Environmental control of growth and flowering of *Rubus idaeus* L. cv. Glen Ample. *Scientia Horticulturae*, 2008, 117(3): 249-256 (doi: 10.1016/j.scienta.2008.05.003).
6. Woznicki T.L., Heide O.M., Remberg S.F., Sønsteby A. Effects of controlled nutrient feeding and different temperatures during floral initiation on yield, berry size and drupelet numbers in red raspberry (*Rubus idaeus* L.). *Scientia Horticulturae*, 2016, 212: 148-154 (doi: 10.1016/j.scienta.2016.09.047).
7. Łabanowska B.H., Cross J. Raspberry cane midge — *Resseliella theobaldi* (Barnes) — flight and egg laying dynamics on raspberry fruiting on two year old canes. *Journal of Fruit and Ornamental Plant Research*, 2008, 16: 315-323
8. Vetek G., Fail J., Penzes B. Susceptibility of raspberry cultivars to the raspberry cane midge (*Resseliella theobaldi* Barnes). *Journal of Fruit and Ornamental Plant Research*, 2006, 14(Suppl. 3): 61-66.
9. Belyaev A.A., Shpatova T.V., Shternshis M.V. Pheromone monitoring of the raspberry cane midge *Resseliella theobaldi* (Barnes) in the conditions of Novosibirskaya Oblast'. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2010, 3: 113-117 (in Engl.).
10. Tanasković S., Milenković S. Monitoring of flight phenology of raspberry cane midge *Resseliella theobaldi* Barnes (*Diptera*: *Cecidomyiidae*) by pheromone traps in Western Serbia. *Acta Entomologica Serbica*, 2010, 15(1): 81-90.
11. Sidorovich A.S., Belyaev A.A., Belova L.B. V sbornike: *Tovarnoe yagodovodstvo: organizatsiya, tekhnologii, sortiment* [In: Commercial berry growing: organization, technology, assortment]. Berdsk, 1990: 28-31 (in Russ.).
12. Dalman P., Malkki S. Experiments on chemicals and cultural control of the raspberry cane midge (*Resseliella theobaldii)* and midge blight. *Annales Agriculturae Fenniae*, 1986, 25(4): 233-241.
13. Prokof'ev M.A. *Zashchita sadov Sibiri ot vreditelei* [Protecting Siberian gardens from pests]. Moscow, 1987 (in Russ.).
14. Milenković S., Retenović D., Antonijević M. Raspberry gall midge *Resseliella theobaldi* (Barnes) (*Diptera*: *Cecidomyidae*) — harmfulness and control. *Journal of Yugoslav pomology*, 2004, 38(3-4): 191-198.
15. Williamson B. Effect of fenitrothion and benomyl sprays on raspberry cane midge (*Resseliella theobaldi*) and midge blight, with particular reference to *Leptosphaeria coniothyrium* in the disease complex. *Journal of Horticultural Science*, 1987, 62(2): 171-175 (doi: 10.1080/14620316.1987.11515766).
16. Łabanowska B.H., Cross J. Raspberry cane midge — flight dynamics, eggs laying and the efficacy of neonicotinoid insecticide acetamiprid on primocane fruiting raspberry. *IOBC/wprs Bulletin*, 2008, 39: 19-25.
17. Gordon S.C., Woodford J.A.T., Birch A.N.E. Arthropod pests of *Rubus* in Europe: pest status, current and future control strategies. *Journal of Horticultural Science*, 1997, 72(6): 831-862 (doi: 10.1080/14620316.1997.11515577).
18. Birch A.N.E., Gordon S.C., Fenton B., Malloch G., Mitchel C., Jones A.T., Griffiths D.V., Brennan R., Graham J., Woodford J.A.T. Developing a sustainable IPM System for high value *Rubus* crops (Raspberry, Blackberry) for Europe. *Acta Horticulturae*, 2004, 649: 289-292 (doi: 10.17660/ActaHortic.2004.649.54).
19. Shternshis M.V. *Zashchita i karantin rastenii*, 2013, 4: 19-22 (in Russ.).
20. Mohamedova M. Field evaluation of three biopesticides for control of the raspberry cane midge *Resseliella theobaldi* (Barnes) in Bulgaria. *Advances in Horticultural Science*, 2017, 31(3): 183-189 (doi: 10.13128/ahs-20575).
21. Shternshis M.V., Belyaev A.A.., Shpatova T.V., Bokova J.V., Duzhak A.B. Field testing of Bacticide®, Phytoverm® and Chitinase for control of the raspberry midge blight in Siberia. *BioControl*, 2002, 47(6): 697-706 (doi: 10.1023/A:1020574914831).
22. De la Rosa W., Lopez F. L., Liedo P. *Beauveria bassiana* as a pathogen of the Mexican fruit fly (*Diptera*: *Tephritidae*) under laboratory conditions. *Journal of Economic Entomology*, 2002, 95(1): 36-43 (doi: 10.1603/0022-0493-95.1.36).
23. Ekesi S., Dimbi S., Maniania N.K. The role of entomopathogenic fungi in the integrated management of fruit flies (*Diptera*: *Tephritidae*) with emphasis on species occurring in Africa. In: *Use of entomopathogenic fungi in biological pest management*. S. Ekesi, N.K. Maniania (eds.). Kerala, India, 2007: 136-172.
24. Mahmoud M.F. Pathogenicity of three commercial products of entomopathogenic fungi, *Beauveria bassiana*, *Metarhizum anisopilae* and *Lecanicillium lecanii* against adults of olive fly *Bactrocera oleae* (Gmelin) (*Diptera*: *Tephritidae*) in the laboratory. *Plant Protection Science*, 2009, 45(3): 98-102 (doi: 10.17221/34/2008-PPS).
25. Shternshis M.V., Shpatova T.V., Borisov B.A., Belyaev A.A. *Mikologiya i fitopatologiya*, 2005, 39(1): 76-82 (in Russ.).
26. Ownley B.H., Pereira R.M., Klingeman W.E., Quigley N.B., Leckie B.M. *Beauveria bassiana*, a

dual purpose biocontrol organism, with activity against insect pests and plant pathogen. In: *Emerging concepts in plant health management*. R.T. Lartey, A.J. Caesar (eds.). Research Signpost, Kerala, 2004: 255-269.
27. Posada F.J., Vega F.E. Establishment of the fungal entomopathogen *Beauveria bassiana* (*Ascomycota*: *Hypocreales*) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia*, 2005, 97(6): 1195-1200 (doi: 10.1080/15572536.2006.11832729).
28. Vega F.E. Insect pathology and fungal endophytes. *Journal of Invertebrate Pathology*, 2008, 98(3): 277-279 (doi: 10.1016/j.jip.2008.01.008)
29. Ownley B.H., Gwinn K.D., Vega F.E. Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl*, 2010, 55(1): 113-128 (doi: 10.1007/s10526-009-9241-x).
30. Dospekhov B.A. *Metodika polevogo opyta (s osnovami statisticheskoi obrabotki rezul'tatov issledovanii)* [Methods of field trials]. Moscow, 1985 (in Russ.).
31. Quesada-Moraga E., Ruiz-García A., Santiago-Álvarez C. Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (*Diptera*: *Tephritidae*). *Journal of Economic Entomology*, 2006, 99(6): 1955-1966 (doi: 10.1093/jee/99.6.1955).
32. Ekesi S., Maniania N.K., Lux S.A. Mortality in three African tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. *Biocontrol Science and Technology*, 2002, 12(1): 7-17 (doi: 10.1080/09583150120093077).
33. Poprawski T.J., Robert P-H., Boivin I.M. G. Susceptibility of *Delia antiqua* (*Diptera*: *Anthomyiidae*) to eleven isolates of entomopathogenic hyphomycetes. *Environmental Entomology,* 1985, 14(5): 557-561 (doi: 10.1093/ee/14.5.557).
34. Kreutz J., Zimmermann G., Vaupel O. Horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* among the spruce bark beetle, *Ips typographus* (Col., Scolytidae) in the laboratory and under field conditions. *Biocontrol Science and Technology*, 2004, 14(8): 837-848 (doi: 10.1080/788222844).

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CHEMICALS-BASED REGULATION OF LEAVES-TO-GRAINS OUTFLOW OF ASSIMILATES TO ENHANCE YIELDS IN RICE (*Oryza sativa* L.) UNDER CONDITIONS OF ITS NORTHERN AREA

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Abstract

Rice is mainly produced in countries with a favorable climate for culture, lying between the equator and 45° latitude, but in recent years, interest in expanding of rice-growing to the north increases. In Russia, the terrotory of rice growing in Krasnodar region is located at the northern border of the crop area. For this reason, in certain years ripening of rice coincides with unfavorable weather conditions that cause an increase in the growing period, which leads to a decrease in the productivity of plants, delay in harvesting and losses of a significant part of the yield. In this regard, there is a need to develop ways to accelerate ripening without reducing the productivity of plants. Such a technique is artificial leaf senescence, a purposeful regulation of metabolism at the final stages of plant development with the use of chemicals. Artificial senescence should be used in case of delayed ripening, which is most often observed in late crops (due to postponement of sowing because of weather conditions), thinned crops or crops receiving excessive nitrogen nutrition. Although at the present time, this technique is little used, mainly due to the long warm period in the long-term cycle of temperature fluctuations, the probability of a period with adverse weather conditions for ripening and harvesting rice in the coming years is high. The effectiveness of this method depends on the composition of the chemical agents, on the terms of treatment and the weather conditions. In addition, to apply this technology to modern rice varieties, it is necessary to estimate its effectiveness and adjust relevant protocols regarding the grain ripening peculiarities. In the present work, we compared some physiological indices of the modern Russian intensive rice variety Khazar plants during ripening, as well as the yield structure and total yielding, as influenced by the composition and timing of the chemicals used under different weather conditions during three years of the observation. It was shown, that the most suitable chemicals are aqueous solutions of ammonium nitrate (15 kg/ha) with the addition of manganese (400 g/ha) or selenium (200 g/ha). Modified selenium solution should be used if it is necessary to start harvesting in 14-20 days. Treatment of plants with a solution modified by manganese stimulates the synthesis and attraction of assimilates in the grain, which is accompanied by a not so rapid completion of ontogenesis, but a significant increase in yield. The treatment of rice crops should be carried out during the stage of milk-wax ripeness of the grain. In the "cold" years, this technilogue allows faster (by 5-6 days) and more (up 0.69-7.39 %) loss of panicle moisture compared to untreted control plants, but also increases yield by 4.2-9.3 %. In the years with favorable weather conditions for rice ripening, artificial senescence provides slight decrease in panicle moisture (by 0.66-2.78 %), but greater increase in yielding (by 7.88-14.73 %). The study of the mechanism of the observed effects can broaden our knowledge about the crop biology and varietal specificity and can be useful in developing technologies for adapting rice plants to north-growing with the use of a new generation chemicals which should be safe for humans and the environment, and in breeding for accelerated maturation under the northern conditions.

Keywords: *Oryza sativa* L., rice, artificial senescence, accelerated maturation, foliar application, urea, ammonium nitrate, Mn, Se

Rice is one of the major crops feeding more than half of the world's population. Rice is mainly produced in countries with a favorable climate for culture, lying between the equator and 45° latitude (South and Southeast Asia yielding up to 90 % of global paddy rice production, primarily China, India, Indonesia, Thailand, Japan, as well as Brazil and the USA) [1, 2]. In Europe, rice is grown in Spain, Italy [3, 4], Greece, and Turkey [5]. In Russia, culture area is limited from up north (42-47° north latitude) with concentration of its major commercial crops in Krasnodar Region (44-45° north latitude) [6]. Recently, there was growing interest in expanding geography of rice planting. Thus, according to researchers, successful growing of rice on experimental fields in the southwestern province of Ontario in Canada at the territory with fairly cold humid continental climate points to a good chance for such country to become a rice producer [7]. Submersion of agricultural fields for rice crops may also fulfill ecologic function creating wetlands as a habitat for waterfowl [7] and ponds for fish farming (similar experience was successful in Krasnodar Region in the 1980s). The issue of rice planting northerning becomes even more important both due to the possibility of global warming, as well as shifting from the period of warmth to the period of cold in the longstanding fluctuation of temperatures.

During plant ontogenesis, competition between organs for metabolites consistently succeeds (shoot—root, leaf—flower, leaf—fruit) [8, 9]. There is a physiologically active center (storage center) to which the largest amounts of assimilatory products are going. At the last stages of plant development, competitive relations in the leaf-fruit system favors the latter, and plastic substances are transported to them. Before cropping, a cascade of signal processes is run in the leaves metabolism with the sharp shift towards strengthening of hydrolytic reactions resulting in the protein and starch breakdown into simpler compounds (amino acids and sugar) which are attracted by generative organs, the fruits and seeds [10-12]. However, assimilatory products, as we know, are not fully attracted, and the extension of the vegetative development period in plants, often occurring under unfavorable weather conditions, is accompanied by a usage of these products for vegetative growth to the detriment of crop [13, 14].

One of the ways to control plant performance is based on a purposeful regulation of outflow of plastic substances from leaves and stems to generative organs. Such agricultural approach accelerating maturing and increase in productivity of agricultural crops was called artificial senescence. Artificial senescence of grain crops results in slow extinction of leaves similar to essential senescence [15]. Mode of action of artificial senescence agents involves acceleration of metabolism in plant tissues and increase in the outflow speed of plastic substances from the leaves and stems to the panicle (spike). The result is better filled grain, reduced blind-seed disease, and an increased grain weight [16]. The more abundant kernel setting during earlier vegetation periods, the more significant is additional yield [17-20]. Artificial senescence has large perspectives for seed-growing (especially thinned) crops with expressed tillering as it stimulates maturing of caryopsis at side shoots, which results in seeds with lesser metrical heterogeneity and higher cropping properties [21].

Theoretic and practical aspects of artificial senescence have been developed at crops of white cabbage [22], maize [16], buckwheat [17], potato [23], sunflower [24], and soya [25-27]. Geographic position of Russia with its climatic conditions determines the increased interest in artificial senescence unlike other countries. In 1970-1980, it was quiet often used in Kuban to accelerate rice maturing. The most effect was achieved upon use of aqueous solution of urea or superphosphate with addition of 2,4-dichlorophenoxyacetic acid (2,4-D) as a growth regulator. It should be noted that along with acceleration of maturing

artificial senescence had promoted the increase of yield and enhancement of rice grain quality [21].

We have proposed physiologically justified way to increase rice crop productivity in conditions of risk rice planting. The novelty of method is in development of the technology of use of new mixtures for artificial senescence since previously applied technical regulations became obsolete due to changing of the cultivar set and ban on use of 2,4-D amine salt (main component of previously applied mixture for artificial senescence). The priority of studies was set out for authors by patent P. 2580162 (RF) MKIZ A01G 16/00. A 01 No. 59/00. Federal State Funded research Institution "All-Union Research and Development Establishment of Rice" (RF) (authors: Sheudzhen A.H., Bondareva T.N., Haritonov E.M., Doroshev I.A., Ladatko M.A., Gish T.H.. Appl. 02.07.2014. Publ. 10.04.2016. Bul. No. 10).

The purpose of research was to study the dynamics of moisture and accumulation of dry substance by rice plants under the influence of various artificial senescence agents for physiological justification of their effective concentrations and modes of application ensuring the accelerated grain maturing.

Techniques. Years of the investigations (2009-2011) varied in hydrothermal conditions of vegetation periods. Field plot experiments were carried out in the irrigation system of Krasnoye Experimental Elite Seed-Production Enterprise (Krasnoarmeyskii District, Krasnodar Territory). The soil was meadow-chernozem, weakly-leached, weakly-humid, and heavy loamy on alluvial deposits. Intensive middle-late Khazar rice (originated by All-Union Rice Research Institute) was grown under growing scheme as follows: perennial grasses as predecessors; row planting; 1.0-1.5 cm depth of seeding-down; seeding rate of 7 mln/ha; fertilization ($N_{90}P_{60}K_{45}$), shortened flood-irrigation.

Artificial senescence was initiated 10 days after the beginning of anthesis of main panicle (milky stage) and at the beginning of milk-wax stage of grain ripeness by sprinkling plants with water solution of urea (20 kg/ha) and ammonium nitrate (15 kg/ha) with addition of manganese (400 g/ha) and selenium (200 g/ha). Manganese sulphate was used for manganese, and sodium selenite for selenium. The rate of working solution was 400 l per ha.

Experiments were arranged in four replications, with 20 m^2 total plot area (10 m^2 sample plot) per test and dactyl scheme of plot location.

Dynamics in plant moisture and dry weigh after artificial senescence was determined at each plot in plants selected with 7-day interval from 0.25 m^2 plots in four replications (1 m^2 in total).

Plant maturing was assessed by changes in moisture and dry weigh of leaves, stems, and panicles upon weighting after 6-hour drying (106 °C). Crop productivity was determined by recalculation of the grain weigh from the sample plot adjusted by standard moisture and purity, 1000-grain weigh was evaluated according to GOST 10842-89, blind-seed disease was estimated by a portion of unfilled spikelets out of their total number in panicle.

Data are presented as means (*M*) and standard error of means (±SEM). The obtained results were assessed by dispersion analysis by *F*-criterion and LSD at 5 % significance level [28].

Results. At the first stage, we had compared over 50 variants of compositions of macro- and micro-fertilizers, plant growth regulators, and desiccants (data are not shown). Of these, aqueous solution of urea (20 kg/ha) and ammonium nitrate (15 kg/ha) modified by manganese (400 g/ha) or selenium (200 g/ha) were the most effective. Selection of agents for artificial senescence was based on the fact that, according to majority of researchers, the main component of such agents should be nitrogen in amido or ammonium form [29], and

nitrogen fertilizers, the urea, ammonium nitrate, and carbamide-ammonium mixture are more often used for these purposes.

Effect of nitrogen treatment on leaf senescence and crop productivity may vary depending on variety, dosage, application mode, and planting conditions [30]. Maturation acceleration in rice upon artificial senescence with nitrogen fertilizers is attributed to action of nitrogen found in plants in approximate 30 minutes after leaf treatment. Such nitrogen serves as additional source of nutrition and is involved in synthesis of organic compounds, due to which N content in leaves significantly increases. This leads to an "overfill" of leaf cells by plastic substances and free ammonium ions which are involved in nitrogen metabolism in plant. It results in accelerated biosynthesis of assimilates and their outflow from vegetative organs to caryopsis. At the same time, degradation of chloroplast [31, 32], weakening of photosynthesis and breath intensity due to ammonium poisoning of plant takes place. With filling of caryopsis, not used (residual) nitrogen in ammonium form is accumulated in cells, which inhibits functional activity of glutamine synthetase and glutamine oxoglutarate aminotransferase (glutamate synthase), the enzymes providing incorporation of ammonium ions in amino acids and amides [33]. Accumulation of ammonium in plant cells [29] which is able to cause destructive processes is considered the cause of accelerated ontogenesis [34, 35].

For more effective senescence, 2,4-D or such important micro elements for a plant as boron, selenium, and manganese are added to the nitrogen fertilizer [29, 36-38]. Role of 2,4-D is to facilitate absorption of ammonium ion or urea molecule through epidermis to leaf mesopfyll, mobilization and strengthening of assimilate outflow from rice caryopsis. Manganese is a cofactor of carbohydrate metabolism of enzymes. When supplied to a plant, it is intensively transported to shoots and is used in oxidation and restoration reactions [39-41]. A number of Mn-stabilizing proteins involved in photosynthesis is found in plants [40, 42, 43]. Main function of Se in plants is participation in regulation of activity of glycolitic and respiratory enzymes [44-46]. Presence of selenium is essential for biosynthesis of formate dehydrogenase, the enzyme of formic acid oxidation decomposition. This element activates fumarase (fumarate-hydratase) playing a catalytic role in dehydration of malic acid in Krebs cycle, and nitrate reductase producing ammonium from nitrate in the course of assimilation. Glutathione reductase, one of the key antioxidant enzymes, also needs selenium [47, 48]. At the same time, high concentrations of this micro element may negatively affect metabolism and constraint vegetation period [44, 49]. For rice plants, critical level of selenium is 19 μg/g dry weight [50].

During 2009-2011, hydrothermal pattern varied when potential senescent agents were tested. In 2009, air temperature during sowing and young growth of rice plants (May to decade I of June) was at the level of long-term annual average values, exceeded them by 1.0-4.3 °C during vegetative growth (June to beginning of August), and in August (filling of caryopsis) was by 0.6-1.3 °C lower than long-term annual average. In 2010, air temperature during rice vegetation had abnormally increased: starting from the decade III of May, it exceeded the long-term annual average values by 3-6 °C. In decades I and II of May in 2011, significant precipitation was fallen, and average daily air temperatures were slightly lower than long-term annual average values, due to which sowing took place later than optimal. In the same year, during plantling period (May to decade I of June) air temperature was by 0.8-1.8 °C higher than long-term annual average, during vegetation growth exceeded it by 1.0-1.6 °C, and in the decade III of August to September, during filling of caryopsis, exceeded it by 0-1.0 °C. In general, temperature was favorable for growth, development, and rice crop for-

mation (except for changes in sowing terms for later terms).

The effects of senescence agents directly depend on temperature, and, thus, we interpreted our findings accounting for weather conditions in a year. An optimal term for artificial senescence was established (earlier treatment significantly reduces the period of caryopsis filling, as a result, the kernels are filled insufficiently whilst the later treatment do not have the expected effect and merely increase the cultivation costs). We compared two treatment terms, i.e. in 10 days after flowering of main panicle and during milk-wax ripeness of grain.

Effectiveness of the chemicals was assessed by a reduction of moisture of vegetative organs and panicles with compulsory further estimation of the grain weigh per panicle and per plant, 1000-grain weigh and crop productivity. Such indicators characterize physiological state of plants during maturing and final result of changes caused by chemicals. In this paper we provided results for one middle-late variety; however, one should take into account that response to chemical senescence varies in varieties of different genotypes and, especially, of different vegetation periods. Thus, additional research is required when technology is adapted to other varieties and weather conditions.

In 2009, upon 10 day-scheme, moisture of panicles during the 1st week had reduced slower than upon natural maturation, which is lawful since the chemical used is based on nitrogen fertilizer. Due to its effect on plant, the panicle moisture was 0.3-2.0 % higher than in control (Table 1). During the 2nd week, the panicle moisture actively reduced (with urea-based chemical it was 2.1-4.2 % lower than in control, with ammonium nitrate by 6.2-8.4 %). In further days, moisture loss slightly decelerated, but after all its speed remained higher under effect of chemicals based on ammonium nitrate (especially with addition of Se and Mn). Thirty days after treatments moisture of panicles in such plants was 2.3-2.8 % lower than in control.

Main purpose of artificial senescence is to promote attraction of assimilates from vegetative organs to caryopsis [10]. In 1 week after application of the chemicals, plant dry weigh exceeded the control by 0.02-0.24 g, or by 1.4-17.0 %. During the next week, when most intensive moisture loss was observed, under the effect of urea and ammonium nitrate solutions, both in pure form and with addition of manganese, dry weigh of panicles was 2.7-5.8 % lower than in control, while addition of selenium made it 0.5-3.6 % higher. Panicle weigh by the end of vegetation did not significantly differ from control only in treating with the urea-based chemicals. Ammonium nitrate, both separately and together with trace elements, even reduced this indicator by 7.0-9.7%. It was due to effect of the chemicals on the rate of assimilation product remobilization to panicle: during the 1st week this rate was higher than in control with further gradual decrease.

The productivity reduced due to altered period of maturation, to the most extent (by 14.3 and 12.7 %) under the effect of the chemicals with Se. Negative consequences of effect of other chemicals were not so significant, but the trend was manifested quietly clear. Thence, artificial senescence promptly after flowering is unreasonable and may be applied only in the critical situation to save at least some part of crop.

Artificial senescence at the beginning of milk-wax ripening (15 days after flowering) had revealed the following trends. During maturing of caryopsis, moisture of leaves and stems in plants had insignificantly changed. It is due to both development biology of rice plants in which, unlike wheat and other grain crops, leaves and stems continue functioning at kernel maturing, as well as to cultivation technology. Rice growing at flooded fields distorts stem moisture value as the plant has leaves and leaf sheaths, dying-off degree of which (and, con-

1. Dynamics of grain maturing and yield of rice (*Oryza sativa* L.) Khazar cv. after artificial senescent during milky ripeness stage (*M*±SEM, Krasnodar Territory, 2009-2011)

Group	Moisture, %			Dry weigh, g/plant			Dry matter accumulation, g/(plant · days)			Yield, t/ha
	Day 6	Day 12	Day 30	Day 6	Day 12	Day 30	Days 0-6	Days 7-12	Days 13-30	
Control (no treatment)	40.6±0.81	40.4±1.01	19.8±0.46	1.42±0.10	2.24±0.11	2.59±0.16	0.052±0.018	0.117±0.005	0.018±0.001	12.6±0.4
Urea	42.6±0.85	38.3±0.96	22.3±0.52	1.44±0.06	2.30±0.12	2.61±0.15	0.055±0.019	0.123±0.005	0.016±0.002	12.5±0.5
Urea + Mn	42.4±0.84	37.3±0.93	20.7±0.49	1.48±0.07	2.37±0.12	2.64±0.12	0.062±0.022	0.127±0.005	0.014±0.001	12.8±0.3
Urea + Se	41.1±0.82	36.2±0.91	19.0±0.44	1.66±0.09	2.23±0.13	2.48±0.11	0.092±0.033	0.081±0.003	0.013±0.003	10.8±0.2
Ammonium nitrate	42.8±0.86	34.2±0.86	17.5±0.41	1.55±0.07	2.33±0.14	2.40±0.15	0.073±0.028	0.111±0.005	0.004±0.0002	12.3±0.4
Ammonium nitrate + Mn	41.2±0.82	33.8±0.85	17.2±0.40	1.54±0.06	2.35±0.12	2.41±0.11	0.072±0.025	0.116±0.005	0.003±0.0004	12.7±0.3
Ammonium nitrate + Se	38.3±0.77	32.0±0.80	17.0±0.39	1.58±0.07	2.16±0.11	2.34±0.12	0.078±0.027	0.083±0.003	0.009±0.0005	11.0±0.4
LSD_{05}				0.11	0.14	0.17				0.40

2. Rate of dry matter accumulation, g/(plant · days), **in panicles of rice (*Oryza sativa* L.) Khazar cv. after artificial senescent during milk-wax ripeness stage** (*M*±SEM, Krasnodar Territory)

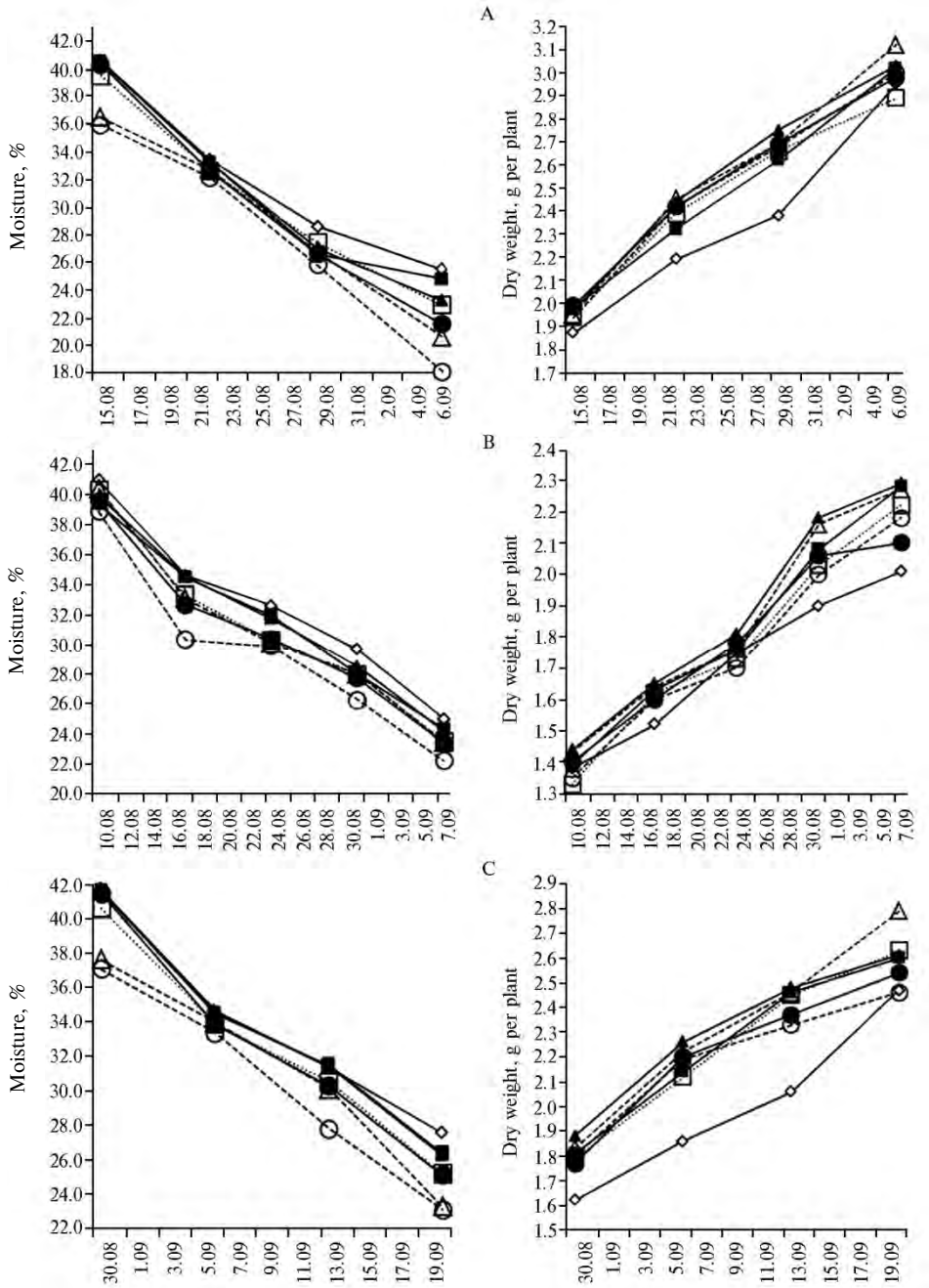
Group	2009				2010					2011			
	Days 0-5	Days 6-12	Days 13-19	Days 20-28	Days 0-7	Days 8-14	Days 15-21	Days 22-28	Days 29-35	Days 0-7	Days 8-14	Days 15-21	Days 22-28
1	0.058±0.003	0.046±0.002	0.027±0.002	0.057±0.003	0.054±0.003	0.020±0.001	0.033±0.002	0.021±0.001	0.016±0.001	0.057±0.002	0.034±0.002	0.029±0.002	0.059±0.002
2	0.080±0.005	0.049±0.002	0.043±0.003	0.040±0.002	0.056±0.003	0.034±0.002	0.019±0.001	0.046±0.002	0.029±0.002	0.084±0.003	0.047±0.003	0.046±0.003	0.020±0.001
3	0.078±0.005	0.067±0.003	0.044±0.003	0.028±0.002	0.063±0.003	0.030±0.002	0.023±0.001	0.053±0.002	0.016±0.001	0.094±0.001	0.054±0.003	0.031±0.002	0.019±0.001
4	0.082±0.005	0.061±0.003	0.039±0.002	0.029±0.002	0.057±0.003	0.029±0.002	0.026±0.001	0.040±0.002	0.006±0.000	0.079±0.003	0.061±0.003	0.024±0.001	0.024±0.001
5	0.072±0.004	0.064±0.003	0.039±0.002	0.023±0.001	0.047±0.002	0.041±0.002	0.016±0.001	0.043±0.002	0.027±0.002	0.081±0.003	0.047±0.003	0.047±0.003	0.026±0.001
6	0.074±0.004	0.071±0.004	0.034±0.002	0.043±0.003	0.061±0.003	0.030±0.002	0.017±0.001	0.057±0.003	0.016±0.001	0.087±0.003	0.056±0.003	0.034±0.002	0.047±0.002
7	0.072±0.004	0.069±0.003	0.037±0.002	0.032±0.002	0.050±0.003	0.036±0.002	0.014±0.001	0.043±0.002	0.026±0.002	0.083±0.003	0.056±0.003	0.020±0.001	0.019±0.001
LSD_{05}	0.006	0.003	0.004	0.004	0.003	0.002	0.001	0.001	0.002	0.002	0.003	0.003	0.001

N o t e. 1 — control (no treatment), 2 — urea, 3 — urea + Mn, 4 — urea + Se, 5 — ammonium nitrate, 6 — ammonium nitrate + Mn, 7 — ammonium nitrate + Se.

3. Productivity and yield structure in rice (*Oryza sativa* L.) Khazar cv. after artificial senescent during milk-wax ripeness stage (*M*±SEM, Krasnodar Territory)

Group	2009				2010				2011			
	Y, t/ha	GWP, g	EG, %	W1000, g	Y, t/ha	GWP, g	EG, %	W1000, g	Y, t/ha	GWP, g	EG, %	W1000, g
1	11.44±0.34	3.19±0.18	0.9±0.1	27.88±1.39	4.82±0.29	2.06±0.12	6.3±0.4	26.95±0.81	8.62±0.34	2.41±0.13	19.9±1.0	28.85±1.15
2	11.49±0.34	3.24±0.18	1.8±0.1	29.85±1.49	4.84±0.29	2.14±0.13	5.2±0.4	27.08±0.81	8.90±0.36	2.56±0.14	18.8±1.1	28.89±1.16
3	11.66±0.35	3.29±0.19	1.7±0.1	29.98±1.50	4.86±0.29	2.17±0.13	5.2±0.4	27.20±0.80	9.30±0.37	2.62±0.14	17.3±1.0	29.20±1.17
4	11.08±0.33	3.21±0.18	1.6±0.1	28.98±1.45	4.23±0.25	2.08±0.12	5.6±0.4	26.02±0.78	8.36±0.33	2.55±0.14	16.7±1.0	29.01±1.16
5	11.58±0.35	3.20±0.18	1.6±0.1	29.12±1.46	5.40±0.32	2.42±0.15	5.2±0.4	27.10±0.81	8.94±0.36	2.61±0.14	19.4±1.2	29.03±1.16
6	11.92±0.36	3.39±0.19	1.6±0.1	29.25±1.46	5.53±0.33	2.43±0.15	5.9±0.4	27.15±0.81	9.42±0.38	2.81±0.15	17.5±1.1	29.37±1.17
7	11.61±0.35	3.09±0.18	1.6±0.1	29.00±1.45	5.20±0.31	2.30±0.14	5.2±0.4	27.00±0.81	8.54±0.34	2.41±0.13	18.9±1.1	28.401.14
LSD_{05}	0.35	0.19		1.49	0.38	0.27		0.74	0.38	0.2		1.12

N o t e. 1 — control (no treatment), 2 — urea, 3 — urea + Mn, 4 — urea + Se, 5 — ammonium nitrate, 6 — ammonium nitrate + Mn, 7 — ammonium nitrate + Se; Y — урожайность, GWP — grain weigh per panicle, EG — empty grains, W100 — 1000-grain weight



Dynamic of panicle moisture (from the left) **and dry weigh** (from the right) in **rice (*Oryza sativa* L.) Khazar cv. after artificial senescent during milk-wax ripeness stage in 2009** (A)**, 2010** (B) **and 2011** (C)**:** —◇— — control (no treatment), —■— — urea, —▲— — urea + Mn, —●— — urea + Se, ···□··· — ammonium nitrate, ···△··· — ammonium nitrate + Mn, ···○··· — ammonium nitrate + Se (Krasnodar Territory).

sequently, water saturation) varies. Nevertheless, it could be stated that with plant maturing differences in moisture of vegetative organs between the control and artificial senescence variants had increased, however did not exceed 1-2% in general. Addition of micro elements to urea and ammonium nitrate solution had resulted in more reduction of stem moisture than at their sole use.

Urea-based preparations had stronger dried leaves promptly after application, while ammonium nitrate-based preparations acted later. Upon the attainment of full ripeness, leaf moisture in all variants was 0.5-5.9 % lower than in control, and added micro elements had strengthened water loss by leaves.

During all years of research, we had not noted valid differences for dry weigh of leaves between the control and treated plants, but with total trend towards reduction of such value in the latter case. Stem weight in treated plants was higher than in control.

Final stage of grain maturation is a complex biochemical process related not only to supply of assimilation products to kernels, but to biosynthesis of reserve substances from low molecule compounds. Upon maturing, stable reduction of moisture with increase of dry matter weight and total number of cells both in germ and in epidermis [51].

At early maturing stages, water is removed from seeds first metabolically and then physically, due to evaporation [52, 53]. Therefore, moisture reduction in kernels serves an easily accounted maturing parameter [54]. From the biological point of view, it is generally understood to account seeds able to enable new generation. Technical ripeness occurs slightly later. Conventionally, full ripeness is fixed at moisture of panicles of 30 % of raw mass.

At artificial senesces during milk-wax ripeness stage, moisture of panicles reduced unevenly and was mainly determined by temperature mode (Fig.). From the treatment to water removal from rice check, panicle moisture had decreased at 0.87 % per day (0.79÷0.97 %) in 2009, at 0.76 % per day (0.73÷0.79 %) in 2010, and at 0.90 % per day (0.85÷0.96 %) in 2011, with 0.77; 0.71 and 0.80 % per day, respectively, for control. The process was mostly extensive during the 1st and 2nd weeks after the treatment, in average 1.47 and 0.89 % per day. Afterwards, panicle moisture reduction rate decreased to 0.46-0.50 % per day. In 2009 and 2011, when weather conditions delayed ripening, differences from control in the rate of water loss by rice panicles increased.

According to our observations, effect of urea-based and ammonium nitrate-based chemicals slightly varies. During the 1st week after treatments, panicles rapidly lost water due to ammonium nitrate-based preparations, within the 2nd week — due to urea-based preparations; afterwards, until the full ripeness the effects of the preparations were approximately equal. By the time of water removal from rice field for harvesting, panicle moisture in treating with ammonium nitrate chemicals was lower compared to urea. It was due to uneven absorption rate of preparations to leaves due to specificities of absorption mechanisms for amide and ammonium nitrogen. Ammonium nitrate penetrates more rapidly to a plant, and its action starts slightly earlier. More energy spent on involvement of ammonium nitrate in metabolism also impacts the effectiveness of chemicals. Mn and Se strengthened the effect of ammonium nitrate and urea, to a greater extent with the use of selenium due to its effect on water regime of plant. It should be noted that increase of selenium concentration in a solution accelerated water loss by panicles and, accordingly, allows earlier harvesting (data are not shown).

In 2009 and 2011, when air temperature during grain filling were close to long-term average annual values, panicles of threated plants attained 30 % moisture 1-6 days earlier than in control. Afterwards, they lost water faster, and before water release from rice checks the panicle moisture was 0.69-7.39 % lower than in control. If the air temperature in this period was significantly higher than long-term average annual values (in 2010), then panicles of treated plants reached 30% moisture 4-10 days earlier. However, in furtherance the rate of water loss by control plants increased, and the differences between all the plants were smoothed

comprising only 0.66-2.78% by the time of draining water from checks.

During maturing, panicle weigh achieves maximum at full grain ripeness. Artificial senescence ensured dry matter accumulation in grain. During the 1st week after treatment dry increased with the rate of 0.072-0.082 g/(plant · day), which is 24.1-41.4 % higher compared to control. In furtherance, until termination of grain filling, dry matter accumulation tended to gradual reduction. In control accumulation of dry matter by panicles had increased during the last week prior to termination of grain filling (Table 2).

During the first 2 weeks of milk-wax ripeness, dry weight of panicles increase faster with slight deceleration of the process later, and by termination of maturing it accelerated again, especially, in control. Upon initiation of artificial senescence by chemicals, dry weight of panicles increased during the 1st week by 35.4%, during the 2nd week by 20.3%, during the 3rd week by 10.6%, and during the 4th week by 12.2%. During the same time, in control plants the values increased only by 27.4; 14.8; 10.8 and 18.4%, respectively (see Fig.).

In 2009, which we had conventionally denoted as cold (with drop of temperature during grain filling period), differences in dry weigh from control in 1 week after treatment reached 5.3-6.4 % for urea (both separately and with Mn and Se) and 3.7-4.3 % for ammonium nitrate. During the next week, the difference increased up to 5.9-11.4 % for urea and up to 9.1-11.9 % for ammonium nitrate, and during the last week of grain filling the flow of assimilates in control plants was 1.3-1.6 times faster^ as a result, the differences were 1.0-5.8 % lower. In 2010 at beginning of grain filling, daily temperatures had risen to 40 °C. Because of combination of temperature and chemicals, differences between plants in panicles dry weigh were significantly less than in 2009, i.e. 0.7-3.6 % for the 1st week, 5.3-8.6 % for the 2nd week, and insignificant for the 3rd week. However, in furtherance in treated plants, assimilates started to be intensively remobilized to caryopsis, and differences from control increased to 4.5-13.9%. In 2011 when temperature during grain filling period was close to 2009 more significant positive effect of chemicals was found at identical trends of dry matter accumulation by panicles. The rate of panicle dry weigh increase in treated plants during the 1st and 2nd weeks was 38.2-79.4% higher than in control, from 14th to 21st day was 58.6 and 62.1% higher for urea and ammonium nitrate, only 6.9-17.2% higher for manganese modification, and decreased by 17.2 and 31.0%m respectively, for selenium modification. During the 4th week, remobilization of assimilates to panicles was 1.3-3.1 times slower than in control. Due to such dynamics of dry matter accumulation, dry weight of panicles in treated plants was higher than in control, in 1 week by 9.3-13.0%, in 2 weeks by 14.0-21.5%, and at full ripeness by 2.9-13.0%. In other words, should it be necessary to start harvesting much earlier, chemically induced artificial senescence could have enabled to save crop.

Our findings evidence that ultimately urea and ammonium nitrate had not significantly differed in the effect on rice grain maturation, regardless of the different moisture and dry weigh dynamics in panicles. Manganese-modified aqueous urea and ammonium nitrate solutions ensure better grain filling. Selenium accelerates water loss by panicles, which slows down the dray matter accumulation at the end of milk-wax ripening period.

Unconditionally, chemically induced intensive outflow of assimilates from vegetative organs as well as faster or higher water loss are advantageous and ensure less costs at harvest and during post-harvesting manipulations. However, in case of favorable weather conditions after chemically induced artificial senescence the shortened period of grain filling may result in crop lost. Thus, when choosing chemicals, we should prefer those unable to reduce crop under favorable temperature as compared to untreated crops. In cold 2009, we had noted in-

significant reduction in productivity caused by urea in combination with Se (Table 3), while ammonium nitrate-based preparations showed the best result. The preparations increased grain yield as a whole, and for ammonium nitrate + Mn the grain yield was significantly higher. The yield has grown due to an increase in grain weight per panicle and 1000-grain weights. In 2010, abnormally high air temperatures during the grain filling period were critical for yield formation. Urea solutions (separately and with Mn) did not block the negative consequences, and rice productivity did not differ from control. Urea solution with Se had reduced productivity by 12.2%, mainly due to lower 1000-grain weight. Ammonium nitrate promoted crop formation which was 7.9-14.7 % higher than in control. To a lesser extent, yield was favored by ammonium nitrate with Se. In 2011, temperature was lower than long-term annual average values and precipitations in May delayed sowing; however, maturing occurred at air temperature close to long-term average, with deviations of 0-1.0 °C. In 2011, urea and ammonium nitrate had not significantly influenced rice productivity, but upon use of solutions modified by manganese, crop yield gain was statistically significant (HCP_{05} = 0.38 t/ha) as compared to control and comprised 7.9 and 9.3 %, while Se showed a trend to reduce grain yield. At the same time, reduction of grain moisture at harvest under artificial senescence is worth attention. Therefore, at least because the cost of chemically induced plant senescence is not higher than that of grain drying to standard moisture, this approach is beneficial.

Induced plant senescence provides purposeful regulation of metabolism at final stages of plant ontogenesis, especially in risky zones of rice cultivation. And although recently induced rice senescence is used little [55, 56], which is mainly due to longstanding warm period in the long-term cycle of temperature fluctuations [57], further period with adverse weather conditions for rice ripening and harvesting is very likely, so the demand for this approach will grow. Universal nature of this agricultural technology deserves attention since it enables, if necessary, to promptly adjust the time of harvesting for a particular variety of any ripeness group. To effectively apply such method on modern rice varieties, one should first study grain maturation under artificial senescence. Novel chemical inducers of artificial senescence should be also safe for humans and environment [39]. The studies to just terms of plant treatment to induce artificial senescence are among key points [40]. According to our data, in these investigations the amount of effective temperatures (for rice over 15 °C) should be referred to [40].
Thus, it was shown than effectiveness of chemically induced artificial senescence of a middle-late rice variety depends on composition of the chemicals, terms of treatment, and weather conditions. The method should be used under maturation delay which more often occurs in late, thinned crops or crops with excessive nitrogen nutrition. The most suitable chemicals are aqueous ammonium nitrate solutions (15 kg/ha) with addition of manganese (400 g/ha) or selenium (200 g/ha). Selenium-modified solution should be used if harvesting should start in 14-20 days; an increase of selenium concentration in the solution accelerates water loss by panicles and reduces the time prior to harvesting. Treatment with manganese-containing solution promotes synthesis of assimilates and their remobilization to grain, which is followed by not so fast termination of ontogenesis as upon selenium-modified solution, but leads to a significant yield growth due to increase of grain yield per plant. Artificial senescence induced at the end of rice flowering is unreasonable as fast termination of vegetation reduces yield. The optimal time for treatment with chemicals is the beginning of milk-wax ripeness stage. In years when air temperature is lower or close to long-term annual average values, artificial senescence not only promotes faster (by 5-6 days) and higher (by 0.69-7.39%) water loss by panicles, but also increases grain yield by 4.2-9.3%. In

years with favorable weather conditions, the method ensures reduction of panicle moisture to time of water release from rice check only by 0.66-2.78 %, but increases productivity by 7.9-14.7 % due to additional supply of nitrogen to plants. Provided sufficient period of favorable temperatures for use of such nitrogen, the additional nutrition promotes increase of productivity of shoots of 2-3rd orders.

REFERENCES

1. *Paddy rice production worldwide 2017-2018, by country.* Available https://www.statista.com/statistics/255937/leading-rice-producers-worldwide/. Accessed 02.06.2019.
2. Chauhan B.S, Jabran K., Mahajan G. *Rice production worldwide.* Springer International Publising AG, Sqitzerland, 2017 (doi: 10.1007/978-3-319-47516-5).
3. Kraehmer H., Thomas C., Vidotto F. Rice production in Europe. In: *Rice production worldwide.* B. Chauhan, K. Jabran, G. Mahajan (eds.). Springer, Cham, 2017: 93-116 (doi: 10.1007/978-3-319-47516-5_4).
4. Tesio F., Tabacchi M., Cerioli S., Follis F. Sustainable hybrid rice cultivation in Italy. A review. *Agron. Sustain. Dev.*, 2014, 34: 93-102.
5. Beser N., Surek H., Sahin M. Search of effective resistant genes to the rice blast pathogen (*Magnaporthe grisea*) under field conditions in Turkey. *Fresenius Environmental Bulletin*, 2015, 24: 791-795.
6. Vorob'ev N.V. *Fiziologicheskie osnovy formirovaniya urozhaya risa* [Physiological basis of rice crop formation]. Krasnodar, 2013 (in Russ.).
7. de Vries G.E. Growing rice in Canada. *Trends in Plant Science*, 2000, 5(1): 8 (doi: 10.1016/S1360-1385(99)01535-6).
8. Clement C., Burrus M., Audran J.-C. Floral organ growth and carbohydrate content during pollen development in Lilium. *American Journal of Botany,* 1996, 83(4): 459-469 (doi: 10.2307/2446215).
9. Lemoine R., La Camera S., Atanassova R., Dédaldéchamp F., Allario T., Pourtau N., Bonnemain J.-L., Laloi M., Coutos-Thévenot P., Maurousset L., Faucher M., Girousse C., Lemonnier P., Parrilla J., Durand M. Source-to-sink transport of sugar and regulation by environmental factors. *Front. Plant Sci.*, 2013, 4: 272 (doi: 10.3389/fpls.2013.00272).
10. Ray S., Choudhuri M.A. Mobilization of metabolites from leaves to grains as the cause of monocarpic senescence in rice. *Plant Physiol.*, 1981, 68: 1345-1348.
11. Lim P.O., Kim H.J., Nam H.G. Leaf senescence. *Annual Review of Plant Biology*, 2007, 58: 115-136 (doi: 10.1146/annurev.arplant.57.032905.105316).
12. Li Z., Pan X., Guo X., Fan K., Lin W. Physiological and transcriptome analyses of early leaf senescence for *ospls1* mutant rice (*Oryza sativa* L.) during the grain-filling stage. *Int. J. Mol. Sci.*, 2019, 20: 1098 (doi: 10.3390/ijms20051098).
13. Bukreeva G.I., Gritsai T.I., Domchenko M.I. *Trudy Kubanskogo gosudarstvennogo agrarnogo universiteta*, 2015, 56: 85-91 (in Russ.).
14. Evdokimov M.G., Yusov V.S., Tatina B.M., Andreeva V.V. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2015, 11(133): 5-9 (in Russ.).
15. Wojciechowska N., Sobieszczuk - Nowicka E., Bagniewska - Zadworna A. Plant organ senescence — regulation by manifold pathways. *Plant Biol. J.*, 2018, 20: 167-181 (doi: 10.1111/plb.12672).
16. Afonin N.M. Effektivnost' desikatsii i senikatsii posevov kukuruzy pri vyrashchivanii na zerno. *Kukuruza i sorgo*, 2010, 3: 14-16 (in Russ.).
17. Kolosova E.N. *Materialy Mezhdarodnoi nauchno-prakticheskoi konferentsii «Nauchnoe obespechenie agropromyshlennogo proizvodstva»* [Proc. Int. Conf. «Scientific support of agroindustrial production»]. Kursk, 2010: 214-216 (in Russ.).
18. Distelfeld A., Avni R., Fischer A.M. Senescence, nutrient remobilization, and yield in wheat and barley. *J. Exp. Bot.*, 2014, 65: 3783-3798 (doi: 10.1093/jxb/ert477).
19. Jibran R., Hunter A., Dijkwel P. Hormonal regulation of leaf senescence through integration of developmental and stress signals. *Plant Mol. Biol.*, 2013, 82: 547-561 (doi: 10.1007/s11103-013-0043-2).
20. Lin M., Pang C., Fan S., Song M., Wei H., Yu S. Global analysis of the *Gossypium hirsutum* L. transcriptome during leaf senescence by RNA-seq. *BMC Plant Biology,* 2015, 15: 43 (doi: 10.1186/s12870-015-0433-5).
21. Kushu A.A. *Povysheniya produktivnosti semenovodcheskikh risovykh polei putem ispol'zovaniya senikatsii v usloviyakh Adygei. Avtoreferat kandidatskoi dissertatsii* [Improving the productivity of seed paddy fields through the use of an artificial aging technikigy in conditions of Adygea. PhD Thesis]. Krasnodar, 1995 (in Russ.).
22. Vorob'ev P.N. V sbornike: *Selektsiya, semenovodstvo i tekhnologiya vozdelyvaniya ovoshchnykh kul'tur* [In: Breeding, seed production and vegetable cultivation technology]. Voronezh, 1989: 77-83 (in Russ.).

23. Molyavko A.A., Marukhlenko A.V., Erenkova L.A., Borisova N.P. *Zashchita i karantin rastenii*, 2017, 11: 30-31 (in Russ.).
24. Goswamy B.K., Srivastava G.C. Effect of foliar application of urea on leaf senescence and photosynthesis in sunflower (*Helianthus annuus* L.). *Photosynthetica*, 1988, 22(1): 99-104.
25. Roman'ko Yu.A., Mel'nik A.V. *Izvestiya Samarskoi gosudarstvennoi sel'skokhozyaistvennoi akademii*, 2016, 1(2): 15-18 (in Russ.).
26. Seren K.D., Ignat'ev L.A. *Agrokhimiya*, 2008, 2: 50-56 (in Russ.).
27. Pavlyutina I.P., Moiseenko I.Ya., Likhachev B.S. *Kormoproizvodstvo*, 2005, 1: 24-27 (in Russ.).
28. Sheudzhen A.Kh., Bondareva T.N. *Agrokhimiya. Ch. 2. Metodika agrokhimicheskikh issledovanii* [Agrochemistry. Part 2. Methods of agrochemical research]. Krasnodar, 2015 (in Russ.).
29. Aleshin E.P., Avakyan K.M., Podlesnyi I.V., Semenova L.M., Korovyanskii A.G., Rybachenko S.I. *Byulleten' NTI VNII risa*, 1986, 36: 37-40 (in Russ.).
30. Craft J.C., Lindsey L.E., Barker D.J., Lindsey A.J. Quantification of soybean leaf senescence and maturation as impacted by soil- and foliar-applied nitrogen. *Crop, Forage & Turfgrass Management*, 2019, 5: 180051 (doi: 10.2134/cftm2018.07.0051).
31. Springer A., Kang C.H., Rustgi S., von Wettstein D., Reinbothe C., Pollmann S., Reinbothe S. Programmed chloroplast destruction during leaf senescence involves 13-lipoxygenase (13-LOX). *PNAS USA*, 2016, 113(12): 3383-3388 (doi: 10.1073/pnas.1525747113).
32. Panda D., Sarkar R.K. Natural leaf senescence: Probed by chlorophyll fluorescence, CO_2 photosynthetic rate and antioxidant enzyme activities during grain filling in different rice cultivars. *Physiol. Mol. Biol. Plants*, 2013, 19(1): 43-51 (doi: 10.1007/s12298-012-0142-6).
33. Avila-Ospina L., Marmagne A., Talbotec J., Krupinska K., Masclaux-Daubresse C. The identification of new cytosolic glutamine synthetase and asparagine synthetase genes in barley (*Hordeum vulgare* L.), and their expression during leaf senescence. *J. Exp. Bot.*, 2015, 66(7): 2013-2026 (doi: 10.1093/jxb/erv003).
34. Britto D.T., Kronzucker H.J. $NH4^+$ toxicity in higher plants: a critical review. *Journal of Plant Physiology*, 2002, 159(6): 567-584 (doi: 10.1078/0176-1617-0774).
35. Bieker S., Zentgraf U. Plant senescence and nitrogen mobilization and signaling. In: *Senescence and senescence-related disorders*. Z. Wang, H. Inuzuka (eds.). Intech Open, 2013: 53-83 (doi: 10.5772/54392).
36. Nawaz F., Naeem M., Ashraf M.Y., Tahir M.N., Zulfiqar B., Salahuddin M., Shabbir R.N., Aslam M. Selenium supplementation affects physiological and biochemical processes to improve fodder yield and quality of maize (*Zea mays* L.) under water deficit conditions. *Front. Plant Sci.*, 2016, 7: 1438 (doi: 10.3389/fpls.2016.01438).
37. Takano J., Wada M., Ludewig U., Schaaf G., von Wirén N., Fujiwara T. The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell*, 2006, 18: 1498-1509 (doi: 10.1105/tpc.106.041640).
38. Socha A.L., Guerinot M.L. Mn-euvering manganese: The role of transporter gene family members in manganese uptake and mobilization in plants. *Front. Plant Sci.*, 2014, 5: 106 (doi: 10.3389/fpls.2014.00106).
39. Sheudzhen A.Kh. *Teoriya i praktika primeneniya mikro- i ul'tramikroudobrenii v risovodstve* [Theory and practice of micro- and ultra-micronutrient fertilizers in rice cultivation]. Maikop, 2016 (in Russ.).
40. Sheudzhen A.Kh., Bondareva T.N., Kharchenko P.N., Doroshev I.A., Khurum Kh.D. Time determination of conducting senication of rice crops taking into account the sum of effective temperatures. *Russian Agricultural Sciences*, 2018, 44(6): 499-504 (doi: 10.3103/S1068367418060149).
41. Page V., Weisskopf L., Feller U. Heavy metals in white lupine: uptake. Root-to-shoot transfer and redistribution within the plant. *New Phytologist*, 2006, 171: 329-241 (doi: 10.1111/j.1469-8137.2006.01756.x).
42. Wyman A. J., Yocum C. F. Structure and activity of the photosystem II manganese-stabilizing protein: role of the conserved disulfide bond. *Photosynthesis Research*, 2005, 85: 359-372 (doi: 10.1007/s11120-005-7385-9).
43. Popelkova H., Yocumb C.F. PsbO, the manganese-stabilizing protein: analysis of the structure—function relations that provide insights into its role in photosystem II. *Journal of Photochemistry and Photobiology B: Biology*, 2011, 104(1-2): 179-190 (doi: 10.1016/j.jphotobiol.2011.01.015).
44. Sheudzhen A.Kh., Bondareva T.N., Doroshev I.A., Esipenko S.V. *Selenovye udobreniya na posevakh risa* [Selenium fertilizer for rice crops]. Maikop, 2017 (in Russ.).
45. Seppanen M., Turakainen M., Hartikainen H. Selenium effects on oxidative stress in potato. *Plant Science*, 2003, 165: 311-319 (doi: 10.1016/S0168-9452(03)00085-2).
46. Irmak S. Effects of selenium application on plant growth and some quality parameters in peanut (*Arachis hypogaea*). *Pak. J. Biol. Sci.*, 2017, 20(2): 92-99 (doi: 10.3923/pjbs.2017.92.99).
47. Couto N., Wood J., Barber J. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. *Free Radic. Biol. Med.*, 2016, 95: 27-42 (doi: 10.1016/j.freeradbiomed.2016.02.028).
48. Trivedi D.K., Gill S.S., Yadav S., Tuteja N. Genome-wide analysis of glutathione reductase (GR) genes from rice and *Arabidopsis*. *Plant Signal. Behav.*, 2013, 8(2): e23021 (doi:

10.4161/psb.23021).
49. Garousi F. Toxicity of selenium, application of selenium in fertilizers, selenium treatment of seeds, and selenium in edible parts of plants. *Acta Universitatis Sapientiae, Alimentaria*, 2017, 10: 61-74 (doi: 10.1515/ausal-2017-0004).
50. Rani N., Dhillon K.S., Dhillon S.K. Critical levels of selenium in different crops grown in an alkaline silty loam soil treated with selenite-Se. *Plant Soil*, 2005, 277: 367-374 (doi: 10.1007/s11104-005-8161-5).
51. Wu X., Li J., Li D., Liu C.M. Rice caryopsis development II: Dynamic changes in the endosperm. *J. Integr. Plant Biol.*, 2016, 58: 786-798 (doi: 10.1111/jipb.12488).
52. Brooking I.R. Maize ear moisture during grain-filling, and its relation to physiological maturity and grain-drying. *Field Crops Research*, 1990, 23(1): 55-68 (doi: 10.1016/0378-4290(90)90097-U).
53. Maiorano A., Fanchini D., Donatellib M. MIMYCS.Moisture, a process-based model of moisture content in developing maize kernels. *European Journal of Agronomy*, 2014, 59: 86-95 (doi: 10.1016/j.eja.2014.05.011).
54. Lin L., He Y., Xiao Z., Zhao K., Dong T., Nie P. Rapid-detection sensor for rice grain moisture based on NIR spectroscopy. *Appl. Sci.*, 2019, 9(8): 1654 (doi: 10.3390/app9081654).
55. Molokov L.G., Pokhno S.L., Zeleneva I.A., Grigor'ev V.A., Dvornik E.D. *Risovodstvo*, 2013, 23: 68-72 (in Russ.).
56. Shkhapatsev A.K. *Novye tekhnologii*, 2006, 2: 44-46 (in Russ.).
57. Sheudzhen A.Kh., Galkin G.A., Bondareva T.N. *Risovodstvo*, 2007, 11: 24-28 (in Russ.).

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COMPARATIVE EFFECTIVENESS OF SHORT-TERM DAILY TEMPERATURE DROP AND PERIODIC DROUGHT AS METHODS TO REGULATE ELONGATION OF CUCUMBER (*Cucumis sativus* L.) PLANTS

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Abstract

Daily short-term temperature drop (DROP) and "periodic drought" (non-lethal water deficit) are used for plant height control as techniques inhibiting plant growth as an alternative to the use of retardants (chemical growth control). However, it is not known which of these two techniques is more effective and whether their combined effect can be stronger. In this paper taking cucumber as an example we have shown for the first time that a temperature drop technique is more effective than "periodic drought". Temperature drops combined with "periodic drought" retard plant growth and enhance plant tolerance, but depending on the relative air humidity may decrease values of some physiological and morphological parameters. The aim of this work was: (a) a comparative assessment of the effectiveness of DROP treatments and "periodic drought", and (b) the study of the combined effects of these two techniques on plant growth and tolerance to chilling temperature and water stress. Cucumber plants (*Cucumis sativus* L.) were exposed daily to a temperature of 10 °C for 2 hours at the end of the night (DROP treatment) for 6 days. The plants were watered daily or watered after the drying of the substrate (once every 2-3 days) creating "periodic drought" (drought treatment). Control plants were watered daily and not exposed to low-temperature treatments. All experiments were carried out at a low (30 %) or high (80 %) relative air humidity (RH). After the termination of the DROP treatments, plants from each treatment (control, DROP, drought, DROP + drought) were subjected to a cold test in the darkness for 1 day at a temperature of 4 °C. The plant height, length of leaf petioles, the area and number of leaves and plant dry mass were determined. The compactness of the plants was determined as the plant dry weight or leaf area per unit stem length (in mg/cm or cm^2/cm). Plant tolerance to low temperature and water stress was estimated by relative electrolyte leakage from leaf tissues and the intensity of lipid peroxidation, as assessed by the content of malonic dialdehyde. Differences between the treatments means were tested with one-way ANOVA followed the least significance difference (LSD) test with $p < 0.05$ level of significance. The obtained results indicate that DROP-treated plants had more dry mass and leaf area per unit length of the stem compared to those treated by "periodic drought". However, DROP treatments were effective in increasing plant compactness only under high (80 %) RH, while low (30 %) RH leveled out the effects of a temperature drop. "Periodic drought" can produce small, but not truly compact plants due to a more significant decrease in the leaf area and plant biomass compared to plant size. Thus, a temperature drop is a more effective technique compared to "periodic drought" that can be used to control plant growth and obtain compact plants. The combination of DROP treatments with "periodic drought" also increases plant compactness and besides enhances plant tolerance to water stress induced by low temperature. However, for a number of parameters (number of leaves, compactness of plants at low RH), the combination of DROP treatments and "drought" led to a worse result than the application of only the first of these two agro-practices.

Keywords: chilling temperature, water stress, plant growth, tolerance, release of electrolytes, lipid peroxidation

For many years, the use of chemical regulators with a retardant effect

was considered the most effective way to control the linear growth of plants [1, 2]. However, over the past 30 years, new legal restrictions on their use have been constantly introduced in the world due to the risk of environmental pollution and the potential danger of chemical residues to human health. This has become a significant incentive to conduct research in order to find new methods and techniques for managing the growth of greenhouse crops [5-8]. In this case, natural factors affecting plant growth, such as temperature, light (intensity and spectral composition), photoperiod, relative air humidity (RH), CO_2 concentration, as well as planting density and container size, were used. The results of manipulation of these factors and their analysis showed that not all methods that effectively reduce the height of plants are applicable in terms of commerce. The most promising method in practical terms was the control of plant growth by changing the temperature and parameters of the moisture regime [2, 4, 9, 10]. So, in the mid-1980s, it was found that with the help of lower daytime temperature, it is possible to reduce the height of plants of many species [3, 5, 7]. However, the practical use of this feature is limited to periods with low air temperature during the year. A little later in greenhouses, a more economical method of daily short-term temperature reduction was applied, called "temperature drop" (in Europe) and "temperature dip" or "cool morning pulse" (in the USA), which inhibited the growth in height of many crops [7, 11, 12]. Usually, in this case, the temperature is reduced at the end of the night, and in the morning, an additional influx of energy is provided using the backlight in order to raise the temperature after the drop.

Similarly to the low-temperature effect, the so-called "periodic drought" (non-lethal water deficit) is also used in crop production to inhibit plant growth as another alternative to retardants [13-16]. It is believed that in this case not only the height of plants is decreased, but also their resistance is increased, so that they better tolerate possible stresses, for example, during transportation, sale or after planting in the ground [17]. Water stress is mainly used in the greenhouse production of seedlings of flower beds [14, 17, 18], in which the height is an important quality indicator, since excessive growth leads to increased transportation costs and greater sensitivity to storage conditions [15, 16].

Therefore, both daily short-term temperature drops (DROP effects) and water stress ("periodic drought") can inhibit the linear growth of plants. However, the literature does not contain data on which of these two agricultural methods is more effective. It is also unclear whether the effect can be enhanced by their combined use, since the mechanisms of action of these factors on plants are not the same.

In this report, for the first time, the authors showed the great effectiveness of the DROP method using the example of cucumber plants. A decrease in temperature, combined with periodic drought, also inhibits linear growth and at the same time increases the resistance of plants to water deficiency, but worsens some physiological and morphological characteristics depending on the relative humidity (RH).

The purpose of the work was to study the separate and combined influence of two natural factors (temperature and humidity) as ways to influence the linear growth and plant resistance in a greenhouse.

Techniques. Cucumber plants (*Cucumis sativus* L., Zozulya F_1 hybrid) were grown in pots (250 ml) with sand in an environmental chamber (Vötsch VB 1014, Vötsch Indusrietechnik GmbH, Germany) under watering with a nutrient solution (in mg/l: 226 N, 55 P, 370 K, 180 Ca, 40 Mg, 45 S, 17 Na, 52 Cl, 2.5 Fe, 0.6 Mn, 0.35 B, 0.3 Zn, 0.15 Cu and 0.05 Mo, pH 6.2-6.4); air temperature 23 °C, photosynthetically active radiation (PAR) 150 μmol/($m^2 \cdot s$), photoperiod

12 h. All experiments were carried out at relatively high (80%) or low (30%) RH. From the 6th day after the soaking of the seeds, when the cotyledonary leaves developed, different watering regimes were applied – daily or after drying of the substrate (sand) (1 time in 2-3 days), thereby creating the conditions of the so-called "periodic drought" ("drought" option). From the 14th day after the soaking of the seeds, when the first true leaf was in the phase of active growth and reached half the final size, some plants with different irrigation regimes were exposed to a temperature of 10 °C for 2 h (plus 30 min to decrease and 30 min to increase temperature) at the end of the night period (DROP variant) for 6 days. The temperature was decreased and increased at a rate of 0.4 °C/min. The control variant was plants receiving daily watering and not exposed to low temperature. In total, two series of experiments under conditions of different RH – high (80%) or low (30%) were conducted, in each of which there were 4 variants with the following conditions: 1st option — constant temperature 23 °C, daily watering (control); 2nd option — DROP exposure, daily watering (DROP); 3rd option — constant temperature 23 °C, "periodic drought" ("drought"); 4th option — DROP exposure, "periodic drought" (DROP + "drought").

Upon completion of DRO exposure, 6 plants from each variant (control, DROP, "drought", DROP + "drought") were exposed to a temperature of 4 °C in the dark chamber with RH of 90-100% within 24 hours ("cold test"), after which they were placed for 24 hours in a chamber with a temperature of 23 °C.

All measurements were carried out 1 day after the completion of DROP exposure or 1 day after the completion of the cold test. The height of the plants, the length of the leaves petioles, the area and number of leaves reaching a length of 10 mm or more, and the dry biomass of the plants were determined. The compactness of plants was calculated as the ratio of the dry mass of the plant to its height (mg/cm) or as the ratio of the area of leaves to the height of the plant (cm^2/cm) [19].

The resistance of leaves to low temperature and water stress was estimated by the relative release of electrolytes (RRE) from leaf tissue, which was determined using an Expert-002 conductivity meter with a probe for microvolumes UEP-P-S (Econix-Expert, Russia), and the intensity of lipid peroxidation, estimated by the content of malondialdehyde (MDA) according to the method of Heath and Packer [20].

Each experiment was repeated twice. The figures show the average values (M) for $n \geq 6$ and their standard errors (±SEM). The difference between the mean values was determined on the basis of the analysis of variance (according to the LSD criterion) using the Statistica software (v. 8.0.550.0, StatSoft Inc., USA) and was considered statistically significant at $p < 0.05$.

Results. The study showed that both DROP exposure and "periodic drought" reduce the height of the plant and the length of leaf petioles at high and low RH (Fig. 1, A, B, Table). In the case of the combination of DROP and "drought", the effect was more intensive, which led to an even greater decrease in the height and length of leaf petioles. It should be noted that the table shows the values of the indicators as a percentage of the control variant, but under the conditions of low RH, the biometric parameters of the control variant plants (plant height, leaf petiole length, leaf area, and dry plant weight) were halved, and the number of leaves was reduced by a third.

The leaf area did not decrease as a result of DROP exposure (with daily watering) at high RH and decreased by 10% at low RH. "Drought" led to a significant ($p < 0.05$) decrease in the leaves area by 23 and 25% under conditions of a correspondingly high and low RH (see Fig. 1, B, Table). In the case of the combined action of DROP and "drought", the effect was intensified (decrease in

the leaf area by 41%, $p < 0.05$) only under the conditions of low RH. DROP exposure and "drought" did not affect the rate of leaf development; however, when these factors were combined, the number of leaves decreased by 20 and 27%, respectively ($p < 0.05$) under the conditions of high and low RH (see Table).

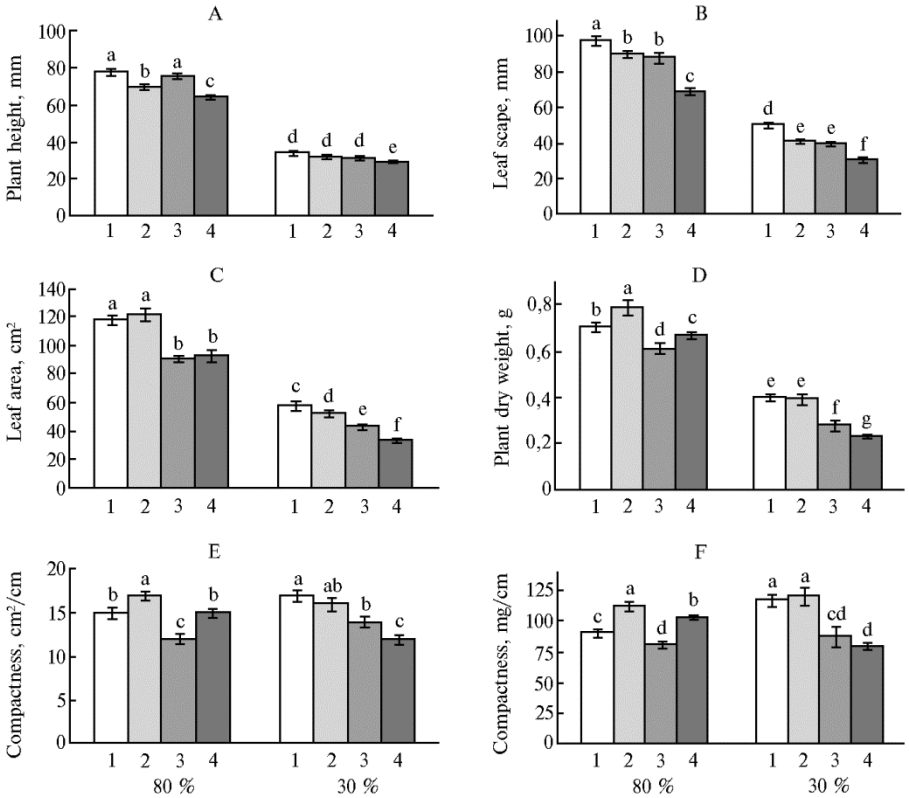

Fig. 1. Plant height (A)**, leaf petiole length** (B)**, area** (C)**, dry weight (D) and compactness** (D, E) of the **control** (1)**, subjected to DROP exposure** (2)**, "drought"** (3) **and the combined action of DROP and "drought"** (4) **plants of the cucumber (*Cucumis sativus* L., Zozulya hybrid F_1) at a relative humidity of 80% or 30%** (pot trials). Different letters indicate the statistical significance of differences in mean values at $p < 0.05$.

Indicators of growth, development, and resistance of cucumber plants (*Cucumis sativus* L., Zozulya hybrid F_1) under the DROP exposure, "periodic drought" and their combination (% of the control variant) (pot trials)

Показатель	Options of the experiment					
	RH 80%			RH 30 %		
	DROP	"drought"	DROP + "drought"	DROP	"drought"	DROP + "drought"
Plant height	90*	97	83*	95	92*	87*
Petiole length	92*	90*	71*	83*	79*	61*
Leaf area	103	77*	79*	90*	75*	59*
Number of leaves	100	100	80*	103	94	73*
Dry weight of plants	112*	87*	95*	98	69*	59*
Compactness						
acc. to biomass	124*	89*	114*	103	75*	68*
acc. to leaves area	113*	80*	100	94	82*	71*
MDA content	95	48*	34*	100	93	83*
RRE	102	57*	21*	65*	76*	65*

N o t e. Indicators of control plants were taken as 100%. Absolute values of the indices of the control plants are shown in Figs. 1 and 2. The content of malondialdehyde (MDA) and the relative release of electrolytes (RRE) are given for plant leaves after a cold test. RH — relative humidity.
* Differences with control are statistically significant at $p < 0.05$.

The dry weight of plants under the influence of DROP exposure increased by 12% compared to the control variant ($p < 0.05$) at high RH and did

not differ from the control variant at low RH (see Fig. 1, D, Table). "Drought" reduced the dry weight of plants under the conditions of high and low RH by 13 and 31%, respectively ($p < 0.05$). In the case of the combined action of DROP and "drought", the dry mass decreased by 5% ($p < 0.05$) at high RH and by 41% ($p < 0.05$) at low RH. Under the influence of DROP exposure and at a high RH, the ratio of dry biomass to plant height increased (by 24%, $p < 0.05$), as well as the ratio of leaf area to plant height (by 13%, $p < 0.05$); at low RH, these indicators did not differ from the control variant (see Fig. 1, D, E, Table). "Drought" in all variants of the experiment led to a decrease in the compactness of plants. The combination of DROP and "drought" caused an increase in the ratio of dry biomass to plant height by 14% ($p < 0.05$) only under the conditions of high RH, and at low RH compactness was even lower than in the case of using these methods separately.

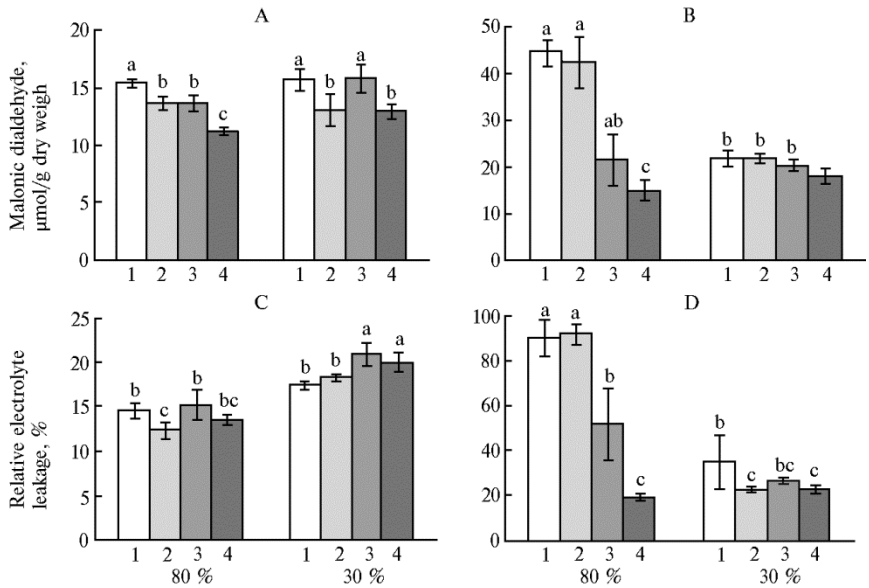

Fig. 2. The content of MDA (A, B) **and the relative release of electrolytes** (RRE) (C, D) **before cold testing** (A, C) **and after it** (B, D) **in the control** (1)**, subjected to DROP exposure** (2)**, "drought"** (3) **and the combined action of DROP and "drought"** (4) **in cucumber plants (*Cucumis sativus* L., Zozulya hybrid F_1)) at a relative humidity of 80% or 30%** (pot trials). Different letters indicate the statistical significance of differences in mean values at $p < 0.05$.

The MDA content, which indicates the intensity of lipid peroxidation, was 11 and 17% lower ($p < 0.05$) in the leaves of plants subjected to DROP exposure, at high and low RH, respectively (Fig. 2, A). "Drought" reduced the content of MDA by 11% ($p < 0.05$) only under the conditions of high PH, without affecting this indicator at low PH. In the case of the combined action of DROP and "drought" and high RH, there was a greater decrease in the content of MDA (by 27%, $p < 0.05$), while at low RH it was 17% ($p < 0.05$), as in the case of a separate DROP exposure. The values of RRE at high RH in plants under the influence of DROP were 15% lower ($p < 0.05$) than the control, but in the variants "drought" and DROP + "drought" these values did not differ significantly from the control (see Fig. 2, B). At low PH, DROP effects did not affect the RRE, but separate "drought" and DROP + "drought" led to an increase in the RRE by 20 and 15%, respectively ($p < 0.05$).

After the cold test (4 °C for 1 day), visually fixed cold injuries (leaf necrosis) in all experimental variants were more pronounced in plants grown under conditions of high RH (Fig. 3). The leaves of the control plants were the most

injured, and the plants subjected to the combined action of DROP and "drought" were the least injured (see Fig. 3). The MDA content in the leaves of plants subjected to DROP exposure did not differ from the control plants (see Fig. 2, B, Table), irrespective of the RH. The leaves of plants subjected to the "drought" effect had a significantly lower (52%) MDA content at high RH and comparable to the control at low RH. In the DROP + "drought" variant, the MDA content was 66 and 17% lower than in the control plants, at high and low RH, respectively.

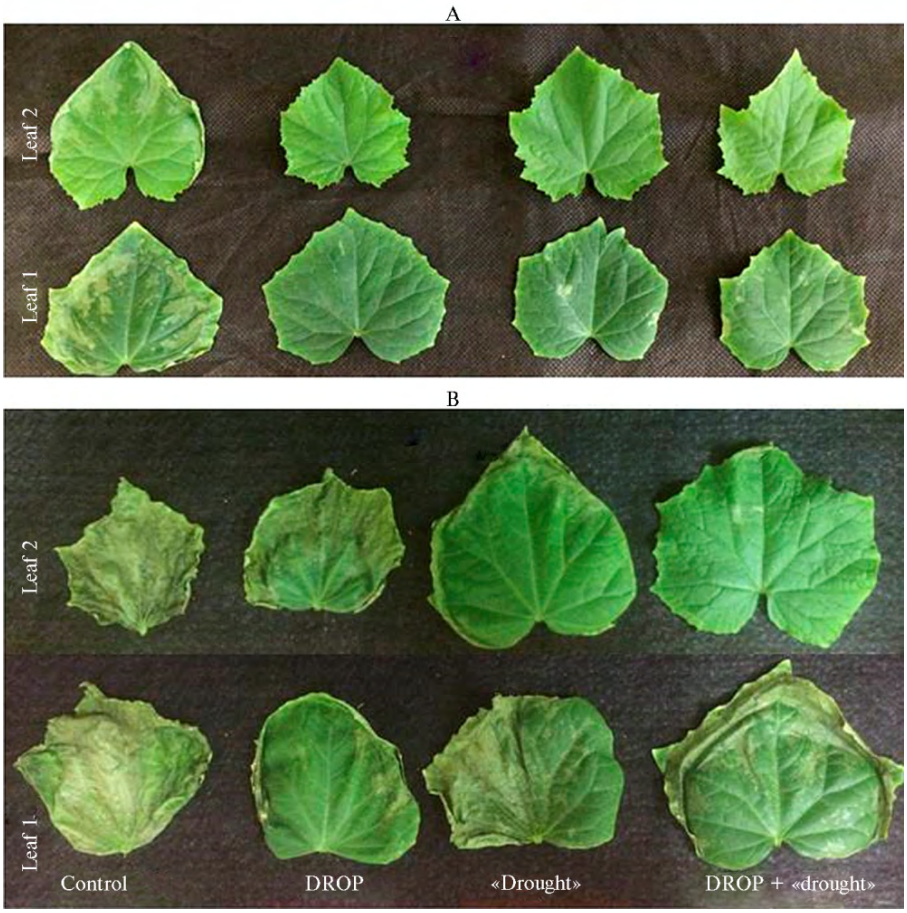

Fig. 3. The first and second true leaves of the control plants (Control) **and plants subjected to DROP exposure** (DROP)**, "periodic drought"** ("drought") **and DROP + "drought" at relative humidity** (RH) **30%** (A) **or 80%** (B) **after a cold test** (4 °C for 1 day) **of cucumber (*Cucumis sativus* L., Zozulya hybrid F_1)** (pot trials).

After the cold test, the RRE was at the control level in plants exposed to DROP at high RH, and 24% less at low RH. In the "drought" and DROP + "drought" variants, the RRE decreased significantly at high RH – by 43 and 79%, respectively, and at low RH – only by 24-35% (see Fig. 2, D, Table).

The results of the study showed that under the conditions of high RH and normal watering, DROP exposure has a pronounced morphogenetic effect, reducing the linear dimensions of plants. At the same time, the dry weight of plants increases slightly, which leads to an increase in the ratio of dry weight to plant height. The ratio of the leaves area to plant height also increases, that is, plants become more compact. A similar effect was previously shown when watering tomato seedlings using cold water (5 °C and 15 °C), which led to an increase in the ratio of dry weight to plant height by 28 and 32% compared with

the variant in which watering was carried out using water at a temperature of 27.5-30.5 °C [21]. The compactness of plants, which is defined not only as a decrease in the linear dimensions of plant organs but also as the ratio of the dry mass of the plant to its height [19], increased as a result of a decrease in plant height while maintaining the biomass accumulation rate, as in the authors' experiment.

At low RH, the authors observed a significant inhibition of all growth processes, both linear growth and biomass accumulation; however, the relative decrease in plant height was greater than the decrease in dry weight, which also led to an increase in the ratio of plant biomass to its height under normal watering. Under these conditions, DROP exposure did not give an additional effect. Consequently, it can be concluded that low RH levels the effect of DROP in terms of plant compactness. The low RH in the authors' experiments led to a significant decrease in plant height, which contradicts the opinion of some authors [22, 23] about the weak effect of RH on plant growth in height, but is consistent with the findings that there is a positive correlation between shoot length and RH [24-26]. However, it should be noted that although growth retardation occurs at a low RH, and the ratio of the plant's dry mass to its height increases, this method can hardly be an alternative to retardants due to a significant reduction in the leaves area and plant biomass.

"Periodic drought" also had a pronounced morphogenetic effect on plants, which involves a decrease in the height and length of leaves petioles, but, unlike DROP exposure, "drought" reduced the leaves area and dry weight of plants. As a result, the ratio of the dry mass of the plant to its height and the ratio of the leaves area to plant height decreased. Similar results, when "drought" reduced the linear sizes of plants, but did not increase their compactness, were also observed in other species [19]. Other side effects of using water stress to control plant growth are also noted in the literature: a decrease in the rate of photosynthesis, a decrease in branching, an increase in the dispersion in terms of height of plants, deterioration of decorative properties (for example, due to a change in the angle of inclination of leaves), later and less abundant flowering [16, 27, 28].

The combined effect of DROP and "drought" increased the inhibition of linear growth compared to their separate application, but due to a decrease of the leaves area and dry mass of plants, this led to an increase in the ratio of dry mass of plant to its height by only 14% ($p < 0.05$) and only under conditions of high RH, while the increase in this indicator under the influence of DROP exposure under normal watering was 24% ($p < 0.05$). In addition, in the case of the combined action of DROP and "drought", a delay in the development of leaves was observed.

It should be noted that, according to the authors' data, both DROP exposure and "periodic drought" do not lead to either an increase in the lipid peroxidation rate, estimated according to the MDA content, or to an increase in the permeability of cell membranes, characterized by the RRE index. The results of the cold test showed that plants subjected to the combined action of DROP and "drought" were the most cold-resistant. It is likely that their increased resistance to low temperature is due to greater resistance to water stress caused by the action of low temperature. Many papers have shown that cooling affects thermophilic plants indirectly through water stress by disrupting plant water metabolism [29]. In such cases, the primary cause of cold damages in thermophilic species is the fall of turgor as a result of the violation of the stomatal conductance control and water loss during transpiration with reduction of the ability of the roots to compensate for these losses. After returning to normal conditions,

necrotic spots appear on the dried parts of the leaves, which was observed in the authors' experiments, especially in control plants grown under the conditions of high RH. It was also previously reported that drought hardening prevents cold damage in thermophilic plants [30]. The higher resistance of plants to low temperature in the variant of combining DROP exposure and "drought" is apparently due to their better ability to regulate stomatal conductance under stress.

Thus, the obtained data indicate the effectiveness of DROP exposure for the inhibition of linear growth of plants with a simultaneous increase in the ratio of the leaves area and plant biomass to its height under the conditions of high RH, that is, DROP makes plants more compact. Under the conditions of low RH, these effects are leveled. Despite the fact that the watering regime, under which the conditions of "drought" are created, leads to a decrease in the size of plants, it reduces the ratio of the leaves area and plant biomass to its height. Therefore, it can be concluded that DROP exposure as an agricultural method is more effective than "periodic drought" and can be used to control the growth and obtain more compact plants as an alternative to retardants. The combination of DROP exposure with "periodic drought" also provides more compact plants, while increasing their resistance to water stress induced by low temperature. However, for a number of parameters (number of leaves, plant compactness under conditions of low RH), the combination of DROP exposure and "drought" has worse results than in the case of using only the first of these two methods.

REFERENCES

1. Rademacher W. Plant growth regulators: Backgrounds and uses in plant production. *J. Plant Growth Regul.*, 2015, 34: 845-872 (doi: 10.1007/s00344-015-9541-6).
2. Bergstrand K.-J.I. Methods for growth regulation of greenhouse produced ornamental pot- and bedding plants — a current review. *Folia Hort.*, 2017, 29(1): 63-74 (doi: 10.1515/fhort-2017-0007).
3. Heins R.D., Erwin J.E. The history of DIF and the use of a morning temperature dip to control plant height. *Minnesota Commercial Flower Growers Bulletin*, 1991, 40: 1-4.
4. Runcle E. Non-chemical height control techniques. *Greenhouse Product News*, 2014, 8: 58.
5. Moe R. Control of plant morphogenesis and flowering by temperature alterations. *Flowering Newsletter*, 1993, 15: 30-34.
6. Bachman G.R., McMahon M.J. Day and night temperature differential (DIF) or the absence of far-red light alters cell elongation in 'Celebrity White' petunia. *Journal of the American Society for Horticultural Science*, 2006, 131: 309-312 (doi: 10.21273/JASHS.131.3.309).
7. Runcle E. Controlling height with temperature drops. *Greenhouse Product News,* 2009, 4: 50.
8. Poorter H., Bühler J., van Dusschoten D., Climent J., Postma J.A. Pot size matters: a meta-analysis of the effects of rooting volume on plant growth. *Functional Plant Biology*, 2012, 39: 839-850 (doi: 10.1071/FP12049).
9. Currey C.J., Lopez R.G. Non-chemical height control. *Greenhouse Grower*, 2010, 11: 24-30.
10. Dean J. Using temperature to control growth. *Greenhouse Management*, 2011, 6: 1.
11. Myster J., Moe R. Effect of diurnal temperature alternations on plant morphology in some greenhouse crops: a mini review. *Scientia Horticulturae*, 1995, 62(4): 205-215 (doi: 10.1016/0304-4238(95)00783-P).
12. Moe R., Heins R.D. Thermo- and photomorphogenesis in plants. In: *Advances in floriculture research. Report no 6/2000.* E. Strømme (ed.). Agricultural University of Norway, Spekter, Oslo, 2000: 52-64.
13. Hendriks L., Ueber E. Alternative methods of regulating the elongation growth of ornamental plants: a current assessment. *Acta Horticulturae*, 1995, 378: 159-167 (doi: 10.17660/ActaHortic.1995.378.20).
14. Latimer J.G., Severson R.F. Effects of mechanical and moisture-stress conditioning on growth and cuticle composition of broccoli transplants. *Journal of the American Society for Horticultural Science*, 1997, 122: 788-791 (doi: 10.21273/JASHS.122.6.788).
15. Liptay A., Sikkema P., Fonteno W. Transplant growth control through water deficit stress — a review. *HortTechnology*, 1998, 8(4): 540-543 (doi: 10.21273/HORTTECH.8.4.540).
16. Carvalho S.M.P., van Noort F., Postma R., Heuvelink E. Possibilities for producing compact floricultural crops. Report 173. Wageningen UR Greenhouse Horticulture, Wageningen, 2008. Available http://library.wur.nl/WebQuery/wurpubs/fulltext/11685. No date.

17. Latimer J.G., Oetting R. Greenhouse conditioning affects landscape performance of bedding plants. *Journal of Environmental Horticulture*, 1998, 16: 138-142.
18. Brown R.D., Eakes J., Behe B.K., Gilliam C.H. Moisture stress: An alternative method of height control to B-nine (daminozide). *Journal of Environmental Horticulture*, 1992, 10: 232-235.
19. van Iersel M.W., Nemali K.S. Drought stress can produce small but not compact marigolds. *HortScience,* 2004, 39(6): 1298-1301 (doi: 10.21273/HORTSCI.39.6.1298).
20. Heath R.L., Packer L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.*, 1968, 125(1): 189-198 (doi: 10.1016/0003-9861(68)90654-1).
21. Chen J.J., Sun Y.W., Sheen T.F. Use of cold water for irrigation reduces stem elongation of plug-grown tomato and cabbage seedlings. *HortScience*, 1999, 34(5): 852-854 (doi: 10.21273/HORTSCI.34.5.852).
22. Mortensen L.M. Effects of air humidity on growth, flowering, keeping quality and water relations of four short-day greenhouse species. *HortScience*, 2000, 86: 299-310 (doi: 10.1016/S0304-4238(00)00155-2).
23. Eveleens B.A., Heuvelink E., Van Noort F.R. *Invoed van EC en RV op de groei en kwaliteitvan Kalanchoe.* Wageningen UR Glastuinbouw, Bleiswijk, 2007.
24. Armitage A.M., Kowalski T. Effect of irrigation frequency during greenhouse production on the postproduction quality of *Petunia hybrida* Vilm. *Journal of the American Society for Horticultural Science*, 1983, 108: 118-121.
25. Mortensen L.M. Effect of relative humidity on growth and flowering of some greenhouse plants. *Scientia Horticulturae*, 1986, 29(4): 301-307 (doi: 10.1016/0304-4238(86)90013-0).
26. Gislerød H.R., Nelson P.V. Effect of relative air humidity and irradiance on growth of *Dendranthema grandiflorum* (Ramat.) Kitamura. *Gartenbauwissenschaft*, 1997, 62: 214-218.
27. Hanssen S.W., Petersen K.K. Reduced nutrient and water availability to hibiscus rosa-sinensis Cairo Red as a method to regulate growth and improve post-production quality. *European Journal of Horticultural Science,* 2004, 69: 159-166.
28. Runcle E. Height control for vegetable transplants. *Greenhouse Product News,* 2010, 2: 50.
29. Janowiak F. Effect of water saturated atmosphere on chilling injuries of maize seedlings (*Zea mays* L.). *Acta Physiologiae Plantarum*, 1989, 11(2): 89-96.
30. Starck Z., Choluj D., Gawronska H. The effect of drought hardening and chilling on ABA content in xylem sap and ABA – delivery rate from root of tomato plant. *Acta Physiologiae Plantarum*, 2004, 20(1): 41-48 (doi: 10.1007/s11738-998-0041-1).

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INTEGRITY OF NUCLEAR DNA AND PHYSIO-BIOCHEMICAL INDICATORS OF *Pisum sativum* L. SEEDS UNDER ACCELERATED AGING

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Abstract

Currently, much attention is paid to understanding the roles of DNA and the main mechanisms for ensuring stability of the genome in maintaining seed viability during aging. It is also shown that significant oxidative damage to DNA occurs during seed swelling, and active DNA restoration processes are a factor that facilitates the initiation of DNA replication and rapid germination of seeds. Our objective was to study, on the example of two pea varieties and their hybrid, the effect of accelerated seed aging on the level of DNA damage (by DNA comet method) and biochemical indicators (lipid peroxidation, peroxidase activity, content of low-molecular antioxidants) in cells of embryos during seed swelling to find a relationship between these parameters and the changes in physiological parameters of seed germination. It is shown that accelerated aging leads to changes in pea seed germination capacity which are varietal specific, as well as in the biochemical indicators studied. The least resistant to the accelerated aging was Melkosemyannyi 2 variety, and the seeds of Saryal variety were medium-resistant. The seeds of a hybrid of these varieties were the most resistant which may be due to the effect of heterosis. Seed aging causes a significant increase in DNA damage assessed as DNA per cent in the tail of the comet and/or atypical comets. The longer the seeds were under aging conditions, the higher was DNA fragmentation in cells of the embryos upon swelling. Under 24 weeks of accelerated aging, there was a 1.6-3.3 % increase in DNA found in the tail of the comet, and the number of atypical comets in the embryo cells increased 17-40-fold depending on the variety (hybrid) as compared to control. Probably, a significant reduction of seed physiological parameters was caused by higher degree of nuclear DNA fragmentation, decreased enzymatic antioxidants activity (in particular, activity of peroxidases) and intensified oxidation in embryos. Intensification of oxidative processes is expressed as a 2.5-fold excess of lipid peroxidation in germs of a rapidly aging variety which is accompanied by low seed germination. It is assumed that the increase in the degree of DNA damage is a consequence of the depletion of antioxidant and repair enzymes and indicates a slowdown or lack of regenerative processes in the embryos of aging seeds.

Keywords: *Pisum sativum*, pea, seeds, accelerated aging, germination energy, lipid peroxidation, peroxidase, low-molecular antioxidants, DNA comet

The seeds' property to stay in a state of physiological dormancy for a long time, to endure adverse conditions while maintaining the viability, and to germinate successfully is the necessary condition for the beginning of the plant's life cycle and subsequent reproduction [1, 2]. It is considered that the preservation of seeds' viability for a certain time largely depends on the genetic characteristics of the species, as well as on the storage conditions. Among the factors influencing on the rate of seeds' aging, the temperature and humidity are con-

sidered the most significant [3, 4]. In addition, the longevity of seeds is conditioned by their quality which, in turn, is determined by the growing and ripening conditions, size of seeds, etc.

However, a long-term storage inevitably leads to the delay in sprouting, reducing of germination or to the complete loss of viability [5, 6]. The main causes of seeds' aging and death include the excessive formation of reactive oxygen species (ROS), inactivation of enzymes, destruction of proteins and lipids, breach of the membranes' integrity and degradation of DNA [6-9].

Currently, much attention is paid to investigating of the role of damaging DNA and of the main mechanisms of maintaining the genome's stability in the preserving of the seeds' viability while aging. The experimental material confirming the intensification of the processes of oxidative damage to DNA while the seeds swelling even their quality is high is being accumulated, and the actively going processes of DNA recovery are considered as a factor contributing to the initiation of replication and to the rapid germination of seeds [10]. The most common damages to the DNA molecule are single-strand and double-strand breaks, as well as the changes in the structure of purine and pyrimidine bases. The slowdown of sprouting and reduction of germination of aging seeds are deemed to be related to the long-term repair of DNA and delayed replicative synthesis of DNA [11, 12].

The investigation of the seeds' physiological and biochemical characteristics, predicting their longevity and storability are mainly performed using the methods of accelerated aging under the conditions conducive to increasing the seeds' moisture and while exposing to high temperature [13, 14]. Under the influence of precisely these factors, as a rule, seeds lose their viability in a short time.

The investigation of the interrelation between the embryo genome's integrity and seeds' quality is of great interest. However, the information on the quantitative assessment of the degree of damage to DNA and its influence on the seeds' germination capacity available in the scientific literature is quite poor. This is probably due to the little experience in application of the DNA comet method on plants while it is widely and efficiently used in the in vivo and in vitro systems for evaluating the genotoxic effects when affecting of various factors of physical and chemical nature on human and animal cells and on microorganisms.

In our information release, we described the genotoxic effects and functions of prooxidant and antioxidant systems accompanying the seeds' aging in two varieties of edible pea (*Pisum sativum* L.) and their intervarietal hybrid, which is important for understanding the physiologic-biochemical changes and for predicting the recovery processes under the conditions of long-term storage. As the result, we have not only confirmed the fact of damages to DNA, but also described them quantitatively, which partially completes the lack of information about the role of damage to DNA in the seeds' aging.

The objective of this work is to investigate the influence of accelerated aging of pea seeds on the indices of their viability, oxidative processes and stability of the nuclear DNA structure in the embryos' cells while germination (in the swelling phase).

Techniques. The seeds of three samples (Saryal and Melkosemyannyi 2 varieties and their hybrid Saryal × Melkosemyannyi 2) of edible pea (*Pisum sativum* L.) have been gotten in the conditions of the experimental station located on the valley side of the Lena river's middle reach. The plot's soil is taiga cryogenic, yellowish, solodized soil, typical for the agricultural zone of Central Yakutia. The growing season has been assessed as arid (HTI [hydrothermal index] = 0.76) and as the most consistent with the long-time average annual data of observations for Central Yakutia (HTI = 0.72).

The seeds were germinated in the containers filled at $^2/_3$ of the height with the sterile quartz sand with the particles size of 0.5-2.0 mm and 80% moisture capacity. The seeds were impressed into the sand with the tamper to the depth equal to their thickness and germinated in the dark at 20 °C. The germination energy was determined on the 4th day, the germination value – on the 8th day. The seeds' moisture was evaluated gravimetrically (MB45 device by Ohaus, Switzerland). The seeds of 10-g samples had been ground for 60 seconds using the rotary grinding system and had been dried for 20 minutes at 150 °C.

The values of seeds' germination and moisture of the initial samples, which have been determined before the beginning of the experiment, were 95-97% and 7% respectively. The accelerated seeds' aging was provoked according to the description [13]. The seeds were moistened in the thermostat at 37 °C and relative air humidity of 98%. The value of final seeds' moisture of each sample was 13.5%. The control of seed moisture was performed gravimetrically as described above. The comparative samples (controls) were the seeds which have not been subjected to additional moistening.

For biochemical and molecular studies the embryos tissues were used. The pea seeds were laid out in Petri dishes in a single layer and filled with distilled water at $^2/_3$; after the seed swelling for 12 hours the embryos were isolated.

The spectrophotometric measurements have been performed using the UV-2600 device (Shimadzu, Japan). The total content of low molecular weight antioxidants (LMWA) was determined according to the technique [15] based on the oxidation of antioxidants with iron chloride (III) with its reduction to iron chloride (II), the amount of which was measured basing on the change in staining intensity when the addition of o-phenanthroline (extinction coefficient $\varepsilon = 52.8\ mM^{-1} \cdot cm^{-1}$ at $\lambda = 510$ nm). The peroxidase activity (POC, EC 1.11.1.7) was evaluated basing on the increase of the optical density due to the formation of the stained product of the o-dianisidine oxidation ($\varepsilon = 30\ mM^{-1} \cdot cm^{-1}$ at $\lambda = 460$ nm) for 1 min [16]. The intensity of peroxidation of lipids (LPO) was evaluated basing on the accumulation of the stained complex of malondialdehyde (MDA) with thiobarbituric acid ($\varepsilon = 155\ mM^{-1} \cdot cm^{-1}$ at $\lambda = 532$ nm) [17].

The DNA fragmentation degree in the isolated embryos was determined using the alkaline version of the DNA comet method (gel electrophoresis of single cells) with some modifications [18], which makes it possible to make the quantitative evaluation of the damages to DNA (single-stranded and double-stranded breaks, alkaline-labile purine and pyrimidine sites) [19]. After the period of seeds swelling, the isolated embryos were placed on ice in the Petri dishes of 60 mm diameter, coated with 250 μl of cold sodium phosphate buffer (pH 7.5), and then the incisions were accurately made on the embryos with the sharp razor blade. The dishes were kept in the ice in tilted position so that the nuclei, which become released from the embryos' cells into the buffer, accumulate in the dish lower part. The nuclei containing suspension was purified from major impurities using the nylon mesh filter with the holes size of 20 μm. Then 60 μl of the resulted suspension was placed in the test tubes with 240 μl of the 1% solution of low-melting agarose and applied on the glass slides previously coated with high-melting agarose. After the agarose solidification at the temperature of 4 °C, the micropreparations were placed in the alkaline buffer for electrophoresis (300 mM of NaOH, 1 mM of EDTA, pH > 13) for 20 min for causing the DNA denaturation and single stranded breaks in the alkaline-labile sites. The electrophoresis has been performed for 20 min at the field density of V = 1 V/cm and the current strength of ~ 300 mA, then the preparations were washed with sodium phosphate buffer (pH 7.5), fixed in the 70% ethanol solution and dried. Immediately prior to the microscopy (LabMed-2L fluorescence microscope,

Russia), the preparations have been stained with SYBR Green I fluorescent stainer (Sigma-Aldrich, USA; concentration 20 μg/ml) for 30 min and examined at ×200 magnification using the excitation and cutoff filters (490 and 530 nm respectively). The obtained images of DNA comets were analyzed using the CASP 1.2.2 software (http://casplab.com/download). The percentage of DNA in the comets' tail (the share of DNA in the comet tail in the total amount of DNA in the comet in %) has been used as the indicator of DNA damage degree. The atypical DNA comets with the absent or practically absent head and with a wide diffuse tail were put into the separate category and their number per 100 DNA comets was calculated [20].

All measurements have been performed in 4 replicates. The experiment results are presented in the form of arithmetic mean (*M*) and its standard error (±SEM). The samples were compared using the one-way analysis of variance (ANOVA), the statistical significance of the differences with control was determined using the Dunnett's test for multiple comparison at the significance level of $p < 0.05$. The calculation was made using the AnalystSoft package, StatPlus v. 2007 (AnlystSoft Inc., Germany).

Results. The brief description of the varieties and their hybrid used in the experiment is given in Table 1. The Melkosemyannyi 2 was originated in the Bashkir Research Institute of Agriculture (Ufa city) in 1961, and since 1963 it has been included in the State Register of Selection Achievements Authorized for Use. The Saryal variety was originated in 2002 in the Yakutsk Research Institute of Agriculture (Yakutsk city) by the method of individual selection for lodging resistance and early ripening on the base of an anonymous sample (USA), in 2015 it was transferred to the State Variety Testing, and since 2019 it has been included in the State Register of Selection Achievements Authorized for Use. The Saryal × Melkosemyannyi 2 hybrid was created in the same institute in 2004 and currently is undergoing the station tests in the breeding nurseries of the Yakutsk Research Institute of Agriculture.

1. Studied forms of edible pea (*Pisum sativum* L.)

Variety, hybrid	Maturing rate	1000 grains weight, g (*M*±SEM)	Seeds		
			size	shape	color
Melkosemyannyi 2	Mid early	141±1.4	Small	Spherical	White matte
Saryal	Mid early	277±3.0	Large	Round	White matte
Saryal × Melkosemyannyi 2	Mid early	201±2.0	Medium	Rounded	White matte

2. Germination energy and germination rate of the edible pea (*Pisum sativum* L.) seeds depending on the period of exposure to the factors determining the accelerated aging (*M*±SEM)

Exposure, weeks	Germination energy, %	Germination rate, %
	Variety Saryal	
0 (control)	86±6	97±3
6	84±7	86±7
12	60±9*	82±8*
24	22±8*	22±8*
	Variety Melkosemyannyi 2	
0 (control)	78±8	96±4
6	48±10*	94±5
12	30±9*	46±10*
24	8±3*	8±5*
	Hybrid Saryal × Melkosemyannyi 2	
0 (control)	62±9	95±4
6	74±9	99±1
12	66±7	84±7
24	26±8*	32±9*

* Differences with the control are statistically significant at $p < 0.05$ (ANOVA, Dunnett's criterion for multiple comparisons).

According to existing concepts, seeds aging conditioned by the oxidation processes in the seed dormancy period, which increases in the swelling period, leads to metabolic changes which, depending on the damage degree, is expressed in the slowdown of germination, reducing the viability and death of seeds [7].

The pea varieties and hybrid used in our researches initially (control) differed in the energy of seeds germination (Table 2). The Saryal variety had a high value (86%), the other two samples had a slightly lower values, 78% for Melkosemyannyi 2 and 62% for the hybrid. The germination of the investigated seeds was in the range of 95-97%.

In the course of accelerated aging after the 6-week exposure, the decrease in germination energy by 30% ($p < 0.05$) was noted only in the Melkosemyannyi 2 variety, that, however, have not affected the germination value which did not significantly differ from the control. In this variety, the exposure for 12 weeks led to the decrease in germination energy by another 18%, in germination value by 50% ($p < 0.05$). In the Saryal variety, the decrease in these indices relative to control (by 26 and 18%, respectively) also occurred. The significant changes in physiological parameters were observed after 24 weeks under conditions determining the artificial aging. In the Melkosemyannyi 2 variety the germination energy and germination value decreased to 8%, in the other two samples the germination energy was about 24%, in the Saryal variety the germination value decreased 4.4 times and in the hybrid 3.0 times as compared to the control. Thus, among the investigated samples, the seeds of the Melkosemyannyi 2 variety showed themselves as rapidly aging, the seeds of the Saryal variety had moderate resistance and the seeds of these varieties' hybrid showed the high resistance that may be conditioned by the heterotic effect. Our data on the variety-specific peculiarity of changing of physiological parameters in the course of seeds aging are consistent with the results of other researchers [21, 22].

During the storage, seeds are characterized by low moisture content which leads to their insignificant metabolic activity, while the autooxidation processes generate free-radical products of reactions [23]. Seed swelling is the most critical stage of seeds germination, which leads to the release of ROS (reactive oxygen species) formed both during the storage and due to the increased respiratory activity [7]. We have investigated the accumulation of MDA (malondialdehyde) (as the end product of lipids peroxidation) in the embryos' cells of the aging pea seeds after the 12-hour swelling (Fig. 1, A).

In the embryo cells of the seeds of two samples characterized by medium and high resistance to aging, a 1.5-fold increase in MDA accumulation as compared to the control (in the Saryal variety after 6 weeks, in the hybrid after 12 and 24 weeks) has been detected. In the Melkosemyannyi 2 variety, such increase has not been noted that (taking into account the highest aging rate in this sample) does not exclude the intense lipids peroxidation (LPO) in the period between 0 and 6 weeks. At the same time, after 24 weeks of exposure to aging factors, in this variety, the LPO intensity was 2.5 times higher than in the control.

It should be noted the absence of the linear dependence of LPO on the duration of accelerated aging.

It is deemed that low molecular weight antioxidants play a decisive role in the inactivation of ROS under the conditions of increasing oxidative stress that is conditioned, among other factors, also by the depletion of the pool and/or of the enzymatic antioxidants' activity in the course of long-time oxidative exposure [7, 24]. We have determined the LMWAs content (Fig. 2) and the peroxidase's activity (see Fig. 1, B) in the embryos' cells while the seeds' swelling at different time points during the artificial aging. Peroxidase is a bifunctional enzyme participating in peroxidation or oxidation and in the release of ROS. Also, this protein is related

with the cell elongation and growth limiting reactions [25, 26].

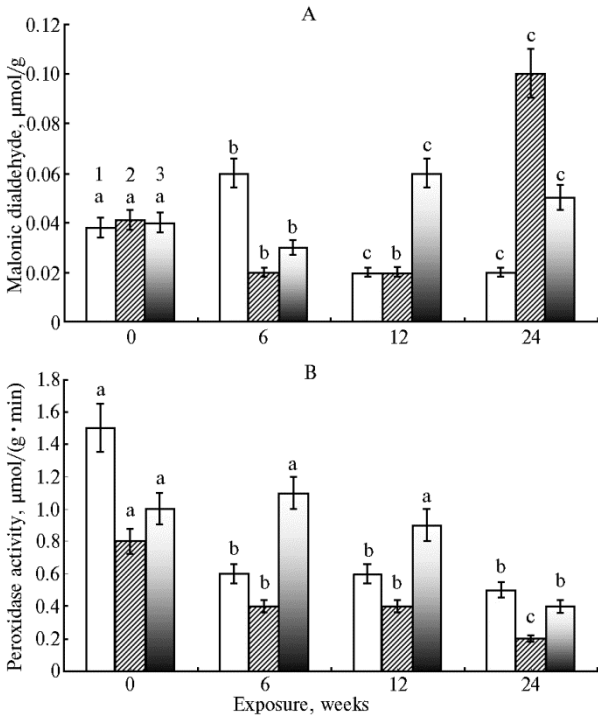

Fig. 1. The accumulation of malondialdehyde (MDA) (A) **and peroxidase activity** (POA) (B) **in the embryos' cells of the swollen seeds of pea (*Pisum sativum* L.) depending on the period of exposure to the factors determining the accelerated aging:** a — the control or the value indistinguishable from it; b — the value differing from the control; c — the value differing both from a and b (the differences are statistically significant at $p < 0.05$ according to the Dunnett's test for multiple comparisons).

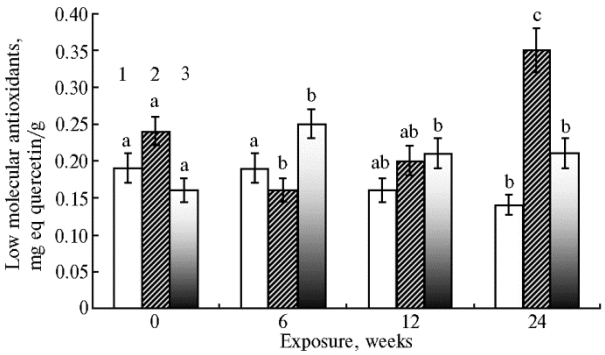

Fig. 2. Content of low molecular weight antioxidants (LMWA) **in the embryos' cells of the swollen seeds of pea (*Pisum sativum* L.) depending on the period of exposure to the factors determining the accelerated aging:** a — the control or the value indistinguishable from it; b — the value differing from the control; c — the value differing both from a and b (the differences are statistically significant at $p < 0.05$ according to the Dunnett's test for multiple comparisons).

The POA activity in the embryo cells of the rapidly aging seeds of the Melkosemyannyi 2 variety decreased as the exposure period extends, in 6-12 weeks 2 times, and after 24 weeks 4 times as compared to the control (see Fig. 1, B). Initially, LMWAs content decreased 1.5 times (6 weeks), and by the end of the 24th week it increased 1.5 times compared to the control (see Fig. 2). In the Saryal variety which showed the medium aging rate (among the investigated samples) the enzyme activity decreased by 60-65% for 6-24 weeks. At the same time, the LMWAs amount decreased by 23% ($p < 0.05$) only at the last term of accelerated aging. The hybrid, which was the most resistant, was characterized by the statistically significant 2.5-fold decrease of peroxidase activity (POA) only after 24 weeks of aging, while the LMWAs content was 1.4 times higher than in the control over the entire observation period.

Thus, the pea seed aging led to the decrease in peroxidase activity in the embryo cells of the swollen seeds. The change in the LMWAs content in the investigated pea samples under the conditions of increasing oxidative stress was multidirectional.

As the result of breach of the oxidation-reduction balance, which is conditioned by the excessive generation of ROS and decrease of the enzymatic activity, the breach of the DNA structure's integrity is initiated [7, 10]. Using the alkaline version of the DNA-comet method in our researches made it possible to make the quantitative comparison of the nuclear DNA fragmentation degree in the embryo cells in the swelling period be-

tween the control samples and the seeds subjected to accelerated aging (Table 3). In the control samples, the share of DNA in the comet's tail was 9-22%. The seed aging led to the statistically significant ($p < 0.05$) increase of the DNA damage degree compared with the control: in the Saryal variety by 13% since the 24th week of exposure, in the Melkosemyannyi 2 variety by 2.2-31.2% since the 12th week and in the hybrid by 3.5-20.5% since the 6 week. The comets with the absent or practically absent head and with a wide diffuse tail (the so-called "ghost cells" or "hedgehogs") have also been detected. The appearance of such atypical DNA comets is deemed an indicator of irreversible processes, i.e. cell death related to the strong oxidative stress, or of the formation of apoptotic cells being at the stage of chromatin fragmentation [27-29].

3. The degree of damage to nuclear DNA in the embryo cells of pea *Pisum sativum* L. seeds depending on the period of exposure to the factors determining the accelerated aging (M±SEM)

Exposure, weeks	DNA in the comet's tail, %	Atypical DNA comets, %
	Variety Saryal	
0 (control)	22.3±1.8	1.0±0.3
6	18.0±2.5	4.7±0.5*
12	20.7±2.9	10.5±0.5*
24	35.3±0.8*	17.0±1.7*
	Variety Melkosemyannyi 2	
0 (control)	17.4±0.5	1.5±1.0
6	19.1±2.9	10.4±2.0*
12	19.6±0.1*	10.3±1.9*
24	48.6±2.7*	60.5±3.0*
	Hybrid Saryal × Melkosemyannyi 2	
0 (control)	8.7±0.6	0.5±0.2
6	12.2±1.8*	2.4*±0.6
12	13.1±1.3*	7.5*±0.9
24	29.2±1.8*	17.0±0.5*

* Differences with the control are statistically significant at $p < 0.05$ (ANOVA, Dunnett's criterion for multiple comparisons).

The experiment showed the increase of the share of atypical comets in all the investigated samples of pea as the period of exposure to the factors of accelerated seeds aging extends. After the 24-week artificial aging, the number of atypical comets increased 17-40 times relative to the control depending on the variety. The comparison of the obtained data showed that when the atypical comet share increased to 10%, the seed germination energy decreased by 25-30% while the germination value either did not differ from the control or decreased slightly. This fact may testify about going of repair processes in the embryos' cells at the seeds' hatching stage and is consistent with the results of the researches [30, 31], in which it has been shown that under the effect of peroxide in the concentration which does not cause a cytotoxic effect, the repair of damaged DNA regions is possible. The further increase of DNA fragmentation led to the decrease of the seeds' germination energy and germination value, which testified about a certain critical level of damages to DNA and a significant decrease of the influence of DNA repair processes.

Thus, the results obtained in this work testify that the accelerated aging exerts different influence on the germination value and germination energy of the seeds, as well as on the biochemical characteristics of the embryo cells of the investigated varieties and inter-variety hybrid of edible pea. The Melkosemyannyi 2 variety showed the lowest resistance to the artificial aging conditions; the seeds of the Saryal variety showed medium resistance. The seeds of the inter-variety hybrid showed the highest resistance, which is probably owing to the heterosis effect. The influence of accelerated aging for 24 weeks led to the 1.6-3.3-fold increase of the DNA share in the comet tail while the number of atypical comets in different samples (varieties and hybrid) increased 17-40 times

compared to the control. The significant decrease in the physiological characteristics in the less resistant pea variety, which has been caused by the aging conditions, could be conditioned by the high degree of the nuclear DNA fragmentation, decrease of the antioxidant enzyme activities (in particular, of peroxidase), and by the increasing intensification of oxidative processes (exceeding the control value of lipids peroxidation by 2.5 times in the rapidly aging variety together with low germination) in the embryo cells. Based on the obtained data, it can be assumed that the increase of the DNA damage degree in the investigated pea varieties occurs due to the "depletion" of enzymatic antioxidant and repair systems and testifies about the slowdown or loss of restoration processes in the embryo cells of aging seeds.

REFERENCES

1. Rajjou L., Duval M., Gallardo K., Catusse J., Bally J., Job C. and Job D. Seed germination and vigor. *Annual Review of Plant Biology*, 2012, 63: 507-533 (doi: 10.1146/annurev-arplant-042811-105550).
2. Bewley J.D. Seed germination and dormancy. *Plant Cell*, 1997, 9: 1055-1066 (doi: 10.1105/tpc.9.7.1055).
3. Walters C., Wheeler L.M, Grotenhuis J.M. Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research*, 2005, 15: 1-20 (doi: 10.1079/SSR2004195).
4. Miura K., Lin Y., Yano M., Nagamine T. Mapping quantitative trait loci controlling seed longevity in rice (*Oryza sativa* L.). *Theor. Appl. Genet.*, 2002, 104: 981-986 (doi: 10.1007/s00122-002-0872-x).
5. Roberts E.H. Storage environment and the control of viability. In: *Viability of seeds.* E.H. Roberts (ed.). Chapman and Hall, London, UK, 1972: 14-58 (doi: 10.1007/978-94-009-5685-8_2).
6. Priestley D.A. *Seed aging. Implications for seed storage and persistence in the soil.* Ithaca, Cornell University Press, 1986.
7. Bailly C. Active oxygen species and antioxidants in seed biology. *Seed Science Research*, 2004, 14: 93-107 (doi: 10.1079/SSR2004159).
8. El-Maarouf-Bouteau H., Mazuy C., Corbineau F., Bailly C. DNA alteration and programmed cell death during ageing of sunflower seed. *Journal of Experimental Botany*, 2011, 62(14): 5003-5011 (doi: 10.1093/jxb/err198).
9. Murthy U.N., Kumar P.P., Sun W.Q. Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. *Journal of Experimental Botany*, 2003, 54(384): 1057-1067 (doi: 10.1093/jxb/erg092).
10. Waterworth W.M., Bray C.M., West C.E. The importance of safeguarding genome integrity in germination and seed longevity. *Journal of Experimental Botany*, 2015, 66(12): 3549-3558 (doi: 10.1093/jxb/erv080).
11. Burgess R.W., Powell A.A. Evidence for repair processes in the invigoration of seed by hydration. *Annals of Botany*, 1984, 53(5): 753-757 (doi: 10.1093/oxfordjournals.aob.a086741).
12. Elder R., Osborne D. Function of DNA synthesis and DNA repair in the survival of embryos during early germination and in dormancy. *Seed Science Research*, 1993, 3: 43-53 (doi: 10.1017/S0960258500001550).
13. Safina G.F., Filipenko G.I. *Trudy po prikladnoi botanike*, 2013, 174: 123-130 (in Russ.).
14. Alekseichuk G.N., Laman N.A. *Fiziologicheskoe kachestvo semyan i metody ego otsenki.* Pod redaktsiei S.I. Griba [Physiological quality of seeds and methods for its assessment. S.I. Grib (ed.)]. Minsk, 2005.
15. Ermakov A.I. *Metody biokhimicheskogo issledovaniya rastenii* [Methods of plants biochemistry]. Leningrad, 1987 (in Russ.).
16. Lebedeva O.V., Ugarova N.N., Berezin I.V. *Biokhimiya*, 1977, 42: 1372-1379 (in Russ.).
17. Vladimirov Yu.A., Archakov A.I. *Perekisnoe okislenie lipidov v biologicheskikh membranakh* [Lipid peroxidation in biological membranes]. Moscow, 1972 (in Russ.).
18. Gichner T., Patkova Z., Szakova J., Demnerova K. Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves. *Mutation Research*, 2004, 559: 49-57 (doi: 10.1016/j.mrgentox.2003.12.008).
19. Tice R.R., Agurell E., Anderson D., Burlinson B., Hartmann A., Kobayashi H., Miyamae Y., Rojas E., Ryu J.-C., Sasaki Y.F. Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, 2000, 35: 206-221 (doi: 10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J).

20. Zhanataev A.K., Nikitina V.A., Voronina E.S., Durnev A.D. *Prikladnaya toksikologiya*, 2011, 2(4): 28-37 (in Russ.).
21. Sisman S., Delibas L. Storing sunflower seed and quality losses during storage. *Journal of Central European Agriculture*, 2004, 4: 239-250 (doi: 10.2298/hel0542115c).
22. Mohammadi H., Soltani A., Sadeghipour H.R., Zeinali E. Effects of seed aging on subsequent seed reserve utilization and seedling growth in soybean. *International Journal of Plant Production*, 2011, 5(1): 65-70 (doi: 10.22069/IJPP.2012.720).
23. McDonald M.B. Seed deterioration: physiology, repair and assessment. *Seed Science and Technology*, 1999, 27: 177-237.
24. Avila M.R., Braccini A.L., Souza C.G.M., Mandarino J.M., Bazo G. L., Cabral Y.C.F. Physiological quality, content and activity of antioxidants in soybean seeds artificially aged. *Revista Brasileira de Sementes*, 2012, 34(3): 397-407 (doi: 10.1590/S0101-31222012000300006).
25. Morohashi Y. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. *Journal of Experimental Botany*, 2002, 53: 1643-1650 (doi: 10.93/jxb/erf012).
26. Passardi F., Penel C., Dunand Ch. Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends in Plant Science*, 2004, 9(11): 534-540 (doi: 10.1016/j.tplants.2004.09.002).
27. Olive P.L., Banath J.P. Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. *Experimental Cell Research*, 1995, 221(1): 19-26 (doi: 10.1006/excr.1995.1348).
28. Yasuhara S., Zhu Y., Matsui T., Tipirneni N., Yasuhara Y., Kaneki M., Rosenzweig A., Martyn J.A. Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis. *Journal Histochem. Cytochem.*, 2003, 51(7): 873-885 (doi: 10.1177/002215540305100703).
29. Zhanataev A.K., Anisina E.A., Chaika Z.V., Miroshkina I.A., Durnev A.D. *Tsitologiya*, 2017, 59(3): 163-168 (in Russ.).
30. Rundell M., Wagner E., Plewa M. The comet assay: genotoxic damage or nuclear fragmentation? *Environmental and Molecular Mutagenesis*, 2003, 42: 61-67 (doi: 10.1002/em.10175).
31. Lorenzo Y., Costa S., Collins A., Azqueta A. The comet assay, DNA damage, DNA repair and cytotoxicity: hedgehogs are not always dead. *Mutagenesis*, 2013, 28: 427-432 (doi: 10.1093/mutage/get018).

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METHODOLOGICAL STUDY ON SELECTION OF CONDITIONS FOR IMPROVING *Taraxacum kok-saghyz* Rodin SEED GERMINATION

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Abstract

Currently, Kok Sagyz is considered to be the most promising source of natural rubber. In many countries significant investments in the development of rubber production technologies based on the use of this plant are being made. Ensuring a high percentage of rapid and uniform germination is important for Kok Sagyz cultivation. In this research we have obtained results significantly extending the idea of temperature as a factor affecting the characteristics of Kok Sagyz seeds germination, which is important for this rubber plant reproduction practice. The aim of our study was to determine a practical and simple method to improve the germination of *Taraxacum kok-saghyz* seed. An orthogonal array design L_9 (3^4)] was used to optimize three factors: concentration of $KMnO_4$ solution (0.07 %, 0.1 %, and 0.2 %), soaking time (1, 2, and 4 h), and germination temperature (4/17, 17, and 23 °C). Germination parameters, including germination percentage, T_{50} (time taken for 50 % of seeds to germinate), germination index, and vigor index, were evaluated. Using analysis of variance, the optimum conditions for germination were determined, i.e. 2 h of soaking time in 0.07 % $KMnO_4$ solution and a germination temperature of 23 °C. The optimum conditions were subsequently validated. Under the optimized conditions, we achieved a germination percentage of 71 %, a T_{50} value of 11 days, and germination and vigor indices of 7.22 and 14.40, respectively. Moreover, we found that in addition to moisture, temperature was the main factor influencing the germination of *T. kok-saghyz* seeds. Solution composition and concentration and soaking time had little or no effect on germination.

Keywords: germination percentage, germination uniformity, $KMnO_4$, potassium permanganate, *Taraxacum kok-saghyz* Rodin, Russian dandelion

Taraxacum kok-saghyz Rodin (Russian dandelion) is a perennial herbaceous plant. The root contains about 2.89 to 27.89% (mass fraction) of natural rubber, molecular weight of which is 2180 [1, 2] and 25-40% (mass fraction) of inulin [3]. It can also be studied as a model plant for revealing mechanism of rubber synthesis [4]. Now it is developing as one of the most promising natural rubber plants, which attracts huge investments in many countries [1, 2, 5].

Securing a high percentage of rapid, uniform and otherwise germination was important for *T. kok-saghyz* production [6]. It is well known that *T. kok-*

saghyz seed enters dormancy during the after-harvest ripening period and so how to break dormancy effectively is the key point to achieve high percentage of rapid and uniform germination. Stratification, prechilling [6], soaking and chemicals methods were found and applied. And relation of temperature, moisture, and several other factors to germination had been revealed [6]. When applying these methods, we found at least 24 hours of pretreatment were needed, which caused some inconveniences.

Some studies showed that $KMNO_4$ (Potassium Permanganate) can sterilize and break seed dormancy [7-9]. So what about effect of $KMNO_4$ solution on seed germination of *T. kok-saghyz*? Moreover, most of previous studies mainly focus on effect of methods on germination percentage of *T. kok-saghyz* seeds, except other germination indices. While germination uniformity, germination index and so on, are also important for seed germination and seedling growth afterwards.

In this study, we'll try to find a practical method to improve seed germination of *T. kok-saghyz* with $KMNO_4$ solution. And answer one question, what effects of temperature, concentration of solution, soaking time are on seed germination of *T. kok-saghyz*? These findings will contribute to facilitating its use as a natural rubber plant. At the same time, this work aims to demonstrate the potential of orthogonal array designs for establishing efficient seed germination protocols.

Our subjective was to optimize the conditions for the germination of Russian dandelion seeds using KMnO4 solution, and also to investigate the effect of temperature, solution concentration, and time for soaking on germination.

Techniques. Seeds used in all germination tests were harvested from k-445 accession of *T. kok-saghyz* which provided by Vavilov All-Russian Institute of Plant Industry. Seeds were extracted from physiologically ripe fruits of plants cultivated in an open trial field of Heilongjiang Academy of Sciences (45°34'59.9"N, 126°34'18.8"E) on June, 2014, when plants were one year of age. The region is characterized by a typical continental climate with black soil. Harvested seeds were air-dried for about two weeks and then hand threshed to minimize seed damage. Round hole sieves (0.45 mm diameter) and a seed blower were used to clean seeds. Seeds were stored for 5 months under room temperature before being treated. Seeds were pre-tested for viability with 1% concentration of TTC solution for 30 min [10].

Depending on the experiment, seeds were germinated in Petri dishes (9.0×2.5 cm) on two layers of filter paper which remained moist. One hundred seeds after being soaked in solutions were placed evenly on filter paper. Seeds for each treatment were sown under different temperatures depending on designs. The Petri dishes were watered regularly to keep the seeds moistened.

The main effects of such three factors studied at three levels were evaluated using an orthogonal array design L9 (3^4) method consisting of nine treatments. For each treatment, three replicates (three Petri dishes, with 50 seeds per Petri dish) were performed. Four factors, concentration, soaking, temperature, blank (for estimating data errors) with three possible levels (level 0, L0; level 1, L1; level 2, L2) for each factor were evaluated for their effect on the germination of *T. kok-saghyz* seeds. The levels for each factor were as follows:

(a) Concentration: L0 = 0.07% (mass fraction) concentration of $KMNO_4$ solution; L1 = 0.1% $KMNO_4$; L2 = 0.2% $KMNO_4$.
(b) Soaking time: L0 = soaking seeds in $KMNO_4$solution for 1 hour; L1= soaking seeds in $KMNO_4$ solution for 2 hours; L2 = soaking seeds in $KMNO_4$ solution for 4 hours.

(c) Temperature: L0 = seeds germinated at 23 °C after being soaked in $KMNO_4$ solution; L1 = seeds germinated at 17 °C; L2 = seeds germinated at 17 °C for 16 hours, and then switch to 4 °C for 8 hours.

Seed germination was evaluated for 20 days after the seeds were placed in Petri dishes for the first experiment, aiming to determine the levels of different factors for improving germination of *T. kok-saghyz*. For the experiments aimed at validating the improved treatment, the seed germination (Petri dishes) was evaluated for 14 days since the seeds were placed in the germination cabinet. Seeds were considered germinated when the radicle was 1 mm or greater.

The following 4 parameters, which evaluate germination process, including time, rate, homogeneity and synchrony [11], were considered for an analysis of variance (ANOVA) statistical evaluation: (a) Germination percentage (%) = (total number of germinated seeds/number of total seeds) × 100%; (b) Germination uniformity, expressed as T_{50}, namely the time for 50% seeds getting germinated. (c) Germination index: ∑(sum of germinated seeds per day/days of germination counted from the first day of testing). (d) Vigour index: germination index × radicle length (mm), reflects growth velocity and biomass comprehensively.

The effect of each factor on the characteristic properties were determined by analysis of variance (ANOVA) and Duncan's multiple range test.Data analyses was made by SPSS (version 19.0, IBM, USA) in which multivariate method of general linear model was applied. The range analysis was employed to discriminate the comparative significance of each factor in OAD. To make results more clearly and intuitive, the average scores of the summations of level1-3 of three factors were shown in line plus symbol shape in graph. To further identify the reliability of the OAD results, additional experiments under the optimum condition of the corresponding parameters plus some control experiments were performed, with three replications of every treatment.

Results. Seed germination indices we estimated are sufficient for a comprehensive assessment of the ongoing processes of growth and biomass accumulation. For three values of each of the factors, a three-way variance analysis L_9 (3^4) was finally designed (Table 1).

1. Orthogonal array matrix L_9 (3^4) to optimize seed germination of *Taraxacum kok-saghyz* Rodin

Treatment	Factors			
	solution $KMnO_4$ (%)	time, h	temperature, °C	Blank
1	1 (0,07 %)	3 (4)	3 (23)	1
2	2 (0,1 %)	2 (2)	3 (23)	2
3	3 (0,2 %)	1 (1)	3 (23)	3
4	3 (0,2 %)	2 (2)	1 (4-17)	3
5	1 (0,07 %)	1 (1)	1 (4-17)	1
6	1 (0,07 %)	2 (2)	2 (17)	2
7	2 (0,1 %)	1 (1)	2 (17)	2
8	2 (0,1 %)	3 (4)	1 (4-17)	3
9	3 (0,2 %)	3 (4)	2 (17)	1

Seeds viability test indicated that 81.57% of seeds were viable.

Impact of three factors on seed germination of *T. kok-saghyz*. Concentration of KMNO4 solution had significant effect on vigour index of seed germination of *T. kok-saghyz* ($p < 0.05$), while no significant effect on germination percentage, germination uniformity, and germination index ($p > 0.05$). Soaking time had no significant effect on all indices of seed germination of *T. kok-saghyz* ($p > 0.05$); Temperature produced significant effect on all indices of seed germination of *T. kok-saghyz* ($p < 0.05$), and highly significant effect on vigour index ($p < 0.01$) (Table 2).

2. Degrees of freedom, the *F*-ratio and its probability obtained from the ANOVA analyses for the effects of treatments and for the orthogonal comparisons between three levels for each factor tested on the *Taraxacum kok-saghyz* Rodin seed germination parameters

Source	Dependent variables	df	Square value	*F*	Sig.
Concentration	Germination percentage (%)	2	34.111	1.415	0.414
	Germination uniformity (days)	2	4.370	1.489	0.402
	Germinability	2	3.356	5.446	0.155
	Germination index	2	10.414	36.596	0.027
Time	Germination percentage (%)	2	15.444	0.641	0.610
	Germination uniformity (days)	2	0.961	0.328	0.753
	Germinability	2	0.200	0.325	0.755
	Germination index	2	2.487	8.738	0.103
Temperature	Germination percentage (%)	2	707.111	29.327	0.033
	Germination uniformity (days)	2	230.633	78.583	0.013
	Germinability	2	14.339	23.266	0.041
	Germination index	2	38.644	135.793	0.007
Error	Germination percentage (%)	2	24.111		
	Germination uniformity (days)	2	2.935		
	Germinability	2	0.616		
	Germination index	2	0.285		

Establishing an improved seed germination treatment. As far as germination percentage was concerned, the bigger is the better. When concentration of $KMNO_4$ increased, germination percentage decreased; more or less soaking time in $KMNO_4$ solution was not adequate for germination percentage; 4 °C-17 °C treatment was better than 4 °C treatment, but not as good as 23 °C treatment. So the best conditions for germination percentage of *T. kok-saghyz* were 1.0% $KMNO_4$ concentration, 2 h soaking time and 23 °C treatment (Fig. 1).

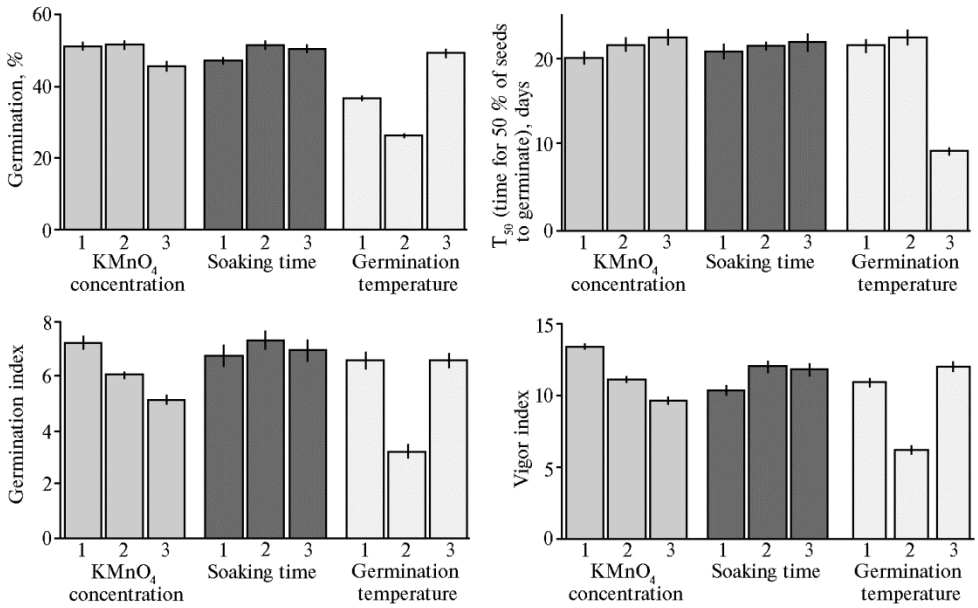

Fig. 1. Effects of three levels of factors on the *Taraxacum kok-saghyz* Rodin seed germination (description of the experimental design see in the section *Techniques*).

T_{50} (time for 50% seeds germinated) stands for germination uniformity. So the smaller one is the better. When concentration of $KMNO_4$ increased, germination uniformity increased; soaking time increased, germination uniformity increased; 23 °C treatment was better than 4-17 °C and 17 °C treatments. So the best conditions for germination uniformity were 0.07% of $KMNO_4$ concentration, 1 h of soaking time and 23 °C of germination temperature (see Fig. 1).

As far as germination index was concerned, the bigger is the better. When concentration of $KMNO_4$ increased, germination index decreased; 2 h of soaking time was better than 4 h, which was better than 1 h; 23 °C of germination temperature was better than 17-4 °C treatment, which was better than 4 °C treatment. So the best conditions for germination index were 0.07% concentration of $KMNO_4$ solution, 2 h of soaking time, 23 °C of germination temperature (see Fig. 1).

As far as vigour index was concerned, the bigger is the better. When concentration of $KMNO_4$ increased, vigour index decreased; 2 h of soaking time was better than 4 h, which was better than 1 h; 23 °C of germination temperature was better than 17-4 °C treatment, which was better than 4 °C treatment. So the best conditions for vigour index were 0.07% concentration of $KMNO_4$ solution, 2 h of soaking time, 23 °C of germination temperature (see Fig. 1).

As above showed, when concentration was 0.07%, germination temperature was 23 °C, all of the germination indices of *T. kok-saghyz* were the best; While when soaking time was 2 h, all of the germination indices were the best too, except germination uniformity, but considering soaking time in $KMNO_4$ solution had no significant effect on all indices of seed germination of *T. kok-saghyz* (see Table 2), 2 h of soaking time could be recognized as the best. We finally concluded that all germination indices of *T. kok-saghyz* were the best when seeds of *T. kok-saghyz* were soaked into 0.07% (mass fraction) concentration of $KMNO_4$ solution for 2 h, and then made germinated under 23 °C.

Validation of the improved germination treatment. The improved germination protocol was not among the treatments tested in the orthogonal array design. To validate the proposed treatment for its germination in Petri dishes, wedesigned and implemented the validated experiment. Some works [13, 14] suggested that low temperature (usually 0-5 °C) may increase germination uniformity of seeds, so we set two low-temperature treatments to determine effect of low temperature on germination of *T. kok-saghyz* seeds and compare results of two different time-disposal treatments. Levitt and Hamm [12] suggested that a certain concentration of KNO_3 solution can improve germination percentage and other indices through promoting physiological maturation of seeds, so we also set a 3% KNO_3 treatment to compare the results between proposed treatments. Finally, we took distilled water treatment as the control (Table 3).

3. Validating experiment design to test suggested protocol for the *Taraxacum kok-saghyz* Rodin seed germination

Treatment	Solution	Concentration, %	Soaking time, h	Temperature
1	$KMnO_4$	0.07	2	23 °C
2	$KMnO_4$	0.07	2	One week under 4 °C, then under 23 °C
3	$KMnO_4$	0.07	2	Two weeks under 4 °C, then under 23°C
4	KNO_3	3.00	24	23 °C
5	Distilled water		2	23 °C

The proposed treatment 1 had the highest germination percentage (71%), compare to other treatments; KNO_3 solution increased germination percentage of *T. kok-saghyz* seeds, but not as high as the proposed treatment, with significant difference.

One-week of 4 °C-disposal increased germination percentage, with no significant difference from control, namely distilled water treatment. Two-week of 4°C-disposal didn't increase germination percentage, with significant difference from one week of 4 °C -disposal (Fig. 2).

One-week 4 °C-disposal decreased germination uniformity (increased time of T_{50}) significantly compared to other treatments, up to 17 days, while there were no significant differences among other treatments.

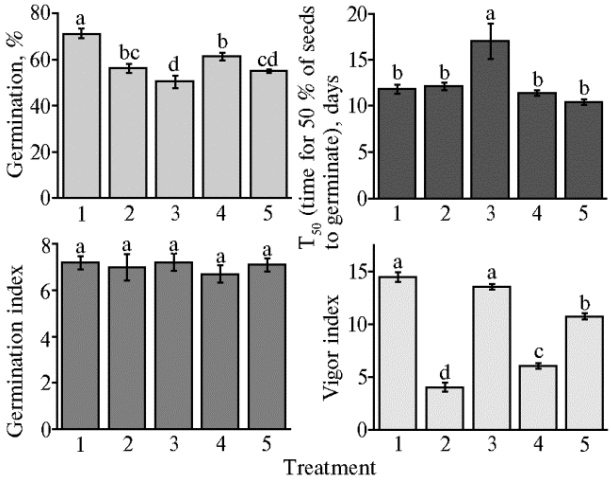

Fig. 2. Validation experiment — effect of treatments on germination of the *Taraxacum kok-saghyz* Rodin seeds: 1, 2, 3, 4, 5 — treatments (see Table 3).

Compared to control, the four treatments didn't affect germination index significantly (see Fig. 2). Considering the five treatments (all germinated under the same temperature), we may make a conclusion: germination temperature maybe the only factor which affects germination index of *T. kok-saghyz* seeds, while no matter what kinds of pretreatments, including sorts of solution pre-soaked orthe concentration of the solution, or the seeds of pre-stored under low temperature or not.

Vigour indexes of the proposed treatment were the highest, up to 14.40; One-week 4 °C disposal treatment got the lowest vigour index, while two-week of 4 °C disposal treatment increased vigour index apparently, up to the same level as the proposed treatment ($\alpha = 0.05$); KNO_3 treatment also decreased vigour index significantly, compare to the control.

In this study, we have shown that temperature had significant effect on all seed germination indices of *T. kok-saghyz*, thus we can state that temperature is the main impact factor on seed germination of *T. kok-sazhyz* besides moisture. The other factors, nature of solution, concentration of solution and soaking time had slight or none effect on seed germination of *T. kok-saghyz*. The conclusion is consistent with Levitt and Hamm's studies [12] in some ways. So if we kept *T. kok-saghyz* seeds certain moisture and made them germinated at suitable temperature (23 °C), we can get desired results in most cases.

The best seed germination conditions for *T. kok-saghyz* is 0.7% concentration of $KMNO_4$ with 2 h of soaking time, 23°C of germinated temperature, which was validated later. Under the condition, germination percentage can be up to 71%, germination uniformity (period for 50% seeds germinated) is about 11 days, germinability is about 20, germination index is about 7.22, vigour index is about 14.40. Germination percentage in our investigation were not as high as previous studies [6, 12], as well as germination uniformity. The most possible reason is seed quality. Because we found almost all the left seeds without germination were empty. And in validating experiment, treatment 4 applied KNO_3 solution to break seed dormancy as Levitt and Hamm [12] suggested, the results were not those their expected. So the only possible reason is seed quality, the maturity, which made some empty seeds and less physiological ripen seeds. And so the method needs more seeds with high quality to validate. Seeds used in the present study were one-year old, so how about two-year old seeds or more. Is the proposed method still applicable? We need more experiments to answer these questions.

According to Roberts [15], there are three separate physiological processed in seeds affected by temperature: first, temperature, together with moisture content, determines the rate of deterioration in all seeds; secondly, temperature affects the rate of dormancy loss in dry seeds and the pattern of dor-

mancy change in moist seeds; and, thirdly, in non-dormant seeds temperature determines the rate of germination. So before discussing effect of temperature on seed germination, we have to answer one question: are *T. kok-saghyz* seeds dormant? *T. kok-saghyz* seed enters dormancy during the after-harvest ripening period. Seed dormancy refers to a state in which the viable seeds fail to germinate when provided with conditions normally favorable to germination such as adequate moisture, appropriate temperature regime and light [16]. Our validating experiment showed that treatment 1 got about 71% of germination percentage, significantly higher than the control (54% of germination percentage) under the same other conditions. This means about 17% seeds were dormant and necessary to be stimulated by $KMNO_4$ to germinate. So we can state that most of one-year old *T. kok-saghyz* seeds are not dormant, only a small amount of seeds (about 17%) are dormant. Therefore, temperature determines the rate of germination of *T. kok-saghyz* seeds.

It has been known for a long time that pretreatment of seeds with Oxidants such as $KMNO_4$, H_2O_2 leads to breaking seed dormancy [17]. In the present study, we found seeds under $KMNO_4$ treatments didn't go moldy, compare to those under no $KMNO_4$ treatments with about 15% of moldy rate. So we stated that $KMNO_4$ has two effects on *T. kok-saghyz* seeds, one is to improve seed germination by breaking seed dormancy. $KMNO_4$ can remove seed coat obstacle, and provide oxygen and Manganese elements, which can strengthen respiration and trophism for germination. Anothereffect is to avoid seeds going moldy by sterilization. Because we found most of the moldy seeds were blank, so $KMNO_4$ increased seed germination of *T. kok-saghyz* mainly by breaking seed dormancy. The concentration should be controlled no more than 0.1% and 0.07% is better, otherwise vitality of germinated seeds maybe decreased.

We suggest 23 °C is the optimal temperature for seeds germination of *T. kok-saghyz*. *T. kok-saghyz* seeds don't germinate at all at 4 °C. The conclusion is not subject to Levitt and Hamm [12], whose investigation showed seeds germination percentage can be up to 71% after pretreatment. As seeds were soaked in $KMNO_4$ solution for 4 hours, they lasted not long as that of Levitt and Hamm's investigation. Because the longest soaking time of seeds were 4 h, far less than that in the soaking time of seeds in Levitt and Hamm [12] studies. So the sole reason maybe is that seeds at 4 °C need more moisture to germinate than that under other temperatures to lessen the effect of low temperature.

In the present study, 4-17 °C treatment showed better than single 17 °C treatment on all germination indices, which is consistent with Hegarty's study [18], which showed that seeds may germinate faster than would be predicted on the basis of thermal time alone in fluctuating temperature environments [19]. So maybe 4-23 °C alternative treatment improve seeds germination better more than single 23 °C treatment. Related experiments can be carried out in the future.

Thus, our findings demonstrate that temperature is the main impact factor on seed germination of *T. kok-saghyz*, besides moisture. Nature, concentration of solutions and soaking time had none or slight effect. The best germination conditions for one-year old seeds of *T. kok-saghyz* are soaking in 0.7% $KMNO_4$ solution for 2 h before germinating at 23 °C. The method need to be validated in field plot. The 23 °C is the optimal temperature for seed germination of *T. kok-saghyz*, 4 °C is not. Most of one-year old seeds of *T. kok-saghyz* are not dormant. These conclusions shall be truly verified by high-maturity seeds.

REFERENCES

1. Mooibroek H., Cornish K. Alternative sources of natural rubber. *Appl. Microbiol. Biotechnol.,*

2000, 53(4): 355-365 (doi: 10.1007/s002530051627).
2. Van Beilen J.B., Poirier Y. Establishment of new crops for the production of natural rubber. *Trends in Biotechnology*, 2007, 25(11): 522-529 (doi: 10.1016/j.tibtech.2007.08.009).
3. Polhamus L.G. *Rubber: botany, production and utilization*. Interscience Publ., New York, 1962.
4. Schmidt T., Lenders M., Hillebrand A., van Deenen N., Munt O., Reichelt R., Eisenreich W., Fischer R., Prufer D., Schulze Gronover C. Characterization of rubber particles and rubber chain elongation in *Taraxacum koksaghyz. BMC Biochemistry*, 2010, 11: 11 (doi: 10.1186/1471-2091-11-11).
5. Venkatachalam P., Geetha N., Sangeetha P., Thulaseedharan A. Natural rubber producing plants: an overview. *African Journal of Biotechnology*, 2013, 12(12): 1297-1310 (doi: 10.5897/AJBX12.016).
6. Whaley W.G., Bowen J.S. *Russian dandelion (kok-saghyz): an emergency source of natural rubber*. US Department of Agriculture, Washington, D.C., 1947.
7. Meng H., Wang X., Tong S., Lv X., Zhang W., Huo L., Cao X., Meng X. Effects of pretreatments on seeds of *Leucaena leucocephala* and *Phragmites australis. Chinese Journal of Ecology*, 2013, 19: 6142-6146.
8. Wang H., Chen Z., Zhang Y., Yang Y., Du S., Guo T. Influence of soaking time and agents on seed germination of *Poa pratensis. Pratacultural Science (China)*, 2014, 11: 2095-2104.
9. Yang, Y., Wang, H., Yang, X., Cheng T. Effect of sterilization on seed germination of *Festuca arundinacea. Acta Agrestia Sinica,* 2014, 02: 380-385.
10. International Seed Testing Association. International Rules for Seed Testing. *Seed Sci. & Technol.*, 1999, 27(Suppl.): 35.
11. Ranal M.A., de Santana D.G. How and why to measure the germination process? *Brazilian Journal of Botany*, 2006, 29(1): 1-11 (doi: 10.1590/S0100-84042006000100002).
12. Levitt J., Hamm P. A method of increasing the rate of seed germination of *Taraxacum kok-saghyz. Plant Physiology,* 1943, 18(2): 288-293.
13. Singh S., Singh P., Sanders D.C., Wehner T.C. Germination of watermelon seeds at low temperature. *Cucurbit Genetics Cooperative*, 2001, 24: 59-64.
14. Yari L., Zareyan A., Sheidaie S., Khazaei F. Influence of high and low temperature treatments on seed germination and seedling vigor of rice (*Oryza sativa* L.). *World Applied Sciences Journal*, 2012, 16(7): 1015-1018.
15. Roberts E.H. Temperature and seed germination. *Symposia of the Society for Experimental Biology*, 1988, 42: 109-132.
16. Schmidt L. *Guide to handling of tropical and subtropical forest seeds*. Danida Forest Centre, Denmark, 2000.
17. Jann R.C., Amen R.D. What is germination? In: *The physiology and biochemistry of seed dormancy and germination*. A.A. Khan (ed.). North Holland Publishing Co., Amsterdam, 1977: 7-28.
18. Hegarty T.W. Temperature relations of germination in the field. In: *Seed ecology*. W. Heydecker (ed.). Butterworths, London, 1973: 411-432.
19. Gavrilova V.A., Konkova N.G., Kutuzova S.N., Gavrilenko T.A., Pendinen G.I., Dunaeva S.E., Podolnaya L.P., Petrosyan I., Fan Y., Zeng X., Zhang J., Shen G. *Kok-saghyz* (*Taraxacum kok-saghyz* Rodin) *cultivation guidelines*. St. Petersburg, 2017.

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SEASONALITY OF ANDROGENETIC RESPONSES IN THE ANTHER CULTURE in vitro IN RICE (*Oryza sativa* L.)

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Abstract

The conditions for growing donor plants in androgegesis in vitro are considered from the point of view of the influence of physical factors on the plants (illumination, length of daylight, temperature, nutrition of plants). A priori it is believed that any period of the year (season) is suitable for plant tissue culture in vitro, and this is the main advantage of in vitro technologies compared to traditional ones. Seasonality is taken into account only when comparing donor plants grown in the field and under controlled conditions. However, we have not found reports on the study of using procedure of anther in vitro culture throughout the year. This paper is the first to show that, under uniform conditions of donor plants growing, the frequency of callus formation and regeneration of rice (*Oryza sativa* L.) plants from anthers in vitro culture differs depending on the month (season) when explants were collected. The aim of the studies was to study the seasonal dependence of in vitro androgenetic responses of *O. sativa* when growing donor plants in a climatic chamber conditions. The 5-9 plants of *O. sativa* subsp. *japonica* Kato, the Cascade variety, were planted monthly during a year and grown in a climatic chamber (at 24 °C and 21 °C, 15000 lux, 60 % humidity and photoperiod of 14 light/10 dark hours) to be the test anther donors. The anthers from naturally grown plants served as controls. As a result, the seasonal dependence of callus formation in the rice anther culture in vitro was revealed. The peak intensity of callus formation occurs in May and June (15.5-28.3 %). When growing donor plants under artificial conditions at both temperatures, in the best period for anther culture in vitro (May-June) callus formation was higher than in the control. The use of the climatic chamber makes it possible to obtain consistently high values of the intensity of callus formation in some months, which is not always possible in the natural conditions. The temperature of donor plant growing affects the frequency of rice callus formation and the regenerative capacity. The temperature which is lower than optimal for rice plants (21 °C) allows for different frequency of callus formation throughout the year, whereas the rice-comfortable temperature (24 °C) leads to a large seasonal dependence and callus formation from May to September and in December and January. At 21 °C vs. 24 °C, four times more calluses with green regenerants are formed (31.6 % vs. 8.8 %), with an increase in the fraction of doubled haploids up to 28.1 % and their number per callus up to 16.6. A moderate correlation was found between the share of calli with green regenerants and the average number of doubled haploids per callus (r = 0.59 at p = 0.05). This means that with an increase in the number of calli with green regenerants, which is observed at 21 °C, the total yield of doubled haploids also increases. As to the number of haploids, no such dependence was found. Thus, greenhouses and climatic chambers may serve not only for growing donor plants by researchers, but also for practical use of the most favorable periods for anthers culture technique.

Keywords: *Oryza sativa* L., androgenesis in vitro, callus formation, regeneration, doubled haploids, seasonality

An in vitro rice anther culture has been used for genetic researches and breeding purposes since 1968 [1]. The works for obtaining doubled rice haploids has been successfully carried out for five decades, however, the breeding of new varieties, hybrids, and lines requires the optimization of anthers cultivation conditions because a significant dependence of callus formation and regeneration on the parent plant's genotype is known. It is considered a key problem in the

plants androgenesis *in vitro* including that in rice [2-4]. Nevertheless, there are also a number of other factors which influence on the androgenesis process: the conditions of donor plants growing, shock treatment of anthers before introduction into the *in vitro* culture, compositions of culture media and conditions of anthers and calli culturing [5-7].

The conditions of donor plants growing are considered from the standpoint of their exposure to the physical factors (light intensity, daylight duration, temperature, plants nutrition) [5, 8]. It is believed that any period of the year (season) is suitable for the in vitro culture of the plants' cells and tissues that constitutes the main advantage of this technology compared to traditional agricultural production [9]. However, experienced researchers know that in certain periods of a year in vitro cultures grow and develop better than in other ones. Some rare experimental data on the plants' seasonal development in the *in vitro* conditions began to appear. Thus, the propagation by cutting of the *Sequoiadendron giganteum* (Lindl.) J. Buchholz in spring (March-May) contributes to the longer preservation of more number of living cuttings [10]. The summer period was the most favorable for the essential oil rose grown in the foothill zone of the Crimea. In this period, the number of developing explants in five varieties reached 92-97%, and in autumn this value was minimal, 40-72% [11]. For roses grown on the southern coast of Crimea the best period for selecting and introducing the explants into the culture were February-March, when the meristems development frequency reached 92-100%, in the autumn-summer period this value did not exceed 10-20% [12]. The authors conclude that such morphogenetic reaction is conditioned by the physiological state of the plant's organ and of the explant secreted from it [12]. We have not found any reports related to the anthers introduction in the in vitro culture in all periods of the year. The seasonality factor is considered solely when comparing the donor plants grown in the field and indoor conditions.

Some researchers introduce the anthers in the in vitro culture year-round without any stable result, but in most cases they try to time the cultivation by the end of spring. There are many information releases about using greenhouses and climate chambers for growing the donor plants for the rice anther culture [13, 14]. The main advantage of using indoor growing is the possibility to regulate the growing conditions [5, 6] and the aseptic purity of anthers introduced into the culture [5, 15]. In all such works, the emphasis is laid on the parent plants' growing conditions and not on the seasonality.

In the consideration of the problems related to the donor plant growing conditions there is also no the clear-cut answer to the question how to get the maximum output of doubled haploids. The relevant researchers are unanimous in the opinion that the conditions must be different for different species, genera, and families [4, 16]. In most cases, the researches try to create the maximally favorable conditions for the growth and development of the parent plants of rice [2, 6] both in the field [17-19] and indoor [13, 14] conditions.

There are some evidences that the stressful conditions of the parent plants growing favorably influence the callus formation or shoots regeneration processes. In the anthers of the rice plant growing in a dry season there are more microspores capable of androgenetic responses [6]. In the plants grown at 18-20 °C, the frequency of calli production and regeneration of green shoots were more than 2 times higher than when growing at 26-28 °C, and the albinos formation was lower [as cited in 20]. The frequency of green regenerants formation turned out to be higher when growing the donor plants in the culture room at 20 °C than when growing in open space [21]. All these experimental data have also been obtained regardless of the seasonal dependence.

This research for the first time showed that the intensity of the callus formation and regeneration of rice plants varies depending on the month when the anthers were introduced in the in vitro culture while the donor plants' growing conditions were uniform throughout the year. The most favorable period for the introduction of anthers in the in vitro culture was May-June. The lower temperature of 21°C made it possible to obtain callus with different intensities throughout the year, and when the temperature of 24 °C which is optimal for rice, the higher seasonal dependence was observed.

The objective of this work was to investigate the seasonal dependence of the androgenetic responses of rice plants *Oryza sativa* L. when the donor plants growing *in vitro* in the controlled conditions of climatic chamber under two temperature schedules.

Techniques. The anthers of rice *O. sativa* subsp. *japonica* Kato Cascade variety were used in the researches (Chaika Far East Federal Research Center for Agrobiotechnology). The 5-9 donors planted in the ground on Day 15 of every month of the year were placed into the climatic chamber MLR-352H (Sanyo, Japan) under the following conditions: temperature 24 °C (in 2015) and 21 °C (in 2016), light intensity 15,000 lux, humidity 60%, photoperiod (light/dark) 14/10 hours. Donor plants grown in pots in the open space were the control. In the control, anther cultures were derived from 400 anthers in August 2015 and 2016.

Rice anthers prior to culture were exposed to low positive temperatures (5 °C) for 7 days by placing the panicles into the cylinders with water. For culture, induction culture medium N_6 was used [2]. A total of 400 to 702 anthers per month were used.

The anthers were cultured at 25-27 °C in the dark until the 1-5 mm calli formation. Then it was transplanted to the N_6-pк medium [23] for the shoots regeneration. The conditions in the culture room were the following: light intensity 4,000 lux, temperature 22-25 °C, daylight/dark duration 16/8 hours. For the rooting the MS medium [24] with the half-amount composition of macrosalts was used as modified by Goncharova [20].

The regenerants with a developed root system were planted in pots and continued to grow in the culture room until the seeds formation. All regenerated plants were divided into groups of haploids (the plants without seeds and with very small flowers), doubled haploids (the plants with seeds), tetraploids (the plants with few very large seeds having the apparent keel and ribbing on the floral squames), the plants without seeds (which have formed flowers of normal size, but not seeds on two or more panicles), and the plants which died during early growth.

The data statistical processing was made using Statistica 10 software (StatSoft Inc., USA). The mean values (*M*), standard errors of means (±SEM) and the correlation coefficient (*r*) were determined. The difference between the variants was evaluated by Student's *t*-test at the significance level of at least 5%.

Results. For 24 °C and 21 °C, 5,172 and 7,278 anther cultures were initiated, 12,450 in total. The seasonal dependence was detected at both temperatures of the parent plants growing; the maximum frequency of callus formation occurred in May under 24 °C (Fig. 1, A) and in May-June under 21 °C (see Fig. 1, B). However, the temperature which is low for the rice plant made it possible to get the callus every month with more or less intensity (1.0-15.5%). At the optimum temperature of 24 °C in the early spring and late autumn periods, the callus formation did not occurred. The monthly callus formation averaged to 4.5% for 24 °C and to 3.9% for 21 °C without statistically significant differences.

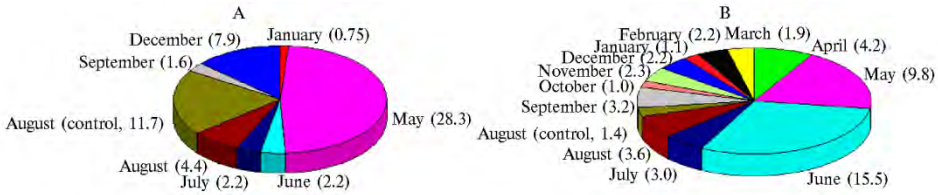

Fig. 1. Callus formation (%) in rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month of anther culture initiation and on the temperature of growing donor plants: A — 24 °C, B — 21 °C.

1. Calli with green and albinos regenerants in rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month depending on the month of anther culture initiation and on the temperature of growing donor plants

Month	Call, %		Green regenerants/albinos
	with green regenerants	albinos	
	Temperature 24 °C		
May	13.7	37.1	3.5
June	7.7	23.1	20.5
July	5.9	26.5	0.8
August (control)	12.8	25.5	1.3
August	5.6	16.7	0.9
September	0	33.3	0
December	15.9	15.9	24.3
Average (*M*±SEM)	8.8±2.1	25.4±3.0	7.3±3.9
	Temperature 21 °C		
January	33.3	16.7	20.0
February	21.4	21.4	23.7
March	50.0	16.7	18.3
April	20.0	20.0	7.6
May	33.3	22.2	18.4
June	56.3	59.8	5.4
July	23.1	7.7	9.8
August	33.3	52.4	2.3
August (control)	0	40.0	0
September	22.2	38.9	8.0
October	60.0	20.0	44.0
November	50.0	16.7	21.1
December	20.0	26.7	50.0
Average (*M*±SEM)	31.6±5.0	27.6±4.3	17.6±4.2

Note. The mean values (*M*) of the share of the calli with green regenerants for 24 °C and 21 °C differ statistically significantly at p = 0.01.

The callus frequency in the control was different in 2015 and 2016. Thus, the conditions of 2015 were typical for parent plants' growth that led to the higher callus formation (11.7%) (see Fig. 1, A). In 2016, we collected panicles in hot period that could be a negative stressor for the callus formation which amounted to 1.4% (see Fig. 1, B), and led to the complete absence of shoot regeneration. Under growing donor plants in a chamber at both temperatures during the best time of the anther culture initiation (May-June), the callus formation was higher than in the control. Thence, using the climatic chamber made it possible to get a stable high callus formation in certain months, which is not always possible under the natural conditions of growing anther donors.

Donors grown under 24 °C produced 8.8% calli capable of green regenerant formation, at 21 °C the average value was 4 times higher ($t = 3.35$, $p = 0.004$), up to 31.6 % (Table 1). Therefore, the temperature which is low for rice plant not only allows callus formation throughout the year, but also influences the frequency of calli with morphogenetic responses. There was tendency to a 2-fold decrease of albinism. Note that when using other methods to increase shoot regeneration from anthers, e.g. different regeneration media, the number of green regenerants also increases due to the greater output of calli capable of morphogenetic responses [23].

Albinism of calli is a problem for plant regeneration in cereal crops [4, 6]. In our research, the albinos were several times less frequent than green regenerants (see Table 1); they appeared only at the initial stage of morphogenesis and could be easily removed from the callus. The next passage of callus aggregates with green initials allowed tens and hundreds of green regenerants to develop normally.

2. The average number of rice (*Oryza sativa* spp. *japonica*) Cascade variety green regenerants per callus depending on the month of anther culture initiation (M±SEM)

Month	Calli	Haploids	Doubled haploids	Tetraploids	Dead plants	Seedless plants
Temperature 24 °C						
May	17	54.1±13.3	14.8±3.9	0	11.2±3.5	0.3±0.2
June	1	157	4	0	44	0
July	2	16.0±10.0	13.5±9.5	0	2.5±1.5	0
August (control)	6	17.2±15.4	3.7±1.8	0	1.7±0.8	0
August	1	14	0	0	1	0
December	7	54.9±24.4	28.0±11.3	1.6±1.6	11.1±8.9	1.4±0.8
Average	5.7±2.5	10.9±5.6	4.6±2.4	0.3±0.3	11.9±6.7	0.3±0.2
Temperature 21 °C						
January	2	90.5±0.5	1.0±0.0	0	8.5±0.5	0
February	3	33.0±31.5	28.3±27.3	0.3±0.3	9.7±2.9	4.3±4.3
March	6	17.3±9.4	23.5±17.9	0	14.2±7.8	0
April	5	27.4±13.3	9.6±5.6	0	9.8±7.2	0.2±0.2
May	17	31.9±8.4	19.7±6.9	0.5±0.5	5.4±1.0	1.9±1.2
June	52	28.0±5.4	10.9±2.5	0.3±0.3	8.9±1.4	2.4±0.6
July	3	3.7±2.0	9.7±7.3	0	3.0±2.1	0
August (control)	7	21.3±13.4	5.9±2.4	0	4.1±1.8	1.4±1.0
September	4	34.0±21.1	18.5±11.2	0	9.8±1.4	1.0±0.6
October	3	3.3±1.3	61.7±30.9	0	7.0±3.4	1.3±0.7
November	6	12.0±5.7	12.3±7.0	0	3.3±3.3	0.8±0.4
December	3	51.7±24.1	15.0±10.0	0	15.0±9.0	0
Average	9.3±4.1	27.2±6.7	16.6±4.3	0.1±0.1	8.2±1.1	1.1±0.4

Note. The average values for doubled haploids at two temperatures differ statistically significantly at p = 0.05; the differences in mean values of the haploids number are statistically insignificant.

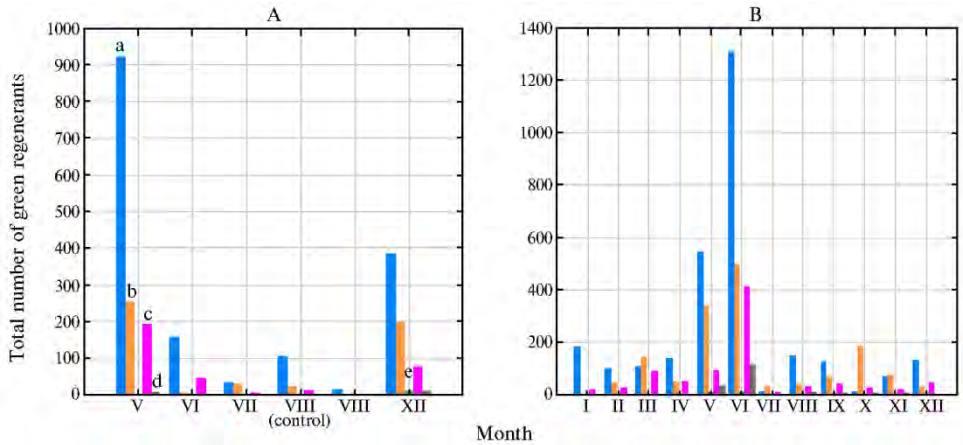

Fig. 2. Regeneration of rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month of anther culture initiation for donor plants growing temperatures of 24 °C (A) **and 21 °C** (B)**:** a — haploids; b — doubled haploids; — dead plants; d — plants without seeds; e — tetraploids.

The average amount of regenerants of various types per callus depending on the temperature of donor plant growing is shown in Table 2. There were no statistically significant differences between haploids and doubled haploids as per months. The statistically significant difference (t = 4.26, p = 0.0001) was only between the average values of doubled haploids in June and October for 21 °C of growing donor plants. The average values of doubled haploids differed (t = 2.39, p = 0.05) between 24 °C and 21 °C temperatures (see Table 2). There was moderate correlation between the portion of the calli with green regenerants and the

average number of doubled haploids per callus (r = 0.59, p = 0.05). It means that as an increase in the number of calli with green regenerants occurred at 21 °C, the total output of doubled haploids also increased. Such dependence by the number of haploids has not been revealed.

For 24 °C and 21 °C the total number of produced regenerants amounted to 2,464 and 5,798, respectively. The maximum number of regenerants was in May-June period in accordance with the callus formation intensity (Fig. 2). For 24 °C, a regenerative ability slightly increased in December.

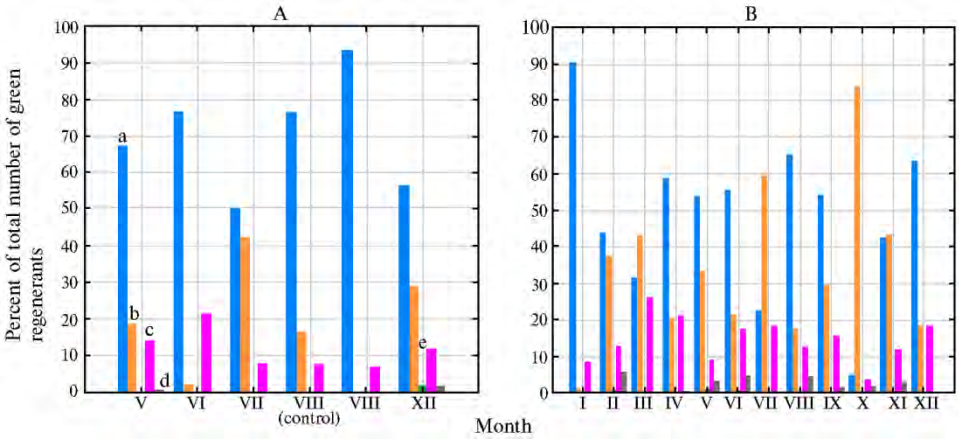

Fig. 3. Groups of regenerants of rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month of anther culture initiation for donor plants growing temperatures of 24 °C (A) **and 21 °C** (B)**:** a — haploids; b — doubled haploids; — dead plants; d — plants without seeds; e — tetraploids.

The number of regenerants of different types was not the same for different temperatures of growing donor plants. At 24 °C, haploids were always the greatest in number, throughout the year up to 65.3% and from 50.0 to 93.3% as per months (see Fig. 3, A). Doubled haploids amounted 20.3% on average per year and varied from 0 to 42.2% as per months. At 21 °C, throughout the year haploids averaged 52.6%, ranging from 4.6 to 90.5%, and doubled haploids reached 28.1%, ranging from 1.0 to 84.1% (see Fig. 3, B). That is, as the temperature used to grow donors decreased, the number doubled haploids increased. This was due to the increase in the average number of regenerants from callus. In certain months (March, July, October and November), if the donor plants were exposed to lower temperature (21 °C), doubled haploids prevailed over haploids (see Fig. 3).

No dependence has been found for nonviable regenerants, tetraploids, and sterile plants of non-haploid origin. Seasons of the year and the temperature of the donors growing did not affect the frequency of their appearance. Apparently, their formation in callus was spontaneous.

The found seasonal dependence of androgenetic responses in rice is well consistent with the general biological laws. Spring and early summer is the most favorable period for active germination of seeds and vegetative propagation of plants. Obviously, even under controlled growing donors, callus formation in anther culture and regeneration intensify precisely in this period. The propagation by cutting of other plants in vitro is also most successful in spring [10-12]. Indoor growing (in greenhouses, climatic chambers) not only ensures the controlled conditions for growing donor plants, which is often practiced by researchers [5, 6], but also enables using favorable time to initiate anther culture. Pershina et al. [25] note the negative response of some wheat varieties in anther culture when growing donor plants in a greenhouse. Perhaps, given the seasonal

dependence of androgenic responses, the result could be positive.

The low temperature during the donor plants growing (21 °C) is an additional stressor which stimulates the androgenetic responses of the rice plants and made it possible to get the regenerants throughout the whole year, while more favorable temperature (24 °C) is effective only during certain months. Shock temperatures (4-12 °C) of anther pretreatment can switch the program of microspore development in cereals from gametophytic to sporophytic [2, 5, 7]. The extremely low temperature of donor growing enhances this effect in rice. A number of researches show that creating the better conditions for the donor plants' growth and development is not necessary as it is commonly believed. A stress including the temperature stress enhances the callus formation and regeneration [as cited in 20, 21].

Thus, there is the seasonal dependence of callus formation in rice anther culture, with the maximum frequency in May to June. The temperature of donor plant growing influences callus formation intensity and regenerative ability. The temperature which is lower than the optimal for the rice plants (21 °C) ensures callus formation with different intensity throughout the whole year, while at the optimal temperature (24 °C) the callus formation is possible only from May to September and in December to January. The anthers of donor plants grown at 21 °C produce practically 4 times more calli with green regenerants (31.6%) as compared to those at 24 °C (8.8%). The number of doubled haploids increases up to 28.1% and averages up to 16.6 per callus.

REFERENCES

1. Niizeki B.H., Oono K. Induction of haploid rice plant from anther culture. *Proceedings of the Japan Academy*, 1968, 44(6): 554-557 (doi: 10.2183/pjab1945.44.554).
2. Mishra R., Rao G.J.N. In-vitro androgenesis in rice: advantages, constraints and future prospects. *Rice Science*, 2016, 23(2): 57-68 (doi: 10.1016/j.rsci.2016.02.001).
3. Xu L., Najeeb U., Tang G.X., Gu H.H., Zhang G.Q., He Y., Zhou W.J. Haploid and doubled haploid technology. In: Advances in Botanical Research-Rapeseed Breeding. S.K. Gupta, M. Delseny, J.-C. Kader (eds.). Academic Press, London, 2007: 181-216 (doi: 10.1016/S0065-2296(07)45007-8).
4. Dunwell J.M. Haploids in flowering plants: origins and exploitation. *Plant Biotechnology Journal*, 2010, 8(4): 377-424 (doi: 10.1111/j.1467-7652.2009.00498.x).
5. Ferrie A.M., Caswell K.L. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. *Plant Cell Tiss. Organ Cult.*, 2011, 104(3): 301-309 (doi: 10.1007/s11240-010-9800-y).
6. Datta S.K. Androgenic haploids: factors controlling development and its application in crop improvement. *Current Science*, 2005, 89(11): 1870-1878.
7. Germanà M.A. Anther culture for haploid and double haploid production. *Plant Cell Organ. Cult.*, 2011, 104(3): 283-300 (doi: 10.1007/s11240-010-9852-z).
8. Bolvell G.P., Vud K.R., Gonzales R.A. et al. *Biotekhnologiya rastenii: kul'tura kletok* /Perevod s angliiskogo V.I. Negruka; s predisloviem R.G. Butenko [Plant biotechnology: cell culture]. Moscow, 1989 (in Russ.).
9. Urazaliev K.R. *Biotekhnologiya. Teoriya i praktika*, 2016, 2: 4-19 (in Russ.).
10. Sultonova M.S., Nimadzhanova K. *Innovatsii i prodovol'stvennaya bezopasnost',* 2014, 4: 77-81 (in Russ.).
11. Egorova N.A., Stavtseva I.V. *Vestnik Udmurtskogo universiteta*, 2016, 26(2): 45-52 (in Russ.).
12. Mitrofanova I.V., Mitrofanova O.V., Brailko V.A., Lesnikova-Sedoshenko N.P. *Prikladnaya khimiya i biotekhnologiya*, 2015, 2(13): 37-48 (in Russ.).
13. Bishnoi U.S., Jain R.K., Gupta K.R., Chowdhury V.K., Chowdhury J.B. High frequency androgenesis in *indica* × Basmati rice hybrids using liquid culture media. *Plant Cell, Tissue and Organ Culture*, 2000, 61(2): 153-159 (doi: 10.1023/A:1006407912576).
14. Lapitan V.C., Redoña E.D., Abe T., Brar D.S. Molecular characterization and agronomic performance of DH lines from the F_1 of *indica* and *japonica* cultivars of rice (*Oryza sativa* L.). *Field Crop Research*, 2009, 112(2-3): 222-228 (doi: 10.1016/j.fcr.2009.03.008).
15. Ilyushko M.V. *Vestnik KrasGAU*, 2014, 6: 143-148 (in Russ.).
16. Smýkal P. Pollen embryogenesis — the stress mediated switch from gametophytic to sporophytic

development. Current status and future prospects. *Biologia Plantarum*, 2000, 43(4): 481-489 (doi: 10.1023/A:1002835330799).
17. Rout P., Naik N., Ngangkham U., Verma R.L., Katara J.L., Singh O.N., Samantaray S. Doubled haploids generated through anther culture from an elite long duration rice hybrid, CRHR32: method optimization and molecular characterization. *Plant Biotechnology*, 2016, 33: 177-186 (doi: 10.5511/plantbiotechnology.16.0719a).
18. Premvaranon P., Vearasilp S., Thanapornpoonpong S., Karladee D., Gorinstein S. In vitro studies to produce double haploid in Indica hybrid rice. *Biologia*, 2011, 66(6): 1074-1081 (doi: 10.2478/s11756-011-0129-8).
19. Mishra R., Rao G.J.N., Rao R.N., Kaushal P. Development and characterization of elite doubled haploid lines from two Indica rice hybrids. *Rice Science*, 2015, 22(6): 290-299 (doi: 10.1016/j.rsci.2015.07.002).
20. Goncharova Yu.K. *Ispol'zovanie metoda kul'tury pyl'nikov v selektsii risa* [Using the anther culture method in rice breeding]. Krasnodar, 2012 (in Russ.).
21. Ilyushko M.V. Effect of growing conditions of rice donor plants on anther culture in vitro. *Journal of Agricultural Science and Technology*, 2015, 5: 686-694 (doi: 10.17265/2161-6256/2015.08.007).
22. Chu C. The N_6 medium and its applications to anther culture of cereal crops. *Proceedings of Symposium on Plant Tissue Culture, 25-30 May 1978, Peking.* Science Press, Peking, 1978: 43-50.
23. Ilyushko M.V. *Izvestiya TSKHA*, 2017, 2: 126-133 (in Russ.).
24. Murashige T., Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 1962, 15: 473-497 (doi: 10.1111/j.1399-3054.1962.tb08052.x).
25. Pershina L.A., Osadchaya T.S., Badaeva E.D., Belan I.A., Rosseeva L.P. *Vavilovskii zhurnal genetiki i selektsii,* 2013, 17(1): 40-49 (in Russ.).

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ENZYMES FOR THE DEGRADATION OF RHAMNOGALACTURONAN I AS VIRULENCE FACTORS OF PHYTOPATHOGENIC BACTERIUM *Pectobacterium atrosepticum*

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Abstract

Plant pathogenic pectobacteria (*Pectobacterium* genus) are well-known all over the world as the causal agents of the cultural plant diseases called soft rots. Rot symptoms are related to the extensive plant tissue maceration due to the production by microorganisms of the plant cell wall degrading enzymes. Most of the pectobacteria-secreted enzymes catalyze the cleavage of homogalacturonan. This polysaccharide, that is a linear homopolymer, consists of galacturonic acid residues and is the most abundant (by mass) pectic polysaccharide of plant cell walls. The knockout of genes of homogalacturonan-degrading enzymes is known to lead to reduced virulence of pectobacteria. In addition, the modification of another pectic compound — rhamnogalacturonan I also occurs in the course of infection process caused by pectobacteria. This compound is a ramified heteropolymer, the backbone of which consists of alternate rhamnose and galacturonic acid residues, and side chains are represented by galactose or arabinose residues. However, the role of pectobacterial enzymes for rhamnogalacturonan I degradation in the development of soft rots has not been previously ascertained. The present study is dedicated to the investigation of the necessity of *P. atrosepticum* SCRI1043 enzymes degrading rhamnogalacturonan I for a full development of soft rots in the plants infected by pectobacteria. By directed mutagenesis, we have obtained mutant forms of *P. atrosepticum* SCRI1043 deficient in genes encoding rhamnogalacturonyl hydrolase (genome locus *eca3749*) that cleaves the backbone of rhamnogalacturonan I, and galactanase (genome locus *eca0852*) that breaks side chains of this polymer. For the target gene knockout, mutant loci were constructed by overlap-extension PCR. Most of the original gene was replaced by kanamicin-resistance cassette. The obtained construction was ligated into a mobilized suicide vector and the resulting plasmid was transferred into donor *E. coli* CC118 strain cells. The recombinant plasmid with the mutant locus was introduced into *P. atrosepticum* SCRI1043 cells by three-parental mating. The *P. atrosepticum* SCRI1043 clones, in which the original locus was replaced by the mutant one, and the donor plasmid was eliminated, were selected on the selective media. The mutant strains *P. atrosepticum* SCRI1043 3749 and *P. atrosepticum* SCRI1043 0852 caused significantly less damage to the plant tissues of *Brassica rapa* spp. *pekinensis* Cha Cha cv. compared to parental wild-type strain. Herewith, the strain mutant in *eca0852* locus encoding galactanase, the enzyme that cleaves side chains of rhamnogalacturonan I, was least virulent. The reduction in virulence, in this case, was not related to the suppression of homogalacturonan-degrading enzyme activity or less motility of bacteria. Thus, we have demonstrated that, first, rhamnogalacturonan I-degrading enzymes may be attributed to virulence factors of phytopathogenic pectobacteria, and second, the hydrolysis of the sides chains of rhamnogalacturonan I contributes more to the process of tissue maceration than the decay of the polymer backbone.

Keywords: *Pectobacterium atrosepticum*, pectic polysaccharides, rhamnogalacturonan I, glycosyl hydrolases

Members of *Pectobacterium* genus are one of the most harmful plant pathogens in the world [1]. These microorganisms cause diseases in plants called soft or wet rot [2, 3]. Key determinants of pectobacteria pathogenity are extracellular enzymes that degrade cell wall polysaccharides, of which enzymes destructing polygalacturonic acid (homogalacturonan) that are more diverse. Such polymer, mainly contained in medial plates, is the most spread pectin polysaccharide of plant cell walls [4]. Its destruction in the course of infection results in tissue maceration [2, 3, 5]. Numerous researches had shown that mutant forms of pectobacteria, in which secretion of polygalacturonan-destructing enzymes is absent or reduced, cannot cause symptoms of soft rots [6-8].

Along with genes encoding the homogalacturonan-destructing enzymes, pectobacteria genome has genes of degrading enzymes of other pectin polysaccharide, the rhamnogalacturonan I (RGU I). Unlike homogalacturonan (linear homopolymer consisting of residues of galacturonic acid), RGU I is a branched heteropolymer. Its backbone consists of the alternate rhamnose and galacturonic acid residues, and side chains attached to rhamnose are galactose or arabinose residues [4].

In our previous studies we had shown that RGU I plays an important role in pectobacteria colonization in vessels of primary xylem, where microorganisms form special biofilm-like multiple cell structures which we call bacterial emboli [9]. As apart from biofilms, in which extracellular matrix is mainly represented by bacterial exopolysaccharides [10-12], primary matrix of bacterial emboli is formed from RGU I [13]. Such polymer is released from plant cell walls due to receptive plant response, and forms certain microcosm for construction of bacterial emboli. With maturing of bacterial emboli, RGU I, as part of extracellular matrix, is replaced by extracellular pectobacteria polysaccharides [14]. It denotes dynamic transformation of RGU I at development of infection. However, for pectobacteria the role of RGU I degradation enzymes in pathogenesis was not demonstrated yet.

We had established for the first time that destruction of RGU I during the infection largely contributes to development of soft rot symptoms caused by *Pectobacterium atrosepticum*.

Purpose of present research is to verify the need for RGU-I destructing enzymes for development of soft rots in plants infected by pectobacteria.

Techniques. Pectobacterium atrosepticum strain SCRI1043 (earlier called *Erwinia carotovora* ssp. *atroseptica* SCRI1043) [15] (by courtesy of E.A. Nikolaychik) (Collection of Belarus State University), *Escherichia coli* and mutants at *eca0852* (Δeca0852) and *eca3749* (Δeca3749) loci were cultured at 28 °C in Luria-Bertani (LB) medium [16] containing 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl (pH 7.5). When necessary, kanamycin (30 μg/ml), streptomycin (100 μg/ml), tetracycline (12.5 μg/ml) were added.

The target enzyme sequences were searched for by BLASTp algorithm in PDB (Protein Data Bank Europe, https://www.ebi.ac.uk/pdbe/) and UniProt (https://sparql.uniprot.org/). Phylogenetic tree was plotted by nearest neighbor method; bootstrap support was indicated on tree branches. Molecule phylogenetic analysis was conducted with MEGA 6.0 software (https://www.megasoftware.net/). Biochemical description of enzymes was taken from CAZy database (Carbohydrate-Active enZyme, http://www.cazy.org/).

Mutant locus for knockout of *eca0852* and *eca3749* genes was established using overlap extension polymerase chain reaction (PCR) method. Most part of gene coding area was removed and insertion in lieu of it kanamycin resistance cas-

sette was inserted as a marker for selection of mutant cells. The obtained structure was ligated into suicidal mobilizable vector pKNG101 (generously provided by Professor L.N. Moleleki, Pretoria University, RSA); the resultant plasmid was electroporated into cells of donor strain *E. coli* CC118. Recombinant mutant loci as part of obtained plasmid were introduced into *P. atrosepticum* SCRI1043 by triparental crossing. Cells with recombinant plasmid were selected on streptomycin- and kanamycin-containing media. Afterwards, cells, in which the second recombination took place with replacement of the target gene and elimination of donor plasmid, were selected on the sucrose-containing medium. It was followed by sampling clones resistant to kanamycin and sensitive to streptomycin. Mutation was verified by PCR method, and nucleotide sequence of mutant locus was determined as described above [8].

Virulence of pectobacteria mutants was assessed as the weight of affected (macerated) leaf tissues of cabbage (*Brassica rapa* spp. *Pekinensis*) Cha Cha cv. inoculated with microorganisms. Pectobacteria were grown in LB medium until the late logarithmic growth phase. Afterwards it was collected by centrifuging and resuspended in 10 mM magnesium sulphate solution less stressful for plants than sodium chloride [17]. Inoculum concentration was adjusted to 1×10^7-3×10^7 CFU/ml by serial dilution. Leaf surface was sterilized by Belizna bleaching solution (0.8% active chlorine) and by 70% ethanol. Next, leaves were rinsed by sterile water with small cuts made, in which 10 μl of bacterial suspension (1×10^5-3×10^5 CFU/ml) or sterile magnesium sulphate solution was injected. Infected leaves were placed in Petri dishes and incubated at 28 °C for 48 hours. Macerated plant tissues were extracted by scalpel and weighted. Results were obtained in minimum 10 biological replications and visualized in form of boxplots plotted by graphic package ggplot2 (https://ggplot2.tidyverse.org/). To assay pectate lyase activity, bacterial cells were cultured in synthetic D5 midium of the following composition with pectin as a sole source of carbon: 13.6 g/l KH_2PO_4, 1.0 g/l NH_4Cl, 0.3 g/l $MgSO_4$ (added as 100× stock solution after sterilization), 1.4 g/l NaOH, 2 g/l pectin, pH 7.5.

Pectate lyase activity was assayed as per described methodology [18]. Cell-free supernatant of cultures (50 μl) grown in D5 midium within 24 hours at 28 °C in a thermostat shaker-incubator (Orbi Safe, Sanyo, Japan) at 160 rpm/min was mixed with 450 μl of reaction mixture (pH 8.5) containing 50 mM Tris-HCl, 0.1 mM $CaCl_2$ and 0.05% polygalacturonic acid, incubated at 37 °C during 5 min. Absorption was measured at λ = 235 μm (spectrophotometer Solar PB2201B, ZAO SOLAR, Belarus). Quantity of enzyme catalyzing conversion of 1 μM substrate per minute was taken as a unit of activity. Specific activity was expressed in u/mg protein.

Swarming motility was assessed upon culturing pectobacteria in semi-liquid D5 midium containing 0.4% Pronadisa agar (Laboratorios CONDA, S.A., Spain) and 2 g/l of sucrose or pectin. At early stationary growth stage, 3 μl of bacterial culture was inoculated in semi-liquid agar and incubated at 28 °C with measuring of macro colony diameter in 24 hours.

Statistical analysis was conducted by standard mathematic methods (calculation of mean M and standard deviation $\pm\sigma$, comparison of means by Student's t-test) in Microsoft Excel 2000. Package ggplot2 was used for visualization of values of macerated tissue mass; confidence level of differences (p-value) was calculated by nonparametric Wilcoxon's test. Differences were statistically significanet at $p < 0.05$.

Results. Used strains of bacteria, plasmids, and primers are characterized in Table 1.

1. Strains, plasmids, and primers used for creation of mutant strains of *Pectobacterium atrosepticum* (*Pba*) deficient in genes encoding rhamnogalacturonyl hydrolase and galactanase

Name	Description
	Strains
Pectobacterium atrosepticum SCRI1043	Wild type [15]
SCRI1043Δ*3749*	Mutant of SCRI1043 with Km resistance cassette insertion in chromosome
SCRI1043Δ*0852*	Mutant of SCRI1043 with Km resistance cassette insertion in chromosome
Escherichia coli CC118	Host of suicidal vector pKNG101 (*ara*, *leu*) *araD lacX* 74 *galE galK PhoA20 thi*-1 *rpsE rpoB argE (am) recA1*, Sm^R (19)
HH26/pNJ5000	Mobilizing strain for conjugative delivery of suicidal vector pKNG101 into cells *Pba*, Tet^R [20]
	Plasmids
pKD4	Matrix for PCR-amplification of kanamycin resistance cassete, Km^R [21]
pKNG101	Suicidal mobilizable vector for inactivation of target genes, *pir*-ori R6K mobRK2 *sacB* Sm^R [22]
pKNG101Δ*3749*	Km^R, Sm^R, *sacB*, contains region of chromosome DNA *Pba* with deleted gene *eca3749*
pKNG101Δ*0852*	Km^R, Sm^R, *sacB*, contains region of chromosome DNA *Pba* with deleted gene *eca0852*
	Primers
upECA3749_F	5′-GCATGTTGACCGAGCTGTCC-3′
upECA3749_KmR	5′-GCCTACACAATCCGACTTCCCAATCCCACTCTTC-3′
dnECA3749_KmF	5′-CCCATGTCAGCCGTTAAGCGATATTCCCAATGTTGCCG-3′
dnECA3749_R	5′-CATGTCCCATCATTTCGCAAC-3′
Km3749_F	5′-GATTGGGAAGTCGGATTGTGTAGGCTGGAGCTGCTTC-3′
Km3749_R	5′-GGAATATCGCTTAACGGCTGACATGGGAATTAGC-3′
chek 3749_F	5′-GTTGCGGTTGGCACGATGG-3′
chek 3749_R	5′-CGAACAGATGGCAATACGTCGG-3′
upECA0852_F	5′-CTAAAGTGTTCTTATTCGATGAGCCC-3′
upECA0852_KmR	5′-CATGTCAGCCGTTAAGTGCTTTACCCAACCAATATCCG-3′
dnECA0852_KmF	5′-CCTACACAATCGCAAATTCTCCAAATGTATAACACCG-3′
dnECA0852_R	5′-CGTCCACTTTCTTACGCCCTC-3′
Km0852_F	5′-GGGTAAAGCACTTAACGGCTGACATGGGAATTAGC-3′
Km0852_R	5′-CATTTGGAGAATTTGCGATTGTGTAGGCTGGAGCTGCTTC-3′
chek 0852_F	5′-GTGTTGCGATTGGGCGGG-3′
chek 0852_R	5′-GTCTGTCGGTAACCAAAGAAAAGCG-3′

Note. Symbols ECA3749 and ECA0852 in primer indices denote gene loci of rhamnogalacturonyl-hydrolase and galactanase, accordingly; F and R are forward and reverse primers, respectively. Symbol up corresponds to amplification of area above the assumed deletion, dn — area next to the deleted fragment. Symbol Km denotes addition of first (F) or resulting (R) fragment of kanamycin resistance cassette of pKD4 plasmid located from the 5′-end of primer (at beginning of index) or at 3′-end of primer (at the end of index). Chek primers initiate amplification from proximal and distal areas of concerned locus to determine its size and sequencing. Detailed scheme of test was earlier described by Datsenko et al. [21].

As per annotations provided for in CAZy and UniProt databases, *P. atrosepticum* SCRI1043 genome has 8 genes encoding enzymes that destruct RGU I. Expression of such genes is increased upon colonization of the host plants by pectobacteria [23]. Genome loci *eca3749* and *eca0852* of *P. atrosepticum*, one of which encodes enzyme that destruct the backbone of RGU I, and the second one that destruct side chains, were selected for knockout of genes encoding RGU I-degrading enzymes. Sequence of the first locus was annotated as encoding rhamnagalacturonyl-hydrolase enzyme that destruct RGU I backbone. Subject to annotation, *eca0852* encodes galactanase that cleave side chains of RGU I. Phylogenetic analysis had proven that target ECA3749 and ECA0852 enzymes were close to protein groups with rhamnagalacturonyl-hydrolase and galactanase activity, respectively (Fig. 1).

The *eca0852* and *eca3749* deficient chromosome mutants of *P. atrosepticum* were constructed by allele exchange with the use of suicidal vector pKNG101 according to earlier described protocol [8]. To assess the influence of target mutations on ability of *P. atrosepticum* to cause maceration in plant tissues, leaves of cabbage were inoculated with wild and mutant pectobacteria. Weight of soft rot produced by such strains within 48 hours varied (Fig. 2). Both mutants caused maceration of plant tissue far less intensively than wild type bacteria. Herewith,

mutation in gene encoding galactanase ECA0852, had considerably stronger effect (see Fig. 2). Most likely, the reason is that hydrolysis of side RGU I chains largely contributes to the tissue maceration process than destruction of polymer backbone. Tissue maceration symptoms were not found in leaves inoculated with sterile 10 mM magnesium chloride solution.

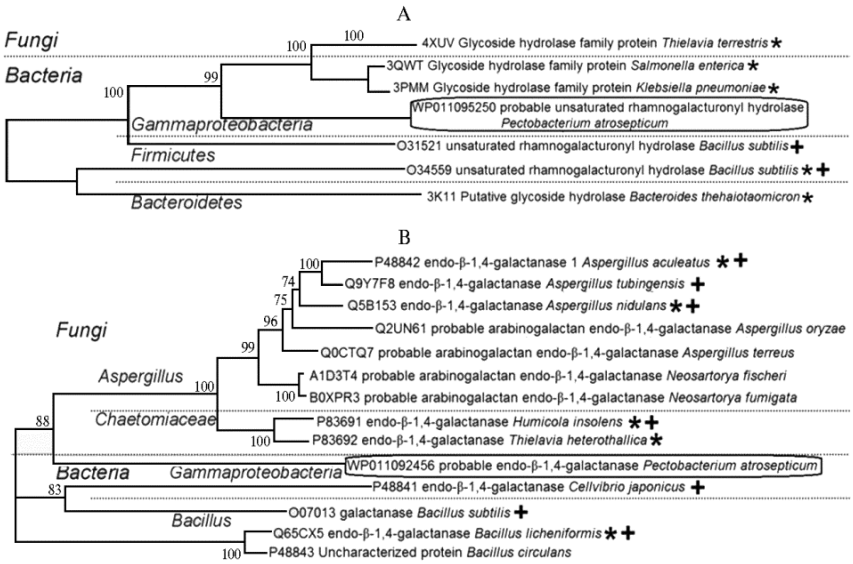

Fig. 1. Cladograms of amine acid sequences foremost similar to rhamnagalacturonyl-hydrolase of *Pectobacterium atrosepticum* (ECA3749, WP011095250) (A) **and β-1,4-endogalactanases of *P. atrosepticum* (ECA0852, WP011092456)** (B: "+" — proteins with known biochemical characteristics, "*" — proteins for which there are X-ray structural analysis in the literature sources.

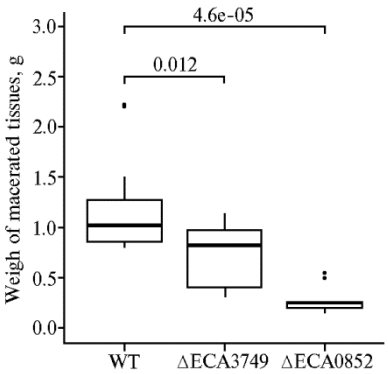

Fig. 2. Box-plots reflecting distribution of weight values in macerated tissues in leaves of cabbage (*Brassica rapa* spp. *Pekinensis*) Cha Cha cv. inoculated by wild-type strain *Pectobacterium atrosepticum* SCRI1043 (WT) **and its mutants for *eca3749* and *eca0852* loci encoding rhamnagalacturonyl-hydrolase and galactanase, respectively.** The dark horizontal line inside the box-plot denotes the median weight value of macerated tissue, upper and lower edges reflect the 1st and 3rd distribution quartiles of the analyzed values of variant, vertical lines indicte extreme value laying within the limits of one and a half interquartile ranges; black points are values exceeding the limits of one and a half interquartile ranges. Difference validities calculated by Wilcoxon's nonparametric test are provided above the parenthesis unifying box-plots.

2. Motility of wild-type strain *Pectobacterium atrosepticum* SCRI1043 and mutants for *eca3749* and *eca0852* loci (0.4 % agar)

Strain	Diameter of colonies, mm ($M \pm \sigma$)	
	sucrose	polygalacturonic acid
SCRI1043	22±0.5	22±0.2
Δ*eca3749*	22±0.5	24±0.6
Δ*eca0852*	23±0.3	23±0.4

Possible effect of mutations in *eca3749* and *eca0852* loci on activity of key virulence factors, the pectate lyases [24], as well as mobility of microorganisms serving their virulence criteria [25-28], was analyzed by relevant testing systems. Extracellular pectate lyase activity of both mutant forms in cultures in vitro, when pectin was used as a sole source of carbon, had not differed from such in a wild type (Fig. 3). In analysis of swarming motility which ensures dis-

tribution of pectobacteria along plant tissues promoting the extension of soft rot area [25] we also had not found any differences between wild and mutant forms of *P. atrosepticum*.

In semi-liquid synthetic medium, whether containing sucrose or polygalacturonic acid, both mutant strains and wild forms moved with similar speed (Table 2). All it means that reduced virulence of strains mutant for *eca3749* and *eca0852* loci is not related to lower motility of microorganisms and lower destruction of homogalacturonan.

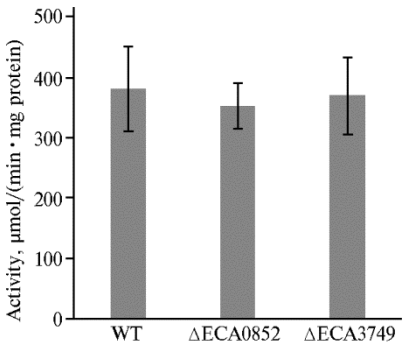

Fig. 3. Pectate lyase in supernatant of *Pectobacterium atrosepticum* SCRI1043 of wild-type (WT) **and mutant forms with inactivated loci *eca0852*** (inactivated ECA0852 enzyme) **and *eca3749*** (inactivated ECA3749 enzyme) **which encode rhamnagalacturonyl-hydrolase and galactanase, respectively**

Although RGU I a minor polymer in pectin fraction as compared to polygalacturonic acid [4], our tests denote the importance of its cleavage for development of soft rots caused by *P. atrosepticum*. Firstly, it could be related to RGU I modification ensuring formation of certain extracellular matrix for pectobacteria [13]. Secondly, regardless of that inactivation of target loci had not resulted in vitro to reduction of the microorganism capacity for cleavage of homogalacturonan (see Fig. 2), in in planta system where various types of pectin substances may represent domains for one molecule [29] inability to destruct RGU I may make certain homogalacturonan areas unavailable for pectobacterial enzymes.

Therefore, we had demonstrated that inactivation of catabolism enzyme genes of rhamnogalacturonan I (RGU I) reduces the capacity of pectobacteria for causing soft rot symptoms in plants. Herewith, it appears that destruction of side chains in such polymer largely contributes to host tissue maceration than backbone hydrolysis, as galactanase mutant is characterized by lesser virulence as compared to not only the parental wild type strain, but also to rhamnagalacturonyl-hydrolase mutant. Our findings allow referring RGU I degradation enzymes to virulence factors of phytopathogenic pectobacteria.

REFERENCES

1. Mansfield J., Genin S., Magori S., Citovsky V., Sriariyanum M., Ronald P., Dow M., Verdier V., Beer S.V.,. Machado M.A., Toth I., Salmond G., Foster G.D. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.*, 2012, 13(6): 614-629 (doi: 10.1111/j.1364-3703.2012.00804.x).
2. Perombelon M.C.M. Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathol.*, 2002, 51(1): 1-12 (doi: 10.1046/j.0032-0862.2001.Short%20title.doc.x).
3. Charkowski A., Blanco C., Condemine G., Expert D., Franza T., Hayes C., Hugouvieux-Cotte-Pattat N., Solanilla E.L., Low D., Moleleki L., Pirhonen M., Pitman A., Perna N., Reverchon S., Rodríguez Palenzuela P., Francisco M.S., Toth I., Tsuyumu S., van der Waals J., Van der Wolf J., Gijsegem F.V., Yang C.-H., Yedidia I. The role of secretion systems and small molecules in soft-rot *Enterobacteriaceae* pathogenicity. *Annu. Rev. Phytopathol.*, 2012, 50: 21.1-21.25 (doi: 10.1146/annurev-phyto-081211-173013).
4. Gorshkova T.A. *Rastitel'naya kletochnaya stenka kak dinamichnaya Sistema* [Plant cell wall as a dynamic system]. Moscow, 2007 (ISBN 978-5-02-035598-9) (in Russ.).
5. Tarasova N., Gorshkov V., Petrova O., Gogolev Y. Potato signal molecules that activate pectate lyase synthesis in *Pectobacterium atrosepticum* SCRI1043. *World J. Microbl. Biot.*, 2013, 29(7): 1189-1196 (doi: 10.1007/s11274-013-1281-9).
6. Walker D.S., Reeves P.J., Salmond G.P.C. The major secreted cellulase, CelV, of *Erwinia carotovora* subsp. *carotovora* is an important soft rot virulence factor. *Mol. Plant Microbe In.*, 1994,

7(3): 425-431 (doi: 10.1094/MPMI-7-0425).
7. Mäe A., Heikinheimo R., Tapio Palva E. Structure and regulation of the *Erwinia carotovora* subspecies *carotovora* SCC3193 cellulase gene *celV1* and the role of cellulase in phytophatogenicity. *Mol. Gen. Genet.*, 1995, 247(1): 17-26 (doi: 10.1007/BF00425817).
8. Moleleki L.N., Pretorius R.G., Tanui C.K., Mosina G., Theron J. A quorum sensing-defective mutant of *Pectobacterium atrosepticum* ssp. *brasiliense* 1692 is attenuated in virulence and unable to occlude xylem tissue of susceptible potato plant stems. *Mol. Plant Pathol.*, 2017, 18(1): 32-44 (doi: 10.1111/mpp.12372).
9. Gorshkov V., Daminova A., Ageeva M., Petrova O., Gogoleva N., Tarasova N., Gogolev Y. Dissociation of a population of *Pectobacterium atrosepticum* SCRI1043 in tobacco plants: formation of bacterial emboli and dormant cells. *Protoplasma*, 2014, 251(3): 499-510 (doi: 10.1007/s00709-013-0546-3).
10. Donlan R.M. Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.*, 2002, 8(9): 881-890 (doi: 10.3201/eid0809.020063).
11. Izano E.A., Amarante M.A., Kher W.B., Kaplan J.B. Differential roles of poly-N-glucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl. Environ. Microb.*, 2008, 74(2): 470-476 (doi: 10.1128/AEM.02073-07).
12. Flemming H.C., Wingender J. The biofilm matrix. *Nat. Rev. Microbiol.*, 2010, 8: 623-633 (doi: 10.1038/nrmicro2415).
13. Gorshkov V.Y., Daminova A.G., Mikshina P.V., Petrova O.E., Ageeva M.V., Salnikov V.V., Gorshkova T.A., Gogolev Y. V. Pathogen-induced conditioning of the primary xylem vessels — a prerequisite for the formation of bacterial emboli by *Pectobacterium atrosepticum*. *Plant Biology*, 2016, 18(4): 609-617 (doi: 10.1111/plb.12448).
14. Gorshkov V., Islamov B., Mikshina P., Petrova O., Burygin G., Sigida E., Shashkov A., Daminova A., Ageeva M., Idiyatullin B., Salnikov V., Zuev Y., Gorshkova T., Gogolev Y. *Pectobacterium atrosepticum* exopolysaccharides: identification, molecular structure, formation under stress and *in planta* conditions. *Glycobiology*, 2017, 27(11): 1016-1026 (doi: 10.1093/glycob/cwx069).
15. Bell K.S., Sebaihia M., Pritchard L., Holden M.T.G., Hyman L.J., Holeva M.C., Thomson N.R., Bentley S.D., Churcher L.J., Mungall K., Atkin R., Bason N., Brooks K., Chillingworth T., Clark K., Doggett J., Fraser A., Hance Z., Hauser H., Jagels K., Moule S., Norbertczak H., Ormond D., Price C., Quail M.A., Sanders M., Walker D., Whitehead S., Salmond G.P.C., Birch P.R.J., Parkhill J., Toth I.K. Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *PNAS USA*, 2004, 101(30): 11105-11110 (doi: 10.1073/pnas.0402424101).
16. Sambrook J., Fritsch E.F., Maniatis T. *Molecular cloning: a Laboratory Manual. 2nd ed.* Cold Spring Harbor, NY, 1989.
17. Lee M.K., van Iersel M. W. Sodium chloride effects on growth, morphology, and physiology of *Chrysanthemum* (*Chrysanthemum morifolium*). *HortScience*, 2008, 43(6): 1888-1891.
18. Shevchik V.E., Robert-Baudoy J., Hugouvieux-Cotte-Pattat N. Pectat lyase PelI of *Erwinia chrysanthemi* belongs to a new family. *J. Bacteriol.*, 1997, 179(23): 7321-7330 (doi: 10.1128/jb.179.23.7321-7330.1997).
19. Herrero M., de Lorenzo V., Timmis K.N. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.*, 1990, 172(11): 6557-6567 (doi: 10.1128/jb.172.11.6557-6567.1990).
20. Grinter N.J. A broad-host-range cloning vector transposable to various replicons. *Gene*, 1983, 21(1-2): 133-143 (doi: 10.1016/0378-1119(83)90155-5).
21. Datsenko K.A., Wanner B.L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS USA*, 2000, 97(12): 6640-6645 (doi: 10.1073/pnas.120163297).
22. Kaniga K., Delor I., Cornelis G.R. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene*, 1991, 109(1): 137-141 (doi: 10.1016/0378-1119(91)90599-7).
23. Gorshkov V., Gubaev R., Petrova O., Daminova A., Gogoleva N., Ageeva M., Parfirova O., Prokchorchik M., Nikolaichik Y., Gogolev Y. Transcriptome profiling helps to identify potential and true molecular switches of stealth to brute force behavior in *Pectobacterium atrosepticum* during systemic colonization of tobacco plants. *European Journal of Plant Pathology*, 2018, 152(4): 957-976 (doi: 10.1007/s10658-018-1496-6).
24. Robert-Baudouy J., Nasser W., Condemine G., Reverchon S., Shevchik V.E., Hugouvieux-Cotte-Pattat N. *Pectic enzymes of Erwinia chrysanthemi, regulation and role in pathogenesis.* The American Phytopathological Society, St. Paul, 2000.
25. Matsumoto H., Umehara M., Muroi H., Yoshitake Y., Tsuyumu S. Homolog of FlhDC, a master regulator for flagellum synthesis: required for pathogenicity in *Erwinia carotovora* subsp. *carotovora*. *J. Gen. Plant Pathol.*, 2003, 69: 189-193 (doi: 10.1007/s10327-002-0029-4).
26. Hossain M.M., Shibata S., Aizawa S.I., Tsuyumu S. Motility is an important determinant for pathogenesis of *Erwinia carotovora* subsp. *carotovora*. *Physiol. Mol. Plant P.*, 2005, 66(4): 134-

143 (doi: 10.1016/j.pmpp.2005.06.001).
27. Duan Q., Zhou M., Zhu L., Zhu G. Flagella and bacterial pathogenicity. *J. Basic Microb.*, 2013, 53(1): 1-8 (doi: 10.1002/jobm.201100335).
28. Pfeilmeier S., Saur I. M, Rathjen J. P., Zipfel C., Malone J. G. High levels of cyclic-di-GMP in plant-associated *Pseudomonas* correlate with evasion of plant immunity. *Mol. Plant Pathol.*, 2016, 17(4): 521-531 (doi: 10.1111/mpp.12297).
29. Harholt J., Suttangkakul A., Vibe Scheller H. Biosynthesis of pectin. *Plant Physiol.*, 2010, 153: 384-395 (doi: 10.1104/pp.110.156588).

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BIOCHEMICAL ASPECTS OF INTERACTIONS BETWEEN FUNGI AND PLANTS: A CASE STUDY OF FUSARIUM IN OATS

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Abstract

Disclosing the mechanisms that build up plant resistance to fungal diseases (pathogenic microorganisms) invariably evokes the need to analyze biochemical factors of resistance. Protective mechanisms are associated with a fairly large number of chemical compounds. *Fusarium* head blight (FHB) affects cereal grains, including wheat, barley and oats. In this work, we report new data on the metabolic profile of oat grains, as influenced by FHB, and a relationship between plant resistance and content of individual metabolites in FHB-susceptible and FHB-resistant varieties. Here, we have challenged the task to assess the connections of FHB-resistance parameters in oat varieties with as many compounds as possible. Such data are required both to understand the mechanisms of resistance and to develop methods for its assessment. Common oat (*Avena sativa* L.) varieties from the collection of the Vavilov Institute (VIR) were studied to evaluate their numerous biochemical characters and their resistance to FHB. The fatty acid composition of oil was analyzed by the method of gas-liquid chromatography with mass spectrometry on an Agilent 6850 chromatographer (USA), and other metabolites were quantitated. Plant resistance was studied under artificial infestation of ears with *F. culmorum* and *F. sporotrichioides* (an experimental field, All-Russian Research Institute for Plant Protection, St. Petersburg—Pushkin, 2015). *Fusarium* Link fungi DNA content and trichothecene mycotoxins were determined in milled grain samples. The amount of DNA of *Fusarium* fungi was measured by real-time PCR techniques using *Tri5* gene-based group-specific primers. Solid-phase competitive enzyme-linked immunosorbent assay (ELISA) was used to measure the content of T-2 toxin and DON in grain. The statistical significance of differences in biochemical parameters, including metabolic profiles, between resistant and susceptible oat varieties was estimated with the Mann-Whitney criterion. For the first time, correlations were found between *Fusarium* resistance and biochemical characteristics of oat. It has been shown that high-protein forms are less affected by FHB, accumulate less toxins, and are more adaptive to biotic stress. Plant resistance to FHB correlates with accumulation of pipecolic acid, monoacylglycegols, tyrosine, galactinol, a number of phytosterols, sugars and adenosine. The values of such correlations and connections between chemical compounds and various parameters of *Fusarium* resistance identified during the study of oat accessions should be regarded as strictly preliminary, since they are the outcome of only one year of field trials. However, the year in consideration was characterized by extremely favorable conditions for the development of parasitic *Fusarium* fungi with all immanent consequences. An assumption can be made that the increased aggressiveness of the latter (kind of a model condition) allowed us to identify with more reliability the connections between a majority of metabolite content and composition parameters and the level of *Fusarium* resistance. Considering the complex polygenic nature of the control over the character "resistance to Fusarium head blight" and, therefore, strong dependence of

its expression on the environments, any future efforts to confirm (or refute) our conclusions will require researching greater intra- and interspecies diversity of this crop's accessions reproduced in various environments and in different years.

Keywords: *Avena sativa* L., oat, varieties, *Fusarium* head blight, *Fusarium*, fungal DNA, PCR, mycotoxins, gas chromatography, mass spectrometry, biochemistry, metabolomics

Fusarium head blight (FHB) is a fungal disease which affects cereals including wheat, barley and oats [1-3]. It leads to yield losses and considered as a global threat to the food security [4]. The most widespread species causing the FHB is *Fusarium graminearum* Schwabe which produces deoxynivalenol (DON). This mycotoxin is the main factor of the fungus aggressiveness, which inhibits, in particular, the translation processes in eukaryotes [5]. Besides the economic damage from yield reduction, the mycotoxins of the *Fusarium* Link genus fungi constitute the threat to the health of people consuming such products [4]. The species of the *Fusarium* genus secrete the toxins having different aggressiveness. Thus, *F. culmorum* (W.G. Sm.) Sacc. is a highly aggressive pathogen producing DON, *F. sporotrichioides* Sherb. is a relatively weak pathogen producing T-2 toxin [6].

The analytical approach based on the chromatography in combination with the mass spectrometry makes it possible to control the changes in many metabolites, including those which synthesis is induced by the pathogen. *F. poae* which produces the nivalenol mycotoxin dominates among the species causing the *Fusarium* disease of oats. This toxin is assumed to be the main factor worsening the nutritional properties of oats. Unfortunately, its control is not carried out for a number of formal reasons [7-9].

The metabolomics approach to the investigation of fungal diseases makes it possible to ensure the reliable identification of components and statistical validity of the quantitative evaluation of their variability in the "plant—pathogen" system [10]. The resistance to the *Fusarium* disease is controlled by the genes of more than a hundred of quantitative trait loci (QTL) and largely depends on the environmental conditions [11, 12]. In the course of evolution, plants have developed the protective reactions to the pathogens' effect. The host plants can produce the chemical compounds which inhibit the pathogen development and have the various strategies of their applying including creating the protective barriers at all stages of the parasite progression [13].

The metabolic response induced by DON includes the loss of chlorophyll [14], as the photosynthesis weakens at the infected area [15]. As a result, the primary (carbohydrate) and secondary (nitrogen) metabolism become modified. The blockage of proteins biosynthesis by the mycotoxin leads to accumulation of free amino acids [16].

One of the priorities of metabolomics is the investigation of the reaction to environmental effects. The obtained profiles (fingerprints) reflect metabolic states including the dynamics of the responses [17].

In this work, new data have been obtained on the *Fusarium* head blight effect on the metabolomic profile of oat grains, the relationship of the resistance indices with the biochemical characteristics (protein content, contents of oil and certain metabolites). The statistically valid differences have been shown of the FHB-resistant (RFs) and FHB-susceptible (SFs) oats from the VIR Collection (Vavilov All-Russian Institute of Plant Genetic Resources).

Our objective was to identify the relationship between the biochemical indices of resistance to *Fusarium culmorum* and *F. sporotrichioides* in oat varieties and the accumulation of the mycotoxins produced by these pathogens (DON and T-2 toxin, respectively).

Techniques. The Russian and foreign varieties, as well as breeding lines of hulled and hulless oats (*Avena sativa* L.) (24 samples in total) were received from

the VIR Collection. Oat plants for seed reproduction and biochemical analysis were field-grown (Pushkin branch of the VIR, Leningrad Region, 2015). The phenological observations and field evaluation were performed according to the methodological guidelines [18], the signs manifestation degree was determined according to the International classifier of the *Avena* L. genus [19]. The seeds were sown on the optimum date for the region (1 sq. m plots, sod-podzolic light loamy soil, and seeding rate of 500 seeds per sq. m). The experiment was arranged in one replication. Potato was tThe precursor crop in the cereal crop rotation.

Protein and oil contents in the seeds was evaluated according to the techniques adopted in the VIR with a Kjeltec Auto 1030 Analyzer (FOSS, Sweden) and Kjeltec 2200 (FOSS, Sweden) for protein assay and a Soxhlet apparatus for oil estimates per dry fat-free residue (20). The oil fatty acids composition was analyzed (gas-liquid chromatography, an Agilent 6850 chromatograph, Agilent 5975B VL MSD quadrupole mass selective detector, Agilent, USA) [20]. Privet variety cultivated in the Leningrad Region as a standard for the main economically valuable parameters.

The plant resistance was investigated after inoculation of the spikes with the *F. culmorum* and *F. sporotrichioides* fungi (the experimental field of the All-Russian Research Institute for Plant Protection — VIZR, St. Petersburg—Pushkin, 2015). Each sample was grown on the plots (1 m row length) in 2 replicates for each infectious background. The plots separated by strips where with Borrus standard oat variety was sown were located on the same field. At heading stage, all samples were inoculated with the suspension of the conidia and mycelium of *F. culmorum* (3×10^7 CFU/ml) and F. *sporotrichioides* (1.3×10^7 CFU/ml) at 50 ml/l rates.

After the harvest and threshing, infection degree, fungal DNA and mycotoxin contents in the grain were evaluated. To assess the infection degree, 100 grains were taken from each average sample; the grain surface was sterilized with the 5% sodium hypochlorite solution for 1-3 min. Then the grains were rinsed with sterile water and placed in Petri dishes with standard potato sucrose agar medium (PSA). After 7 day incubation (in the dark at 24 °C) *Fusarium* fungi were counted. DNA was isolated from grain flour (200 mg) as per adapted CTAB method [21-23]. The typical *F. graminearum* and *F. poae* strains from the VIZR Laboratory of mycology and phytopathology collection were grown on PSA, and DNA were isolated from aerial mycelium with Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania). In the milled grain samples, the amount of the fungal DNA was determined by quantitative PCR (polymerase chain reaction) method, T-2 toxin and DON contents — by the enzyme-linked immunosorbent assay (ELISA) as per description [24].

Basing on the assessment of FHB resistance [24, 25], 10 samples were selected for metabolomic analysis. The grains was weighed and homogenized with methanol, 1/10 (w/v). The samples were allowed for 30 days at 5-6 °C. The 100 μl extracts were evaporated (CentriVap® Concentrator, Labconco, USA). The dry residue was silylated for 40 min at 100 °C with bis(trimethyl-silyl)trifluoroacetamide. Samples were analyzed using gas-liquid chromatography with mass spectrometry (gas-liquid chromatography-mass-spectrometry, Agilent, USA) on HP-5MS capillary column (30.0 m, 250.00 μm, 0.25 μm; Agilent, USA) with 5% phenylmethyl polysiloxane and Ome-gawax^{TM250} fused silica capillary column (30.0 m, 250.00 μm, 0.25 μm, Supelco®, Sigma-Aldrich, USA). Other parameters were as follows: 1.5 ml/min helium flow rate; initial and final temperatures of 70 °C and 220 °C, respectively, the heating rate of 4 °C/min; of the mass spectrometer detector temperature of 250 °C, the injector temperature of 300 °C; 1 μl sample aliquotes. The tricosan in pyridine (1 μg/μl) was an inter-

nal standard. The obtained results were processed with UniChrom software (Restek Corporation, USA) [9].

The data were statistically processed with Statistica 7.0 (StatSoft. Inc., USA) and Microsoft Excel 2010 software. The mean values (*M*) and standard errors of the mean (±SEM) were calculated. The statistical significance of the differences between the oat forms was estimated with Mann-Whitney and Tukey tests. The differences were recognized statistically significant at $p \leq 0.05$.

Results. The weather conditions during the growing season (2015) were mainly favorable for oat plants, as well as for the development of *Fusarium* infection.

The investigated samples are given in Table 1.

1. Oat (*Avena sativa* L.) from the VIR Collection (Vavilov All-Russian Institute of Plant Genetic Resources) tested for economically valuable biochemical traits and indices of resistance to *Fusarium* infection (St. Petersburg–Pushkin, 2015)

No. according to the VIR catalog	Species, subspecies	Variety, line	Origin
k-14648	*A. sativa* var. *mutica*	Argamak	Kirov Region
k-11840	*A. sativa* var. *aurea*	Borrus	Germany
k-14960	*A. sativa* var. *inermis*	Vyatskii	Kirov Region
k-15068	*A. sativa* var. *mutica*	Konkur	Ul'yanovsk Region
k-14851	*A. sativa* var. *inermis*	Numbat[r]	Australia
k-10841	*A. sativa* var. *aurea*	Bisuandorodu	Sakhalin Region
k-14329	*A. sativa* var. *aristata*	Kouzan Zaizai	Japan
k-13911	*A. sativa* var. *mutica*	Kambulinskii	Leningrad Region
k-14911	*A. sativa* var. *mutica*	Belinda	Sweden
k-15297	*A. sativa* var. *aurea*	Geszti	Hungary
k-15305	*A. sativa* var. *chinensis*	Gehl[r]	Canada
k-15301	*A. sativa-A. byzantina*	CDC Dancer	Canada
k-15442	*A. sativa* var. *mutica*	Zalp	Moscow Region
k-15496	*A. sativa* var. *mutica*	Stipler	Ul'yanovsk Region
k-15444	*A. sativa* var. *mutica*	Sapsan	Kirov Region
k-15494	*A. sativa-A. byzantina*	Medved'	Kirov Region
k-15348	*A. sativa* var. *mutica*	Hurdal	Norway
k-15353	*A. sativa* var. *aurea*	Odal	Norway
k-15611	*A. sativa* var. *aurea*	Bessin	Norway
k-15612	*A. sativa* var. *aurea*	Valer	Norway
k-15347	*A. sativa* var. *mutica*	Gere	Norway
k-15326	*A. sativa* var. *mutica*	KSI 432/08	Ul'yanovsk Region
k-15327	*A. sativa* var. *mutica*	KSI 731/01	Ul'yanovsk Region
k-15506	*A. sativa* var. *mutica*	Fux	Germany
k-14787	*A. sativa* var. *mutica*	Privet	Moscow Region

N o t e. [h] — hulless oats.

The *F. culmorum* and *F. sporotrichioides* DNA concentrations reflect the degree of plants affection by these pathogens [26]. We based the evaluation of resistance to *Fusarium* and mycotoxin accumulation on the combination of the following three indicators: the degree of fungal infection in grain, fungal DNA concentration and the amount of the fungal toxic metabolites in grains (Table 2).

As per the results, the samples were divided into two groups, FHB-resistant (RFs) and FHB-susceptible forms, that was necessary for the further evaluation of the influence of FHB pathogenicity factors on the biochemical parameters including metabolomic analysis (Tables 3, 4). The samples with the *F. culmorum* and *F. sporotrichioide* DNA levels not more than 0.22 and 0.29 pg/kg respectively, and the T-2 toxin and DON values no higher than 10 and 100 μg/kg were classified as FHB-resistant; the rest were constituted as FHB-susceptible ones. The correctness of group formation has been confirmed by the Tukey test for unequal sample sizes. In breeding resistant varieties of high quality it is important to regard the relationship between the FHB-resistance parameters and metabolomics data together with other biochemical characteristics of the grain [27].

2. Characterization of the samples of oat (*Avena sativa* L.) from the VIR Collection (Vavilov All-Russian Institute of Plant Genetic Resources) for resistance to the *Fusarium* head blight (n = 3, M±SEM, Pushkin, 2015)

No. according to the VIR catalog	Variety, line	*Fusarium sporotrichioides*				*Fusarium culmorum*			
		infection indicators		T-2 toxin production		infection indicators		T-2 toxin production	
		DNA, pg per kg flour	resistance to infection	μg/kg	resistance to accumulation	DNA, pg per kg flour	resistance to infection	μg/kg	resistance to accumulation
k-14648	Argamak[m]	0.08	R	0	R	0.13	MR	213	S
k-11840	Borrus[m]	0.11	MR	14	S	0.15	MR	64	MR
k-14960	Vyatskii[hm]	0.06	R	5	MR	0.15	MR	128	S
k-15068	Konkur	0.31	S	7	MR	0.42	S	491	HS
k-14851	Numbat[hm]	0.13	MR	59	HS	0.91	HS	285	S
k-10841	Bisuandorodu	0.04	R	5	MR	0.05	R	131	S
k-14329	Kouzan Zaizai	0.05	R	0	R	0.2	MR	148	S
k-13911	Kambulinskii	0.03	R	0	R	0.1	MR	45	R
k-14911	Belinda[m]	0.25	MR	60	HS	0.14	MR	456	HS
k-15297	Geszti	0.03	R	4	MR	0.04	R	25	R
k-15305	Gehl[hm]	0.05	R	23	S	0.03	R	20	R
k-15301	CDC Dancer	1.29	HS	5	MR	0.1	MR	41	R
k-15442	Zalp[m]	0.63	HS	30	S	0.16	MR	163	S
k-15496	Stipler	0.10	MR	0	R	0.03	R	33	R
k-15444	Sapsan[m]	0.12	MR	19	S	0.16	MR	257	B
k-15494	Medved’	0.33	S	13	S	0.69	HS	72	MR
k-15348	Hurdal[m]	0.85	HS	17	S	0.34	S	30	R
k-15353	Odal	0.34	S	7	MR	0.35	S	50	R
k-15611	Bessin	0.39	S	49	S	0.57	HS	331	HS
k-15612	Veler	0.29	MR	0	R	0.5	S	77	MR
k-15347	Gere	0.67	HS	133	HS	0.1	MR	121	S
k-15326	KSI 432/08	0.86	HS	8	MR	3.64	HS	1179	HS
k-15327	KSI 731/01[1]	1.93	HS	45	S	0.22	MR	97	MR
k-15506	Fux	0.40	S	10	MR	0.09	R	331	HS

N o t e. T-2 — T-2 toxin; DON — deoxynivalenol; R — resistant; MR — medium resistant; S — susceptible; HS — highly susceptible; [h] — hulless oats; [m] — samples used for the metabolomic analysis.

3. Characterization of the samples of oat (*Avena sativa* L.) from the VIR Collection (Vavilov All-Russian Institute of Plant Genetic Resources) for biochemical parameters of quality

No. according to the VIR catalog	Variety, line	Concentration, %		Fatty acids, %					Total amount, %		Ratio	
		protein, N × 5.7	oil	$C_{16:0}$	$C_{18:1}$	$C1_{8:2}$	$C_{18:3}$	$C_{20:1}$	SFAs	UFAs	UFAs/SFAs	18:1/18:2
k-14648	Argamak[m]	11.5±0.02	5.0±0.01	19.3±0.02	31.3±0.00	44.4±0.03	2.0±0.01	0.5±0.01	21.6±0.02	78.4±0.01	3.6	0.7
k-11840	Borrus[m]	12.8±0.01	4.1±0.01	20.0±0.02	30.9±0.02	45.0±0.01	1.4±0.00	0.5±0.00	22.0±0.03	78.0±0.02	3.5	0.7
k-14960	Vyatskii[hm]	14.6±0.01	6.5±0.00	20.0±0.01	40.6±0.03	31.3±0.01	3.5±0.01	0.4±0.01	24.1±0.02	75.9±0.01	3.1	1.3
k-15068	Konkur	12.7±0.01	5.1±0.01	20.9±0.02	31.4±0.05	43.0±0.01	1.0±0.02	0.6±0.02	23.9±0.00	76.1±0.02	3.2	0.7
k-14851	Numbat[hm]	14.9±0.02	7.9±0.00	19.4±0.03	34.3±0.01	42.0±0.02	1.3±0.01	0.7±0.01	21.6±0.02	78.4±0.01	3.6	0.8
k-10841	Bisuandorodu	14.6±0.00	3.9±0.01	16.7±0.01	38.8±0.02	40.2±0.02	1.2±0.01	0.7±0.01	19.1±0.03	80.9±0.01	4.2	1.0
k-14329	Kouzan Zaizai	13.5±0.00	4.3±0.03	18.1±0.00	32.4±0.02	44.6±0.02	1.6±0.00	0.8±0.02	20.4±0.03	79.6±0.01	3.9	0.7
k-13911	Kambulinskii	13.0±0.02	3.8±0.01	20.5±0.00	29.5±0.02	45.4±0.01	1.7±0.03	0.6±0.01	22.7±0.01	77.3±0.02	3.4	0.7
k-14911	Belinda[m]	11.6±0.00	5.2±0.02	19.0±0.02	35.0±0.00	41.2±0.01	1.3±0.02	0.6±0.00	21.7±0.01	78.3±0.01	3.6	0.9
k-15297	Geszti	13.3±0.01	5.8±0.02	16.1±0.01	44.4±0.03	32.7±0.01	2.4±0.04	0.5±0.00	19.9±0.03	80.2±0.01	4.0	1.4
k-15305	Gehl[hm]	15.5±0.01	6.7±0.02	16.8±0.01	44.8±0.01	34.8±0.01	1.6±0.06	1.0±0.02	17.8±0.02	82.2±0.01	4.6	1.3
k-15301	CDC Dancer	10.3±0.01	3.9±0.01	17.6±0.01	40.2±0.01	38.4±0.01	0.7±0.02	0.7±0.01	19.9±0.03	80.1±0.02	4.0	1.0
k-15442	Zalp[m]	11.7±0.01	4.5±0.01	16.6±0.01	43.2±0.02	36.6±0.01	1.0±0.01	0.6±0.01	18.6±0.01	81.4±0.01	4.4	1.2
k-15496	Stipler	11.5±0.01	3.3±0.02	17.7±0.02	38.6±0.02	38.4±0.02	1.9±0.02	0.8±0.01	20.3±0.01	79.7±0.00	3.9	1.0
k-15444	Sapsan[m]	12.5±0.00	4.0±0.01	16.5±0.02	40.3±0.02	39.1±0.02	1.2±0.00	0.7±0.02	18.6±0.01	81.4±0.02	4.4	1.0
k-15494	Medved’	12.7±0.01	4.5±0.01	18.6±0.03	39.1±0.02	37.7±0.02	1.4±0.01	0.6±0.02	21.2±0.01	78.8±0.01	3.7	1.0
k-15348	Hurdal[m]	11.8±0.01	5.1±0.01	19.1±0.02	33.7±0.02	42.6±0.01	1.6±0.01	0.6±0.03	21.4±0.01	78.6±0.01	3.7	0.8
k-15353	Odal	12.2±0.01	5.3±0.01	18.1±0.02	42.1±0.01	35.5±0.01	1.7±0.01	1.0±0.01	19.7±0.02	80.3±0.01	4.1	1.2
k-15611	Bessin	10.6±0.00	4.1±0.02	16.1±0.00	44.0±0.02	35.4±0.01	2.0±0.01	0.8±0.00	17.8±0.01	82.2±0.02	4.6	1.2
k-15612	Veler	10.9±0.03	5.3±0.01	16.1±0.02	45.0±0.02	34.2±0.01	2.4±0.01	1.0±0.01	17.4±0.01	82.6±0.01	4.7	1.3
k-15347	Gere	11.6±0.01	5.6±0.03	17.5±0.03	36.4±0.01	42.6±0.02	1.5±0.01	0.5±0.01	19.0±0.00	81.0±0.02	4.3	0.9
k-15326	KSI 432/08	11.0±0.01	4.6±0.02	15.9±0.05	43.4±0.01	36.6±0.00	1.0±0.01	0.7±0.01	18.2±0.00	81.8±0.02	4.5	1.2
k-15327	KSI 731/01[1]	12.1±0.01	5.0±0.01	15.0±0.03	42.5±0.01	39.3±0.02	0.5±0.02	0.7±0.01	16.9±0.01	83.1±0.00	4.9	1.1
k-15506	Fux	12.5±0.01	3.0±0.01	19.7±0.02	42.8±0.02	31.9±0.01	0.4±0.02	0.4±0.00	24.5±0.00	75.6±0.01	3.1	1.3

Note. $C_{16:0}$ — palmitic acid; $C_{18:1}$ — oleic acid; $C_{18:2}$ — linoleic acid; $C_{18:3}$ — linolenic acid; $C_{20:1}$ — eicosenoic acid; SFAs — saturated fatty acids; UFAs — unsaturated fatty acids; [h] — hulless oats; [m] — samples used for the metabolomic analysis.

In our researches, grain proteins and oil in average amounted to 12.5 and 4.9%, respectively (see Table 3). The prevailing saturated fatty acid (SFA) contained in the oat grain was palmitic acid (up to 20.9%), and prevailing unsaturated fatty acids (UFAs) were oleic and linoleic acids (38.5 and 38.9%, respectively). The total amount of UFAs was quite high for cereal crops (up to 83.1%), and the ratio of linoleic and oleic acids was close to 1. Such values testify about high nutritional quality of the investigated oat samples that was confirmed previously [27].

By a nonspecific metabolomic analysis, we have identified over 100 components, i.e. organic acids, free amino acids (including non-protein ones), polyols (including phytosterols), fatty acids, nucleosides, monosaccharides and oligosaccharides, as well as phenol-containing compounds. A pairwise comparison of the resultes for RFs and SFs oats with Mann-Whitney coefficient (at $p < 0.05$) revealed significant differences between the RFs and SFs oats.

According to other authors, fungal pathogens affect almost all stages of primary and secondary metabolism that has an impact on changing of the main biochemical parameters including the metabolomic profile [28].

We noted the inverse relationship between the protein content in the RFs oats and the amount of DNA of *F. sporotrichioides* ($r = -0.5$), *F. culmorum* ($r = -0.6$) and DON toxin ($r = -0.5$). In the SFs, only the direct relationship was found with DNA concentration of *F. sporotrichioides* ($r = 1$). The oil level in the RFs correlated negatively with the concentrations of T-2 toxins ($r = -0.6$) and DON ($r = -0.6$), in the SFs it had the direct relationship with the amount of DNA of *F. sporotrichioides* ($r = 0.5$) and *F. culmorum* ($r = 0.5$). The ratios we determined in the RFs oat allow us to assume that high-protein forms are less affected and accumulate less toxins. At the same time, in the SFs, the high oil content in the grains contributed to the fungal development. Our assumptions are confirmed by the paper [29] which established the nature of the FHB toxin influence on the grain protein and oil levels in cereals.

It was reported [29] that FHB exerts a significant influence on the total content of free amino acids. In our researches, the *F. sporotrichioides* DNA levels in SRs oat directly depended on the free amino acids content ($r = 0.5$) that is most likely related to the damage to ribosomes and destruction of the plant tissues due to the affection by FHB [26]. The inverse correlation between the DON content and the total amount of amino acids in the RFs oat ($r = -0.8$) has been found that may reflect the activation of the plant protection mechanisms. In the SFs oat we have not found such relationships. The DON content in the RFs had the direct dependence on the concentration of asparagine ($r = 0.7$) and glutamine ($r = 0.6$), and in the SFs it had the inverse dependence (the r values were –0.4 and –0.8, respectively). In addition, in the RFs, there was an inverse relationship between *F. sporotrichioides* DNA concentration and the amount of asparagine ($r = -0.5$), T-2 toxin and glutamine ($r = -0.5$). An increase in asparagine and glutamine in the RFs cereals in response to the toxin inflow into the plant tissues was confirmed by other researchers [26] who suppose that such changes are related to plant protection from FHB. It was discussed [29] that the proline content is expected to be increased proportionally to the toxin concentration because proline participates in the inactivation of free radicals which accumulate in the plant tissues affected by FHB. However, in our researches we revealed inverse correlation between the proline accumulation and T-2 toxin production ($r = -0.6$) in the SFs group.

The tyrosine and phenylalanine amino acids are involved in the shikimate pathway the products of which, e.g. phenylpropanoids, actively participate in plant protection. As per available data [26], the content of tyrosine, phenylal-

anine and secondary metabolites, which are the products of the shikimate pathway, directly depended on the indices of affection by FHB. In the SFs, we have found such relationship between the content of tyrosine and *F. sporotrichioides* DNA of (r = 1); in other cases, the inverse relationship between the tyrosine and DON concentrations, as well as between the phenylalanine and DON concentrations has been found (the r values were –0.5 and –0.7, respectively). The negative correlation between alanine and DNA amounts and the FHD toxins both in RFs and SFs oat was confirmed by other authors [29]. The experimental data [16] testify about the increase in serine concentration in response to the effect of FHB toxins that has not been confirmed in our research. On the contrary, the serine indices were decreasing, and the correlation coefficients between their values and T-2 and DON production amounted to –0.6 and –0.5, respectively.

In the discussing of the obtained data, the special attention should be paid to a pipecolic acid. The role of this non-protein amino acid is considered to be related to the mechanisms of plant protection from stressful environmental factors including FHB [26, 29]. We have found the positive correlation (r = 0.5) between the content of this acid and *F. sporotrichioides* and *F. culmorum* DNA concentrations, as well as DON content in SFs; the DON accumulation in the RFs oat inversely correlated with the pipecolic acid level (r = –0.8). The different directions and values of these correlations may be explained by the different immune response in the RFs and SFs oats.

In the samples of FHB-susceptible forms, we have found the negative relationship between the amount of DNA of *F. sporotrichioides* and linolenic acid (up to r = –0.7) and the positive relationship with the total amount of unsaturated and saturated fatty acids (the r values were up to 0.7 and 0.7, respectively). The positive relationship has also been found between *F. sporotrichioides* DNA and MAG2-18:2 (monoacylglycerol) (r = 0.6), between *F. culmorum* DNA and MAG-2-18:2 (r = 0.6), as well as between *F. culmorum* DNA and MAG-16:0 (r = 0.5) (Table 4). In the previous paper [9] we assumed that monoacylglycerols may be related to the plant adaptability, in particular, to resistance to biotic and abiotic factors that was confirmed by other researchers [29]. The content of DON toxin positively correlated with the amounts of linoleic (r = 0.6), eicosanoic (r = 0.5) and eicosanic (r = 0.5) acids and negatively — with the oleic (r = –0.5) and linolenic (r = –0.5) acids. Perhaps, the positive and negative values of the correlation coefficients are related to the different roles of certain fatty acids in the fungal vital activity.

In the group of FHB-resistant samples, the negative correlation between DON toxin and linolenic acid concentrations increased up to r = –0.9 compared to the FHB-susceptible ones. The relationship between the linolenic acid content and FHB-resistance has been confirmed [28]. The negative correlation was observed between the T-2 toxin and eicosenoic acid contents (r = –0.5). The negative correlation between the amounts of *F. sporotrichioides* DNA and linolenic acid decreased to r = –0.5 as compared to the values in the FHB-susceptible samples.

We have not found any relationship between the total content of organic acids and FHB-resistance, which has been noted by other authors. The direct correlation between the content of nicotinic acid and the FHB characteristics (up to r = 0.7), and the inverse relationship between the content of erythronic and methylmalonic acids (up to r = –0.9) were found. The concentration of lactic acid positively correlated with DON (r = 1) and T-2 toxin (r = 1) in RSs oats, and with *F. sporotrichioides* DNA (r = 0.7) in SFs. The negative correlation between the accumulation of lactic acid and amount of the *F. sporotrichioides*

DNA (r = –0.7) has been found in RSs, and between the DNA of *F. culmorum* (r = –0.5) and DON (r = –0.7) in SFs. The changes in the content of the main organic acids of primary metabolism including the acids of the Krebs cycle under the impact of FHB are confirmed by other authors [26, 29].

4. Характеристика устойчивых и неустойчивых к фузариозу форм овса (*Avena sativa* L.) из коллекции Всероссийского института генетических ресурсов растений им. Н.И. Вавилова по средним показателям накопления основных метаболитов (n = 25, M±SEM, г. Пушкин, 2015 год)

Main metabolites, mg/100 g	Forms	
	resistant	susceptible
Lactic acid	2.28±0.76	1.89±0.51
Methylmalonic acid	2.85±0.82	2.98±0.69
Glyceric acid	0.58±0.21	0.61±0.18
Erythronic acid	0.828±0.31	0.92±0.36
Nicotinic acid	0.198±0.08	0.20±0.10
Glycine	14.42±1.97	12.67±2.33
α-Alaniin	4.88±1.63	5.16±1.80
Valine	1.95±0.77	2.28±1.16
Leucine	0.59±0.14	0.61±0.14
Proline	5.02±2.63	4.87±2.65
Serine	2.42±1.12	3.00±1.38
Threonine	1.64±0.95	1.84±1.11
Oxyproline	0.80±0.63	0.62±0.48
Phenylalanine	1.72±1.04	1.66±0.81
Tyrosine	32.51±17.58	27.37±16.33
Glutamine	2.25±1.15	2.12±1.06
Asparagine	7.79±2.24	9.62±3.82
Total amount of free amino acids	78.15±17.66	74.01±16.52
Ethanolamine	2.16±0.56	2.17±0.67
Pipecolic acid	1.56±0.89	1.78±1.13
Adenosine	3.22±0.75	3.29±0.78
MAG-16:0	14.4±2.81	14.23±2.69
MAG-2-18:2	31.75±6.73	30.13±5.97
Dulcitol	18.38±3.72	16.33±2.41
Galactinol	38.41±13.43	36.87±11.5
Chiroinositol	0.37±0.12	0.74±0.57
Total amount of polyols	192.11±38.84	188.17±24.61
Cholesterol	0.64±0.19	0.86±0.39
Campesterol	0.69±0.23	0.68±0.17
Stigmasterol	0.47±0.17	0.55±0.20
β-Sitosterol	8.22±2.41	9.51±2.41
Total phytosterols	14.32±3.53	17.95±5.24
Glyceroaldehyde-3-phosphate	10.72±3.85	11.67±3.67
Total monosugars	974.22±303.73	843.34±175.47
Total disaccharides	2123.87±480.62	2543.03±477.91

The total content of polyols inversely correlated with the amount of DON toxin, at r = –0.8 in SFs and r = –0.5 in RFs. The dulcitol content in SFs positively correlated with the accumulation of DON and T-2 toxins (the r values were 0.6 and 0.7, respectively) and with the amount of *F. culmorum* DNA (r = 0.7). In the RFs group, we have found the inverse relationship between the galactinol and DON toxin, as well as with the *F. sporotrichioides* DNA concentration (r = –0.7). The inverse relationship between the DON and T-2 toxins concentrations and the total content of polyols confirmed by other authors [29] is due to the FHB influence on the primary metabolism. At the same time, the increase in the galactinol content [28] is related to the plant defense mechanisms under the influence of abiotic environmental factors; however, in this case, the change in the galactinol concentration is obviously due to the FHB influence.

In SFs, phytosterols, cholesterol and β-sitosterol have the inverse relationship with the amount of the *F. sporotrichioides* DNA (the r values were –0.7; –0.7 and –0.5, respectively). In the RFs group, we have also found the inverse relationship between the DON content and phytosterols, the campes-

terol (r = –0.6) and stigmasterol (r = –0.6). The dependence of the amount of *F. sporotrichioides* DNA on the β-sitosterol level (r = 0.5) turned out to be direct, in contrast to that in the FHB-resistant samples. The increase in the ergosterol amount under the *Fusarium* affection of tobacco leaves [29] is due to phytosterols participation in plant protection from biotic stresses, which is confirmed by our data.

In the SFs and RFs, we have found the inverse relationship between the FHB indices (accumulation of DNA of *F. sporotrichioides*, *F. culmorum* and DON) and the total amount of monosaccharides (up to r = –0.9). The total amount of disaccharides in the RFs group showed a direct dependence on the DON content (r = 0.9) and the inverse dependence on the amount of *F. sporotrichioides* DNA and glyceroaldehyde-3-phosphate (r = –0.5). In several researches, the changing in saccharides content under the influence of *Fusarium* causing agents has been noted. The inverse relationship between the DON and saccharides contents may be related to the inactivation of this toxin owing to the synthesis of the form conjugated with saccharides and the increase in the activity of glycolysis and the pentose-phosphate cycle [26].

The correlations between the contents of adenosine and the *F. sporotrichioides* DNA (r = –0.5), as well as DON (r = – 0.5) have been found only in SFs oat. It is obvious that this dependence is conditioned by the fungal influence on the synthesis of nucleic acids and protein as a whole in the affected plant tissues; there are no any information about detecting such relationships in other authors' papers.

All the r values obtained by us were at $p < 0.05$.

So, FHB exerts the influence practically on the all stages of primary metabolism including the synthesis of protein, oil, sugars, polyols, and activates the synthesis of the compounds related to the plants' protection against FHB, which, in particular, include pipecolic acids, acyl glycerols and phytosterols. The correlation between the protein content and amount of DNA of the main FHB infection agents, which have been found by us in the RFs oat, gives us the reason to suggest that in high-protein forms, the damage caused by pathogens is lower and, as a result, the toxins accumulate in a less amount, while in the SFs the high oil content in the grains contributes to the infection development. The unequal changes in the content of certain metabolites and their groups, which has been noted by us, may be related both to the synthesis of the compounds necessary for the fungal vital activity and to the peculiarities of the immune response in plant tissues. The comparing of the composition and content of the most important chemical groups of compounds including minor ones in the grains with the FHB-resistance indices is the stage necessary when investigating the biochemical processes occurring when the plants became infected by pathogenic microorganisms.

The physiological and biochemical factors of a passive immunity include the metabolic particularities, presence and content in the plant tissues of chemical compounds playing the protective role, physicochemical particularities of the tissues and plants themselves. The resistance related to the plants' physiological and biochemical properties may be explained by the absence in their tissues of the nutrients and physiologically active substances necessary for the pathogen as well as by the pathogen inhibition by toxic matters or other factors which are unfavorable. In the optimal case, the plants may contain the components which are harmful for the pathogenic fungus, for example, the glycosides, i.e. phenolic, cyanogenic and other compounds, as well as synthesize phytoalexins in response to the microbial infection [13, 30, 31].

The metabolome of a plant (grain) may be at different extent unfavorable

for the pathogen development, as it has been shown above in the analysis of the oat varieties with different resistance to FHB. The levels of certain substances have different extents and patterns of correlation with plant resistance characterized by the content of the fungal mycotoxins and DNA in grain. It is appropriate to recall the adaptive role of prolamins (avenins) in cereal resistance to biotic and abiotic factors [32-34]. The biochemical approach combined with the genetic investigations can provide the researchers with the valuable information about the mechanisms of plant protection against *Fusarium* fungi [35]. We have noted the relationship between the FHB-resistance indices and the accumulation of pipecolic acid, monoacylglycegols, tyrosine, galactinol, a number of phytosterols, sugars and adenosine in the plants.

So, we have obtained the data on the relationship of a wide range of compounds with different indices of FHB resistance in oat varieties, that is important not only for understanding the nature of this trait, but also for developing the diagnostic methods. These results of one-year field tests should be considered as preliminary, however, it should be noted that 2015 was extremely favorable for *Fusarium* infection. It can be assumed that the increased aggressiveness of *Fusarium* fungi made it possible for us to more reliably identify the dependences of the metabolite composition and content on the indices of plant resistance to FHB. Considering the complex polygenic nature of the control of FHB resistance and strong dependence of this trait manifestation on environmental conditions, for the confirmation or refutation of our conclusions, it is necessary to investigate the greater intraspecific and interspecific diversity of the oat samples reproduced under different conditions and in different years. Nevertheless, we have succeed in the identification of a number of dependencies between the resistance indices and the accumulation of certain metabolites, that makes it possible to select directly, at the initial stage, the samples with high content of such compounds for breeding oat varieties which combine resistance to *Fusarium* and high nutritional quality. Further researches will make it possible for us to expand the list of the metabolomic profile components which are promising when selecting the oat samples for resistance not only to the *Fusarium*, but also to other pathogens.

REFERENCES

1. Loskutov I.G. *Oves (Avena L.). Rasprostranenie, sistematika, evolyutsiya i selektsionnaya tsennost'* [Oats (*Avena* L.). Distribution, taxonomy, evolution and breeding value]. St. Petersburg, 2007 (in Russ.).
2. Baum B.R. *Oats: wild and cultivated. A monograph of the genus Avena L. (Poaceae). Monogr. No. 14.* Ottava, Canada, 1977.
3. Gagkaeva T.Yu., Gavrilova O.P., Levitin M.M., Novozhilov K.V. *Prilozhenie k zhurnalu «Zashchita i karantin rastenii»*, 2011, 5: 70-82 (in Russ.).
4. Chakraborty S., Newton A.C. Climate change, plant diseases and food security: an overview. *Plant Pathology*, 2011, 60(1): 1-14 (doi: 10.1111/j.1365-3059.2010.02411.x).
5. Pestka J. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology*, 2010, 84(9): 663-679 (doi: 10.1007/s00204-010-0579-8).
6. Gagkaeva T.Yu., Gavrilova O.P., Levitin M.M. Bioraznoobrazie i arealy osnovnykh toksinoprodutsiruyushchikh gribov roda *Fusarium*. *Biosfera*, 2014, 6(1): 36-45 (in Russ.).
7. Fiehn O., Kopka J., Dörmann P., Altmann T., Trethewey R.N., Willmitzer L. Metabolite profiling for plant functional genomics. *Nature Biotechnology*, 2000, 18(11): 1157-1161 (doi: 10.1038/81137).
8. Hill C. B., Roessner U. Metabolic profiling of plants by GC—MS. In: *The handbook of plant metabolomics* /W. Weckwerth, G. Kahl (eds.). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2013: 1-23.
9. Loskutov I.G., Shelenga T.V., Konarev A.V., Shavarda A.L., Blinova E.V., Dzyubenko N.I. *Vavilovskii zhurnal genetiki i selektsii*, 2016, 20(5): 642-648 (doi: 10.18699/VJ16.185) (in Russ.).
10. Choo T.M. Breeding barley for resistance to *Fusarium* head blight and mycotoxin accumulation. *Plant Breeding Reviews*, 2006, 26: 125-169 (doi: 10.1002/9780470650325.ch5).

11. Buerstmayr H., Ban T., Anderson J.A. QTL mapping and marker-assisted selection for *Fusarium* head blight resistance in wheat: a review. *Plant Breeding*, 2009, 128(1): 1-26 (doi: 10.1111/j.1439-0523.2008.01550.x).
12. Lemmens M., Scholz U., Berthiller F., Dall'Asta C., Koutnik A., Schuhmacher R., Adam G., Buerstmayr H., Mesterházy Á., Krska R., Ruckenbauer P. The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for *Fusarium* head blight resistance in wheat. *Molecular Plant-Microbe Interactions*, 2005, 18(12): 1318-1324 (doi: 10.1094/MPMI-18-1318).
13. D'yakov Yu.T., Tikhonovich I.A., Shcherbakova L.A., Ozeretskovskaya O.L., Dzhavakhiya V.G., Provorov N.A., Bagirova S.F. *Fundamental'naya fitopatologiya* /Pod redaktsiei Yu.T. D'yakova [Fundamental phytopathology. Yu.T. D'yakov (ed.)]. Moscow, 2012 (in Russ.).
14. Bushnell W.R., Perkins-Veazie P., Russo V.M., Collins J., Seeland T.M. Effects of deoxynivalenol on content of chloroplast pigments in barley leaf tissues. *Phytopathology*, 2009, 100(1), 33-41 (doi: 10.1094/phyto-100-1-0033).
15. Bolton M.D. Current review: Primary metabolism and plant defense — fuel for the fire. *Molecular Plant-Microbe Interactions*, 2009, 22(5), 487-497 (doi: 10.1094/MPMI-22-5-0487).
16. Warth B., Parich A., Bueschl C., Schoefbeck D., Katharina N., Neumann N., Kluger B., Schuster K., Krska R., Adam G., Lemmens M., Schuhmacher R. GC—MS based targeted metabolic profiling identifies changes in the wheat metabolome following deoxynivalenol treatment. *Metabolomics*, 2015, 11(3): 722-738 (doi: 10.1007/s11306-014-0731-1).
17. Sitkin S.I., Tkachenko E.I., Vakhitov T.Ya., Oreshko L.S., Zhigalova T.N. *Eksperimental'naya gastroentoroskopiya*, 2013, 12: 77-90 (in Russ.).
18. Loskutov I.G., Kovaleva O.N., Blinova E.V. *Metodicheskie ukazaniya po izucheniyu i sokhraneniyu mirovoi kollektsii yachmenya i ovsa* [Study and preservation of the world collection of barley and oats: Guidelines]. St. Petersburg, 2012 (in Russ.).
19. *Oat Descriptors*. IBPGR, Rome, 1985.
20. *Metody biokhimicheskogo issledovaniya rastenii* /Pod redaktsiei A.I. Ermakova [Methods of plant biochemistry. A.I. Ermakov (ed.)]. Leningrad, 1987 (in Russ.).
21. Halstensen A.S., Nordby K.C., Eduard W., Klemsdal S.S. Real-time PCR detection of toxigenic *Fusarium* in airborne and settled grain dust and associations with trichothecene mycotoxins. *Journal of Environmental Monitoring*, 2006, 8(12): 1235-1241 (doi: 10.1039/b609840a).
22. Yli-Mattila T., Paavanen-Huhtala S., Jestoi M., Parikka P., Hietaniemi V., Gagkaeva T., Sarlin T., Haikara A., Laaksonen S., Rizzo A. Real-time PCR detection and quantification of *Fusarium poae, F. graminearum, F. sporotrichioides* and *F. langsethiae* in cereal grains in Finland and Russia. *Archives of Phytopathology and Plant Protection*, 2008, 41(4): 243-260 (doi: 10.1080/03235400600680659).
23. *European commission. Community reference laboratory for GM food and feed. Event-specific for the quantitation of maize line NK603 using real-time PCR. 2005.* Available http://gmo-crl.jrc.ec.europa.eu/summaries/NK603report_mm.pdf. No date.
24. Gagkaeva T.Yu., Gavrilova O.P., Orina A.S., Blinova E.V., Loskutov I.G. Diversity of *Avena* species by morphological traits and resistance to *Fusarium* head blight. *Russian Journal of Genetics: Applied Research*, 2018, 8(1): 44-51 (doi: 10.1134/S2079059718010070).
25. Perkowski J., Stuper K., Busko M., Goral T., Kaczmarek A., Jelen H. Differences in metabolomic profiles of the naturals contaminated grain of barley, oats and rye. *Journal of Cereal Science*, 2012, 56: 544-551 (doi: 10.1016/j.jcs.2012.07.012).
26. Nussbaumer T., Warth B., Sharma S., Ametz C., Bueschl C., Parich A., Pfeifer M., Siegwart G., Steiner B., Lemmens M., Schuhmacher R., Buerstmayr H., Mayer K.F.X., Kugler K.G., Schweiger W. Joint transcriptomic and metabolomic analyses reveal changes in the primary metabolism and imbalances in the subgenome orchestration in the bread wheat molecular response to *Fusarium graminearum*. *Genes, Genomes, Genetics*, 2015, 5(12): 2579-2592 (doi: 10.1534/g3.115.021550).
27. Konarev A.V., Shelenga T.V., Perchuk I.N., Blinova E.V., Loskutov I.G. *Agrarnaya Rossiya*, 2015, 5: 2-10 (in Russ.).
28. Balmer D., Flors V. , Glauser G., Mauch-Mani B. Metabolomics of cereals under biotic stress: current knowledge and techniques. *Front. Plant Sci.*, 2013, 4(82): 1-12 (doi: 10.3389/fpls.2013.00082).
29. Heuberger A.L., Robison F.M., Lyons S.M.A., Broeckling C.D., Prenni J.E. Evaluating plant immunity using mass spectrometry-based metabolomics workflows. *Front. Plant Sci.*, 2014, 5(291): 1-11 (doi: 10.3389/fpls.2014.00291).
30. Hollywood K., Brison D.R., Goodacre R. Metabolomics: current technologies and future trends. *Proteomics*, 2006, 6(17): 4716-4723 (doi: 10.1002/pmic.200600106).
31. Zilić S., Hadzi-Tasković Sukalović V., Dodig D., Maksimović V., Maksimović M., Basić Z. Antioxidant activity of small grain cereals caused by phenolics and lipid soluble antioxidants. *Journal of Cereal Science*, 2011, 54(3): 417-424 (doi: 10.1016/j.jcs.2011.08.006).
32. Semikhov V.F., Aref'eva L.P., Novozhilova O.A. *Fiziologiya rastenii*, 2000, 3: 303-321 (in Russ.).
33. Allard R.W. Genetic basis of the evolution of adaptedness in plants. In: *Adaptation in plant*

breeding. P.M.A. Tigerstedt (ed.). Kluwer Academic Publishers, 1996: 1-11.
34. Schauer N., Fernie A.R. Plant metabolomics: towards biological functions and mechanism. *Trends Plant Sci.*, 2006, 11(10): 508-516 (doi: 10.1016/j.tplants.2006.08.007).
35. Kluger B., Bueschl C., Lemmens M., Michlmayr H., Malachova A., Koutnik A., Maloku I., Berthiller F., Adam G., Krska R., Schuhmacher R. Biotransformation of the mycotoxin deoxynivalenol in *Fusarium* resistant and susceptible near isogenic wheat lines. *PLoS ONE*, 2015, 10(3): e0119656 (doi: 10.1371/journal.pone.0119656).

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THE VIRULENCE OF THE BARLEY LEAF RUST PATHOGEN IN THE NORTH CAUCASUS IN 2014-2017

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Abstract

Barley leaf rust caused by *Puccinia hordei* Otth. is a harmful disease of barley. If the crop is severely damaged, the yield loss may be of 20 to 80 %. Population studies of the pathogen abroad are actively conducted in countries for which protection against the disease is of particular importance (Australia, New Zealand, the United States, Europe, the countries of Northern Africa). This paper describes the North Caucasian *P. hordei* population virulence in 2014-2017 to 17 differentiator varieties and barley lines of international and Australian kits containing currently known pathogen resistance genes, shows. Winter barley leaves (*Hordeum vulgare* L.) of different varieties affected by *P. hordei* which were collected during route surveys in 2014-2017 in the territory of the North Caucasus served as a biomaterial. The selection and reproduction of mono-pustule isolates was carried out according to the common procedure. Barley plants were grown hydroponically with the use of Knop's nutrient solution. To assess the virulence of the fungus, 17 varieties-differentiators and lines from the international and Australian sets containing the currently known genes of resistance to the pathogen were used. A total of 208 mono-pustule isolates of the fungus were analyzed most of which were virulent to the testers with *Rph* genes, the *Rph1, Rph2, Rph3, Rph3 + Rph7, Rph4, Rph5, Rph7, Rph8, Rph 9 + Rph2.* During the four years of study, no isolates virulent to the line with *Rph13* gene were detected. In 2016-2017 as compared to 2014-2015, there was a decrease in the number of isolates virulent to the lines with genes *Rph9, Rph19,* and an increase in clones virulent to testers with genes *Rph19 + Rph2, Rph21 + Rph2.* The frequency of *P. hordei* isolates that are virulent to varieties and lines of barley with genes *Rph5 + Rph2, Rph6 + Rph2* remained moderate throughout all the years of the research. In 2016, due to unfavorable conditions for the pathogen and the collection of spore material from a limited set of winter barley varieties affected by the pathogen, the frequency of isolates virulent to varieties and lines of barley significantly reduced. In 2014, 2015 and 2017, isolates with a large number of virulence alleles, from 11 to 15, prevailed in the population and reached 52.2 %, 39.5 % and 50.0 %, respectively. The portion of isolates, avirulent to all used plants with genes *Rph*, was 1.1 %, 2.1 % and 2.8 %, respectively. In 2016, the fungal isolates with moderate and low virulence alleles prevailed. The Nei index indicates a high similarity of the structure of North Caucasian pathogen population by virulence in 2014, 2015 and 2017 (N = 0.02-0.05) and its minor changes in 2016 (N = 0.14-0.19). The obtained statistical results indicate the stability of the North Caucasian *P. hordei* population by virulence. The level of its diversity in the frequency of virulence alleles remained medium (H_S = 0.26-0.40) throughout the entire study period.

Keywords: winter barley, leaf rust, *Puccinia hordei*, virulence

Barley leaf rust (the causative agent *Puccinia hordei* Otth.) is a widespread and harmful disease of barley, which leads to grain shrinkage and yield decrease. If the plants are damaged severely the yield loss may be more than 20% [1]. The economic importance of *P. hordei* in the world agriculture depends

on the region of the crop cultivation and varies by years [2]. In recent years it has increased [3). The effect of this pathogen is especially severe in the regions of the East and Midwest of the USA, North Africa, New Zealand, Europe, Australia, and some Asian countries [4]. In a number of countries, especially in the regions where crops ripen late, significant yield losses have been observed in the susceptible varieties [5, 6]. In Russia, the barley leaf rust is most harmful in the Volga, North Caucasus and Central Black Earth regions, Western and Eastern Siberia and in the Far East where it emerges almost annually. The epiphytoties in the North Caucasus, Central Black Earth and Volga regions occur 1-2 times per 10 years [7, 8].

Breeding and cultivation of highly productive varieties resistant to diseases including the barley leaf rust possess the leading role among barley protection methods. Their creation and application require a comprehensive study of the gene pool of the host plant resistance and of the pathogen virulence. To date, 21 genes conferring juvenile resistance (*Rph1-Rph19*, *Rph21* and *Rph22*) and 3 genes for age-related resistance (*Rph20*, *Rph23* and *Rph24*) which have been found in *Hordeum vulgare*, *H. vulgare subsp. spontaneum* and *H. bulbosum* are known [9, 10].

Abroad, the population studies of the pathogen are actively conducted in the countries for which the protection against the disease is especially urgent. Cotterill et al. [11] have proved that most of the known genes are ineffective against the isolates identified in Australia from 1966 to 1995, and only *Rph3* and *Rph7* genes remained effective. Subsequently, the *P. hordei* isolates which are virulent for the lines with these genes emerged in different countries [12, 13]. The virulence of the fungal isolates to the line having the *Rph15* gene has been reported by Sun [14]. Nevertheless, the gene of a wild barley species *H. vulgare* subsp. *spontaneum*, like the *Rph16* gene [15] is still considered effective and is actively used in breeding programs [16). The juvenile genes *Rph11* and *Rph14* are ineffective, and the isolates which are virulent for their carriers are found in many parts of the world [17]. Currently, *Rph20*, *Rph23*, and *Rph24* are recognized effective genes of adult plant resistance to *P. hordei* [18].

In the territory of the former USSR, until 1964, the race and phenotypic composition of *P. hordei* has not been studied. The first investigations of its race composition with a set of differentiator varieties selected by Levin and Cherevik were published in 1968 by Rogozhina et al. [19]. A total of 18 races, 16 of which were listed in the international registry and two ones (X and Y) were described for the first time were discovered. Subsequently, the study of the race diversity of different geographical populations of the pathogen was continued [20, 21], and another two new races (1L, 2L) and two races listed in the international register [22, 23] were found.

In this study, we for the first time established that most of the mono-pustule isolates of the barley leaf rust causative agent from the North Caucasus population are virulent to the testers with *Rph1*, *Rph2*, *Rph3*, *Rph3* + *Rph7*, *Rph4*, *Rph5, Rph7*, *Rph8*, and *Rph9* + *Rph2* genes. No virulent isolates for the line with *Rph13* gene have been found.

The objective of this work was to study the structure of the North Caucasian population of barley leaf rust causative agent (*Puccinia hordei*) for virulence.

Techniques. The leaves of different varieties of winter barley (*Hordeum vulgare* L.) affected by *P. hordei* were collected in the North Caucasus in the course of routine surveys in 2014-2017. A total of 208 mono-pustule isolates of the fungus have been isolated and analyzed.

The isolation, reproduction, and differentiation of mono-pustule isolates were performed according to the described technique [24]. The barley plants

were grown hydroponically using the Knop's nutrient solution [22].

To assess virulence, 17 differentiator varieties and lines from the international and Australian kits [18] which contain the known to date genes of resistance to the pathogen were used. Seeds of the differentiators were germinated in Petri dishes. The germinated seeds were sown, 5 seeds per 25 ml plastic flowerpots with wet sand. On Days 5 to 7, the differentiator plants were inoculated with aqueous suspension of *P. hordei* spores of each mono-pustule isolate. On Days 10 to 14, the types of plant response (in points) were evaluated according to the Levin and Cherevik scales. The plants with response types of 0, 0; 1 and 2 points were classified as resistant, with 3 and 4 points — as susceptible. In case if the type of reaction was slightly higher or lower than the above points, the additional designations "+" or "–" were introduced [25].

The diversity of the *P. hordei* population was described for virulence genes using Nei's H_s index [26]:

$$H_s\,(P) = \Sigma[1 - q_i^2 - (1 - q_i)^2]/k,\ 1 \le i \le k,$$

where q_i is the frequency of the i allele in the population, k is the number of alleles.

The inter-population differences (Nei's N index) were evaluated as the frequency of virulence alleles using the genetic distance (D) according to Nei [26, 27]:

$$D = -\ln IN,$$

$$IN = \Sigma\Sigma\, x_{ij}\, y_{ij}/\sqrt{\Sigma\Sigma\, x_{ij}^2\, \Sigma\Sigma\, y_{ij}^2},$$

where x_{ij} and y_{ij} are the frequencies of the i allele of the j year in the compared populations x and y.

The statistical processing was performed using Statistica 10.0 software (StatSoft, Inc., USA).

Results. The barley differentiator varieties and lines used in the work are shown in Table 1.

1. The differentiator varieties and lines of winter barley (*Hordeum vulgare* L.) with known genes of resistance to barley leaf rust used to investigate virulence of *Puccinia hordei* Otth. North Caucasian population isolates

Set of differentiators	Variety/line	Origin	Resistance gene(s)
International	Sudan	C.I. 6489	*Rph1*
International	Peruvian	*Hordeum vulgare*	*Rph2*
International	Estate	*Hordeum vulgare*	*Rph3*
International	Midas	*Hordeum vulgare*	*Rph3 + Rph7*
International	Gold	*Hordeum vulgare*	*Rph4*
International	Magnif 104	*Hordeum vulgare*	*Rph5*
Additional Australian	Quinn	*Mains' Quinn,* C.I. 1024	*Rph5 + Rph2*
International	Bolivia	*Hordeum vulgare*	*Rph6 + Rph2*
International	Cebada Capa	*Hordeum vulgare*	*Rph7*
International	Egypt 4	*Hordeum vulgare*	*Rph8*
International	Triumph	*H. vulgare* ssp. *spontaneum*	*Rph12*
International	Abyssinian	*Hordeum vulgare*	*Rph9*
Additional Australian	PI 531849	*H. vulgare* ssp. *spontaneum*	*Rph13*
Additional Australian	Prior	*Hordeum vulgare*	*Rph19*
Additional Australian	Reka 1	C.I. 5051	*Rph19 + Rph2*
Additional Australian	Ricardo	*Hordeum vulgare*	*Rph21 + Rph2*
Additional Australian	Cantala	*Hordeum vulgare*	*RphC*

In 2014, 2015 and 2017, the conditions for the pathogen development were favorable, while 2016 was unfavorable due to weather conditions.

Over the four years of study, the *Rph13* gene had been demonstrating the absolute effectiveness (Table 2). The source of this gene is the wild species of barley *H. vulgare* ssp. *spontaneum*. However, despite its effectiveness in the North Caucasus, the isolates which are virulent for carriers of this gene have been found in several regions of the world [18]. In all years of study, most testers with *Rph1*, *Rph2*, *Rph3*, *Rph3 + Rph7*, *Rph4, Rph5*, *Rph7*, *Rph8*, and *Rph9 + Rph2*

resistance genes were affected by isolates of the North Caucasian *P. hordei* population with the high frequency. In 2016-2017, compared to 2014-2015, there was a decrease in the number of isolates virulent to the lines with the *Rph9* and *Rph19* genes, and an increase in the number of clones virulent for the testers with *Rph19* + *Rph2* and *Rph21* + *Rph2* genes. The number of isolates virulent for the barley varieties and lines with *Rph5* + *Rph2* and *Rph6* + *Rph2* genes remained medium in all years of research. In 2016, due to the conditions unfavorable for the pathogen and due to the collection of spore material from the limited set of varieties affected by the pathogen, the frequency of isolates virulent to barley varieties and lines was significantly lower.

2. Frequency of *Puccinia hordei* Otth. isolates of North Caucasian population virulent to winter barley (*Hordeum vulgare* L.) lines and varieties with *Rph* genes

Testers with *Rph* genes	Frequency, %			
	2014	2015	2016	2017
Rph1	76.1	60.5	51.3	83.3
Rph2	75.0	73.7	33.3	72.2
Rph3	81.5	63.2	15.4	80.6
Rph3 + *Rph7*	79.3	60.5	20.5	86.1
Rph4	68.5	63.2	28.2	83.3
Rph5	73.9	55.3	30.8	75.0
Rph5 + *Rph2*	30.4	50.0	35.9	36.1
Rph6 + *Rph2*	34.8	36.8	12.8	47.2
Rph7	80.4	55.3	15.4	83.3
Rph8	78.3	57.9	25.6	88.9
Rph9	56.5	47.4	2.6	8.3
Rph12	50.0	52.6	5.1	38.9
Rph13	0.0	0.0	0.0	0.0
Rph19	26.1	10.5	2.6	8.3
Rph19 + *Rph2*	42.4	50.0	46.2	86.1
Rph21+ *Rph2*	0.0	0.0	0.0	25.0
Rph C	41.3	34.2	2.6	75.0
Number of isolates	92	38	39	39
Intrapopulation diversity index H_s	0.36	0.40	0.26	0.30

Therefore, most of the currently known resistance genes were ineffective against *P. hordei* in all years of researches. The *Rph13* gene, no virulent isolates to which have been detected since 2012, retained its high efficiency [8, 21].

The statistical analysis of the pathogen population diversity for the frequencies of virulence alleles showed that in 2016 the population was the least diverse (Hs = 0.26) that was determined by the conditions unfavorable for the pathogen (Table 2). In general, the diversity of the North Caucasian population of *P. hordei* remained medium (Hs = 0.26-0.40 (Hs = 0.26-0.40) as per the frequencies of virulence alleles throughout the entire survey period.

3. Frequency of isolates with different number of virulence alleles attacking lines and varieties of winter barley (*Hordeum vulgare* L.)

Number of virulence alleles	Isolates with different number of virulence alleles, %			
	2014	2015	2016	2017
0	1.1	2.1	30.8	2.8
1-5	22.8	28.9	35.9	11.1
6-10	23.9	26.3	33.3	36.1
11-15	52.2	39.5	0.0	50.0
Number of isolates	92	38	39	39

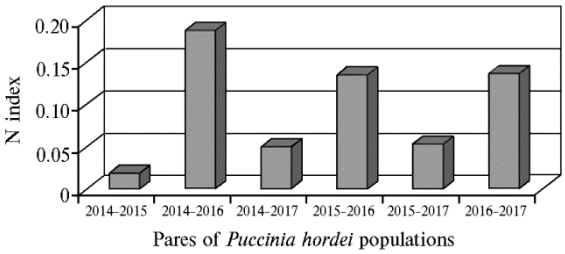
The dynamics of frequency of *P. hordei* isolates with different number of virulence alleles showed that in 2014, 2015 and 2017, the isolates with a large number of virulence alleles (11 to 15) predominated in the pathogen population (Table 3), with 1.1 to 2.8% of isolates avirulent to all the lines with *Rph* genes

used in our researches. In 2016, 33.3-35.9% isolates had medium and low number of virulence alleles, and no isolates with high number of virulence alleles (11-15 genes) were found. Moreover, almost a third of the isolates were avirulent to all studied testers with *Rph* genes.

The decrease in the number of virulence alleles and their frequencies toward most testers could be the consequence of the weather conditions unfavorable for the pathogen during the growing season of 2016. In average, the emergence of *P. hordei* throughout the region had not exceed 5%; the spores were collected from the limited set of winter barley varieties affected by the pathogen that could also affect the reduction of the pathogen population diversity. The obtained results are consistent with the existing theory that the isolates with minimum number of virulence alleles survive under adverse conditions. This is due to the fact that the abundance of "excessive" alleles affects the viability of the rust causative agents. On the contrary, if the conditions are favorable for the pathogen, the isolates with medium and high number of virulence alleles predominate (28].

The values of the Nei's N index which characterizes the differences between populations points out on the high similarity of the structure of the pathogen North Caucasian population by virulence in 2014, 2015 and 2017 (N = 0.02-0.05) and its insignificant changes in 2016 (0.14-0.19) (Fig.).

The revealed differentiation may be due to the reasons described above.



The values of the Nei's N index characterizing the differences between the North Caucasian populations of *Puccinia hordei* Otth. in the frequency of virulence alleles.

The comparison of the frequency of *P. hordei* isolates of the North Caucasian population which are virulent to the carriers of *Rph* genes with the data obtained by other researchers testifies about the discordance in the effectiveness of most resistance genes. Thus, in Australia, *Rph7, Rph11*, *Rph14*, *Rph15*, and *Rph18* [29] genes and later mapped *Rph21* gene [23) remain effective. The *Rph3* gene has retained its effectiveness in Australia since 1992 [9], but in 2009 the isolates virulent to lines with this gene were discovered [30]. *Rph7* and *Rph16* genes in Europe [2, 3] and *Rph3* and *Rph7* genes in Ethiopia [31] are deemed effective. The frequency of *P. hordei* isolates of the North Caucasian population virulent to lines with *Rph3* and *Rph7* genes ranged from 15.4% to 83.3% (see Table 2). The *Rph13* gene effective against the pathogen of North Caucasian population is ineffective in Europe and Australia [3, 9, 29].

So, the differences in virulence of the North Caucasian population of *Puccinia hordei* Otth. in 2014, 2015 and 2017 were insignificant (N = 0.02-0.05) that testifies about stability of the pathogen in virulence. In 2016 which was unfavorable for *P. hordei*, insignificant intrapopulation changes occurred (N = 0.14-0.19). During four years, no isolates virulent to the line with the *Rph13* gene were found. Most isolates turned out to be saturated with virulent alleles. The diversity of the North Caucasian population of *P. hordei* in the virulence allele frequency (H_s) ranged from 0.26 to 0.40 throughout the entire survey period.

REFERENCES

1. Kuznetsova T.E., Serkin N.V. *Selektsiya yachmenya na ustoichivost' k boleznyam: monografiya* [Barley breeding for disease resistance: a monograph]. Krasnodar, 2006 (in Russ.).

2. Niks R.E., Walther U., Jaiser H., Martinez F., Rubiales D. Resistance against barley leaf rust (*Puccinia hordei*) in West European spring barley germplasm. *Agronomie*, 2000, 20(7): 769-782 (doi: 10.1051/agro:2000174).
3. Czembor H.J., Czembor J.H. Leaf rust resistance in winter barley cultivars and breeding lines. *Plant Breed. Seed Sci.*, 2007, 56: 47-56.
4. *Compendium of barley diseases* . D.E. Mathre (ed.). The American Phytopathological Society, St. Paul, MN, 1982: 32-41.
5. Shtaya M.J.Y., Sillero J.C., Rubiales D. Search for partial resistance against *Puccinia hordei* in barley landraces from the Fertile Crescent. *Plant Breeding*, 2006, 125: 343-346 (doi: 10.1111/j.1439-0523.2006.01239.x).
6. Woldeab G., Fininsa C., Singh H., Yuen J. Virulence spectrum of *Puccinia hordei* in barley production systems in Ethiopia. *Plant Pathology*, 2006, 55: 351-357 (doi: 10.1111/j.1365-3059.2006.01357.x).
7. *Karlikovaya rzhavchina yachmenya* [Barley dwarf rust]. Available https://rosselhoscenter.com/2014-02-28-11-39-42/2011-11-16-12-58-47/vozbuditeli-boleznej/1754-karlikovaya-rzhavchina-yachmenya. Accessed 24.09.2018 (in Russ.).
8. Danilova A.V., Volkova G.V. *Zashchita i karantin rastenii*, 2015, 7: 46-48 (in Russ.).
9. Park R.F. Pathogenic specialization and pathotype distribution of *Puccinia hordei* in Australia, 1992 to 2001. *Plant Disease*, 2003, 87(11): 1311-1316 (doi: 10.1094/PDIS.2003.87.11.1311).
10. Singh D., Dracatos P., Derevnina L., Zhou M., Park R.F. *Rph23*: a new designated additive adult plant resistance gene to leaf rust in barley on chromosome 7H. *Plant Breeding*, 2014, 134(1): 62-69 (doi: 10.1111/pbr.12229).
11. Cotterill P.J., Park R.F., Rees R.G. Pathogenic specialization of *Puccinia hordei* Otth. in Australia, 1966-1990. *Australian Journal of Agricultural Research*, 1995, 46(1): 127-134 (doi: 10.1071/AR9950127).
12. Cromey M.G., Viljanen-Rollinson S. Virulence of *Puccinia hordei* on barley in New Zealand from 1990 to 1993. *New Zealand Journal of Crop and Horticultural Science*, 1995, 23(2): 115-119 (doi: 10.1080/01140671.1995.9513877).
13. Shtaya M.J.Y., Sillero J.C., Rubiales D. Screening for resistance to leaf rust (*Puccinia hordei*) in a collection of Spanish barleys. *Breeding Science*, 2006, 56: 173-177 (doi: 10.1270/jsbbs.56.173).
14. Sun Y. *Study of Puccinia hordei and its host resistances in Hordeum vulgare. PhD Thesis.* North Dakota State Univ, Fargo, ND, 2007.
15. Perovic D., Stein N., Zhang H., Drescher A., Prasad M., Kota R., Kopahnke D., Graner A. An integrated approach for comparative mapping in rice and barley with special reference to the *Rph16* resistance locus. *Functional & Integrative Genomics*, 2004, 4(2): 74-83 (doi: 10.1007/s10142-003-0100-z).
16. Bernardo L., Prinsi B., Negri A.S., Cattivelli L., Espen L., Valè G. Proteomic characterization of the *Rph*15 barley resistance gene-mediated defense responses to leaf rust. *BMC Genomics*, 2012, 13: 642-658 (doi: 10.1186/1471-2164-13-642).
17. Fetch T.G., Steffenson B.J., Jin Y. Worldwide virulence of *Puccinia hordei* on barley. *Phytopathology*, 1998, 88: 28-34.
18. Park R.F., Golegaonkar P.G., Derevnina L., Sandhu K.S., Karaoglu H., Elmansour H.M., Dracatos P.M., Singh D. Leaf rust of cultivated barley: pathology and control. *Annual Review of Phytopathology*, 2015, 53: 565-589 (doi: 10.1146/annurev-phyto-080614-120324).
19. Rogozhina E.M., Trofimovskaya L.Ya. V sbornike: *Byulleten' VIR* [In: VIR newsletter]. Leningrad, 1968: 58-63 (in Russ.).
20. Shchelko L.G. *Trudy po prikladnoi botanike, genetike i selektsii*, 1974, 53(2): 105-112 (in Russ.).
21. Danilova A.V., Volkova, G.V., Danilov R.Yu. *Politematicheskii setevoi elektronnyi nauchnyi zhurnal KubGAU*, 2014, 7(101). Available http://ej.kubagro.ru/2014/07/pdf/73.pdf. No date (in Russ.).
22. Smirnova L.A., Alekseeva T.P. *Usovershenstvovannyi metod vyrashchivaniya vskhodov zernovykh kul'tur dlya immunologicheskikh issledovanii: metodicheskie rekomendatsii po izucheniyu rasovogo sostava vozbuditelei rzhavchiny khlebnykh zlakov* [Improved method of growing seedlings of cereals for immunological research: guidelines for the study of the race composition of cereal rust pathogens]. Moscow, 1988 (in Russ.).
23. Sandhu K.S., Forrest K.L., Kong S., Bansal U.K., Singh D., Hayden M.J., Park R.F. Inheritance and molecular mapping of a gene conferring seedling resistance against *Puccinia hordei* in the barley cultivar Ricardo. *Theoretical and Applied Genetics*, 2012, 125(7): 1403-1411 (doi: 10.1007/s00122-012-1921-8).
24. Anpilogova L.K., Volkova G.V. *Metody sozdaniya iskusstvennykh infektsionnykh fonov i otsenki sortoobraztsov pshenitsy na ustoichivost' k vredonosnym boleznyam (fuzariozu kolosa, rzhavchinam, muchnistoi rose)* [Artificial infectious methods and assessing wheat varieties for resistance to harmful diseases (ear fusarium, rust, powdery mildew)]. Krasnodar, 2000 (in Russ.).
25. Babayants L., Meshterkhazi A., Vekhter F., Neklksa N., Dubinina L., Omel'chenko L., Klechkovskaya E., Slyusarenko A., Bartosh P. *Metody selektsii i otsenki ustoichivosti pshenitsy i*

yachmenya k boleznyam v stranakh-chlenakh SEV [Methods of breeding and assessing the resistance of wheat and barley to diseases in the CMEA member countries]. Praga, 1988 (in Russ.).
26. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 1978, 89(3): 583-590.
27. Kosman E., Leonard K.J. Conceptual analysis of methods applied to assessment of diversity within and distance between populations with asexual or mixed mode of reproduction. *New Phytologist*, 2007, 174(3): 683-696 (doi: 10.1111/j.1469-8137.2007.02031.x).
28. D'yakov Yu.T. *Populyatsionnaya biologiya fitopatogennykh gribov* [Population biology of phytopathogenic fungi]. Moscow, 1998 (in Russ.).
29. Sandhu K.S., Singh D., Park R.F. Characterization of leaf rust resistance in international barley nurseries. *Journal of Plant Breeding and Crop Science*, 2016, 8(8): 117-125 (doi: 10.5897/JPBCS2016.0587).
30. Park R.F., Williams M. Barley leaf rust (caused by *Puccinia hordei*). In: *2010-2011 Cereal Rust Survey — Annual Report*. The University of Sydney, Plant Breeding Institute, Cobbitty, 2010: 7-8. Available https://sydney.edu.au/content/dam/corporate/documents/sydney-institute-of-agriculture/research/plant-breeding-and-production/cereal_rust_survey_2010_11.pdf. No date.
31. Woldeab G., Fininsa C., Singh H., Yuen J. Virulence spectrum of *Puccinia hordei* in barley production systems in Ethiopia. *Plant Pathology*, 2006, 55(3): 351-357 (doi: 10.1111/j.1365-3059.2006.01357.x).

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VIABILITY AND VIRULENCE OF WHEAT LEAF RUST AGENT (*Puccinia triticina* Eriks.) ISOLATES AFTER LONG TERM PRESERVATION

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Abstract

The State Collection of phytopathogenic microorganisms (ARRIP) accumulates a great number of wheat leaf rust agent (*Puccinia triticina* Eriks.) isolates, an extremely harmful and epiphytotic pathogen. Annually the collection is replenished with new leaf rust isolates from different populations. Annual estimation of the virulence genes' frequency in isolates makes it possible to track the dynamics of the fungal populations. One of the main tasks of the State collection is to preserve the isolates of the fungus without losing their biological properties to involve these isolates in further laboratory and field experiments. For this purpose, the viability and virulence of *P. triticina* collection isolates was evaluated during 10-year preservation in a household refrigerator (+4 °C) and in a REVCO freezer (–80 °C, Revco, USA). We used 124 *P. triticina* isolates collected in 2005, 2006, 2008, 2009, 2010 and 2012 from damaged wheat (*Triticum aestivum* L.) samples in the Central, North Caucasus and West Siberian regions of the Russian Federation. The isolates differed in virulence and were assigned to 74 phenotypes. The viability of the isolates after storage was determined by spore germination on 2 % water agar plates and by inoculation of susceptible wheat cultivars. Storing of the leaf rust uredospores at low positive temperatures quickly led to a weakening of the viability of the fungus, up to complete destruction. After 1-2 months at +4 °C, the isolates had a high germination capacity, from 48 to 95 %, which decreased in 6 months to 3.0-22.7 %. The correlation between the number of germinated spores on 2 % agar-agar and the duration of storage at a low positive temperature was 0.79. Leaf rust isolates remained viable during storage for 3-10 years under low negative temperatures (–80 °C). The number of germinated spores of different isolates regardless of the preservation period was 25-79 %, and the disease intensity reached 25-100 %. Many factors influence viability of isolates. These mostly are improper sample processing prior to putting into storage and during reviving from an anabiotic state, or disturbance of storage of technical character. However, storage of *P. triticina* isolates at low negative temperatures ensured a rather high survival rate for 10 years. Checking the virulence of the isolates after 7-year storage with the use of susceptible wheat cultivar and *Lr9* and *Lr19* lines showed identical indicators before and after the storage. The method of storing spores at –80 °C allows rather high rate of preservation without changing fungal viability and virulence.

Keywords: collection of microorganisms, leaf rust, isolate, population, virulence, preservation

The State Collection of Phytopathogenic Microorganisms of the All-Russian Research Institute of Phytopathology is intended for the long-term storage of pathogens of agricultural crops in a viable state [1-3]. The Collection fund contains about 5,500 strains of the causative agents of plant diseases (fungi, bacteria and viruses). For the recent 10 years, more than 1,000 isolates of *Puccinia triticina* Eriks. from different wheat cultivation regions of the Russian Federation have been collected. The virulence spectrum of the collected samples of this fungus makes it possible to preserve the diversity of the natural populations of *P. triticina*, to use them for predicting the dynamics of the resistance gene frequency and tests during wheat breeding [4, 5].

Monitoring of *P. triticina* population virulence makes it possible to reveal new resistance genes. In the nature, mutations and genetic recombination, as well as spore migration, significantly influence virulence of the fungus populations [6-8]. Due to joint evolution in the "host-pathogen" system, the permanent selection of virulent clones of leaf rust goes on in the resistant wheat varieties. Virulent clones accumulate in *P. triticina* populations; the avirulent clones become displaced and eventually either eliminate or remain in small amounts. The annual variations in the frequency of leaf rust races are influenced by weather conditions and by the set of cultivated wheat varieties [5, 9]. The annual sampling of *P. triticina* isolates to the collection makes it possible to compare the genetic material of different years and thus to trace the population changes in the frequency of virulence genes, as well as to determine the influence of the cultivated wheat varieties on the appearance and spread of the pathogen's races [10, 11]. As the result of monitoring the virulence of *P. triticina* populations, new potentially dangerous races are detected. Basing on the study of the dynamics and frequency of virulence genes, the effectiveness of wheat *Lr* genes in Russian regions is determined to predict their inclusion in breeding programs. Information about the gene pool of the pathogen populations makes it possible to use proper pathogen compositions for artificial inoculants and to evaluate the wheat genotype resistance to the leaf rust pathogen [12, 13].

The main factor influencing the emergence and frequency of new *P. triticina* races is natural selection which occurs in fungal populations under the influence of the varieties with the race-specific resistance [14]. In this regard, the information about finding out of the clones of wheat leaf rust pathogen which overcomes the resistance of host varieties having the effective resistance genes becomes relevant for the selection of samples which can be used in the breeding for immunity to the disease.

It is known that more than 90% of microorganisms, including rust fungi, cannot be cultured on an artificial medium. The *P. triticina* fungus is an economically important fungus, the storage of isolates of which often causes certain difficulties. The studies related to the necessity to preserve such microorganisms imply the choice of the conditions of conservation and reactivation under which the restoration of the pathogen viability is possible. The maintaining of the isolates in working state and preserving their valuable properties are of importance for practical use [15-17]).

The creators of the collection were faced with the task not only to maintain the *P. triticina* isolates in a viable state, but also to optimize the conditions for maintaining physiological properties of the fungus [18, 19]. It is known that traditional methods of storage of rust fungi (drying and sealing of uredospores in ampoules) do not guarantee the maintenance of high viability for a long time.

Storing in liquid nitrogen requires significant costs. Storing the rust spores in household refrigerators at low positive temperatures allows them to remain viable for several months, and in the form of herbarium material — up to a year. In a routine daily work which consists of continuously repeating cycles requiring the short-term maintenance of the fungus in a viable state, storing the spores at low positive temperatures is sufficient. However, the long-term storing of the fungus requires different conditions.

One of the most widely used contemporary methods of preserving biomaterial without changing its viability is storage in freezers at ultra-low temperatures [20, 21].

It is recommended for many mycological objects to place them in freezing chambers at –80 °C. Low negative temperatures stops biochemical processes in cells including the metabolic process. Using the freezers with ultra-low tem-

peratures ensures the preservation of rust fungi spores without changing their biological properties for 7 years or more [22-24].

In this work, for the first time, the correlation relationship between the viability of leaf rust isolates and the duration of storage at low positive and ultra-low temperatures has been established. As the time of spores storing at a low positive temperature extended, the viability of the isolates decreased that had been expressed in the reduction of spore germination on 2% starvation agar and in the decrease of the intensity of wheat seedlings infecting. Under the conditions of ultra-low temperatures, regardless of the storing period, the isolates had been retaining the ability to germinate on the starvation agar and to infect the plants. The duration of storage at ultra-low temperatures had not influenced the changing of the virulence sign.

The goal of our researches was the comparative assessment of the viability and virulence of the collected isolates of *Puccinia triticina* at the long-term storage under conditions of low positive (+4 °C) and low negative temperatures (−80 °C).

Techniques. The material for the 10-year study was 124 isolates of *P. triticina* taken from the infected wheat samples (*Triticum aestivum* L.) in 2005, 2006, 2008, 2009, 2010 and 2012 in the Central, North Caucasus and West Siberian regions of the Russian Federation.

Isolation, reproduction and identification of virulence in single-pustule isolates of *P. triticina* were performed under optimal conditions for the development of plants and pathogen, the relative average daily air temperature +20 °C, relative air humidity 60% (daytime) and 70% (in night), light intensity 10-15 thousand lux, and 16-hour photoperiod.

The plants of the susceptible Khakasskaya line and the wheat lines with the single resistance genes were grown according to the standard technique in hydroponic culture [12]. The virulence genes in the *P. triticina* isolates were determined with Thatcher *Lr* lines having juvenile resistance genes. The set with juvenile resistance genes contained 42 *Lr* lines, i.e. *Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr9*, *Lr10*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr19*, *Lr20*, *Lr21*, *Lr23*, *Lr24*, *Lr25*, *Lr26*, *Lr27+Lr31*, *Lr28*, *Lr29*, *Lr30*, *Lr32*, *Lr33*, *Lr36*, *Lr38*, *Lr39*, *Lr40*, *Lr 41*, *Lr 42*, *Lr44*, *Lr45*, *Lr46*, *Lr47*, *Lr51*, *Lr53*, and *LrB* [25].

In 25 5-6-day old seedlings lower side of the leaf was inoculated with aqueous suspension of uredospores (0.5 mg spores per 1 ml of water). For better adhesion, one or two drops of Tween^{R} 20 were added to the water. The spores were applied using the scalpel after the wax removal from the leaves with fingers. The infected wheat plants were placed into the humid chamber at +18-20 °C for 16-20 hours, and then into the artificial climate chamber with controlled conditions of temperature, humidity and lighting. In 5-7 days after the inoculation, the plants were examined to detect the disease signs which were evaluated according to the common international practice [26-28]. After the differentiation by virulence, each isolate of *P. triticina* was propagated on the susceptible Khakasskaya line until 4-5 mg biomass accumulation and placed for storage to the State collection of the All-Russian Research Institute of Phytopathology.

Spores were stored for 1-10 months at +4 °C in a household refrigerator in test tubes and for 3, 4, 5, 6, 7, and 10 years at –80 °C in the REVCO freezing chamber (Revco, USA) in plastic containers. Before each inspection of the material, the uredospores stored in the freezers were recovered from the anabiosis by heating at +45 °C for 5 minutes [29].

The viability of the isolates was determined by germination on plates with 2% starvation agar and by inoculation of the seedlings of susceptible wheat

variety with spore suspension [30]. In the first case, the molten 2% starvation agar was poured onto the glass slides laid out in the sterile Petri dishes. The spores were plated using a preparation needle. The dissemination evenness was achieved by slight knocking with the needle on the Petri dish's edge. Then the dishes were closed and kept at room temperature. After 6 hours, the glasses were examined under the microscope at high magnification and the number of germinated spores in 100 examined samples (triple replication) was counted out. The viability of the spores of each isolate was expressed in percentage value.

In order to test the ability to infect the 5-6-day-old wheat seedlings of the universally susceptible Khakasskaya line, the plants were inoculated with spores taken from storage and recovered from anabiosis. The formation of pustules on the leaf surface served as the indicator of the spore viability.

In the statistical processing of the results, the coefficients of correlation (*r*) and coefficients covariance were calculated using the Microsoft Excel program [2, 3].

Results. All *P. triticina* isolates used in this work have been marked with the stating of their origin and virulence. The differentiation of the *P. triticina* isolates with the wheat lines having the juvenile resistance genes has revealed significant differences in virulence. The isolates contained different number of virulence genes and were classified into 74 phenotypes. Although the isolates derived from the *P. triticina* populations in different years, all of them were characterized by the presence of the same 12 virulence genes: *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p17*, *p18*, *p21*, *p30*, *p33*, and *pB*. The *P. triticina* isolates differed among themselves by the presence of genes *p1*, *p2a*, *p2b*, *p2c*, *p9*, *p11*, *p15*, *p16*, *p19*, *p20*, *p23*, *p25*, *p26*, *p27* + *p31*, *p28*, *p32*, *p36*, *p38*, *p39*, *p40*, *p44*, and *p46*. No *p24*, *p29*, *p41*, *p42*, *p45*, *p47*, *p51*, and *p53* genes have been detected in isolates from the Collection.

While storing the uredospores of the leaf rust pathogen under low positive temperatures, their viability weakened rapidly (Table 1). Thus, after the storing at +4 °C for 1-2 months, the isolates demonstrated a high (from 48 to 95%) germinability of spores, and after 6 months this value decreased to 3.0-22.7%. After 10 months of storing, the spores turned to be completely non-germinable. A 0.79 correlation was found between the number of spores germinated on 2% starvation agar and the duration of storage in the household refrigerator. The similar results have been also obtained when infection of the seedlings of the susceptible Khakasskaya variety with the spore suspensions: as the duration of storing extended, the intensity of affection of the wheat seedlings decreased.

1. Viability of *Puccinia triticina* Eriks. uredospores on 2% starvation agar and on seedlings of susceptible Khakasskaya wheat variety after 1-10 month storing at +4 °C

Storing, months	Isolates	Germinated spores (min-max), %	Affection of the wheat seedlings (min-max), %
1	8	55,9-89	60-100
1,5	5	69,4-84,6	60-100
2	32	48,0-95,0	60-100
3,5	12	23,0-76,0	25-60
6	23	3,0-22,7	1-20
8	5	2,3-7,3	1-5
8,5	3	0-2,3	0-1
9	4	0,7-1,3	0-1
10	5	0	0
Correlation coefficient		–0,79	–0,65
Covariance coefficient		0,62	0,42

At –80 °C the isolates remained viable for 3-10 years. Regardless of the storing duration, in different isolates the percentage of germinated spore was 25-

79% and the plants affection reached 25-100%.

The viability values of isolates at the same storing duration had been influenced by many factors, the main of which could be faults when the preparation of the material before putting for storage and technological violations of the storage conditions. Nevertheless, the storage of the Collection samples of *P. triticina* at low negative temperatures ensured a rather high survival rate for 10 years (Table 2).

2. Viability of *Puccinia triticina* Eriks. isolates on 2% starvation agar and on seedlings of susceptible Khakasskaya wheat variety depending on duration of storing in the freezer

Years	Storing, years	Number of isolates	Spores germinated on the agar (min-max), %	Affection of the wheat seedlings (min-max), %
2005-2015	10	15	34-69	25-80
2008-2015	7	8	34-56	25-60
2006-2012	6	6	45-48	40
2006-2011	5	10	25-60	25-80
2006-2010	4	10	45-68	40-80
2009-2012	3	11	50-67	40-80
2010-2012	3	16	35-79	25-100
2012-2015	3	9	53-78	60-100
Correlation coefficient			–0,22	–0,41
Covariance coefficient			0,05	0,17

The storing of the *P. triticina* isolates at low negative temperatures does not affect their virulence. This has been confirmed by the results of inoculation of the susceptible Khakasskaya line and two monogenic lines of the Thatcher variety (*Lr9* and *Lr19*) with isolates taken from the storage (Table 3). The *Lr9* and *Lr19* lines were used as the indicators of virulence of the isolates with the *p9* and *p19* genes. The isolates of *P. triticina* (34 ones in total) collected in the Krasnodar Territory, Omsk and Moscow regions demonstrated the same response on the tester lines as when putting for storage (see Table 3).

Thus, the comparison of isolates 729-5 (from the Omskaya 32 variety), 730-1, 730-2, 730-4, 730-6, 730-11 (from the Chernyava variety), 733-3 (from the Chernyava 13 variety) and 732-1 (from the Omskaya 29 variety) collected in the West Siberian region did not reveal differences in the virulence to *Lr9* and *Lr19* lines before and after the storage. The uredospores collected in 2010 in Moscow region from the Pamyati Fedina, Moskovskaya 39, Mironovskaya 808 and Moskovskaya 39 winter wheat varieties and in 2008 in the Krasnodar Territory from Batko, Valeria, Krasnodar 99, Kupava, Delta, Michigan Amber varieties, in 2015 year, as when putting for storage, were avirulent to *Lr9* line. In the virulence formulas of 648-4, 648-11, 649-2 and 676-14 isolates, the *p19* gene has been noted. Testing of the virulence of these isolates after 7 years storage showed the identical reactions on the tester lines.

Despite the significant progress in genetics, biochemistry, physiology, and ecology of microorganisms, the mechanisms responsible for the reversible transition of cells to the anabiotic state are still studied insufficiently. For the many years of creating the collections of bacteria and fungi, general but not always clear concepts about managing the processes of conservation and restoration of the viability of each specific organism have accumulated. The interest to the works on investigation of the structural and functional cell transformations of microorganisms under the influence of conservation-reactivation factors appeared after the finding of alive microbes in the Arctic ice [31]. The experience of working with collections testifies that many contemporary methods of conservation turn to be relatively effective in maintaining laboratory cultures of microorganisms. However, the conservation at ultralow temperatures with the complete preservation of populations and genomes is the most effective especially if

3. Virulence of the *Puccinia triticina* Eriks. isolates before and after the storing at low negative temperatures (–80 °C)

Years	Name code of the isolate	Virulence formula (presence of the virulence genes)	Virulence lines having the resistance gene	
			Lr9	*Lr19*
		Krasnodar Territory, North Caucasus		
2008-2015	670-2	*p1*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p25*, *p26*, *p27*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	R/R
2008-2015	670-6	*p1*, *p2c*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p14b*, *p17*, *p18*, *p21*, *p25*, *p26*, *p27* + *p31*, *p30*, *p33*, *p36*, *pB*	R/R	R/R
2008-2015	670-4	*p1*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p19*, *p21*, *p23*, *p25*, *p26*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	S/S
2008-2015	670-7	*p1*, *p2b*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p25*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	R/R
2008-2015	670-8	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p19*, *p21*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	S/S
		Omsk Region, West Siberia		
2008-2015	648-4	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p11*, *p14a*, *p14b*, *p15*, *p16*, *p17*, *p18*, *p19*, *p20*, *p21*, *p25*, *p26*, *p27* + *p31*, *p28*, *p30*, *p32*, *p33*, *pB*	R/R	S/S
2008-2015	648-9	*p1*, *p2c*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p14b*, *p17*, *p18*, *p21*, *p23*, *p25*, *p30*, *p33*, *pB*	R/R	R/R
2008-2015	648-11	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p17*, *p18*, *p19*, *p21*, *p23*, *p25*, *p28*, *p30*, *p33*, *p36*, *pB*	R/R	S/S
2008-2015	649-2	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p17*, *p18*, *p19*, *p20*, *p21*, *p25*, *p26*, *p27* + *p31*, *p30*, *p33*, *pB*	R/R	S/S
2008-2015	653-1	*p1*, *p2c*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p21*, *p25*, *p27* + *p31*, *p28*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	R/R
2008-2015	676-5	*p1*, *p2c*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p16*, *p17*, *p18*, *p20*, *p21*, *p26*, *p27* + *p31*, *p30*, *p32*, *p33*, *pB*	R/R	R/R
2008-2015	676-6	*p1*, *p2c*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p15*, *p16*, *p17*, *p18*, *p20*, *p21*, *p26*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	R/R
2008-2015	676-7	*p1*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p16*, *p17*, *p18*, *p20*, *p26*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *pB*	R/R	R/R
2008-2015	676-12	*p1*, *p3a*, *p3bg*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p20*, *p25*, *p30*, *p32*, *p33*, *pB*	R/R	R/R
2008-2015	676-14	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p19*, *p20*, *p26*, *p30*, *p32*, *p33*, *pB*	R/R	S/S
2010-2012	729-1	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p17*, *p18*, *p20*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	R/R	R/R
2010-2012	729-5	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p15*, *p17*, *p18*, *p19*, *p20*, *p21*, *p26*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	R/R	S/S
2010-2012	729-6	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p15*, *p17*, *p18*, *p20*, *p21*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p44*, *pB*	R/R	R/R

Table 3 continued

2010-2012	730-1	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p20*, *p21*, *p23*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	S/S	R/R
2010-2012	730-2	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p14a*, *p17*, *p18*, *p20*, *p23*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	S/S	R/R
2010-2012	730-4	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p11*, *p14a*, *p14b*, *p17*, *p18*, *p19*, *p20*, *p21*, *p23*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *p46*, *pB*	S/S	S/S
2010-2012	730-6	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p14a*, *p14b*, *p15*, *p17*, *p18*, *p23*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *p46*, *pB*	S/S	R/R
2010-2012	730-11	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p11*, *p14a*, *p14b*, *p15*, *p17*, *p18*, *p20*, *p21*, *p23*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	S/S	R/R
2010-2012	733-3	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p14a*, *p14b*, *p15*, *p17*, *p18*, *p20*, *p21*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	S/S	R/R
2010-2012	732-1	*p1*, *p2a*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p9*, *p10*, *p11*, *p14a*, *p14b*, *p15*, *p17*, *p18*, *p20*, *p21*, *p23*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*		
		Moscow Region, Central Russia		
2010-2012	728-3	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p17*, *p18*, *p19*, *p21*, *p23*, *p25*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	R/R	S/S
2010-2012	728-1	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	R/R	R/R
2010-2012	720-4	*p1*, *p2b*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p19*, *p20*, *p21*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p44*, *pB*	R/R	S/S
2010-2012	728-4	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p17*, *p18*, *p19*, *p20*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	R/R	S/S
2010-2012	728-8	*p1*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p17*, *p18*, *p19*, *p20*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *p39*, *p40*, *pB*	R/R	S/S
2010-2012	718-3	*p1*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p15*, *p16*, *p17*, *p18*, *p19*, *p20*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p39*, *p40*, *pB*	R/R	S/S
2010-2012	720-2	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p19*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *p39*, *p40*, *p44*, *pB*	R/R	S/S
2010-2012	724-1	*p1*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p16*, *p17*, *p18*, *p19*, *p21*, *p23*, *p25*, *p26*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *p39*, *p40*, *pB*	R/R	S/S
2010-2012	718-1	*p1*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p14a*, *p14b*, *p15*, *p16*, *p17*, *p18*, *p19*, *p20*, *p21*, *p23*, *p25*, *p30*, *p32*, *p33*, *p36*, *p39*, *p40*, *p46*, *pB*	R/R	S/S
2010-2012	720-5	*p1*, *p2b*, *p2c*, *p3a*, *p3bg*, *p3ka*, *p10*, *p11*, *p14a*, *p14b*, *p15*, *p16*, *p17*, *p18*, *p21*, *p23*, *p25*, *p27* + *p31*, *p30*, *p32*, *p33*, *p36*, *p39*, *p40*, *p44*, *p46*, *pB*	R/R	R/R

Note. R — resistance, S — susceptibility (types of plant responses to *P. triticina* isolates before/after the storage).

considering the phenomenal physiological diversity of microorganisms [19-22].

During the period of practical tests on the conservation of microorganisms, the techniques of turning vegetative cells into the anabiotic state have been developed [23, 24], however, the works on finding out the clearer criteria when managing the processes of conservation and restoration of the viability of certain microorganisms are still topical.

Thus, we have established that low temperatures (– 80 °C) is effective for the long-term (from 3 to 10 years) storing of the collection of wheat leaf rust isolates. Even while storing for 10 years, low temperatures had not been decreasing the pathogen viability and for 7 years had not been affecting its virulence. Low positive temperatures (+4 °C) are unsuitable for the long-term storing of the *Puccinia triticina* isolates and may be used for preserving only up to 2-3 months that makes it impossible to use these spores in the next growing season. The maintaining of *P. triticina* isolates in usable condition and preserving their valuable properties are important not only for population genetics, but also for selection studies.

REFERENCES

1. *Katalog shtammov Gosudarstvennoi kollektsii fitopatogennykh mikroorganizmov i sortov rastenii — identifikatorov (differentsiatorov) patogennykh shtammov mikroorganizmov* [Catalog of strains of the State collection of phytopathogenic microorganisms and differentiating varieties]. Bol'shie Vyazemy, 2009. Available http://www.vniif.ru/vniif/structure/collection/. No date (in Russ.).
2. Zhemchuzhina N.S., Kiseleva M.I., Abramova S.L., Makarov A.A. *Zashchita i karantin rastenii*, 2014, 1: 48-50 (in Russ.).
3. Kiseleva M.I., Zhemchuzhina N.S., Kovalenko E.D., Dubovoi V.P., Makarov A.A. *Zashchita i karantin rastenii*, 2014, 1: 50-52 (in Russ.).
4. Kolmer J.A. Genetics of resistance to wheat leaf rust. *Annual Review of Phytopathology*, 1996, 34(1): 435-455 (doi: 10.1146/annurev.phyto.34.1.435).
5. Singh R.P., Huerta-Espino J., William H.M. Genetics and breeding for durable resistance to leaf and stripe rusts in wheat. *Turk. J. Agric. For.*, 2005, 29: 121-127.
6. Kovalenko E.D., Zhemchuzhina A.I., Kiseleva M.I., Kolomiets T.M., Shcherbik A.A. *Zashchita i karantin rastenii*, 2012, 9: 19-22 (in Russ.).
7. Oelke L.M., Kolmer J.A. Characterization of leaf rust resistance in hard red spring wheat cultivars. *Plant Disease*, 2004, 88(10): 1127-1133 (doi: 10.1094/PDIS.2004.88.10.1127).
8. DadrezaieS., Lababidi S., Nazari K., Goltapeh E., Afshari F., Alo F., Shams-Bakhsh M., Safaie N. Molecular genetic diversity in Iranian populations of *Puccinia triticina*, the causal agent of wheat leaf rust. *American Journal of Plant Sciences*, 2013, 4(7): 1375-1386 (doi: 10.4236/ajps.2013.47168).
9. Kosman E., Ben - Yehuda P., Manisterski, J. Diversity of virulence phenotypes among annual populations of wheat leaf rust in Israel from 1993 to 2008. *Plant Pathol.*, 2014, 63: 563-571 (doi: 10.1111/ppa.12117).
10. Kovalenko E.D., Kolomiets T.M., Kiseleva M.I., Zhemchuzhina A.I., Smirnova L.A., Shcherbik A.A. *Metody otsenki i otbora iskhodnogo materiala pri sozdanii sortov pshenitsy ustoichivykh k buroi rzhavchine* [Methods for assessing and selecting the source material when creating wheat rust resistant varieties]. Moscow, 2012: 1-93 (in Russ.).
11. Kolmer J.A., Liu J.Q. Simple inheritance of partial leaf rust resistance in two wheat cultivars. *Plant Pathology*, 2001, 50(5): 546-551 (doi: 10.1046/j.1365-3059.2001.00607.x).
12. Kovalenko E.D., Zhemchuzhina A.I., Kiseleva M.I., Kolomiets T.M., Shcherbik A.A. *Zashchita i karantin rastenii*, 2012, 9: 19-22 (in Russ.).
13. Liu T.-G., Ge R.-J., Ma Y.-T., Liu B., Gao L., Chen W.-Q. Population genetic structure of Chinese *Puccinia triticina* races based on multi-locus sequences. *Journal of Integrative Agriculture*, 2018, 17(8): 1779- 1789 (doi: 10.1016/S2095-3119(18)61923-9).
14. Bolton M.D., Kolmer J.A., Garvin D.F. Wheat leaf rust caused by *Puccinia triticina*. *Molecular Plant Pathology*, 2008, 9: 563-575 (doi: 10.1111/j.1364-3703.2008.00487.x).
15. Morgan C.A., Herman N., White P.A., Vesey G. Preservation of micro-organisms by drying; a review. *Journal of Microbiological Methods*, 2006, 66(2): 183-193 (doi: 10.1016/j.mimet.2006.02.017).
16. Strei W.R., Schmitz R.A. Metagenomics — the key to the uncultured microbes. *Current Opinion in Microbiology*, 2004, 7(5): 492-498 (doi: 10.1016/j.mib.2004.08.002).
17. Miyamoto-Shinohara Y., Sukenobe J., Imaizumi T., Nakahara T. Survival of freeze-dried bacteria. *The Journal of General and Applied Microbiology*, 2008, 54(1): 9-24 (doi: 10.2323/jgam.54.9).

18. Pokhilenko V.D., Baranov A.M., Detushev K.V. *Izvestiya vysshikh uchebnykh zavedenii. Povolzhskii region. Meditsinskie nauki*, 2009, 4(12): 99-121 (in Russ.).
19. Smith D., Onions H.S. *The preservation and maintenance of living fungi.* CAB International, Wallingford, UK, 1994.
20. Volkov V.Ya. *Mikrobiologiya*, 1994, 63(1): 5-16 (in Russ.).
21. Kitamoto Y., Suzuki A., Shimada S., Yamanaka K. A new method for the preservation of fungus stock cultures by deep-freezing. *Mycoscience*, 2002, 43(2): 143-149 (doi: 10.1007/S102670200021).
22. Muldrew K., McGann L.E. *Cryobiology — a short course.* University of Calgary, Alberta, Canada, 1999.
23. Wolfw J., Bryant G. *Cryobiology and anhydrobiology of cells.* RMIT University, Melbourn, Australia, 2004.
24. Tedeschi R., De Paoli P. Collection and preservation of frozen microorganisms. In: *Methods in Biobanking, Methods in Molecular Biology, Vol. 675.* J. Dillner (ed.). Humana Press, Totowa, NJ, 2011: 313-326 (doi: 10.1007/978-1-59745-423-0_18).
25. McIntosh R.A., Dubcovsky J., Rogers W.J., Morris C., Appels R., Xia X.C. *Catalogue of gene symbols for wheat, 2013.* Available https://wheat.pw.usda.gov/GG2/Triticum/wgc/2013/. No date.
26. Hanzalová A., Bartoš P. Physiologic specialization of wheat leaf rust (*Puccinia triticina* Eriks.) in the Czech Republic in 2001-2004. *Czech J. Genet. Plant Breed.*, 2006, 42(4): 126-131.
27. Peterson R.F., Campbell A.B., Hannah A.E. A diagrammatic scale for estimating rust intensity of leaves and stem of cereals. *Canadian Journal of Research*, 1948, 26c(5): 496-500 (doi: 10.1139/cjr48c-033).
28. Kolmer J.A. Physiologic specialization of *Puccinia triticina* in Canada in 1998. *Plant Disease*, 2001, 85: 135-138.
29. Uzunova-Doneva T., Donev T. Anabiosis and conservation of microorganisms. *Journal of Culture Collections*, 2005, 4(1): 17-28.
30. *Maintenance of microorganisms. A manual of laboratory methods.* B.E. Kirsop, J.J.S. Snell (eds.). Academic Press, London, UK, 1984.
31. McCann C.M., Wade M.J., Gray N.D., Roberts J.A., Hubert C.R.J., Graham D.W. Microbial communities in a high Arctic polar desert landscape. *Front. Microbiol.*, 2016, 7: 419 (doi: 10.3389/fmicb.2016.00419).

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EFFECT OF PRETREATMENT OF LIGNOCELLULOSIC SUBSTRATES ON PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF SOME SPECIES OF EDIBLE AND MEDICAL MUSHROOMS

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Abstract

The development of new effective substrates for mushroom cultivation is relevant not only in order to obtain high-quality food products, but also as a need for rational use of natural resources. Chemical pretreatment can modify the chemical composition of various types of lignocellulosic materials and remove the growth-inhibiting compounds which can affect the physiological and biochemical processes in fungi. The present work provides the first comprehensive assessment of growth and biochemical characteristics of xylotrophic basidiomycetes cultivated on substrates that contain chemical pretreated lignocellulosic materials. Edible mushrooms *Hericium erinaceus* (Bull.) Persoon and *Flammulina velutipes* (Curtis) Singer and medicinal mushroom *Ganoderma lucidum* (Curtis) P. Karst. were selected for study. Pine (*Pinus sylvestris* L.) and beech (*Fagus orientalis* Lipsky) sawdust of particle size 0.24-0.315 mm were used. Pretreatment of lignocellulosic material with 2 % wt. solutions of hydrochloric acid, sulfuric acid, sodium hydroxide and hydrogen peroxide was carried out in autoclave at 120 °C for 60 min. Sodium hydroxide pretreatment resulted in a statistically significant ($p < 0,05$) increase in the easily hydrolysable carbohydrates content in pine sawdust by 5 % and in beach sawdust by 4 % compared to control. Acid pretreatment instead led to an increase in hardly hydrolysable carbohydrates content in lignocellulosic materials by 20 %. Hydrogen peroxide pretreatment had no significant effect on composition of pine and beech sawdust. Chemically pretreated pine and beach sawdust were used as the basis for substrates for cultivation of xylotrophic basidiomycetes. Substrates, in addition to sawdust, contained 10 % wheat bran and 1 % $CaCO_3$. Alkaline and acid pretreatments contributed to increased bioavailability of lignocellulosic materials for the vegetative growth of *G. lucidum*. The colony of *G. lucidum* after nine days of growth on untreated pine sawdust was 43±4 mm in diameter, on hydrochloric acid-, sulfuric acid-, sodium hydroxide- and hydrogen peroxide-pretreated pine sawdust were 58±7, 52±3, 63±4 and 49±7 mm in diameter, respectively. Acid pretreatments of sawdust improved the growth rate and density of *F. velutipes* and *H. erinaceus* vegetative mycelium. Hydrochloric acid pretreatment of pine sawdust led to an increase in the colony diameters of *F. velutipes* and *H. erinaceus* from 51±1 and 19±2 mm to 62±1 и 23±4 mm, respectively, of beach sawdust — from 48±4 and 19±2 mm to 50±4 и 32±3 mm, respectively. No growth of *H. erinaceus* occurred on substrate with alkali-pretreated pine sawdust. Fruit bodies of *H. erinaceus* were harvested from substrates containing acid-pretreated and untreated pine and beach sawdust. Acid pretreatments of pine and beach sawdust decreased the cultivation time twice. The second wave of fructification occurred during the cultivation of *H. erinaceus* only on substrates containing hydrochloric acid-pretreated sawdust. This provided an increase in yield of fruit bodies compared to control. The total yield of fruit bodies of the first and second waves cultivated on substrates with pretreated pine sawdust was 8.0 % dry wt., on substrate with untreated sawdust — 3.9 % dry wt. The yield of fruit bodies reduced significantly during cultivation of *H. erinaceus* on a substrate with beech sawdust after sulfuric acid pretreatment. For the first time, a significant difference in the protein and polysaccharide contents of fruit bodies of the first and second waves is shown. In the case of growing mushrooms on a substrate with hydrochloric acid-pretreated sawdust fruit bodies of the second wave had 1.3 times higher protein content and 1.7 times higher polysac-

charide content than fruit bodies of the first wave. Thus, a comparative study of different pretreatments of lignocellulosic materials revealed a high efficiency of hydrochloric acid pretreatment step in preparing pine sawdust substrates for cultivation of *H. erinaceus*.

Keywords: basidiomycetes, *Hericium erinaceus*, *Ganoderma lucidum*, *Flammulina velutipes*, solid-phase cultivation, substrate, pine sawdust, beech sawdust, pretreatment

The involvement of hardly processible wastes from agriculture, food and wood processing industry in technological processes is a key aspect of the rational use of natural resources [1, 2]. Sawdust of coniferous trees, leaves, fir needles and bark are valuable raw materials for inclusion in existing production cycles [3, 4]. However, besides cellulose, hemicelluloses and lignin such wastes contain the polyphenolic and tanning substances which negatively affect the raw materials processing [5, 6]. The unwanted components can be removed in the course of preprocessing using the physical [7], chemical [8, 9] or biological methods [10]. As the result, the reactive capacity of the key components increases owing to the breaking of intermolecular bonds, decrease of crystallinity degree, increase of the pore size and available surface area of the raw material (11-13). The successful example of the introduction of new technologies which include the stage of preprocessing of lignocellulosic raw materials is the ethanol production [14-16]. When using the sugar cane bagasse pretreated with ammonia the yield of alcohol is 6 times higher compared to using the untreated one (17).

In growing the fruit bodies of edible xylotrophic mushrooms, the sawdust of deciduous trees, straw, sunflower husk and corncobs which do not require modifying the chemical composition are used [18, 19]. It can be assumed that the pretreatment of such substrates will contribute to the increase of their bioavailability. Using the untreated sawdust of coniferous trees for growing fruit bodies is impractical because the resins contained in their wood inhibit the fungi growth and worsen the quality of final products.

There is no information in the scientific literature on the comparing various types of chemical pretreatment of lignocellulosic raw materials in the development of effective substrates for growing fruit bodies of mushrooms.

In this work, for the first time, the assessment of the growth and biochemical characteristics of xylotrophic mushrooms grown on the substrates containing chemically pretreated lignocellulosic raw materials has been made. It is shown that the share of carbohydrates in the sawdust composition decreases after the acid pretreatment and increases after the alkaline pretreatment. The growth and density of the mycelium of *Ganoderma lucidum* (Curtis) P. Karst. increases after all types of pretreatment. For *Flammulina velutipes* (Curtis) Singer and *Hericium erinaceus* (Bull.) Persoon the substrates after the acid pretreatments are more accessible. The pretreatment of pine and beech sawdust with hydrochloric acid and the pretreatment of pine sawdust with sulfuric acid ensures the second wave of fruiting in *H. erinaceus*. Wherein, the fruit bodies of the first and second waves significantly differe in the content of proteins and polysaccharides.

The objective of this work was to establish the influence of the methods of pretreatment of deciduous and coniferous trees' sawdust on the growth and biochemical characteristics of some species of edible and medicinal fungi.

Techniques. The sawdust of pine (*Pinus sylvestris* L.) and beech (*Fagus orientalis* Lipsky) was grinded into 0.24-0.315 mm particles and dried at 60 °C for 48 hours. 100 grams of dried sawdust were mixed in 750 ml Erlenmeyer flasks with 2% hydrochloric acid, sulfuric acid, sodium hydroxide and hydrogen peroxide (all reagents of the Ruskhim LLC, Russia) in the 1:1 proportion. The flasks were kept in the autoclave (steam sterilizer VK-75-01, TZMOI JSC, Russia) at 120 °C for 60 minutes. The pretreated sawdust was separated by filtration through the ashless filter paper under vacuum, washed with the distilled water up to pH 7 and dried at

60 °C for 48 hours.

Dried preprocessed sawdust (1 g) was mixed with 1.5% hydrochloric acid (45 ml) in conic 100 ml flasks. The flasks were autoclaved at 120 °C for 120 minutes. The reaction mixture was air-cooled for 30 minutes up to room temperature and filtered through ashless paper filters under vacuum. Easily hydrolyzable carbohydrates in the pretreated sawdust was determined by measuring the amount of reducing substances (RS) in the hydrolysate filtrates by the standard spectrophotometric method with the 3,5-dinitrosalicylic acid (Acros Organics B.V.B.A., Belgium) [20].

The strains of the medicinal basidiomycete *G. lucidum* and edible basidiomycetes *F. velutipes* and *H. erinaceus* which also have medicinal properties were received from the collection of the Gause Institute of New Antibiotics. The workable cultures were stored on the slant potato-glucose agar at 4 °C.

The solid substrate containing 4.5 g of sawdust, 0.5 g of wheat bran and 0.05 g of $CaCO_3$ (Ruskhim LLC, Russia) were placed in Petri dishes and then 8 ml of hot (90 °C) distilled water was added. The substrate pH was adjusted to 6.0±0.2. The Petri dishes were sterilized at 120 °C for 1 hour, inoculated with the mycelial-agar blocks (3 mm diameter) of the 10-day-old fungus cultures and incubated at 25 °C for 9 days, and the diameters of colonies were measured.

To grow the fruit bodies of *H. erinaceus*, the solid substrate containing 18 g of sawdust, 2 g of wheat bran and 0.2 g of $CaCO_3$ was placed into conical flat-bottomed 200 ml flasks and then 32 ml of hot (90 °C) distilled water was added. The pH was adjusted to 6.0±0.2. To assess the effect of sawdust pretreatment on the yield and biochemical composition of fruit bodies, the content of an obligatory additional source of nitrogen (bran) was half of the standard according to the generally accepted recommendations [21]. The flasks were sterilized at 120 °C for 60 min, inoculated with the mycelial-agar blocks (3 mm diameter) of the 10-day-old culture of *H. erinaceus* and incubated in the dark at 25 °C for 3 weeks until the complete coverage of the substrate with the mycelium. After that, the cotton-gauze plugs were removed and the flasks were incubated at 20 °C and 80% relative humidity for 3 months in incubation chamber at 0.1 rpm and 8-hour daylight (150 lx, LED lamps). Fruit bodies that reached commercial maturity were harvested, dried (freeze dryer LS-500, Prointekh LLC, Russia) and grinded.

The total polysaccharide content was determined by phenol-sulfuric acid method with glucose (NTK DIAEM LLC, Russia) as a standard [22]. The proteins were extracted with 0.1 M phosphate buffer (pH 7.4) for 3 hours under constant stirring. The total content of proteins was evaluated by the Bradford method [23] with bovine serum albumin (NTK DIAEM LLC, Russia) as a standard.

All experiments were performed in 3 biological and 3 analytical replicates.

The statistical processing of the results was made using Microsoft Excel 2013 software package. The figures and tables show the mean values (*M*) and standard errors of the mean (±SEM). The statistical significance of the differences between the average values of the parameters was evaluated according to the Student's *t*-test, the differences at $p \leq 0.05$ were considered statistically significant.

Results. The chosen basidiomycetes are economically valuable producers of nutritious and bioactive compounds [24-28].

Easily hydrolyzable carbohydrate content was 30% in the untreated pine sawdust and 37% in the beech sawdust (Fig. 1) that corresponds to the content of hemicelluloses in the raw material [29]. The pretreatment with the sodium hydroxide solution contributed to a 7% increase in the level of easily hydrolyzable carbohydrates in the pine sawdust and a 4% increase for the beech sawdust ($p \leq 0.05$).

Most likely, this was the consequence of the removal of lignin and a part

of hemicelluloses that corresponds to the reported data [30]. The hydrogen peroxide did not significantly influenced the content of easily hydrolyzable carbohydrates in the beech sawdust, while the pretreatment of the pine sawdust led to their a 5% decrease. It is possible that the differences in the results of the pretreatment with hydrogen peroxide are due to the difference in the chemical structure of hemicelluloses and lignin in coniferous and deciduous trees [31].

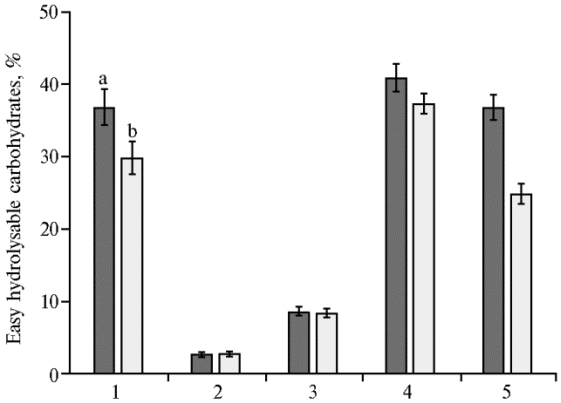

Fig. 1. Easily hydrolyzable carbohydrates in the sawdust of beech *(Fagus orientalis)* (a) **and of pine (*Pinus sylvestris)*** (b) **before and after chemical pretreatments:** 1 — control, 2 — 2% hydrochloric acid, 3 — 2% sulfuric acid, 4 — 2% sodium hydroxide, 5 — 2% hydrogen peroxide.

The acid pretreatment caused the sharp increase in concentration of hardly hydrolyzable compounds in the raw materials, i.e. the content of easily hydrolyzable carbohydrates in sawdust of pine and beech decreased by 20% ($p \leq 0.05$). Since the loss in substrate weight exceeded the portion of easily hydrolyzed sugars, acid pretreatment seemed to cause not only the hydrolysis of hemicelluloses and the amorphous parts of cellulose but also the lignin partial removal. A similar result of chemical changes under the influence of mineral acids has been obtained when studying the pretreatment of poplar wood [32].

At the next stage, we evaluated the effect of the pretreatment on the substrates bioavailability for xylotrophic basidiomycetes causing white rot, which are able to utilize lignin. The rate of the substrate consumption and the intensity of mycelium development were determined (Table 1).

1. Diameter of 8-day-old colonies (d) **and visual assessment of the density of xylotrophic basidiomycetes mycelium** (q) **grown in Petri dishes under different chemical pretreatments of the substrate** (M±SEM)

Substrate	Pretreatment	*Ganoderma lucidum*		*Flammulina velutipes*		*Hericium erinaceus*	
		d, mm	q	d, mm	q	d, m	q
Pine	Control	43±4	2	51±1	2	19±2	2
	HCl, 2 %	58±7	3	62±1	2	23±4	2
	H_2SO_4, 2 %	52±3	3	62±2	2	29±2	2
	NaOH, 2 %	63±4	3	58±2	1	–	–
	H_2O_2, 2 %	49±7	3	54±1	1	24±2	1
Beech	Контроль	34±1	2	48±4	3	19±2	2
	HCl, 2 %	49±6	3	50±4	3	32±3	2
	H_2SO_4, 2 %	38±3	3	57±2	3	32±3	2
	NaOH, 2 %	60±8	3	63±4	1	24±1	1
	H_2O_2 2 %	49±7	3	42±5	1	26±2	1

N o t e. The mycelium density is given in points; "–" means no growth.

All types of chemical pretreatment contributed to higher growth rate and density of the mycelium of *G. lucidum*. That is, *G. lucidum* with equal efficiency assimilated the substrates enriched both with lignin (acid pretreatment) and with carbohydrates (alkaline and peroxide pretreatment). In case of *G. lucidum*, the increase of bioavailability of the substrates containing the preprocessed sawdust is probably related to the destruction of the intermolecular bonds of the lignocellulosic complex as a result of chemical exposure. For *F. velutipes* and *H. erinaceus* the substrates after the acid pretreatments were more accessible. Wherein, the enrichment of the substrates with lignin led to the increase in the growth rates of basidiomycetes compared to the control both on the pine sawdust and on the beech

sawdust. The peroxide and alkaline pretreatments did not contributed to the increase of the substrate bioavailability for *F. velutipes* and *H. erinaceus*. While the slight increase in the growth rate of *F. velutipes* on the sawdust enriched with carbohydrates, the mycelium density decreased. There was no growth of *H. erinaceus* on the substrate containing the pine sawdust after the alkaline pretreatment.

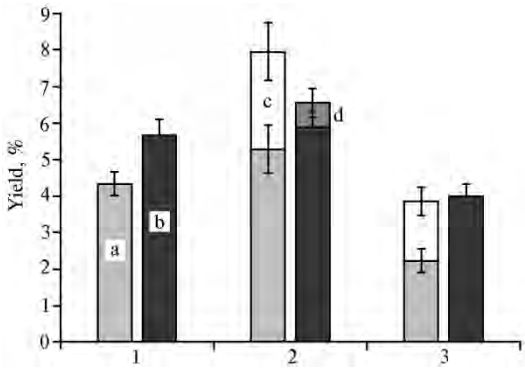

Fig. 2. Yield of *Hericium erinaceus* fruit bodies in the control (1)**, on substrates pretreated with 2% hydrochloric** (2) **and sulfuric** (3) **acids** (expressed as dry matter)**:** a — pine, first wave; b — beech, first wave; c — pine, second wave; d – beech, second wave

The analysis of the research papers shows that numerous technologies are developed for *G. lucidum* and *F. velutipes* the cultivation [18]. At the same time, the edible fungus *H. erinaceus*, which has medicinal properties, is a valuable food product and a source of biologically active substances, such as immunomodulating and antitumor polysaccharides, antioxidants, and neuroactive compounds [24, 26, 33].

For the entire period of the experiment on the untreated sawdust of pine and beech, we got only one wave of the *H. erinaceus* fruit bodies which have reached the commercial maturity after 63 days of cultivation (Table 2). The acid pretreatment led to significant acceleration of fruiting of *H. erinaceus*. During the 2-month experiment, on the substrates containing all variants of the pretreated sawdust, except for beech sawdust pretreated with sulfuric acid, there were two fruiting waves. The fruit bodies of the first and second waves were harvested at commercial maturity on Day 35 and Day 63, respectively. The increase of the yield compared to controls occurred on pine sawdust pretreated with hydrochloric acid ($p \leq 0.05$) (Fig. 2, 3).

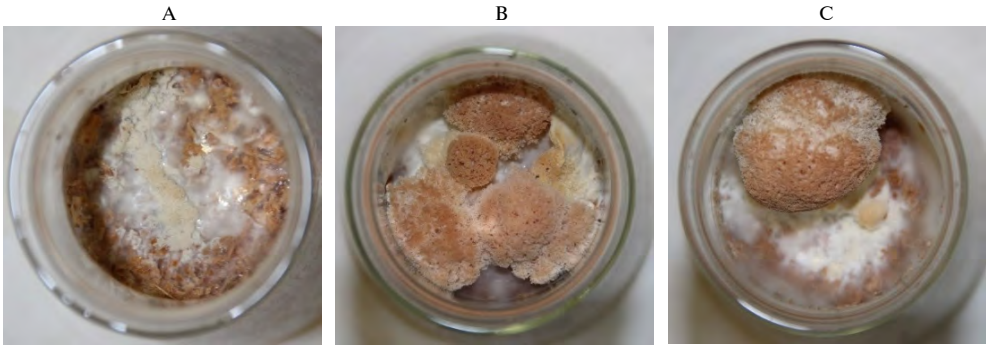

Fig. 3. *Hericium erinaceus* growth on Day 35 upon different chemical pretreatment of the substrates for cultivation: A — mycelium on the untreated pine sawdust, B — fruit bodies on pine sawdust pretreated with hydrochloric acid, C — fruit bodies on pine sawdust pretreated with sulfuric acid.

Perhaps this is due to the fact that chloride anions are able to break the intermolecular hydrogen bonds of lignocellulosic complex and to increase lignin bioavailability [34]. Despite the fact that as a result of the pretreatment of pine sawdust with sulfuric acid two waves of fruiting have been gotten, the total yield turned to be comparable to the yield of the first wave on the untreated substrate.

At the final stage, we compared the contents of proteins and polysaccharides in fruit bodies of *H. erinaceus*. The pretreatment of the pine sawdust with sulfuric acid reduced mass fraction of proteins from 13.5 to 6.8% and of poly-

saccharides from 41.6 to 23.9% during the first wave (Table 3). On the pine sawdust pretreated with hydrochloric acid and on the untreated pine sawdust, the fruit bodies had the statistically significantly equal content of proteins and polysaccharides ($p > 0.05$). The pretreatment of beech sawdust with hydrochloric acid led to almost 2-fold decrease of the proteins content in fruit bodies as compared to the control, however, the polysaccharide content was maximum on this substrate. It has been established that the fruit bodies of the second wave differed from the basidiomas of the first wave in the higher content of proteins. Such trend has not been yet described in special literature and requires further study.

2. Fruiting of *Hericium erinaceus* as depends on chemical pretreatment of the wood substrates

Substrate	Pretreatment	Commercial maturity, days	
		first wave	second wave
Pine	Control	63	–
	HCl, 2 %	35	63
	H_2SO_4, 2 %	35	63
Beech	Control	63	–
	HCl 2, %	35	63
	H_2SO_4, 2 %	35	–

N o t e. The commercial maturity is the stage at which fruit bodies shall be harvested for sale. "–" means no fruiting.

3. The contents of proteins and polysaccharides in *Hericium erinaceus* fruit bodies as depend on chemical pretreatment of the wood substrates (M±SEM)

Substrate	Pretreatment	The first wave		The second wave	
		proteins, %	polysaccharides, %	proteins, %	polysaccharides, %
Pine	Control	12.7±0.4	41.6±3.9	–	–
	HCl, 2 %	12.0±2.2	42.5±5.3	16.0±2.5	24.3±1.6
	H_2SO_4, 2 %	9.7±3.9	23.9±3.4	16.4±0.5	16.0±1.3
Beech	Control	13.5±0.5	35.4±0.9	–	–
	HCl, 2 %	6.8±1.2	43.6±2.3	12.7±1.7	29.7±1.9
	H_2SO_4, 2 %	12.4±0.6	29.4±0.1	–	–

N o t e. "–" means no fruiting.

Our results shows that, for developing the compositions of new substrates for solid-phase cultivation and for growing the fruit bodies of *H. erinaceus*, the pretreatment of raw materials with hydrochloric acid is the most effective that makes it possible to increase the yield capacity, to accelerate the process of getting the basidiomas and to use pine sawdust for *H. erinaceus* cultivation. Further experiments will be aimed at studying the influence of the pretreatment with hydrochloric acid of sawdust of other coniferous trees on the *H. erinaceus* growth and fruiting. The pretreatment of sawdust from deciduous trees is not practical because it does not lead to the increase in biotechnological and biochemical parameters.

Thus, the acid pretreatment declines the level of easily hydrolyzable carbohydrates in the sawdust of pine and beech. The alkaline pretreatment contributes to the partial removal of lignin and thereby increases the content of total carbohydrates. All types of chemical pretreatment lead to an increase in the growth rate and density of *Ganoderma lucidum* mycelium. The bioavailability of sawdust for *Flammulina velutipes* and *Hericium erinaceus* is higher after the acid pretreatments. At that, the highest growth rate and density of the mycelium occur on sawdust substrates pretreated with hydrochloric acid. The acid pretreatment of sawdust shortens the time until fruiting in *H. erinaceus* from 63 days to 35 days compared to the untreated sawdust. A significant increase in yield capacity ($p \leq 0.05$) was typical for *H. erinaceus* on the substrates containing the pine sawdust pretreated with hydrochloric acid. The substrate containing beech sawdust pretreated with sulfuric acid decreases the yield. The fruit bodies of the second wave are higher in protein content and lower in polysaccharides as com-

pared to the first wave fruit bodies.

REFERENCES

1. Ingrao C., Bacenetti J., Bezama A., Blok V., Geldermann J., Goglio P., Koukios E., Lindner M., Nemecek M., Siracusa V., Zabaniotou A., Huisingh D. Agricultural and forest biomass for food, materials and energy: bio-economy as the cornerstone to cleaner production and more sustainable consumption patterns for accelerating the transition towards equitable, sustainable, post fossil-carbon societies. *Journal of Cleaner Production*, 2016, 117: 4-6 (doi: 10.1016/j.jclepro.2015.12.066).
2. Gokhberg L., Kuzminov I., Chulok A., Thurner T. The future of Russia's agriculture and food industry between global opportunities and technological restrictions. *International Journal of Agricultural Sustainability*, 2017, 5(4): 457-466 (doi: 10.1080/14735903.2017.1335572).
3. Grebner D.L., Grala R.K., Joshi O., Perez-Verdin G. Physical and economic aspects to assessing woody biomass availability for bioenergy production and related supply constraints. In: *Handbook of bioenergy*. S. Eksioglu, S. Rebennack, P. Pardalos (eds.). Springer, Cham, 2015: 299-321 (doi: 10.1007/978-3-319-20092-7_13).
4. Latva-Mäenpää H. *Bioactive and protective polyphenolics from roots and stumps of conifer trees (Norway spruce and Scots pine)*. Unigrafia Oy, Helsinki, 2017.
5. Ogawa M., Okimori Y. Pioneering works in biochar research, Japan. *Soil Research*, 2010, 48(7): 489-500 (doi: 10.1071/SR10006 489-5006).
6. Jylhä P. *Harvesting of undelimbed Scots pine (Pinus sylvestris L.) from first thinnings for integrated production of kraft pulp and energy. Dissertationes Forestales 133.* Helsinki, 2011 (doi: 10.14214/df.133).
7. Ewanick S.M., Bura R., Saddler J.N. Acid-catalyzed steam pretreatment of lodgepole pine and subsequent enzymatic hydrolysis and fermentation to ethanol. *Biotechnology and Bioengineering*, 2007, 98(4): 737-746 (doi: 10.1002/bit.21436).
8. Jin S., Zhang G., Zhang P., Li F., Fan S., Li J. Thermo-chemical pretreatment and enzymatic hydrolysis for enhancing saccharification of catalpa sawdust. *Bioresource Technology*, 2016, 205: 34-39 (doi: 10.1016/j.biortech.2016.01.019).
9. Lai C., Yang B., He J., Huang C., Li X., Song X., Yong Q. Enhanced enzymatic digestibility of mixed wood sawdust by lignin modification with naphthol derivatives during dilute acid pretreatment. *Bioresource Technology*, 2018, 269: 18-24 (doi: 10.1016/j.biortech.2018.08.086).
10. Cuilty K.R., Ballinas-Casarrubias L., de San Miguel E.R., de Gyves J., Robles-Venzor J.C., González-Sánchez G. Cellulose recovery from *Quercus* sp. sawdust using Ethanosolv pretreatment. *Biomass and Bioenergy*, 2018, 111: 114-124 (doi: 10.1016/j.biombioe.2018.02.004).
11. Al'myasheva N.R., Novikov A.A., Kozhevnikova E.Yu., Golyshkin A.V., Barkov A. V., Vinkurov V.A. Ethanol production from lignocellulosic biomass using xylotrophic basidiomycetes. *Chemistry and Technology of Fuels and Oils*, 2015, 51(5): 516-525 (doi: 10.1007/s10553-015-0633-6).
12. Kim T.H., Choi C.H., Oh K.K. Bioconversion of sawdust into ethanol using dilute sulfuric acid-assisted continuous twin screw-driven reactor pretreatment and fed-batch simultaneous saccharification and fermentation. *Bioresource Technology*, 2013, 130: 306-313 (doi: 10.1016/j.biortech.2012.11.125).
13. Mosier N., Wyman C., Dale B., Elander R., Lee Y.Y., Holtzapple M., Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, 2005, 96(6): 673-686 (doi: 10.1016/j.biortech.2004.06.025).
14. Galbe M., Zacchi G. Pretreatment of lignocellulosic materials for efficient bioethanol production. In: *Biofuels. Advances in biochemical engineering/biotechnology, Vol. 108*. L. Olsson (ed.). Springer, Berlin, Heidelberg, 2007: 41-65 (doi: 10.1007/10_2007_070).
15. Sun S., Sun S., Cao X., Sun R. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresource Technology*, 2016, 199: 49-58 (doi: 10.1016/j.biortech.2015.08.061).
16. Okamoto K., Uchii A., Kanawaku R., Yanase H. Bioconversion of xylose, hexoses and biomass to ethanol by a new isolate of the white rot basidiomycete *Trametes versicolor*. *SpringerPlus*, 2014, 3(1): 121 (doi: 10.1186/2193-1801-3-121).
17. Aita G.A., Salvi D.A., Walker M.S. Enzyme hydrolysis and ethanol fermentation of dilute ammonia pretreated energy cane. *Bioresource Technology*, 2011, 102(6): 4444-4448 (doi: 10.1016/j.biortech.2010.12.095).
18. Stamets P. *Growing gourmet and medicinal mushrooms*. Ten Speed Press, California, USA, 2000.
19. Rodriguez Estrada A.E., Royse D.J. Yield, size and bacterial blotch resistance of *Pleurotus eryngii* grown on cottonseed hulls/oak sawdust supplemented with manganese, copper and whole ground soybean. *Bioresource Technology*, 2007, 98(10): 1898-1906 (doi: 10.1016/j.biortech.2006.07.027).
20. Miller G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 1959, 31(3): 426-428 (doi: 10.1021/ac60147a030).

21. Atila F., Tüzel Y., Faz Cano A., Fernandez J.A. Effect of different lignocellulosic wastes on *Hericium americanum* yield and nutritional characteristics. *Science of Food and Agriculture*, 2017, 97(2): 606-612 (doi: 10.1002/jsfa.7772).
22. Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 1956, 28(3): 350-356 (doi: 10.1021/ac60111a017).
23. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 1976, 72(1-2): 248-254 (doi: 10.1016/0003-2697(76)90527-3).
24. Avtonomova A.V., Bakanov A.V., Leont'eva M.I., Vinokurov V.A., Usov A.I., Krasnopol'skaya L.M. *Antibiotiki i khimioterapiya*, 2012, 57(7-8): 7-11 (in Russ.).
25. Avtonomova A.V., Krasnopol'skaya L.M. *Mikologiya i fitopatologiya*, 2013, 47(1): 3-11 (in Russ.).
26. Al'myasheva N.R., Yarina M.S., Golyshkin A.V., Dzhavakhyan B.R., Krasnopol'skaya L.M. *Antibiotiki i khimioterapiya*, 2017, 62(7-8): 8-12 (in Russ.).
27. Krasnopol'skaya L.M., Yarina M.S., Avtonomova A.V., Usov A.I., Isakova E.B., Bukhman V.M. *Antibiotiki i khimioterapiya*, 2015, 60(11-12): 29-34 (in Russ.).
28. Krasnopol'skaya L.M., Shuktueva M.I., Avtonomova A.V., Yarina M.S., Dzhavakhyan B.R., Isakova E.B., Bukhman V.M. *Antibiotiki i khimioterapiya*, 2016, 61(11-12): 16-20 (in Russ.).
29. Räisänen T., Athanassiadis D. *Basic chemical composition of the biomass components of pine, spruce and birch*. BioFuel Region AB, 2013. Available http://biofuelregion.se/wp-content/uploads/2017/01/1_2_IS_2013-01-31_Basic_chemical_composition.pdf. Accessed 19.05.2019.
30. Jönsson L. J., Martín C. Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, 2016, 199: 103-112 (doi: 10.1016/j.biortech.2015.10.009).
31. McMillan J.D. Pretreatment of lignocellulosic biomass. *ACS Symposium Series*, 1994, 556(15): 292-324 (doi: 10.1021/bk-1994-0566.ch015).
32. Sun Q., Foston M., Meng X., Sawada D., Pingali S.V., O'Neill H.M., Li H., Wyman C.E., Langan P., Ragauskas A.J., Kumar R. Effect of lignin content on changes occurring in poplar cellulose ultrastructure during dilute acid pretreatment. *Biotechnology for Biofuels*, 2014, 7(1): 150 (doi: 10.1186/s13068-014-0150-6).
33. Friedman M. Chemistry, nutrition, and health-promoting properties of *Hericium erinaceus* (Lion's Mane) mushroom fruiting bodies and mycelia and their bioactive compounds. *Journal of Agricultural and Food Chemistry,* 2015, 63(32): 7108-7123 (doi: 10.1021/acs.jafc.5b02914).
34. Foston M., Ragauskas A. J. Biomass characterization: recent progress in understanding biomass recalcitrance. *Industrial Biotechnology*, 2012, 8(4): 191-208 (doi: 10.1089/ind.2012.0015).

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TOXIN-PRODUCING FUNGI OF THE GENUS *Penicillium* IN COARSE FODDERS

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Abstract

The search for toxin-producing microscopic fungi, affecting plant and animals products, food and feed continues to be relevant in scientific research. A long-term study of this problem has shown that the threat of the occurrence of toxicoses of humans and animals is associated mainly with micromycetes of genus *Fusarium*, *Aspergillus* and *Penicillium* (CAST, 1989). In coarse feed, which form the basis of the ration of ruminants, these fungi have a leading position. Among *Fusarium* fungi the highly toxic species *F. sporotrichioides* dominates in hay and straw (E.A. Piryazeva et al., 2016), and 7 species of *Aspergillus* are capable to contaminate that with CPA, STE and MPA (G.P. Kononenko et al., 2017). The purpose of this work was to elucidate the toxin-forming potential of 11 species of the genus *Penicillium* fungi which prevail in mycobiota of coarse feed. The strains were cultured for 7 days at 25 °C on a panel including Czapek Dox agar (CDA), wort agar (WA), Czapek Yeast Autolysate Agar (CYA), yeast extract sucrose agar (YES) and moistened rice grain (RG). Further, in the extracts, the amounts of ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR-toxin (PR), cyclopiazonic acid (CPA), emodin (EMO) and ergot alkaloids (EA) were determined by indirect competitive enzyme-linked immunosorbent assay (ELISA) using certified commercial and research test systems. On the basis of obtained results, the species *P. aurantiovirens*, *P. palitans*, as well as *P. martensii* and *P. meleagrinum* are classified as non-producing. Among the representatives of *P. cyclopium*, we found weak producers of MPA and CIT, as well as isolates that do not form any of the analyzed mycotoxins. High accumulation (10 μg/g and more) is revealed in *P. brevicompactum*, *P. stoloniferum* (MPA), *P. roqueforti* (PR + MPA), *P. chrysogenum* (PR) and average level (1-10 μg/g) is characteristic of *P. urticae* (CPA) and *P. expansum* (CIT). Isolates lacking production capacity were found only among *P. chrysogenum* and *P. expansum*, toxin production by other species was stable. In the representatives of *P. roqueforti*, producing jointly PR and MPA, the amount of MPA was, as a rule, less than PR. EMO, OA and EA in the metabolites of fungi were not found. In this paper it was shown for the first time that the species *P. expansum*, *P. brevicompactum*, *P. stoloniferum*, *P. roqueforti*, *P. chrysogenum* and *P. urticae* from a typical complex of fungi of this genus can be related to extensive and intensive contamination of coarse fodder by CIT, MPA, PR and CPA. It has been established that the use of a growth media panel for testing the toxicity of *Penicillium* fungi is an indispensable technique for an exhaustive assessment of their potential, and, in addition to agar media, it is expedient to use substrates of plant origin. Virtually all producing species had the highest intensity of toxin accumulation on RG, and for *P. chrysogenum*, PR production could be detected only on this substrate. Specific features of the profile of toxic metabolites in isolates belonging to one species are discussed, as well as the problem of the possible contribution of other species of *Penicillium* to the contamination of coarse fodder by mycotoxins.

Keywords: mycobiota, feeds, *Penicillium* fungi, mycotoxins

The search for mycotoxin producers among microscopic fungi which affect food and forage crops, animal products, food stuffs and feeds is still topical in scientific researches [1, 2]. The greatest threat of toxicoses occurrence is related to the micromycetes of the *Fusarium, Aspergillus* and *Penicillium* genera [3, 4]. In roughage fodder which forms the basis of the ration of ruminants the fungi of these taxa have a leading position, however, the diversity of the botanical

composition of herbages and differences in storage conditions after the harvesting imply the conduction of regional surveys with the evaluation of the individual toxin-forming ability of the species being a part of the mycobiota.

The world's data on this issue are very poor. In Russia, the data on mycological analysis of the samples from production batches of hay and straw harvested in livestock farms of the Bryansk, Moscow and Chelyabinsk regions and subsequent testing of taxonomically classified isolates show that among *Fusarium* fungi the highly toxic *F. sporotrichioides* species dominates [5], and the complex consisting of 7 *Aspergillus* species may be related to the contamination of the fodder with cyclopiazonic acid, sterigmatocystin and mycophenolic acid [6]. According to the data of mycological examination of the production batches of hay and straw harvested in the Moscow and Belgorod regions in 2011 and 2013, more than half of the samples were contaminated with *Penicillium* fungi which belong to 28 species from five sections of this genus, 11 species of were being found with the frequency of 3.8-30.8% (7).

In this paper, for the first time, we showed that the *P. brevicompactum, P. stoloniferum*, *P. roqueforti*, *P. chrysogenum*, *P. urticae*, and *P. expansum* species belonging to *Penicillium* genus may be related to the extensive and intensive contamination of roughage fodder with mycophenolic acid, PR toxin, cyclopiazonic acid and citrinin.

Our objective was to investigate the toxin-producing ability of *Penicillium* species predominating in roughage fodder mycobiota in the experimental conditions ensuring the most complete performance of biosynthetic capabilities of these fungi.

Techniques. We studied 55 *Penicillium* isolates of *P. aurantio-virens* Biourge, *P. brevi-compactum* Dierckx, *P. chrysogenum* Thom, *P. cyclopium* Westling, *P. expansum* Link, *P. martensii* Biourge, *P. notatum* Westling, *P. palitans* Westling, *P. roqueforti* Thom, *P. stoloniferum* Thom, *P. urticae* Bainier species, which have been isolated from the roughage fodder harvested in the livestock farms of the Bryansk and Moscow regions [7], as well as 134 strains of the same species from the Collection of the Feed Mycotoxicology and Sanitation Laboratory of the All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology. The identification of the species was based on the cultural and morphological properties according to the taxonomic system [8] with the use of species epithets from the up-to-date nomenclature database of the MycoBank (http://www.mycobank.org/) and with the following changes: *P. aurantiovirens* (=*P. aurantiovirens*), *P. brevi-compactum* (=*P. brevicompactum*) и *P. notatum* (=*P. meleagrinum* Biourge) [9].

The evaluation of toxin formation included the preparation of the inoculum and substrate, inoculation, culture, extraction, and analysis of mycotoxins. The 10-day-old cultures on Czapek-Dox agar (CDA, HiMedia Laboratories Pvt., Ltd., India) were used for inoculums. Approximately equal size pieces taken from the agar surface with the mycological hook was placed in 3 replicates on the grain substrate or on solid culture media in 15 ml glass bottles with the bottom diameter of about 18 mm. The sterile crushed rice (1 g) previously moistened with 1 ml of water was a grain substrate (RG). The CDA, wort agar (WA, Liofilchem, Italy), as well as Czapek Yeast Autolysate (CYA) and yeast extract sucrose agar (YES) [10] prepared of the commercial components (HiMedia Laboratories Pvt., Ltd, India) were used as growth media (1.5 ml each).

After adding of the inoculum, the bottles were closed with cotton-gauze plugs, which were wrapped with a laboratory film (Parafilm "M"® PM-996, Pechiney Plastic Packaging, USA). The culturing was performed in the dark for 7 days at 25 °C. Then the mixture of acetonitrile and water (84:16 v/v) was add-

ed to each bottle and intensively shaken at the beginning and in the end of the 14-hour stationary extraction. The content of ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), cyclopiazonic acid (CPA), PR toxin (PR), emodin (EMO) and ergoalkaloids (EA) in the extracts was determined by the enzyme-linked immunosorbent assay (ELISA) using certified test systems [8], the lower limits of detection corresponded to 85% antibody binding.

The data were processed using Microsoft Excel 2013 for descriptive statistics, the results were expressed as the absolute content of mycotoxin or as the arithmetic means of the obtained values (*M*) with a standard error of the mean (±SEM).

Results. In recent years, a polyphase approach has been widely used in the systematics of microscopic fungi. Along with the morpho-cultural and molecular-genetic characterization, this approach takes account of biochemical properties. There is a quite large database on the *Penicillium* fungi profile of secondary metabolites jointly called "extrolites" which include physiologically active substances and mycotoxins [12-15]. However, detecting of the substances in a semi-quantitative assessment mode visually or using instrumental physicochemical methods does not make it possible to conclude about the intensity of their biosynthesis and about the completeness of the obtained information. In this regard, at the preparatory stage of the work, we quantified toxin formation in a standard experiment on WA with collection cultures of 11 the most abundant species of roughage fodder mycobiota from *Penicillium* genus isolated from different subjects (Table 1).

1. Toxin formation in the collection strains of 11 species of the *Penicillium* fungi (WA, 23 °C, 7 days)

Penicillium species (*n*)	Mycotoxin	n^+ (mycotoxin content min-max, μg per 1 g of the substrate)
P. aurantiovirens (12)	CIT	4 (0.1-0.3)
P. brevicompactum (7)	CIT	7 (40-440)
P. chrysogenum (8)	PR	4 (0.8-40)
	EMO	2 (0.1; 0.6)
P. cyclopium (24)	PR	1 (0.7)
	CIT	3 (30, 40, 50)
P. expansum (3)	CIT	2 (13, 16)
P. martensii (24)	CPA	1 (0.6)
P. meleagrinum (3)	–	–
P. palitans (11)	–	–
P. roqueforti (16)	PR + CIT	7 (35-135)+(0.2-25)
	PR	1 (20)
P. stoloniferum (3)	CIT	3 (2, 22, 76)
P. urticae (23)	CPA	17 (0.2-4)
	CIT	1 (40)

N o t e. WA — wort agar; *n* — number of investigated strains, n^+ — number of producers; CIT — citrinin, MPA — mycophenolic acid, CPA — cyclopiazonic acid, EMO — emodin, PR — PR toxin; "–" means that no producers were found.

In all species except *P. meleagrinum* and *P. palitans*, the producers of CIT, MPA, CPA and EMO have been found. However, only in *P. brevicompactum* and *P. stoloniferum* showed a complete realization of the potential; in other species, the strains which are unable for the production were found: CIT was found only in four of 12 *P. aurantiovirens* strains, and CPA only in one of 24 *P. martensii* strains (see Table. 1). The producers of different toxins were identified among *P. chrysogenum, P. cyclopium* and *P. urticae*; most of the *P. roqueforti* strains synthesized both PR and MPA, and one strain only PR. This indicates the existence in these species of intraspecific chemotypes or subspecies with the specific profile of mycotoxins [13] or of atypical forms with specific features of metabolic ways.

In order to assess the toxin formation potential of fungi isolated from roughage fodder, groups were formed, 5 cultures of each of 11 species per group,

and a panel of 5 culture media was composed. Given our previous data on metabolic response of fungi isolated from the same subjects, we used WA and RG taken for fungi of the *Fusarium* genus [5], and CDA that we earlier applied for testing *Aspergillus* [6]. Among the solid media recommended for growth and identification of *Penicillium* [10, 12-15], we chose CYA and YES on which two *P. stoloniferum* strains from the Collection showed more accumulation of MPA (38±8 and 73±8 μg/g, 230±36 and 305±22 μg/g, respectively) than on the agar with the malt extract (MEA) (2.0±0.4 and 22±3 μg/g).

In these experiments, we did not find any toxin formation in *P. aurantiovirens* (n = 5) and *P. palitans* (n = 5) isolates. This result was expected because the collection strains of the first type had extremely weak production of CIT, and the strains of the second type did not produced CIT at all (see Table 1). Nevertheless, it is important to note that there are many papers on taxonomy which report the ability of *P. palitans* to synthesize CPA [15], therefore, we should admit the probability of some uncertainty in the identification of this species basing on morphological properties. In *P. martensii* (n = 5) and *P. meleagrinum* (n = 5) we could determine CIT only sporadically on WA and RG in the amounts less than 1 μg/g, and we have not found CPA, although it has been found in small amount in one of 24 collection strains of *P. martensii* (see Table. 1). In *P. cyclopium*, the toxin formation was also weakly expressed and multivariate. Particularly, on CDA, WA, and RG one isolate produced small amounts of MPA (0.3-1.0 μg/g), while other isolate produced CIT (0.03-0.8 μg/g), moreover, according to data of chemotaxonomic researches, this species is not capable of biosynthesis of the analyzed mycotoxins at all [15]. The results indicated that the contribution of these five species (*P. aurantiovirens*, *P. cyclopium*, *P. martensii*, *P. meleagrinum*, and *P. palitans*) to contamination of roughage fodder with the sanitarily significant mycotoxins is inconsiderable.

In the rest six species (*P. brevicompactum*, *P. stoloniferum*, *P. roqueforti*, *P. chrysogenum*, *P. urticae*, and *P. expansum*), high production of MPA and PR (more than 10 μg/g) and medium production of CPA and CIT (up to 10 μg/g) has been confirmed (Table. 2). As expected, *P. brevicompactum* and *P. stoloniferum* were characterized by stable biosynthesis of MPA; no other toxins from the list of analyzed ones have been found. The significant intensity of accumulation of this toxin allowed us to attribute these isolates to highly active producers and highly probable sources of feed contamination [16].

It has been confirmed that all isolates of *P. roqueforti* from roughage fodder are able to produce PR in combination with MPA (see Table 2), which is deemed a hallmark of the *P. roqueforti* var. *roqueforti* subspecies [13]. The reaction of the tested cultures to the growth medium was very peculiar. On the depleted CDA substrate, the intensity of PR accumulation was significantly higher than that on WA, although it was previously reported that the isolates of this species from silage feeds had almost no differences in toxin formation on CDA and WA, and MPA were also found in much smaller amounts than PR [17].

The reason of this discrepancy could well be the using in the said work of the substrate prepared of beer wort in laboratory conditions, and the observed sharp increase in biosynthesis of PR on the analogue of a commercial standardized medium undoubtedly deserves attention. The metabolic response of the *P. roqueforti* isolates to CYA and YES generally was weakly expressed and similar in terms of the proportion of PR and MPA amounts. The differences between the strains have been noted only on CYA, and on the other solid media were insignificant. Nevertheless, recently, when testing for YES with visual TLC detection, the strains of *P. roqueforti* from different cheese varieties, which jointly produce PR and MPA, showed significant differences in the intensity of PR

accumulation and minor differences in MPA [18]. It is quite possible that the morphologically identical cultures from different subjects have genetically determined features and, as a consequence, show dissimilar metabolic responses to changes in growth conditions.

2. Toxin formation in isolates of *Penicillium* fungi species from roughage fodder on solid media and on the rice grain substrate (25 °C, 7 days)

Mycotoxin	Isolate No.	Mycotoxin concentration, μg/g substrate (M±SEM)				
		CDA	WA	CYA	YES	RG
		P. brevicompactum (n^+/n = 6/6)				
MPA	40/2	6.0±0.9	6,0±1,1	76±15	110±20	124±25
	181/1	9.0±1.8	17±3	34±7	174±35	302±70
	254/1	8.0±1.6	15±3	28±6	100±18	169±32
	4/3	–	128±30	126±32	120±18	722±101
	340/1	–	81±10	144±25	192±25	1117±91
	16/1	–	53±8	19±4	72±8	792±19
		P. chrysogenum (n^+/n = 4/5)				
PR	172/1	0	0	0	0	14±3
	373/1	0	0	0	0	13±2
	592/1	0	0	0	0	5,7±1,3
	639/5	0	0	0	0	2,0±0,4
		P. expansum (n^+/n = 1/5)				
CIT	88/4	5.0±0.8	3.0±0.5	7.0±1.7	3.3±0.6	11±2)
		P. roqueforti (n^+/n = 5/5)				
PR + MPA (the values are separated with slash)	88/2	50±9/ 1±0	2.8±0.5/ 3.0±0.5	4.3±0.7/ 3.1±0.6	5.7±1.8/ 0.13±0.02	150±24/ 63±13
	118/1	45±8/ 1±0	0.9±0.2/ 2.5±0.5	1.7±0.3/ 0.9±0.2	2.0±0.3/ 0.13±0.02	95±14/ 48±8
	648/5	48±9/ 0.2±0.0	10±2/ 3.3±0.6	6.7±1.0/ 4.7±0.9	3.0±0.4/ 0.07±0.01	780±72/ 67±14
	393/1	29±)/ 0.3±0.1	2.1±0.4/ 5.3±1.0	50±9/ 8.7±1.5	4.7±0.8/ 3.7±0.6	329±43/ 98±17
	557/1	22±3/ 0	4.7±0.6/ 2.7±0.4	34±6/ 3.4±0.7	2.3±0.4/ 0.1±0.02	224±38/ 55±8
		P. stoloniferum (n^+/n = 5/5)				
MPA	631/4	0	11±2	43±5	64±13	100±20
	631/3	0	14±4	41±10	91±10	175±39
	317/4	10±2	193±39	115±22	151±30	1330±250
	602/1	5.7±1.7	24±5	40±6	272±54	332±60
	602/3	0	9±2	23±4	110±20	145±22
		P. urticae (n^+/n = 5/5)				
CPA	349/1	1±0	0.1±0	5.7±0.6	2.3±0.5	4.0±0.8
	584/4	2.0±0.2	0.1±0	12±2	1±0	10±2
	201/1	2±0	0.1±0	7±1	3.3±0.6	12±1
	216/5	0	0	0.43±0.07	0.23±0.05	0.37±0.07
	434/4	2±0	0.33±0.06	5.3±0.6	5±0	6.7±1.3

N o t e. CDA — Czapek-Dox agar, WA — wort agar; CYA — Czapek yeast autolysate agar; YES — yeast extract sucrose agar, RG — rice grain; n — number of investigated strains. n^+ — number of producers; CIT — citrinin, MPA — mycophenolic acid, CPA — cyclopiazonic acid, PR — PR toxin; "–" means that the mycotoxin detection was not performed.

All representatives of *P. urticae* demonstrated the ability to synthesize CPA although with the accumulation of the amount less than 10 μg/g. This is consistent with the data of TLC testing (synthetic medium, 28 °C, 8 days) of 13 isolates of the same species (isolated from dried beans and macaroni products), which also turned out to be the producers of this toxin [19]. In accordance with the new taxonomic approach, *P. urticae* is deemed the synonymous of *P. griseofulvum* Dierckx [14], and CPA is deemed one of the specific metabolic markers [15].

In *P. chrysogenum* and *P. expansum* species, the full realization of the potential has not been noted. All members of *P. chrysogenum*, except for one, produced PR in a wide quantitative range and only on the grain substrate (see Table 2). The absence of toxins formation on the wide panel of solid media was unexpected, because in the aforementioned work [12] it was reported that, on CYA and YES, 50-80% isolates of this species of 87 investigated ones are capable of PR biosynthesis, and most strains from the All-Russian Collection of Mi-

croorganisms of the Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS formed this toxin although in small amounts [17].

Only one of the five *P. expansum* strains synthesized CIT on all media (see Table 2); an analogous partial production was noted when testing the collection strains on WA (see Table 1). Apparently, "zero" chemotypes of this fungus species are found in fodder subjects quite often. However, it should be noted that when TLC screening (CYA, YES) of *P. expansum* from different sources, CIT was detected in almost all strains of different origin (95-99%, $n = 91$) [9].

The obtained results show that the choice of growth media panel is necessary for a comprehensive evaluation of toxin formation in microscopic fungi, and using of standardized commercial media for investigating biochemical processes is rather necessary than preferable. A particular attention should be paid to the qualitative differences in the fungal response when changing the growth medium (for example, as in case of *P. roqueforti* and *P. chrysogenum*). In recent years, the structure of the genome regions which are responsible for the synthesis of toxic metabolites has been investigated in detail for these species, and the important role of the extra-cluster regulators, the activity of which is determined by growth conditions, in particular, by the properties of growth media, has been shown [21-23]. In our experiments with the fungi of *Penicillium* genus, more active accumulation of MPA, PR, CPA, and CIT occurred on the rice grain substrate. Only on this substrate, four of five *P. chrysogenum* strains produced PR which is considered one of its chemotaxonomic markers. Expanding the range of natural substrates will make it possible in the future to create the analogous formulations with controlled composition which will ensure the maximum realization of the metabolic potential of fungi.

The data set forth in the work testify that the *P. brevicompactum*, *P. stoloniferum*, *P. roqueforti*, *P. chrysogenum*, *P. urticae*, and *P. expansum* species can be related to an extensive and intensive contamination of roughage fodder with MPA, PR, CPA and CIT [24]. However, it is known that the fungi of this genus, when living in anthropogenically disturbed and extreme conditions, can form morphologically modified phenotypes of an adaptive nature [25]. Thus, when testing some strains of *P. cyclopium* and *P. urticae* on WA, we found the highly active producers of a metabolite which is atypical, the MPA (see Table 1). These producers were isolated from caked layers of crumbled feed affected by long-term self-heating. In the described case, it is quite possible that the species attribution based on micro- and macromorphological characters will be wrong.

Moreover, the participation of other representatives of the *Penicillium* genus can hardly be completely excluded, because the species which have been selected for the assessment as the most represented in the mycobiota comprise only less than half of their total number. In particular, for the isolate attributed to the *P. steckii* Zaleski (=*P. steckii* K.M. Zalesski) [9], (=*P. citrinum* Thom) [26] species rarely occurred in such feed the active production of CIT has been shown (our own unpublished data).

The mycotoxins frequently occurred in roughage feed, i.e. OA, EMO, and EA, have not been found in the tested isolates. Nevertheless, among the rarely occurred species, we have identified P. viridicatum [7] which includs, as it has been proved, several lines (in the status of a species or subspecies) with distinct differences in toxin formation. Some of these are P. verrucosum and P. nordicum which produce OA [27]. The frequent detection and high content of EMO in feed still have no satisfying explanation in the mycological aspect. The arguments for attribution of EMO to the associated metabolites of higher plants and of specific endophytic fungi prevail [28], although several species of the *Penicillium* genus, in particular *P. islandicum*, *P. brunneum*, *P. janthinellum*, and

P. herquei, have been attributed to the producers of this metabolite [29]. The sources of extensive contamination of roughage feed with EA also remain unknown. Among the taxa classified as non-traditional sources of peptide EA, only the imperfect fungi of the *Aspergillus*, *Botrytis*, *Curvularia* and *Geotrichum* genera, lower fungi (*Cinnighamella blakesleana, Mucor hiemalis* and *Rhyzopus* spp.), as well as the grass endophytes belonging to ascomycetes (*Balansia* spp., *Epichloe typhina*, *Hypomyces aurantius*, *Sepedonium* sp.) are named [30]. However, recently in an isolate from mixed cereal-bean hay harvested in 2005 in the Perm Territory, which has been identified as *P. palitans* according to cultural properties, the active accumulation of these metabolites (2.7±0.2 μg/g) on WA has been detected (our own unpublished data).

It cannot be ruled out that the *Penicillium* fungi are related to the contamination of such feeds also with other toxins from the list of frequently occurred ones. Thus, in the separate experiment, alternariol was found in small amounts in the isolate of *P. steckii* and two isolates of *P. urticae* grown on MEA, CYA, and YES (our own unpublished data). It was also previously reported about the ability of *P. coprophilum* to synthesize this toxin [15].

The important outcome of the presented work is the understanding that the improvement of the methodology of evaluating the toxins formation in microscopic fungi must be continued. For many decades, the science has accumulated the rich experience in the study of toxins formation in microscopic fungi, but the information about the potential of the populations which vital activity is related to agricultural plants is still very poor, and the available experimental data are often the subject of incorrect interpretations and conclusions. One of the main reasons of it is the underestimation of the complexity of this problem and the absence of a generally accepted methodology of conducting the research works. Toxin-producing ability should be evaluated under unified conditions to make the results comparable [31]. In recent years, the simple technology of short-term growing on nutrient substrates with screening analysis ensuring wide diapason of measurement is gaining the increasing recognition. The distinction level of 10 μg/g and relevant terms, i.e. “weak producers” with less accumulation and “highly active producers” with accumulation above the said threshold value, have already been proposed to differentiate isolates for their intensity of toxin formation [32]. The ratio of the producers number to the total number of investigated strains denoted as n^{+}/n (in percent) is usually called the toxin formation potential in the analyzed aggregate of isolates, and the accumulation diapason denoted as min-medium-max (μg/g) is called the toxin formation intensity. The term “toxin formation pattern” may be applied to identify qualitative differences in metabolic profiles within a set of producers.

The *Penicillium* fungi prevailing in the composition of roughage feed mycobiota produce the mycotoxins and thus can suppress microbial activity in ruminants, which leads to serious violations of the rumen function and provoke animal intoxication [33]. In addition, microscopic fungi along with bacteria and protozoa actively participate in assimilation of polysaccharides of the fodder plant cell walls. Recently it was shown that fungi of the *Aspergillus* and *Penicillium* genera, one of which is *P. brevicompactum* [34], dominate in the partially digested cellulose in cow abomasum. The consequences of long-term anaerobic habitation of toxigenic fungi in the digestive tract of such animals at each of the four successive stages may be very dangerous and need to be studied in details.

Thus, six species of *Penicillium* fungi (*P. brevicompactum*, *P. stoloniferum*, *P. roqueforti*, *P. chrysogenum*, *P. urticae* and *P. expansum*), represented in the composition of the roughage feed mycobiota are capable of active producing a complex of mycotoxins and may be related to massive and intensive contamina-

tion by citrinin, mycophenolic acid, PR-toxin and cyclopiazonic acid. It has been established that the choice of the panel of growth media for testing toxin formation in *Penicillium* fungi is necessary to comprehensively evaluate their potential. Along with solid media, substrates of plant origin should be used. Knowing of the biosynthetic capabilities of microscopic fungi is important not only for solving such global practical problems as animal safety and safe products of agriculture, but also for the further development of fundamental molecular genetics and evolutionary concepts about the vital activity of these organisms.

REFERENCES

1. Pitt J.I. Toxigenic fungi and mycotoxins. *British Medical Bulletin*, 2000, 56: 184-192 (doi: 10.1258/0007142001902888).
2. Egbuta M.A., Mwanza M., Babalola O.O. Health risks associated with exposure to filamentous *fungi. International Journal of Environmental Research and Public Health*, 2017, 14: 719 (doi: 10.3390/ijerph14070719).
3. *Mycotoxins: economic and health risks. Task Force Report. R 116.* November 1989. CAST, Ames, 1989. Available http://www.cast-science.org/publications/?mycotoxins_economic_and_health_-risks&show=product&productID=2869. Accessed 10.06.2019.
4. Milićević D.R., Škrinjar M., Baltić T. Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins*, 2010, 2: 572-592 (doi: 10.3390/toxins2040572).
5. Piryazeva E.A., Kononenko G.P., Burkin A.A. Affection of coarse fodders by toxigenic *Fusarium* fungi. *Agricultural Biology* [Sel'skokhozyaistvennaya Biologiya], 2016, 51(6): 937-945 (doi: 10.15389/agrobiology.2016.6.eng).
6. Kononenko G.P., Piryazeva E.A., Zotova E.V., Burkin A.A. Toxicological characteristics of *Aspergillus* fungi from coarse fodders. *Agricultural Biology* [Sel'skokhozyaistvennaya Biologiya], 2017, 52(6): 937-945 (doi: 10.15389/agrobiology.2017.6.eng).
7. Piryazeva E.A. *Problemy veterinarnoi sanitarii, gigieny i ekologii*, 2017, 4(24): 42-45 (in Russ.).
8. Raper K.B., Thom C., Fennell D.J. *A manual of the Penicillia.* Williams &Wilkins Company, Baltimore, 1949.
9. *MycoBank database. Fungal databases, Nomenclature & Species Banks.* Available http://www.mycobank.org/mycotaxo.aspx. No date.
10. *Introduction to food- and airborne fungi.* R.A. Samson, E.S. Hoekstra, J.S. Frisvad, O. Filtenborg (eds.). CBS, Utrecht, 2000.
11. GOST 31653-2012 *Korma. Metod immunofermentnogo opredeleniya mikotoksinov* [GOST 31653-2012 Fodder. Enzyme-linked immunosorbent assay of mycotoxins]. Moscow, 2012 (in Russ.).
12. Frisvad J.C., Filtenborg O. Classification of *Terverticillate penicillia* based on profiles of mycotoxins and other secondary metabolites. *Appl. Environ. Microbiol.*, 1983, 46(6): 1301-1310.
13. Frisvad J.C., Filtenborg O. *Terverticillate penicillia*: chemotaxonomy and mycotoxin production. *Mycologia*, 1989, 81: 837-861 (doi: 10.2307/3760103).
14. Frisvad J.C., Samson R.A. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate *Penicillia* and their mycotoxins. *Studies in Mycology*, 2004, 49: 1-174.
15. Frisvad J.C., Smedsgaard J., Larsen T.O., Samson R.A. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium. Studies in Mycology*, 2004, 49: 201-241.
16. Burkin A.A., Kononenko G.P. Producers of mycophenolic acid in ensiled and grain feeds. *Applied Biochemistry and Microbiology*, 2010, (46)5: 545-550 (doi: 10.1134/S0003683810050145).
17. Burkin A.A., Kononenko G.P., Kochkina G.A., Ozerskaya S.M. Enzyme-linked immune sorbent assay for PR-toxin in taxonomical assessment of fungi belonging to the genus *Penicillium* Link. *Applied Biochemistry and Microbiology*, 2007, 43(4): 453-458 (doi: 10.1134/S0003683807040175).
18. Fernández-Bodega M.A., Mauriz E., Gymez A., Martín J.F. Proteolytic activity, mycotoxins and andrastin A in *Penicillium roqueforti* strains isolated from Cabrales, Valdeyn and Bejes-Tresviso local varieties of blue-veined cheeses. *International Journal of Food Microbiology*, 2009, 136: 18-25 (doi: 10.1016/j.ijfoodmicro.2009.09.014).
19. Trucksess M.W., Mislives P.B., Young K., Bruce V.R., Page S.W. Cyclopiazonic acid production by cultures of *Aspergillus* and *Penicillium* species isolated from dried beans, corn meal, macaroni and pecans. *Journal of Association of Official Analytical Chemists*, 1987, 70(1): 123-126.
20. Rao V.K., Shilpa P., Girisham S., Reddy S.M. Incidence of mycotoxigenic penicillia in feeds of Andhra Pradesh, India. *International Journal for Biotechnology and Molecular Biology Research*, 2011, 2(2): 46-50.
21. Brock N.L. Dickschat J.S. PR-toxin biosynthesis in *Penicillium roqueforti. ChemBioChem*, 2013, 14(10): 1189-1193 (doi: 10.1002/cbic.201300254).

22. Hidalgo P.I., Ullán R.V., Albillos S.M., Montero O., Fernández-Bodega M.A., Garcia-Estrada C., Fernández-Aguado M., Martin J.-F. Molecular characterization of the PR toxin gene cluster in *Penicillium roqueforti* and *Penicillium chrysogenum*: cross talk of secondary metabolite pathways. *Fungal Genetics and Biology*, 2014, 62: 11-24 (doi: 10.1016/j.fgb.2013.10.009).
23. Hidalgo P.I., Poirier E., Ullán R.V., Piqueras J, Meslet-Cardière L., Coton E., Coton M. *Penicillium roqueforti* PR toxin gene cluster characterization. *Applied Genetics and Molecular Biotechnology*, 2017, 101: 2043-2056 (doi: 10.1016/j.fgb.2013.10.009).
24. Kononenko G.P., Burkin A.A. Mycotoxin contaminations in commercially used hay. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2014, 4: 120-126 (doi: 10.15389/agrobiology.2014.4.120rus) (in Russ.).
25. Kozlovsky A.G., Zhelifonova V.P., Antipova T.V. Secondary metabolites in the taxonomy of fungi of the subgenus *Penicillium*. *Microbiology*, 2009, 78(5): 618-623 (doi: 10.1134/S0026261709050142).
26. *Index Fungorum*. Available http://www.indexfungorum.org/Names/Names.asp. No date.
27. Larsen T.O., Svendsen A., Smedsgaard J. Biochemical characterization of ochratoxin A-producing strains of the genus *Penicillium*. *Applied and Environmental Microbiology*, 2001, 67(8): 3630-3635 (doi: 10.1128/AEM.67.8.3630-3635.2001).
28. Izhaki I. Emodin — a secondary metabolite with multiple ecological functions in higher plants. *New Phytologist*, 2002, 155(2): 205-217 (doi: 10.1046/j.1469-8137.2002.00459.x).
29. Refai M., Abo El-Yazid H., Tawakkol W. Monograph on the genus *Penicillium*. A guide for historical, classification and identification of penicillin, their industrial applications and detrimental effects, 2015. Available https://scholar.cu.edu.eg/?q=hanem/files/monograph_on_the_genus_penicillium.pdf. No date.
30. Kozlovskii A.G. *Prikladnaya biokhimiya i mikrobiologiya*, 1999, 35(5): 536-545 (in Russ.).
31. Vinokurova N.G., Ivanushkina N.E., Kochkina G.A., Arinbasarov M.U., Ozerskaya S.M. *Prikladnaya biokhimiya i mikrobiologiya*, 2005, 41(1): 95-98 (in Russ.).
32. Varga J., Rigo K., Lamper C., Téren J., Szaby G. Kinetics of ochratoxin A production in different *Aspergillus* species. *Acta Biologica Hungarica*, 2002, 53(3): 381-388 (doi: 10.1556/ABiol.53.2002.3.14).
33. Gallo A., Giuberti G., Frisvad J.C., Bertuzzi T., Nielsen K.F. Review on mycotoxin issues in ruminants: occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effects. *Toxins*, 2015, 7: 3057-3111 (doi: 10.3390/toxins7083057).
34. Obire O., Okigbo R.N. Ojim C.F. Fungal population and diversity in partially digested cellulose from the abomasum of beef cows. *Journal of Agricultural Technology*, 2010, 6(4): 783-792.