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## CONTENTS

### REVIEWS, CHALLENGES

- Roumiantseva M.L. Root nodule bacteria: perspectives of monitoring symbiotic properties by applying genetic markers (review) ........................................ 847
- Chesnokov Yu.V. Biochemical markers in genetic investigations of cultivated crops: the pros and cons (review) ................................................................. 863
- Shecherbakova L.A. Fungicide resistance of plant pathogenic fungi and their chemosensitization as a tool to increase anti-disease effects of triazoles and strobilurines (review) ........................................... 875

### GRAIN CROPS

#### GENETICS AND BREEDING

- Kharitonov E.M., Goncharova Yu.K., Gontcharov S.V. et al. Molecular markers associated with high early growth rate of Russian rice (*Oryza sativa* L.) varieties ................................................................. 892
- Novokhatin V.V., Dragavtsev V.A., Leonova T.A. et al. Creation of a spring soft wheat variety Grenada with the use of innovative breeding technologies based on the original theory of eco-genetic arrangement of quantitative traits ........................................ 905

#### TRITICALE

- Kroupin P.Yu., Chernook A.G., Karlov G.I. et al. Molecular markers associated with high early growth rate of Russian rice (*Oryza sativa* L.) varieties ................................................................. 892
- Yerzhebayeva R.S., Abdurakhmanova M.A., Bastaubayeva Sh.O. et al. Effect of Zeatin on in vitro embryogenesis and plant regeneration from anther culture of hexaploid triticale (*Triticosecale* Wittmack) ........................................ 934

### PHYSIOLOGY OF ADAPTATION

- Tsypurskaya E.V., Kazantseva V.V., Fesenko A.N. et al. Growth of buckwheat (*Fagopyrum esculentum* Moench) seedlings and the accumulation of primary and secondary metabolites under various mineral nutrition conditions .................................................. 946

### POTATO FARMING: SCIENCE AND TECHNOLOGIES

- Klimenko N.S., Antonova O.Yu., Zheltova V.V. et al. Screening of Russian potato cultivars (*Solanum tuberosum* L.) with DNA markers linked to the genes conferring extreme resistance to potato virus Y .................................................. 958
- Dyachenko E.A., Kulakova A.V., Meleshin A.A. et al. Allele variability of amylase inhibitor gene *AI* in potato varieties and lines .................................................. 970
- Pakul V.N., Lapshinov N.A., Gantimurova A.N. et al. Donors of potato (*Solanum* L.) plasticity and yield stability traits in the environmental conditions of north forest steppe of Western Siberia .................................................. 978
- Titova J.A., Novikova I.I., Boykova I.V. et al. Novel solid-phase multibiorecycled biologics based on *Bacillus subtilis* and *Trichoderma asperellum* as effective potato protectants against *Phytophthora* disease .................................................. 1002

### FUTURE FARMING SYSTEMS

#### SYMBIOTIC INTERACTIONS

- Kitaeva A.B., Tsygagov V.E. Influence of mutation in the gene *Sym26* of the garden pea (*Pisum sativum* L.) on the organization of tubulin cytoskeleton in nodules .................................................. 1014

#### BIOPREPARATIONS AND BIOCONTROL

- Kolesnikov L.E., Popova E.V., Novikova I.I. et al. Multifunctional biologics which combine microbial anti-fungal strains with chitosan improve soft wheat (*Triticum aestivum* L.) yield and grain quality .................................................. 1024
- Smirnova I.E., Sadanov A.K. Cellulolytic bacteria and association of effective microorganisms for biocontrol of root rot infections in sugar beet (*Beta vulgaris* L.) .................................................. 1041
ROOT NODULE BACTERIA: PERSPECTIVES OF MONITORING SYMBIOTIC PROPERTIES BY APPLYING GENETIC MARKERS (review)

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Abstract

Alfalfa and soybeans are widely cultivated economically valuable fodder and leguminous crops the yield of which directly depends on bacterial microsymbionts. The legume seeds inoculation by nodule bacteria (rhizobia) is significantly increased the productivity of the plant-microbial system both in typical and in adverse growing conditions, for example, on degraded soils, including those subjected to salinization, waterlogging, aridity, etc. That is why obtaining new strains capable of forming highly productive and stress-tolerant symbiotic systems with leguminous plants is highly requested in agriculture. Modern technologies for the production of highly productive and environmentally friendly varieties of legumes require the use of a biogeocenotic approach which primary takes into account the symbiotrophic indicators (Z.S. Shamsutdinov, 2014). The formation of highly productive plant-microbial systems is based on the principle of complementarity of the interaction of macro- and microsymbiont genomes (I.A. Tikhonovich et al., 2015), that is ensured their successful introduction into agrocenoses, which differ in agroclimatic and soil conditions. Virulence, competitiveness, host specificity and effectiveness of nitrogen-fixing activity, which root nodule bacteria exhibit in relation to a certain species and sometimes to the variety of legume host plant, are among the symbiotically significant and genetically determined properties of rhizobia. All of the above symbiotically significant characteristics are determined by numerous groups of rhizobia genes. The review presents an analysis of data on the genes of soybean and alfalfa microsymbionts, the participation of which in the control of symbiotic activity and stress tolerance has been experimentally proven. Nodule bacteria of the species Sinorhizobium meliloti, S. fredii and Bradyrhizobium japonicum, contrastingly differing in the genetic and morphophysiological characteristics are the most studied. Analysis of recently published data on the main groups of symbiotically significant genes (i.e. nod genes involved in the synthesis and decoration of the Nod factor signal molecule initiating the nodulation process during plant-microbial interaction, the nif, fix, and eff groups of genes responsible for the nitrogen fixation and symbiotic effectiveness) indicates a continuing high degree of incompleteness and fragmentation data for both fast- and slow-growing rhizobia species. At the same time, according to published data, allelic polymorphism for these genes is a factor that plays an important role in varying signaling, host specificity, and symbiotic effectiveness in both fast and slow-growing species of nodule bacteria. It is concluded that a coupled analysis of sequences of genes from functionally different groups relating to the formation of highly effective stress-tolerant symbioses, which are represented by sym genes (symbiosis), srg (stress related genes; genes of resistance to stress factors), QS (quorum sensing genes), or sym-srg-QS genes, is promising for the search and creation of molecular markers associated with the symbiotic and adaptive properties of nodule bacteria and it is promising for monitoring them under laboratory conditions and in microbiome of agroecosystems.

Keywords: nodule bacteria, Sinorhizobium meliloti, S. fredii, Bradyrhizobium japonicum, alfalfa, soybean, genes of symbiotic activity, effectiveness, resistance to abiotic stresses

Alfalfa and soybeans are the most widely cultivated economically valuable crops worldwide, therefore, their bacterial microsymbionts are in the spot-
light. Modern technologies for breeding highly productive and environmentally friendly varieties of legumes suggest the use of a biogeocenotic approach based on the symbiotic selection method [1]. This technology involving genetically selected nodule bacteria (rhizobium) considerably shortens the time to create new alfalfa varieties.

The method is based on a complementarity interaction of macro- and microsymbiont genomes [2, 3], which predetermines suitability of a plant-microbial system for the environmental conditions and agroecosystems. Virulence, competitiveness, capability to nodulate of a certain legume host and nitrogen-fixing activity are symbiotically significant and genetically determining properties of nodule bacteria. Seed treatment with rhizobia strains provides an increase in symbiotic productivity of legume plants both in typical and in adverse growing conditions, for example, on degraded soils, including salinization, waterlogging, aridity, etc. [4-6]. That is why obtaining new strains capable of forming highly productive and stress-tolerant symbiotic systems with leguminous plants are extremely relevant [7, 8].

Traditionally, obtaining effective nodule bacteria necessitates long-term plots experiments and field trials. In the result strains providing significant positive biometric (height, development of root system, etc.), biochemical (nitrogen content) and symbiotrophic (an increase in plant green biomass or dry matter) changes in plants [1, 4, 5, 9, 10] were selected. These are deemed qualitative and quantitative indicator parameters of symbiotic activity and effectiveness of root nodule bacteria strains. However, an improved plant green mass yield or dry matter production resulting from inoculation by selected strains may eventually decline [11], wherefore such strains are subjected to a supporting selection with isolation of new clones and the analysis of their symbiotic properties in multiple micro-vegetation and then vegetation experiments [12, 13]. Therefore, the development of molecular genetic approaches to search, select and monitor strains with high symbiotic properties seems extremely urgent.

In the modern view, the cause of decrease or loss of symbiotic activity and effectiveness in rhizobia strains may be the instability of their symbiotic genome [14]. The latter means the complex of sym genes (structural and regulatory) responsible for various stages of plant-microbial interaction which formed due to the coevolution of nodule bacteria with legume hosts. As per published data, the symbiotic genome of slow-growing and fast-growing nodule bacteria (i.e. \textit{Bradyrhizobium} and \textit{Sinorhizobium} genera, respectively) comprises at least five hundred sym genes [15]. Of the genes related to virulence and, thence, nodule formation, a group of common nod genes stands out. These genes determine synthesis of specific signal molecules, the Nod-factors, involved in plant-microbe interaction and are found in virtually all known rhizobia species [16]. Genes determining symbiotic activity are normally located on one or several plasmids or in a genomic island located on a chromosome. Such type of location could be a reason for potential loss of individual sym genes as well as their clusters, especially under the influence of various abiotic stress factors [17, 18]. Therefore, genotyping of strains for genes determining formation and functioning symbiosis and involved in stress tolerance of bacteria appears imperative.

This review focuses on genes which are experimentally proven to be involved in control of symbiotic activity and stress tolerance of root nodule bacteria of \textit{Sinorhizobium} and \textit{Bradyrhizobium} genera which are contrastingly different genetically and phenotypically, but most well studied. Genes related to symbiosis, but common for fast- and slow-growing rhizobia species may be promising in searching for candidate genes and molecular markers to facilitate analysis of genome stability and inheritance of symbiotic traits in nodule bacteria.
Alfalfa and soybean symbionts. Slow-growing *B. japonicum* and *B. elkanii* rhizobia form highly effective symbiosis with *Glycine max* (L.) Merr. soybean cultivar in neutral or sub acid soils [19-21]. In alkaline soil, some soybean cultivars form symbiosis with fast-growing *S. fredii*, the typical symbionts of *G. soja* a wild soybean relative widely used in genetic improvement of *G. max* for many economically valuable traits. There is an opinion that use of fast-growing *S. fredii* bacteria in inoculation of soybean cultivars may be of environmental and practical importance due to an increase in agricultural production [22]. Symbionts of perennial tetraploid alfalfa (*Medicago varia*) are fast-growing *S. meliloti* rhizobia, however, in natural conditions, strains of closely related *S. medicae* species mainly forming effective symbiosis with annual diploid alfalfa cultivars, can also be a symbionts of *M. varia*.

Thus, both soybeans and alfalfa form an effective symbiosis with certain species of nodule bacteria, but also could form an ineffective one with bacteria of closely related and quite often with unrelated rhizobia species, which naturally results in considerable yield loss. Hence, the species attribution of nodule bacteria strains is essential for evaluation of their potential symbiotic effectiveness. The method of nucleotide sequence analysis of 16S rRNA gene proved to be the best as it enables characterization of strains not only at the species level but also identification of rhizobia genospecies [23, 24]. Analysis of ribosomal operon *rrn*-rfl intergenic sequence (ITS) or its part, e.g. *hin* region, is also frequently used [25-27]. The analysis of these sequences is useful not only for identification of strains species allocation but also for strain-specific characterization at the level of chromosomal markers [28], which, however, does not guarantee inheritance by strain(s) of genetic determinants of stress tolerance and symbiotic activity.

Genes determining symbiotic activity are located differently in genomes of bacteria of *Bradyrhizobium* and *Sinorhizobium* genera. In fast-growing *S. meliloti* and *S. fredii* species, *sym* genes are on one to two high-molecular megaplasmids exceeding 0.8-1.0 million bps in size but may also be found on chromosome (≥ 3.6 million bps) and on cryptic plasmids (30 to 600 kbps), the number of which in different strains varies from 0 to 5 [29]. In slow-growing *Bradyrhizobium*, *sym* genes are detected in so-called symbiotic island located on a chromosome which size varies from 643 to 998 kbps [30, 31]. Symbiotic islands have mosaic structure in which sequences determining symbiotic activity alternate with sequences that do not affect symbiotic properties or are senseless [30, 31]. Symbiotic islands are related to the type of genomic islands, which is also found in fast-growing nodule bacteria. However, in the latter, the genes relating to symbiotic activity or rhizobia fitness are sporadic and much less frequent [32]. Genomic islands of nodule bacteria are able to substantially affect the functional activity of genes located on a chromosome and/or on plasmids and may be involved in horizontal gene transfer [32-34]. Thence, detection of genomic islands in genomes of highly effective nodule bacteria is essential and is directly related to the selection of genetically stable strains for manufacturing biologicals.

**Nod gene group.** This group determines/regulated the synthesis of signal molecules (or Nod-factors) required to initiate nodule formation on the roots of the host plant, is best studied in rhizobia species considered in the review.

By the example of *B. japonicum* USDA110 strain it is shown that slow-growing bradyrhizobia contain two operons, *nodYABCUSIJnolMNO* and *nolYZ*, responsible for synthesis of core part of signal molecule, while fast-growing symbionts of soybean *S. fredii* (the strains HH103 and USDA257) have one operon united *nodABCIJnolOnoeI* genes [16, 35]. In case of alfalfa symbionts, *nod* genes are arranged into five operons, *nodABCIJ*, *nodFEGPQ*, *nodH*, *nod-MnolFGnodN* and *nodLnoeAB* (the strain *S. meliloti* Rm1021) [36]. It should be
noted that the location and functional role of nod genes known now is a result from studying of a single strains including the above mentioned. Disruption of one of so-called common nod genes in the result of mutation or targeted alteration results in significant changes in nodulation and, normally, in decrease in or loss of symbiotic activity [37]. Activity of common nod genes of rhizobia is regulated by the product of nodD1 gene which in S. meliloti plays a more significant role than its orthologs nodD2 and nodD3 [38]. The inducers of nodD1 are flavonoids present in root exudates of the host plant, but inducers can also be betaines (osmo-protectors) found in alfalfa root exudates too. The homology of nodD1 genes of S. meliloti Rm2011 strain and reference strains B. japonicum and B. elkanii does not exceed 75% (at amino-acid level), while homology between the above genes of B. japonicum and B. elkanii is 92%. In B. japonicum and B. elkanii nodD1 genes are activated by different flavonoids, drawing to a suggestion that analysis of allelic polymorphism of nodD1 genes in native bradyrhizobia strains may be benefit to identify strains differ in host specificity [39]. An important role in regulation of nod genes of bradyrhizobia is played by nodD2 gene, which, in its turn, is controlled by nolA gene [40].

On the example of B. japonicum USDA110 strain it was shown the involvement of nolA gene in control of host specificity, since a corresponding mutants formed nodules on soybean plant roots much later than the parent strain, and were not capable to form nodules on the roots of Vigna unguiculata [41]. A two-component system of regulatory genes nodVW activated by flavonoid genistein are also related to host specificity of B. japonicum [39]. Activity of these genes is required for nodulation of siratro (Macroptilium atropurpureum) and vigna roots by bradyrhizobia, but not soybeans [39]. In addition to regulators reviewed above, the secretory protection system TTSS (Type III Secretion System) that is under control of nodD1 in B. japonicum and S. fredii is also involved in interaction of rhizobia with certain plant hosts [42-44]. High activity of tts genes was noted at early stages of nodule formation, while the tts gene mutants were unable to form nodules on the roots of a number of plant hosts [42-44].

Particular role play nod genes involved in structural modification (or so-called decoration) of the core of Nod-factor, which makes the signal factor molecule species-specific. For example, the presence of sulfate group on reducible end of S. meliloti Nod-factor molecule is required to form symbiosis with alfalfa, which had been proven in studying respective mutants [45]. The Nod-factor sulfating is carried out by nodH gene product in connection with products of two other genes, nodP and nodQ. However, native strains capable to form symbiosis with alfalfa in acidic soils possess signal molecules which lack sulfate group [45]. In S. fredii, signal molecules are sulfate-terminated too, while decoration of Nod-factor includes fucosylation and methylation of fucosylated residue on reducible end of signal molecule for which the activity of noeJ gene and nolK-noeL-nodZ-noeK gene cluster are required [46]. Similar cluster is also present in B. japonicum but it includes also another two genes, nolL and noeE, involved in methylation of signal molecule.

Signal molecules of fast- and slow-growing rhizobia symbionts of soybeans have some structural differences. In bradyrhizobia they are acetylated while in sinorhizobia non-carbamylated at non-reducible end of the molecule [47]. Carbamylation is controlled by nolO and nodU genes. It was found that all of the studied so far S. fredii strains (HH103, 042B, USDA192, USDA193 and USDA257) had an insertion and a stop codon in nolO gene, and only in the first two mentioned strains structural modifications in nodU gene were found as well [47]. Obviously, the genes involved in signal molecule decoration, such as nolO
and nodH, may be of interest as candidate genes for searching marker sequences required to facilitate express search for fast-growing symbionts of soybeans and alfalfa, respectively.

It should be noted that the relationship of structural polymorphism of nod genes with the structure of signal molecules and their indirect impact on symbiotic potential of root nodule bacteria remains very poorly understood. Investigations carried out in our laboratory revealed high level of the structural polymorphism of common nod genes and species-specific nodH gene in geographically isolated native populations of S. meliloti as compared to reference strain Rm1021 [48]. It was shown that native S. meliloti strains harbouring divergent alleles (in comparison with the reference strain) formed symbiosis mainly with annual alfalfa species under typical growth conditions, while under model salinity conditions tested strains were differed in ability to form effective symbiosis with different alfalfa species [49]. For different species of rhizobia, a positive correlation between nodA gene structure and the type of synthesized Nod factors was shown according in silico analysis (50). Based on the data obtained, the authors proposed to use the analysis of the nodA gene to search symbiotically active rhizobia strains with a certain host specificity in various ecological niches (50). Thus, the analysis of the literature data on the structural organization of nod genes involved in synthesis and decoration of Nod-factor allows us to consider allele polymorphism of nod genes as an important factor of signaling and host specificity variations in both fast- and slow-growing nodule bacteria species.

Nif, fix and eff gene groups. These nodule bacteria genes are responsible, respectively, for nitrogen fixing and symbiotic activity. The nif genes determine the synthesis of nitrogenase responsible for conversion of nitrogen into compounds available for plant metabolism. Of 20 nif genes described for Klebsiella pneumonia, two genes, nifDK and nifH encoding an enzyme complex, are mainly studied [39, 51]. The structure of nif genes is almost identical in taxonomically different microorganisms, while their arrangement and regulation differ. Analysis of these genes is often used to assess the potential nitrogen fixing ability in newly discovered microorganisms [52].

Fix and eff gene groups are characterized as genes determining so-called central intermediate metabolism [39, 41]. B. japonicum or S. meliloti genes highly expressed in bacteroids comprise about 15-16% of their number in saprophytic bacteria. The genes of B. japonicum or S. meliloti with high expression in bacteroids make up about 15-16% in relation to their number in saprophytic forms of bacteria. Total number of genes involved in regulation of symbiotic activity differs significantly in rhizobia species, also varies between the strains of the same species and depends on the experimental conditions [53]. Thus, the number of genes the activity of which changes (increases or decreases) as a result of symbiotic interaction is 982 or 1288 for S. meliloti, and 1234 or 2778 for B. japonicum [53]. If we compare sinorizobia and bradyrhizobia, then the portion of genes for which the expression is increased is 37% and 54%, correspondingly, while the functions of the vast majority of these genes remain unknown (53, 54). PubMed NCBI database (https://www.ncbi.nlm.nih.gov/pubmed/) contains over sixty papers that include the information about the genes of Bradyrhizobium and Sinorhizobium genera involved in symbiotic effectiveness which can be classified as fix and eff genes, however, the number of genes of each genus directly studied in this respect does not go above twenty. Up to this time, there is no systematization of data for these genes, apparently due to the fact that their activity is mediated and/or that their products are involved in different cellular processes. For instance, fixNOQP genes determine the synthesis of cbb3-cytochrome oxidase playing a key role in generation of reducing-oxidation potential (redox potential)
in bacteroids [55]. We have correlated the fixB, fixC, fixU, fixX, fixO and fixQ genes products of *B. japonicum* with the corresponding COG groups (cluster of orthologous groups) of widely-used protein product classification system [56]. It turned out that the products of these genes are predominantly belong to C group (metabolism associated with energy processes), while fixK2 and fixN gene products belong to group T (signal transduction mechanisms) and P (transport of inorganic ions and metabolism), respectively. Moreover, among the genes affecting symbiotic activity of *B. japonicum* there are genes determining the synthesis of ferrodoxins (fixN; COG group C), ACC-deaminase (acdS; group E), hydro-lase (blr6420; group C) homologous to the pobA gene product (involved in hydroxybenzoate conversion), and transcription factor (blr6378; group K). Therefore, *B. japonicum* genes involved in control of symbiotic effectiveness mainly belong to COG groups associated with amino acid and energy metabolism.

Similar groups of *fix* and *eff* genes in *S. melliloti* are located on megaplasmids and on a chromosome. Genetic analysis of Tn5 mutants of streptomycin resistant CXM1-105 strain which, in turn, is a derivate of 425a strain widely used in manufacturing biologicals in 1980-1990s, revealed 12 new genes involved in control of symbiotic effectiveness [57-59]. Increase in dry mass yield of alfalfa plants inoculated with such transposants was ranged from 15 to 34%. Analysis of products of these genes showed that 42% are responsible for transport and metabolism of inorganic ions (COG group P), while the rest are involved in transport and metabolism of amino acids (COG group E) and carbohydrates (COG group G). Also the products of some genes are related to cellular processes and signaling (COG group M), and to storing and processing of information (COG group K). Therefore, *S. melliloti* genes involved in control of symbiotic effectiveness are numerous, and their products belong to a larger number of different COG groups than in *B. japonicum* species considered above.

Thus, genes involved in control of symbiotic effectiveness of plant-microbial systems of soybeans or alfalfa are numerous in both cases. At the same time, genes mentioned above are primarily involved in metabolic processes in *B. japonicum*, while in *S. melliloti* they are involved in various cellular processes. In addition, homologous genes in the reviewed rhizobia species may or may not be related to the regulation of symbiotic effectiveness. As was recently demonstrated, *rirA* gene in *S. fredii* HH103 is involved in iron metabolism but directly affects symbiotic effectiveness as well, while nothing like that was found in *S. melliloti* [60]. The described differences between the considered species in genes involved in symbiotic effectiveness control rather indicate utmost lack of our knowledge about this process. It should be noted that native strains may not harbour some of genes or carry different alleles of genes of interest in which functionally significant structural changes have occurred, as it was reported [47, 61]. Apparently, analysis of native polymorphism of alleles of the discovered above candidate genes is one of the approaches to studying the regulation of symbiotic effectiveness in rhizobia.

**Group of genes involved in bacteria fitness control.** Fitness means the ability of microorganisms to occupy various environmental niches, and the formation of mutualistic symbiosis is also viewed as a part of the latter. Sustainability under different biotic and abiotic factors of soil environment (temperature, humidity, medium pH, pollutants, osmotic stress, particularly, salinization) promotes an increase of the population density. At the same time, bacterial tolerance to drying (temperature, humidity, osmotic stress) is an important technical parameter for routine seed treatment [62-64]. Therefore, enhancement of strain tolerance to various abiotic factors is of practical significance. Aridity and salinity can be attributed to the most common model abiotic factors [64, 65].
Accumulation of various ions or compounds, including K⁺, amino acids (e.g. glutamate), carbohydrates (including trehalose) or osmoprotectors (ectoin, glycine, betaine and choline) by bacteria increases their tolerance to high osmolality. *S. meliloti* rhizobia may be attributed to moderate halotolerants as over 71% of native strains grow well at 0.6 M NaCl [65]. For now, bacteria of this species have revealed at least six groups of genes involved in responses to different types of stresses, which are further designated as *srg* (stress related genes) genes.

Resistance to salinization is predetermined by the activity of *bet, pro, tre* groups of genes, and to low pH by *act, ots* and *hpr* groups of genes. Interestingly, the products of these genes relating to various metabolic processes also affect symbiotic activity. For instance, *S. meliloti* betaine synthesis genes (*bet* genes) are involved in carbon and nitrogen pathways [65]. Microvegetation tests at normal conditions and under salinization revealed that native strains of *S. meliloti*, in which certain alleles of the *bet* gene were detected, have a salt tolerant phenotype (tolerant to high salt concentrations) and significantly more often form symbiosis with increased effectiveness [47, 65].

*B. japonicum*, on the contrary, grow poorly even at 0.05 M NaCl, thus, bradyrhizobia have a salt-sensitive phenotype. As it was recently demonstrated, *B. japonicum* with higher catalase activity was able to grow in 0.15 M NaCl media [66]. According to transcriptomic analysis, 441 genes of *B. japonicum* changed activity at 0.05 M NaCl [53]. For 13 genes, the highest increase in activity was detected, and these genes (except *rpoH2*) were characterized as genus-specific since in *S. meliloti* they were not associated with salt tolerance [53]. Apparently, bradyrhizobia sensitivity to osmotic stress probably was caused by lack of transport systems like BCCT (betaine, carnitine, choline) which are typical, for *S. meliloti* [67]. Experimentally was shown that *B. japonicum* strains capable to utilize trehalose formed highly effective symbiosis [67].

It has been suggested that the selection of bradyrhzobia strains by *ecfG*, *nepR* and *phyR* genes which products are involved in the cascade regulation of bacterial tolerance to general stresses, as well as by genes of *bl1/r1465-69* locus involved in the response to temperature shock and UV-light tolerance, can be promising for identification of stress-tolerant strains [68, 69], and these candidate genes can be used to create appropriate marker sequences.

It should be concluded that *srg* genes involved in development of stress-tolerance are present in the genomes of not only fast-growing and salt tolerant sinorhizobia, but in slow-growing salt sensitive bradyrhizobia. Activity of these genes in bacteria of both genera is associated with the central metabolism and directly affects the symbiotic effectiveness and nitrogen fixation. All of the above proves that evaluation of genes involved in resistance to various stress factors is promising in practical aspect.

Tolerance of soil bacteria to adverse environmental factors also depends on the ability to synthesize polysaccharides. These are various macromolecules whether bound or not bound to cellular wall having a wide range of biological functions, from signaling to protection. Polysaccharides provide bacteria protection from drying, temperature changes and from bacteriophages, as well as from specific and non-specific host plant immunity response, etc. These compounds may have adhesive properties that enable bacteria to colonize various surfaces by forming microcolonies and/or biofilms. Thus, polysaccharides ensure rhizobia fitness enabling them ability to occupy almost any ecological niche, including colonizing legume roots, and are crucial for overcoming of abiotic stresses by bacteria [70-72].

*S. meliloti* synthesize various polysaccharides, i.e. capsular polysaccha-
rides, exopolysaccharides such as EPSI (succinoglucan) and EPSII (galactoglucan), lipopolysaccharides and cyclic glucans [36, 73-75]. Genes determining polysaccharide synthesis (exo-exp-lps) are located primarily on the second megaplasmid which does not contain nod-nif-fix genes. It has been shown that mutations in some polysaccharide synthesis genes result in disruption of plant-microbe interaction event at early stages, wherefore nodules do not form [58, 67, 74, 76]. With that, mutations in some genes (e.g. exoB, exoY or lpsL) can, on the contrary, enhance nodule formation [77]. In fast-growing soybean rhizobia *S. fredii*, a cluster of genes similar to that of *S. meliloti* which determines the polysaccharide synthesis and is also located on megaplasmid was found [16]. However, *S. fredii* strains do not synthesize EPSII and do not bind soybean lectin. Moreover, bacteria of this species are more sensitive to salinization, less mobile and adhere better to non-biotic surfaces than *S. meliloti* bacteria.

Bradyrhizobia polysaccharides bind soy lectin and accumulate polysaccharides in peribacteroid space. These polysaccharides may play an important role in survival of bacteria which were not transformed into bacteroids and, after destroying nodules, release into environment. In *B. japonicum* the lps locus including rfaD, rfaF, lpcC and galE genes (their products are heptose epimerase, heptosyltransferase, mannosyltransferase and glucose epimerase, respectively) is directly involved in receptor and nodulation [78]. Of vast interest is rfaL (or waal) gene the product of which is a key enzyme in biosynthesis of cell wall in bradyrhizobia. The product of this gene participates in providing bacterial tolerance to various stresses and escaping from plant defense mechanisms under inoculation was shown [78].

Important that rhizobia could utilize inositol and its derivatives which are widespread in environmental systems as a source of carbon. Inositol is a part of plant cell wall, it is a main form of storage of phosphorus in seeds, and also can be a signal molecule [79]. Inositol derivatives attract attention due to their therapeutic effect in diabetes and Alzheimer’s disease. Extended clusters of iol genes are discovered in sino- and bradyrhizobia, but the best studied gene is idhA in *S. fredii*. The activity of this gene is interconnected with formation of nitrogen fixing nodules, while similar gene of *S. meliloti* is related to competitiveness [79].

Among the genes associated with bradyrhizobia fitness, a special focus belongs to nosZ. The nosZ gene mutants of *B. japonicum* have higher N₂O-reductase activity (N₂O reduction to N₂) and remain capable to form highly effective nitrogen fixing symbiosis with soybeans [80, 81]. Use of biofertilizers for soybean crops based on native strains harbouring modified nosZ gene, in the authors’ opinion, may contribute to reduction in nitrogen oxide (greenhouse gas) emissions [82]. There is also an opinion that allelic polymorphism of hup genes (responsible for hydrogen accumulation), nap genes (encoding periplasmic nitrate reductase), and nos genes (encoding nitrite reductase) can be used to search native effective soybean symbionts [7, 81, 82].

Of direct relation to bacteria fitness are also so-called quorum-sensing (QS) systems. They are function as a global regulation factors detected in almost all known bacteria species and can be involved in the coordinating interaction between prokaryotes and eukaryotes. During growing bacterial cell population upon to certain density the synchronous synthesis, accumulation and secretion of chemical signals, the acylated homoserine lactones (AHLs), occurs [83]. Several QS systems are found in *S. meliloti*, one of which is sinR/sinI responsible for synthesis of homoserinelactones which regulate EPSII synthesis. Strains with mutated sin gene form nitrogen fixing nodules on *M. sativa* roots in considerably less amount and much later. QS system in *S. fredii* plays important role in devel-
oping biofilm on *Glycine max* roots. Apparently, similar system is also present in *B. japonicum* but it differs substantially from those described above and those found in other rhizobia species. It has been demonstrated that with high density of bradyrhizobia cells the virulence, on the contrary, goes down due to production of bradyoxetin, a compound structurally similar to some antibiotics and siderophores [70]. For bradyrhizobia, the inhibition of *nod* genes activity at high cell density and presence of flavonoids were established; the process also involves *nodD2* and *nolA* genes [84] discussed above. Data currently accumulated evidence that QS systems are extremely diverse and have different regulatory mechanisms, but the investigation of their role in plant-microbe interactions is actually in its early stage.

Summarizing the above, it should primarily be noted that information on main groups of symbiotically significant genes of nodule bacteria are still limited, fragmented and hardly comparable not only for different genera and species, but even for the strains of the same species. The available data confirm the fact that for each type of nodule bacteria that differ in genetic and phenotypic characteristics, it is necessary to analyze species-specific and strain-specific genes, for example, the 16S rRNA gene and genes which activity is associated with the formation of symbiosis and the ability to withstand to abiotic stresses (sym, srg and QS genes). Confirmation of the stable inheritance of sym-srg-QS genes alleles related to the formation of highly effective and stress tolerant plant-microbe symbiotic systems will increase the likelihood of the strain retaining symbiotically significant properties during its lab storage, and such approach can also be used for monitoring of the strain in microbiome of agroecosystem. Conversely, the identification of structural modifications in sequences of one or several candidate genes, for example, according to PCR data (in particular RFLP), will indicate at a possible change or loss of the corresponding phenotypic properties by the strain. A joint analysis of sequences of genes of interest belonged to functionally different groups of genes involved in the formation of highly effective stress-tolerant symbioses can be used to develop genetic markers using the SNP technology adapted for haploid genomes (85). The introduction of such markers in modern molecular genetic studies on symbiogenomics will promote targeted design of highly productive nitrogen-fixing plant-microbial systems with extensive adaptive potential for the development of organic agriculture in geographically different Russian regions.

Thus, evaluation of joint inheritance of alleles of genes of root nodule bacteria responsible for the formation of highly effective and stress tolerant symbiotic systems with host legumes will enable express testing of symbiotically significant and adaptive properties of rhizobia even during lab storage. This also makes it possible to develop protocols providing strain viability and genetic stability in biologicals and in microbiomes of agroecosystems.

REFERENCES

5. Dragomir N., Pe I., Dragomir C.P.A., Toth S., Ravdan S. Enhancement of the capacity of


Torres T.G., Del Papa M.F., Soria-Diaz M.E., Draghi W., Lozano M., Giusti M.L.,...


BIOCHEMICAL MARKERS IN GENETIC INVESTIGATIONS
OF CULTIVATED CROPS: THE PROS AND CONS
(review)
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A b s t r a c t

This literature review summarizes the accumulated knowledge and the author’s own research data about suitability of seed storage proteins, allozymes and isozymes as biochemical genetic markers. These markers have a huge potential, since it allows researchers to distinguish genotypes from other in a short time. Therewith, biochemical markers are usually tissue- and organ-specific. The advantages that these markers possess over morphological markers are shown. So biochemical markers can be used on a much larger number of experimental objects than morphological ones. Protein markers are usually characterized by a greater correspondence between genotype and phenotype, and, besides, the path to the implementation of genetic differences into phenotypic ones for protein markers is much shorter than for morphological ones. In addition, metabolites (sugars, carbohydrates, secondary metabolites, etc.), which are identified biochemically after isolation from the organs or tissues of the studied organism and purification, are also referred to biochemical genetic markers. Though more than half a century has passed since the first description of biochemical markers, the physicochemical bases to their detection and identification have hardly changed methodologically. This gives some limitations on their use in genetic investigations. For example, it is shown, that plant protein polymorphisms revealed by one-dimensional electrophoresis can be subjected to quality and quantity changes because of ecological stresses such as nutrition deficiency or temperature deviations. Researchers also must take into account casual destructive changes and breaks of the analyzed molecules for various reasons, including due to non-standard conditions for protein and polypeptide extraction and purification, as well as during electrophoretic separation, which leads to non-specific electrophoretic spectra. Because of degeneracy of the genetic code and the fact that not every amino acid substitution leads to a change in charge and the molecular weight of the protein, only 30 % of nucleotide substitutions can cause electrophoretically detected protein polymorphism. Only strict observance of all methodological, biological and other restrictions, as well as established requirements, allow the correct and skillful use of biochemical markers in genetic research.

Keywords: seed storage proteins, allozymes, isozymes, polymorphism, electrophoretic patterns, biochemical markers

The search for molecular markers to solve practical tasks of plant genetics and selection began in 1960-s. At that time, DNA technologies were still absent and scientists began using protein polymorphism to evaluate and study genetic diversity. The markers based on identifying the genetic product or product of its activity the visualization of which requires biochemical analysis became known as biochemical markers. This category of markers includes not only proteins of different types (reserve, transport, construction proteins, different enzymes, etc.), but also metabolites (sugars, carbohydrates, secondary metabolites, etc.), which are identified biochemically after isolation from the organs or tissues and purification. Reserve proteins of plant seeds or enzymes are primarily used as markers. Metabolites did not gain a broad recognition as markers due to the specifics of them identifying, which require expensive and often specialized equipment (spectrophotometers, fluid analyzers and high and low pressure gas analyzers, distillation
stills, etc.). However, the application of this group of biochemical markers contains a significant potential because it allows researchers to differentiate between genotypes in a relatively short time; furthermore, metabolites, as a rule, are tissue- and organ-specific. Even in spite of the fact that they are generally all dominant, metabolites as biochemical markers are successfully used in analysis of genetic diversity of plants preserved in collections of genetic resources [1-4].

The biochemical mutations are nominally considered to belong to the marker class in question [5]. Their carriers also have specific organic molecules identified by biochemical methods; however, since manifestation of such mutations can be directly observed on plants (without resorting to biochemical manipulations), it is more properly to associate such mutant forms with a class of morphological markers identified phenotypically. We follow the rule according to which additional expensive manipulations (biochemical or molecular) should be avoided to determine variations between genotypes when simple external phenotype description suffices.

The significant breakthrough in using the marker biochemical analysis developed in the second half of 1960-s is primarily connected with a widespread distribution of electrophoretic separation of different proteins (including enzymes) in genetic studies of different objects.

The protein variations in terms of electrophoretic mobility attributable to allelic substitutions in the gene determinant can be used to analyze changes of genotypic composition of populations similar to the morphological variations related to marker loci. At the same time, protein markers, along with certain shortcomings discussed below, possess a number of advantages over common morphological markers [6]. Firstly, the electrophoretic biochemical markers can be used on a much wider number of experimental objects than morphological markers. For this, collections of marker mutants do not have to be created, which takes a long time, since numerous protein variants are much easier to detect in the available experimental material. Secondly, protein markers are usually characterized by more consistency between genotype and phenotype. Furthermore, at monolocus level homozygotes and heterozygotes are distinguishable, for instance, with the help of isoenzyme analysis, whereas morphological mutations are often recessive, less frequently they are dominant, therefore, usually it is impossible to differentiate a homozygote from a heterozygote, as has been mentioned earlier. Thirdly, the path of implementing genetic differences into phenotypic for protein markers is much shorter than for morphological trait. Fourthly and finally, the number of phenotypic classes morphologically distinguishable during hybrid segregation is determined by the number of heterozygotic marker loci \((n)\) and with complete dominance constitutes \(2^n\). At the same time, genotypes with new components in the protein spectrum are sometimes observed during analysis of electrophoretic differences (allelic forms), specifically, in the progeny of remote hybrids, which are not observed in either parent forms (\(P_1\) or \(P_2\)) or in \(F_1\). Furthermore, the specimens are segregated, whose specter misses the lines characteristic for both parents and \(F_1\) [7]. Therefore, the electrophoresis method allows identifying recombinants with brand new protein spectrums, which cannot be predicted because they are formed not as a result of simple combination of spectrum lines \(P_1\), \(P_2\) and \(F_1\).

Whereas the modifiers do not affect the qualitative composition of proteins determined by structural genes, the modifiers alter the activity of structural genes and, therefore, the qualitative ratio between protein fractions. Registering these ratios that can be construed as common quantitative attributes allows providing a more complete characterization of the genotype. The significance of the last circumstance can be estimated based on data obtained during drosophila
tests: intensive artificial selection for increase of fly resilience against ethanol results in significant change of quantitative ratio of alcohol dehydrogenase fractions without affecting their qualitative composition [8, 9]. The authors indicate that these results are connected with the changes in different regulatory loci. Similar changes of protein spectrum during lifetime of one generation occur in case of adaptive metabolism reformation in response to stresses [7, 10].

The reserve proteins of plant seeds, allo- and isoenzymes. For the first time, the methodological approaches to study of genetic biodiversity using proteins were developed in 1960-1970s and since then have undergone almost no changes. In order to detect and describe biochemical markers associated with protein polymorphism, polyacrylamide gel electrophoresis (PAGE) with subsequent staining is most frequently used. The reserve proteins of plant seeds are primarily used for this purpose; otherwise, identification of activity of specific enzymes is performed.

The study of plant seed proteins as molecular markers, which began in 1972-1973 in Vavilov All-Union Research Institute of Plant Breeding and ISTA (International Seed Testing Association, https://www.seedtest.org/en/home.html), laid the groundwork for identification of varieties and selective registration according to electrophoretic mobility of reserve seed proteins. In 1980, 19 Congress of ISTA recommended these methods for seed farming and seed control, and in 1983 they were accepted as wheat and barley variety identification standards [11]. Rrs14 gene responsible for resistance of barley seeds against Rhynchosporium secalis [12] pathogen was successfully mapped and genetic maps for Pinus pinaster [13-15] were constructed based on analysis of reserve seed proteins. Nevertheless, this approach did not receive a wider spread in international practice, since such markers don’t encompass all linkage groups [12, 16]. During molecular-genetic identification of plant genotypes this cheap and simple express test, as a rule, is used for evaluation of large commercial and seed farming lots or for studies of parent material in seed farming and in view of specific problems of preserving plant genetic resources. Primarily, this is due to the fact that according to the central dogma of molecular biology proteins are not a direct manifestation of genes. mRNA acts as intermediary between DNA (primary carrier of genetic information) and protein (biochemical product of gene expression). Just like DNA, mRNA can be exposed to various endogenous (recombination, splicing, mutations, etc.) and/or exogenous (for instance, environmental) factors [17]. Consequently, changes in the structure and activity of translated proteins are possible, which means that proteins do not fully comply with the genetic marker requirements [18], which somewhat limits their use in this role.

The electrophoretic analysis of allozymes has been successfully used since 1960s on different groups of organisms from bacteria to many types of animals and plants [19]. The allozymes were used in physiological, biochemical, genetic and selection research to solve different tasks, including study of the structure of populations, polyploidy, hybridization and hybrid analysis, in systematics, etc. [20, 21]. The allozyme analysis is relatively simple and easy to use. As a rule, a tissue homogenate is prepared to conduct the allozyme analysis, and obtained essence is fractioned in polyacrylamide or starch gel. Furthermore, the proteins in the essence are successively divided by charges and sizes. After electrophoresis the gel is stained in accordance with the activity of the separated enzyme by adding the substrate and stain. A characteristic pattern is formed as a result of staining (in accordance with the migratory position of enzyme protein in the gel). Depending on the number of loci, their condition (homo- or heterozygosity) and enzyme molecule configuration, it can have from one to several bands.
The bands can be pleomorphic, and, therefore, informative to determine the gene loci and linkage groups.

The isoenzymes are also used for marker analysis. For instance, in tomatoes, they were used to study genetic diversity [22, 23], to localize agriculturally important genes [24], to monitor hybrid seed purity [25], to identify gene introgression and wild type chromosomes [26], to conduct pollen selection [27], and to screen haploid genotypes regenerated in cell and tissue culture in vitro [28]. However, there exist several limitations not allowing widespread usage of isoenzymes as molecular markers [29]. We have already described general molecular marker requirements [18]; the additional mandatory conditions (specifically when generating molecular genetic maps), include, firstly, the availability of a sufficient number of marker loci equally distributed in a genome at a distance of no more than 10-15 cM from each other, secondly, marker locus should be polymorphic to ensure that hybridization would identify segregation according to marker locus at the discretion of the researcher [30]. The isoenzymes do not meet these two molecular genetic marker requirements specifically. Furthermore, the purely technical inability to detect and qualitatively evaluate the activity of the enzymes due to lack of the required stains (there are significantly fewer of them than identified enzymes, and less than half of such stains are suitable for analysis of plant isoenzymes) [31].

Sometimes, the terms ‘isoenzymes’ and ‘allozymes’ are used to substitute each other, which cannot be deemed correct. The isoenzymes identify and segregate the same substrates, but are not necessarily the products of the same gene. The isoenzymes can be active in different cells, tissues and/or organelles or at different stages of organism development. An isoenzyme variant includes allozymes, which are the products of orthologic genes. Due to their allelic differences, the amino acid composition of allozymes does not coincide for one or several amino acids. Subsequently, we will be using the term ‘isoenzymes’ (taking into account the described differences).

Protein separation in polyacrylamide gel using one-dimensional electrophoresis method. Protein markers (along with undisputable advantages that set them apart from morphological markers) have a number of limitations. First and foremost, we will note that proteins are not classified as primary carriers of genetic information and represent products of transcription and translation of genes encoding them. Moreover, protein polymorphism identified via one-dimensional electrophoresis can be subjected both to qualitative and quantitative change due to impact of environmental stress on plants attributable, for instance, to shortage of feed elements, which is described for barley [32], peas [33], chickpea [34], soybeans [35] and other cultures [36, 37] (Fig. 1) or change of air temperature (Fig. 2) [6].

A possibility of violation of the structure and integrity of analyzed molecules due to various reasons should be taken into account [18], including failure to meet the standard conditions of polypeptide protein extraction during extraction and purification, and during electrophoretic separation, which can result in identification of non-specific electrophoretic spectra [36, 38-41]. The degeneracy of genetic code also serves a limitation because it significantly narrows the polymorphism spectrum identified at the level of amino acid sequences as compared with polymorphism of primary nucleotide sequences, and, therefore, of analysis possibilities. Furthermore, it is a known fact that just a third of the genome (or even less) encodes and expresses certain proteins. Consequently, genomic changes in the non-coding or regulatory parts of the genes (more than 70% of the genome) are overlooked during study of protein products.
Fig. 1. The impact of sulfur shortage (+−) on formation of whole proteins of colza seeds (A), sunflower seeds (B) and barley seeds (C). Of interest is the presence of fractions of sulfur-rich low molecular weight proteins (apparently, 2S albumen) for colza and sunflower and sulfur-poor barley hordeins [36].

Fig. 2. The impact of temperature on the electrophoretic spectrum of leaf water-soluble proteins of seedlings of different genera of Lycopersicon species: 1 — L. hirsutum var. glabratum, 2 — L. pimpinellifolium, 3 — L. esculentum (Mo500) × Solanum pinellii (F1), 4 — L. esculentum (Teplichny 200 variety); a — 25 °C, b — 40 °C [6].

Nevertheless, using proteins as biochemical markers still remains an attractive tool [11, 42-44]. This is due to three key advantages of reserve seed proteins and isoenzymes as compared with DNA markers. First, this is due to relative simplicity of protein analysis using comparatively cheap electrophoretic methods. Secondly, in a sufficiently large number of individuals (genotypes) reserve proteins or isoenzymes can be analyzed during a relatively short time. Thirdly, the isoenzymes and some reserve seed proteins are codominant markers, because both alleles in a diploid organism are usually clearly distinguishable and heterozygotes can be separated from homozygotes. As a rule, this is sufficient to determine allele frequency (specifically, in population genetics). Does the resolving power of one-dimensional electrophoretic separation method allow detecting and accurately evaluating the polymorphism of amino acid sequences constituting the basis of marker biochemical analysis?

The one-dimensional electrophoresis of protein dissociated sodium dodecyl sulfate (SDS-PAGE) belongs to the most frequently used methods of studying polymorphism of plants using reserve seed proteins as markers [11, 12, 43, 44]. This method allows identifying variations of polypeptide molecular mass occurring as a result of indels (insertions/deletions) in the coding region of a respective gene; however, it is insensitive to the changes of polypeptide charges. Therefore, the applicability of reserve seed proteins as molecular markers is determined by indel frequency and their dimensions, which should be rather large to identify variations of molecular mass of encoded polypeptide. This problem is aggravated by heterogeneity of reserve protein polypeptides formed as a result of macroevolution of plant orders, families and genera. In other words, the task of analyzing the reserve protein intraspecific polymorphism with the help of SDS-PAGE should include the description of modification of molecular mass of each of polypeptides formed as a result of macroevolution events.

Consequently, the intraspecific polymorphism of reserve protein can be described only if evolutionary relations between the polypeptides of which it is comprised are known, at least at the level of plant families and genera. For instance, one of the studies [44] analyzed seed proteins of 11 varieties of blue lupine and seed parents of some of these varieties using a method of one-dimensional denaturating electrophoresis in polyacrylamide gel. Whereas pro-
teins from seeds were separately extracted using tris glycine electrode buffer (pH 8.3) and additional purification was not performed (using chromatography or any other method), the authors identified the heterogeneity and polymorphism not only of α- and β-conglutins, but of some other proteins as well, which have identical physical and chemical properties and are, therefore, extracted simultaneously with reserve plant seed proteins (Fig. 3, 4). Furthermore, in analyzed seed proteins polymorphism was discovered not for all varieties, and authors used microsatellite DNA analysis to clarify data of protein analysis, which showed polymorphism in varieties, for which the electrophoretic analysis of analyzed seed proteins did not give a positive result. The authors reason that during selection of parent plants the electrophoretic analysis of their proteins will allow excluding obstruction of homogenous varieties and preserving the composition and ratio of biological types for multicomponent varieties [44]. However, it is known that today the completely homogeneous varieties almost do not exist, and population varieties used in the agriculture are rather heterogeneous. The same goes for samples of genetic plant resources preserved in gene banks or primary breeding material. Nevertheless, reserve plant seed electrophoresis still remains a simple and reliable method of controlling purity of seed material lots and trademark safety, i.e., to determine the predominantly mechanical contamination of seed lots, which is declared, for instance, by ISTA (http://www.seedtest.org/en/home.html) as its goal. At the same time, global international organization Bioversity International (Italy) (http://www.bioversityinternational.org), which has studied genetic diversity since 1990-s has been recommending to perform genetic analysis with the help of molecular DNA markers [45, 46].

![Fig. 3. Separation and purification of legumin-like protein from soybean seeds.](image)

The results identical with the data of Eggi et al. [44] were obtained in 1969-1970 and 2006-2007 by two independent groups of scientists in Russia during experiments to determine the impact of nitrogenous nutrition on blend composition of gliadine in winter wheat [17, 48, 49]. Interestingly, the cereal prolamines, which include wheat gliadine, have a rather broad polymorphic blend composition and, as a rule, belong to multigene families, which sets them apart from globulins of the dicotyledons. However, the blend composition of prolamines, as with any other plant seed protein, can be subject to change due to impact of the environment and conditions of cultivation (see Fig. 1), which was made clear by studies of the Russian scientists [17, 48, 49]. Wheat was cultivated in field conditions at different seeding rates, and with various doses of mineral fertilizers and pesticides. Significant changes in the blend composition of gliadine were observed in case of introduction of increased nitrogen doses [17, 48, 49]. The molecular genetic analysis determined that different genetically deter-
mined components in the electrophoretic spectrum of wheat gliadines have uneven response to changes of the environmental [49]. Up to 30% components manifested very high dependence of synthesis on plant cultivation environment. It is quite probable that quantitative changes in synthesis of these proteins are determined by the degree of stability of corresponding mRNA [17].

In all fairness it has to be pointed out that essentially any vegetable protein can be used as genetic marker for description of intraspecific polymorphism with the help of SDS-PAGE, if this protein satisfies the following formalized conditions: it is sufficiently conservative to be identified as a member of vegetable protein family; however, it is sufficiently variable for its microevolution changes to be identified using the SDS-PAGE method; ideally the genome should contain a single gene of potential marker protein or a small number of its clearly distinguishable variants formed as a result of the macroevolution.

It has to be made specifically clear that the required condition of applicability of SDS-PAGE method for description of polymorphism of any proteins is the purity of preparations in question (Fig. 4). As a rule, the unpurified or crudely purified preparations contain polypeptides of protein admixtures close in terms of molecular mass to studied proteins, for instance, to reserve plant seed proteins (see Fig. 4), which complicates identification of their macro- and microevolution variants.

Rough fractionation (sedimentation and reprecipitation) by various types of salts provides only a preliminary purification (because along with the proteins in question other proteins are segregated, which are contained in plant tissues and have similar physical and chemical properties) and serve as the first stage of pure protein segregation, which should be followed by the second stage, i.e. purification by chromatographic or other methods (Fig. 5) [38-40, 50].

If purification via chromatography or another method is not performed, it is strictly necessary to provide evidence that the analyzed preparation does not contain admixtures of other proteins, or heterogeneity of protein essence being analyzed should be specified. Furthermore, keep in mind that variability of elec-
trophoretic spectrum of rough protein spectrum in case of one-dimensional electrophoresis can be the result of genotype-environment interaction [17, 34, 37, 52, 53] of varying degree of seed plumpness [54] and change of gene activity regulation [36, 49]. It has to be additionally pointed out that resolving power and specificity of bioinformatics methods used to describe protein polymorphism at the level of encoding nucleotide and amino acid sequences is higher by far compared to any electrophoretic method of their analysis [41, 55-57], which is another evidence against using the SDS-PAGE method. And finally, isoenzyme analysis is commonly used to identify an insignificant degree of variability for no more than 20-50 enzymes visualized with the help of biochemical staining [24], and common protein polymorphism, as a rule, depends on a number of endogenous and exogenous factors, which was discussed earlier, and covers only the expressed part of the genome. In some instances, it was displayed that isoenzymes vary in terms of one or several physiological properties [58] due to which they cannot be evolutionary neutral.

A more practical aspect resides in the fact that plant tissues designed for analysis of isoenzymes or other proteins immediately after collection should be used for extraction of these components, because along with the other proteins in such samples isoenzymes are usually not very stable.

In summary, a number of limitations exist that do not allow isoenzyme analysis and any other protein analysis to become universal for identifying of genetic variability in spite of its easiness of use and low cost. The new allele can be identified as polymorphic only if nucleotide replacement in DNA results in replacement of amino acid in protein, which, in turn, entails changes in electrophoretic mobility of protein molecules in question. The genetic code is degenerated and not every amino acid replacement will result in charge change and significant change of protein molecular mass; therefore, only 30% of all nucleotide replacements are manifested as protein polymorphism detected by electrophoresis. The electrophoretic spectrum of proteins depends on genotype-environment and genetic interaction (for instance, on changing nutrition conditions during protein biosynthesis conditions, impact of various stresses, year, location and time of plant cultivation, etc.) and even on the terms of protein extraction and electrophoresis. Consequently, by analyzing allozymes and isoenzymes and/or other proteins it is impossible to fully determine and evaluate the genetic variability. Another problem resides in the fact that many plant species are polyploid, and, as it is known, for polyploid types the analysis of isoenzymes can be significantly complicated. Furthermore, isoenzymes can vary in terms of one or several physiological properties and in this case cannot be neutral in terms of evolution. The samples selected for analysis of protein polymorphism should be promptly used for their segregation and fractionation. Quite the opposite, the methods based on DNA analysis allow conducting research a long time after collection of plant tissues or DNA isolation due to the ability of long-term preservation of samples and preparations without property loss. If plant material collection is performed at considerable distance from the laboratory, it is quite obvious that DNA analysis is preferable.

REFERENCES


34. Charcosset A., Gallais A. Application of markers in selection. In: Molecular markers in plant
56. Chesnokov Yu.V. Geneticheskie resursy rastenii i sovremennye metody DNK-tipirovaniya [Plant genetic resources and modern DNA typing techniques]. St. Petersburg, 2007 (in Russ.).
57. Stefanov V.E., Mavropulo-Stolyarenko G.R. Analiz struktury belkov metodami bioinformatiki [Bioinformatic analysis of proteins structure]. St. Petersburg, 2007 (in Russ.).
FUNGICIDE RESISTANCE OF PLANT PATHOGENIC FUNGI AND THEIR CHEMOSENSITIZATION AS A TOOL TO INCREASE ANTI-DISEASE EFFECTS OF TRIAZOLES AND STROBILURINES
(review)

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Abstract

The chemical method for plant protection is still the most reliable way to provide the high yield of economically significant crops and ensure its quality. In the world agriculture, at least 150 different fungicidal compounds with different mechanisms of action are now used, and the number of products developed and registered on their basis is much more. Triazoles and strobilurins belong to fungicides, which have expanded the opportunities to control causative agents of the most damaging diseases (D. Fernández-Ortuño et al., 2008). Nevertheless, multiple applications of fungicides during each new growing season are often required to achieve an effective control of fungal and oomycete pathogens. Such extensive applications of fungicides exacerbate negative impact on environment, and promote developing the resistance by these pathogens, representing the most disturbing consequence of fungicidal treatments (J.A. Lucas et al., 2015) that makes them relatively short-lived and eventually uneconomical (K.J. Brent et al., 2007; R.P. Oliver, 2014). Attempts to combat resistant forms of plant pathogenic fungi and oomycetes by increasing the dosage of fungicides and treatment numbers are futile, as they cause accumulation of more and more resistant strains in fungal populations. Therefore, control of these pathogens by minimal effective dosages of fungicides, without any decrease in the fungicidal efficacy, and search for ways to overcome the plant pathogen resistance to fungicides are dominant trends in plant protection for current sustainable agriculture. At the same time, the rejection of modern fungicides with high and medium risk of the resistance, including strobilurins and triazoles, does not seem to be practically rational, since they provide a highly effective control of a wide range of diseases and have several other advantages (A.V. Filippov et al., 2016). Chemosensitization of plant pathogens by natural compounds to increase efficacy of fungicidal treatments is an approach to solving the aforementioned problems. Chemosensitization can be accomplished by combining a commercial fungicide with a certain non- or marginally fungicidal substance at concentrations where, alone, neither compounds would be effective, while after their co-application a synergistic fungicidal effect is achieved, sometimes at a level significantly exceeding that of the fungicide dosages to which resistant strains are insensitive (B.C. Campbell et al., 2012; V.G. Dzhavakiya et al., 2012). Since biochemical and structural targets of chemosensitizing substances differ from those targeted by fungicides, chemosensitization do not contribute to the selection of resistant pathogenic form, and reduces the toxic impact on the environment by lowering effective dosage levels of toxic fungicides. In this review, the promise of chemosensitization as an antiresistant strategy to improve efficacy of the protective fungicidal effect is exemplified by experiments with several economically significant phytopathogenic fungi, which sensitivity to strobilurins and triazoles was demonstrated to enhance significantly by co-application of these fungicides with secondary plant or microbial metabolites and their synthetic analogues. In addition, the problem of the development of resistance in plant pathogenic fungi and the methods for its management are briefly described, information on the types and main mechanisms of resistance, in particular, those responsible for resistance to triazoles and strobilurins as well as data on the mechanisms of action of some chemosensitizers are presented.

Keywords: chemosensitization, plant pathogenic fungi, resistance to fungicides, triazoles, strobilurins, fungicide stress-responsive metabolic pathways, resistance overcoming
In the context of intensively developing horticulture the stability of agricultural crops, especially commercially cultivated, cannot be ensured without taking steps to fight crop disease. Plant pathogenic fungi and oomycetes are some of the most dangerous pathogens [1, 2]. In the vast majority of cases fungicidal treatment still remains (and for a long time will remain) the most desirable method of combating pathogens that guarantees high yielding crops, preservation and quality of agricultural products.

At the same time, it is a known fact that wide-scale application of fungicides is connected with serious environmental and medical risks, and with pathogen resistance to these antifungal agents. In spite of the fact that integrated plant protection systems aimed at environmentalization of agricultural production is dominated by the tendency of reduced application of fungicides, multiple fungicidal treatment during each season is often required for reliable protection of crops and control of many plant pathogenic fungi and oomycetes. For instance, potato farming in Russia has 9-11 fungicidal treatments per season, and in some European countries — up to 18 sprayings with fungicides per season. Such an intensive application of fungicides, in spite of strict regulations developed with the purpose of risk minimization, increases pesticide load in agrobiocenosis, deteriorates the environmental situation and is accompanied by development of resistance. Arguably, the accumulation of persistent forms of fungi and oomycetes in natural populations is the most formidable undesirable consequence of fungicidal treatment [3], which in many cases makes them ineffective and economically unjustified [5, 6], and sometimes results in removal of entire classes of fungicides from circulation [7].

It has to be pointed out that resistance phenomenon is of general biological nature. Resistance to fungicides is a particular case of natural biological evolution of organisms capable of adapting to changing environmental conditions, which creates problems not only for horticulture. For instance, resistance of bacteria and fungi to medical preparations significantly complicates treatment of patients suffering from bacterial disease and mycosis [8, 9]. In general, resistance of pathogenic microorganisms remains one of the major problems of our age, and reduction of resistance of pathogenic microorganisms in phytopathogenic fungi is a pressing task of agricultural science. In 1994, the Fungicide Resistance Action Committee (FRAC, https://www.frac.info/) was established. This Committee supervises monitoring of resistant mutations in populations of phytopathogens and coordinates the development of anti-resistance defense technologies and gives recommendations for resistance development risk mitigation when using fungicides against pathogens of agricultural plants of economic significance in different countries. EuroBligh (http://agro.au.dk/forskning/inter-nationale-platorme/euroblight/) specifically pursues the issue of resistance of Phytophthora infestans and early blight of potato (Alternaria solani). Whereas potato is a high demand culture, this network combines not only the scientists, but also agricultural producers interested in obtaining maximum crops via multiple treatments using various fungicides. In Russia, the problem of antibiotic and fungicide resistance is also emphasized. For instance, a suitable strategy has been developed and recently approved (Resolution of the Government of the Russian Federation No. 2045-p dated September 25, 2017) to prevent advancement of resistance of pathogenic organisms, including pathogenic organisms affecting plants.

The problem of pathogenic fungi resistance of plants in agriculture. At least 150 chemical formulations with various mechanisms of action (MA) presently divided into 46 classes are used as fungicide active agents in agriculture worldwide. Furthermore, 12 groups of multisite fungicides are combined in a separate class [3, 4, 10]. The triazoles (class G1) and strobilurins
(class C3) belong to three groups of active agents, commercialization of which in 1980-1990s ensured a breakthrough in the fight against pathogens of the most maleficent plant diseases in many countries [11].

The triazoles or DMI-fungicides suppress synthesis of fungal strains by inhibiting $14\alpha$-demethylase (CYP51, cyp51/erg11 gene), which belongs to cytochrome P450 superfamily. This enzyme is responsible for cleavage of $14\alpha$-methyl group of lanosterol, the ergosterine precursor [12] (primary sterol component of fungal membranes, which the plants do not have). The inhibiting effect of this enzyme results in deficit of ergosterine and accumulation of toxic $14\alpha$-methyl sterol, high concentrations of which intensify the oxidative stress, cause damage to the membrane and, consequently, death of fungal cell [13]. The strobilurins or QoI-fungicides act as blocking agents of ubiquinone oxidase (cytochrome bc1, CYTB, cyt b gene). For the first time, strobilurins A and B were detected in culture liquid of *Strobilurus tenacellus* basidiomycete, following which their chemical derivatives were obtained with an identical mechanism of action, which are divided into 9 groups [11, 14]. The strobilurins inhibit fungal mitochondrial respiration by connecting to the external bc1 cytochrome site (complex III) and inhibiting the transfer of electrons between cytochromes b and c. As a result, strobilurins suppress ATF synthesis, thus causing deficit of energy in fungal cells, which resulting in their stasis.

Numerous fungicidal agricultural products were developed and registered based on various triazoles and strobilurins and their combinations with active agents from different classes; triazoles are also used in medicine as medicinal products.

**Fungicide resistance development.** Until the turn of 1970s, reports about reduced efficiency of fungicides after prolonged periods of use due to emergence of resistant phytopathogen forms were relatively rare [15]. Therefore, it seemed that in agriculture the resistance problem does not have practical value and can be solved with the help of tank mixtures, development of new preparative forms and treatment rules based on experience of application of active agent groups [16] predominant in the 1960s. However, as new systemic and contact fungicides emerge, especially with single-site activity type (including DMI- and QoI-fungicides), the number of cases of reduced or lost sensitivity and cross-resistance of different fungi [17-21], including those affecting potato [22], and oomycetes [23, 24] grew significantly [4, 25], and the time required for the emergence of resistant forms in many cases decreased significantly — sometimes to 2 years after the first commercial application [11, 26, 27].

The attempts to counter resistant forms by increasing dosages, usage rates and repetition factor of new fungicide treatment, which were recommended for use in relatively small volumes due to high activity against phytopathogens only aggravated the problem causing accumulation of ever more resistant strains and stimulating their spread in populations [3]. This tendency was first observed in practice when the efficiency of chemical protection is reduced, and later proven experimentally. In particular, the laboratory research validated a positive correlation between concentration increase of triazole fungicide and accumulation of resistant forms, and development of intraspecific cross-resistance of winter wheat leaf blotch pathogenic agent (*Zymoseptoria tritici* = *Mycosphaerella graminicola*) [27]. It was also demonstrated that isolate frequency resistant to this QoI-fungicide [28] increases in case of increasing dosages and azoxystrobin usage rate by the end of the growing season in the population of powdery mildew (*Blumeria graminis* f. sp. *hordei*). Similarly, the sensitivity of *P. infestans* to mancozeb and chlorothalonil gradually decreases in the course of their multiple use, although the risk of resistance development to these multisite fungicides is low [29].
Presently, there have been registered cases of emerging resistance to almost all key classes of fungicides in the most diverse phytopathogen types [10]. In this context, triazoles and strobilurins are not an exception, and according to FRAC rating the risk of resisting them is evaluated correspondingly as average and high. It is believed that fast development of resistance to QoI-fungicides is due to the fact that ubiquinone oxidase is encoded with mitochondrial DNA, which possesses less expressed reparation ability than nuclear DNA [25]. The forms of potato blight pathogens (A. solani and A. alternata) resistant to azoxystrobin for the first time were identified as early as in 2000 (the preparations on its basis came into use at the end of 1990s). The intraspecific resistance was identified for all active agents of QoI-fungicides. For DMI-fungicides it is usually marked for those active agents that are active against the same pathogen, and are not detected with regard to inhibitors of strain synthesis from different classes.

**Primary mechanisms of fungal resistance to fungicides. Resistance types.**

The molecular research of stable strain genome changes of various fungi and oomycetes enable understanding the resistance mechanisms and showed that in most cases it is caused by gene mutations encoding metabolic targets of fungicides, and is connected with different adaptation mechanisms triggered by chemical stress.

Quality resistance [7] usually develops relatively fast and with a high degree of probability occurs in the first instance typical for single-site fungicides. When it spreads, only fully resistant strains accumulate in populations of pathogens, whereas forms with average resistance are absent (complete loss of fungicide efficiency). However, forms with quality type resistance can be preserved only provided that gene mutations encoding structural and metabolic fungicide targets do not interfere with pathogen viability. In this way, strains resistant to QoI-fungicides cannot be detected in populations of Rhynchosporium secalis and Puccinia spp. in spite of regular use of QoI-fungicides in the course of many years. Due to special structure of cytochrome b gene, in fungi, the G143A mutation typical for resistance to strobilurins disrupts mRNA processing and expression of cytochrome non-functional protein, as a result of which the mutants with the allel carrying G143A do not survive.

The quantitative resistance can impart resistance to fungicides with different mechanism of action and result in existence of forms with incomplete resistance in populations (reduced fungicide efficiency). This is due to several adaptation mechanisms the effect of which is aimed at maintaining nonlethal concentrations of fungicides in a cell. This is achieved by strengthening the expression of ABC transporter genes (proteins that remove molecules of pharmaceutical and other toxic substances from a cell into extracellular space; in some phytopathogens MFS-transporters are also involved in this process), plasmatic membrane modifications (reduced permeability for fungicides), and synthesis of enzymes that destroy a fungicide or transform its molecules into compounds that are not toxic for the fungus [7, 30-32]. Furthermore, the overexpression of genes encoding biochemical or structural targets of fungicides [33] and use of alternative metabolic pathways by pathogens can also make a contribution to ensuring stability of the qualitative type [34, 35].

**Mechanisms of resistance to triazoles and strobilurins.** Several mechanisms of phytopathogenic fungi and oomycetes take part in the development of resistance and tolerance to triazoles and strobilurins resulting in both qualitative and quantitative resistance. Primarily, point mutations V136A, Y136F, Y137F, A379G, I381V in cyp51(erg 11) gene and its promoter and activation of ABC-transporters are responsible for resistance to triazoles. For instance, it has been demonstrated that Y136F, the point mutation in the 136-th
codon resulting in replacement of phenylalanine with tyrosine, causes the development of resistance to triadimenol in powdery mildew pathogens in grapes (Uncinula necator) and barley (Erysiphe graminis f. sp. hordei) [10, 36]. Almost all possible cyp51 single nucleotide changes were detected in high-, weak and moderately resistance strains of winter wheat leaf blotch pathogen Z. tritici (M. graminicola) from France and the UK, and some strains of the pathogen with moderate or high resistance contained an insertion in the promoter of this gene or combinations of point mutations [37]. At the same time, it was determined that resistance to DM-inhibitors of field isolates of some phytopathogenic fungi is not always related with amino acid replacements in CYP51 protein [12]. For instance, in mutant phenotype of Z. tritici (M. graminicola) high multiple resistance to DMI-fungicides, apparently, is also ensured by overexpression of genes of transporter proteins [37].

Resistance to strobilurins is primarily due to two mutations of cyt b target gene resulting in glycine replacement with alanine (G143A) and phenylalanine with leucine (F129L), as well as adaptation mechanisms, specifically, over-expression of alternative oxidase functioning in circumvention of respiratory complex III [10, 12]. None of the named mutations causes negative impact on viability of pathogens; therefore, resistant strains with these point replacements are often encountered in populations of different fungi, including potato, rice, barley, wheat and gourd family pathogens [12]. The G143A point mutation has been identified in A. alternata, but still not identified in A. solani [38]. Quite the opposite, phenotype with F129L mutation are known for both types causing potato blight. The degree of resistance of such mutants A. alternata is usually lower than for phenotypes with G143A, but in American, Canadian and Swedish populations of A. solani the contribution of isolates with F129L into reduction of efficiency of azoxystrobin usage against early potato blight has been observed [39, 40].

For more detailed information refer to surveys [7, 11, 12, 41, 42], which are specifically dedicated to resistance mechanisms, and to experimental articles analyzing various mutations related with resistance to DM- [37], Qo-inhibitors and some other fungicides [43-48], as well as the role of ABC-transporters in the processes of their detoxication [49, 50]. It has to be pointed out that accumulation of mutations in natural populations occurs gradually. Furthermore, mutants that are tolerant or completely resistant to fungicides often do not display any phenotypical differences or weakening of pathogenicity or other defects [45, 51]; therefore, loss of treatment efficiency due to development of resistance usually becomes noticeable after resistant strains begin to dominate in the population. Similarly, the populations of P. infestans, which are fully resistant to fungicides of acrylanine and carbamate group [29] developed in the territory of potato growing regions.

“Best practices” antiresistant strategy. The realization of a problem of a mounting decline of biological and economical effectiveness of fungicidal products resulted in the development of a strategy of fungicide resistance management, which is based on experience of their most efficient and rational practical application (best practices). The fungicide resistance management is a body of rules of using fungicides and methods of cultivating treated cultures aimed at deceleration of selection of stable pathogen forms and mitigation of the risk of spreading resistance [6, 52]. The essential elements of this strategy are the following: firstly, proper selection of fungicides (if possible, priority is given to the so-called low risk fungicides) and their rational usage (combining preparations with single-site and multisite active agents); secondly, treatment with fungicides with varying mechanisms of actions, so that if a pathogen develops resistance to one of them it would become controlled by a partner preparation with different
mechanisms of action, and periodic rotation of preparation with different mechanisms of action. Furthermore, one must take into consideration the prohibitions to combine or consecutively use of several fungicides, regulations for a summary dose and number of treatments, strict compliance with the instruction for a method of using the preparation and not violate the usage rates listed in it. Also, in fungicide resistance management great value is attached to the selection of resistant varieties, crop rotation (preceding crop should not contribute to infection accumulation) and alternative (non-chemical) plant protection methods, as well as agricultural practices adequate to the region of culture growing to avoid insufficient or excessive irrigation and/or fertilization because both of these factors can contribute to the disease development.

Appropriately, for a specific combination of crop-disease-geographical region the tactics of resistance management in scope of strategy description and its efficiency can vary significantly. For instance, in Northern Ireland the introduction of fungicide resistance management in potato growing practices resulted in containing the selection of \textit{P. Infestans} strains, which are resistant to phenylamides [53]: their share in pathogen populations in 1999-2001 reached 76%, and after introduction of fungicide resistance management in 2002 it was reduced after 3 years to 22% [54]. In this region phenylamides are successfully used to combat potato blight, and it is assumed that their application against the background of the antiresistant technology will continue [54]. In a similar manner in 2004-2005 they managed to change the tendency of spreading resistance to azoxystrobin. However, in a number of regions of the UK and USA adherence to a rational scheme of preventive or curative spraying against potato early blight and late blight, although successful for the development of disease at the level not yielding economic damage, at the same time stimulated the selection of strains with hyposensitivity to phenylamides of \textit{P. infestans} or strobilurin in \textit{A. solani} [38]. Furthermore, there are voiced concerns that even if the recommendations of fungicide resistance management are adhered to, the risk of resistance development of certain fungi and oomycetes, especially those affecting perennial plants can remain quite high [55].

However, according to FRAC, the abandonment of modern systemic and contact single-site fungicides from high and medium resistance risk groups, including strobilurins and triazoles, is not deemed successful from the practical point of view, because they ensure highly efficient control of a wide range of diseases and have a number of other advantages [29]. For instance, strobilurins preserve their efficiency with regard to winter wheat leaf blotch pathogen (\textit{Z. tritici}) in Italy [56]. The results of analyses made in 2015 under project EUROwheat (http://agro.au.dk/forskning/internationale-platforme/eurowheat/) also demonstrated that triazoles that have been used in the Northern and Central Europe for more than 35 years to fight Septoria spot (\textit{Z. tritici}), yellow (\textit{P. striiformis}) and brown (\textit{P. triticina}) rust successfully protect wheat from these diseases, including Septoria spot, in spite of detecting in it a pathogen of six different mutations of 14α-demethylase gene and intraspecific cross-resistance [57].

Under the circumstances the development of additional approaches seems rather promising, which would allow preserving or even strengthening the protective effect of modern fungicidal preparations with the help of environmentally friendly compounds, and thus reduce their selective action in phytopathogen populations facilitating the selection of resistant forms without increasing the dosages or the number of treatments. Chemosensitization of phytopathogenic fungi to agricultural fungicides can be one of such approaches.

Chemosensitization as an advanced antiresistant strategy of increasing the efficiency of protective effect of fungicides. The term
“chemosensitization” is borrowed from medicine where initially it was introduced to designate an approach preventing resistance of cancer cells to radiation and chemotherapeutic anticancer agents with the help of chemosensitizers — chemical compounds or natural substances. Synergism is often observed in their use, due to which the efficiency of medicines and radiation exposure increases [8, 58].

It turned out that key mechanisms determining the resistance of cancer cells [59] are in many respects identical to resistance mechanisms of microorganisms, including fungi, which are pathogenic for humans. Therefore, chemosensitization began to be actively developed in medicine to overcome the resistance of mycosis pathogens and increase of their sensitivity to antimycotic agents with the help of natural and synthetic compounds nontoxic or insufficiently toxic to completely suppress the development of the mentioned pathogens [60]. In the meantime, the chemosensitization approach can be useful not only for improvement of antifungal chemotherapy of human mycosis, but also to fight phytopathogenic fungi. It could become an essential component of antiresistant strategy of protecting cultivated plants with the help of fungicides because it would facilitate weakening or overcoming the resistance of commercial preparations used presently. However, only isolated attempts have been made so far to apply a similar strategy to plant protection and adapt it to overcome resistance of phytopathogenic fungi to commercial fungicides [8, 61, 62]. Whereas the need to reduce unfavorable environmental effects is taken into consideration, natural compounds or their alternatives degradable in the environment are usually used as sensibilizators.

The natural or synthetic compounds functioning as chemosensitizers are either nontoxic for fungi or possess weak fungitoxicity, which is at least an order lower than that of active agent fungicides. Potentially fungitoxic substances are also very effective sensibilizators (for instance, such secondary plant metabolites as thymol or berberine) used in minimal concentrations, which have insignificant inhibiting (subfungicidal) effect on the pathogen. In other words, during chemosensitization the used concentrations of both fungicide and sensibilizator are such that acting alone these agents are inefficient; however, when used collectively they suppress a pathogen, including levels significantly surpassing the effect of fungicide dosages, to which resistant strains are insensitive [8, 61]. This phenomenon is based on the ability of chemosensitizers to cause various stresses, disrupt cellular structures or otherwise weaken phytopathogenic fungi, thus increasing their sensitivity to antifungal preparations and strengthening their effect. The interaction of chemosensitizers with fungicides can be additive, but in most cases, it is synergetic, which allows significantly reducing effective concentrations of active agents (by one or even two orders) [8, 61]. Moreover, as a result of changes of metabolism weakening the pathogen and caused by chemosensitizers even its resistant forms can become more sensitive to fungicide. Subsequently, the suppression of resistant strains and effectively inhibiting their accumulation can be achieved without increasing the recommended dosages of fungicidal preparation, which enables reducing the risk of selecting resistant forms and their spread in populations of phytopathogenic fungi.

A wide-scale screening of natural compounds aimed at identifying their sensibilizing activity to overcome cancer cell, bacteria and fungi resistance to medicines resulted in identifying target activity of a number of secondary plant metabolites and certain microorganisms. Potential chemosensitizers have been identified in plants among phenolic acids, tannins, terpenoids (including mono-, di- and triterpenes, the saponins), steroids, alkaloids, flavonoids, catechines, as well as in some other groups of secondary metabolites [63] and their synthetic
equivalents [61, 64, 65]. Many of these substances are of interest as components of medicines efficient against resistant hospital strains of mycosis pathogens [8, 66]. As for naturally growing fungi, the ability of cinnamic aldehyde, which is almost nontoxic for basidiomycetes, is known to strengthen its activity manifold versus tinder *Laetiporus sulphureus* in combination with vegetable phenols: eugenol, quercetine and catechine [67, 68].

The mechanisms of action of chemosensitizers responsible for amplification of fungicidal effect are not always clear. Nevertheless, the research of synergism of natural and synthetic compounds with medicinal antimycotic agents summarized in a review of Campbell at al. [8] demonstrated that many of these compounds affect the ability of fungi to respond to stress. This stress can be caused both by environmental factors (for instance, UV radiation, salinity, drought, etc.) and by impact of fungicidal preparations. Using molecular genetics methods in these studies allowed identifying links in the systems of fungal protection against stress, which could become biochemical or structural targets of chemosensitizers. In particular, it was determined that sensitivity of pathogenic yeast and aspergilli to antimycotic preparations can be significantly increased in case of exposure to compounds violating the defenses of these fungi against oxidative and osmotic stress [69-71]. It has also been demonstrated that adding the aforementioned cinnamic aldehyde, which causes osmotic stress, to the cultures of four types of xylotroph fungi synergistically amplifies the inhibition of fungal growth by octyl gallate, a plant phenol, which disrupts the cellular membrane structure [72] and causes apoptosis.

By contrast to medical studies, there are few works, in which metabolites of germs and plants are used for sensibilization of phytopathogenic fungi to fungicides in order to increase the efficiency of the latter, including against resistant strains. So far, these experiments [73-75] have not included treatment of plants infected with resistant strains, and with the exception of two reports [62, 76] are performed during growing of phytopathogenic fungi in the culture. Nevertheless, the authors convincingly proved the presence of synergism when using certain concentrations of these metabolites and their synthetic equivalents, and fruitfulness of chemosensitization approach to increase fungicidal effect against phytopathogens was confirmed.

The first detailed studies of sensibilization of fungi that are saprotrophic and potentially pathogenic for plants via secondary metabolites of plant origin were undertaken by employees of a scientific center of the United States Department of Agriculture, USDA in the state of California. These studies were performed using cultures of several aspergilli, which, although are optional human pathogens, have agricultural significance, and *Penicillium expansum* fungus causing mildew of apples. These experiments showed the efficiency of 2,3-dioxybenzaldehyde (2,3-DOBA), 4-oxybenzaldehyde (4-OBA), thymol and 2,5-dihydroxybenzoic acid as sensibilizators *P. expansum*, certain types of *Aspergillus* and toxigenic strains *A. flavus* (69, 77). It was discovered that in vitro usage of 2,3-DOBA or gallic acid along with strobilurin fungicides kresoxim-methyl and fludioxonil results in synergetic increase of their activity against *A. flavus* and *A. parasiticus* (78). When using *Saccharomyces cerevisiae* with point deletions in *sodA* gene encoding mitochondrial superoxide dismutase (Mn-SOD) as mutant models it was determined that salicylic aldehyde, gallic, ascorbic and chlorogenic acids make *A. flavus* more sensitive to kresoxim-methyl because they disrupt the functioning of metabolic fungus systems responsible for protection from oxidative stress caused by this fungicide [79]. The increase of fungicidal activity of fludioxonil towards *A. flavus* was demonstrated for berberine (barbery alkaloid) and certain phenol compound that also affect the antioxidant fungal systems [69]. It was
determined that aldehyde activity of salicylic acid and other benzole analogues was based on inhibiting HOG1-signal system controlling the defense against osmotic stress of A. flavus and P. expansum [64, 70, 79]. Furthermore, as exemplified by kojic acid produced by many filamentous fungi it was demonstrated that, in spite of the common mechanism of action, some of the sensibilizers can be species- and/or strain-specific [80, 81]. To summarize it was demonstrated that alkylgallates are capable of amplifying the sensitivity to fludioxonil in resistant strains of P. expansum [73].

Generally speaking, as a result of studies conducted by aforementioned group of American scientists understanding was gained that presently known sensibilizers attack those paths of fungal metabolism that control their defense response to oxidative stress, and amplify it by provoking generation of reactive oxygen species toxic for fungi and disrupt the integrity of cellular and vacuolar membranes causing osmotic stress and apoptosis. However, the significance of these mechanisms was so far demonstrated only for a small number of fungal pathogens, and many aspects of phytopathogenic chemosensitization to agricultural fungicides remain unstudied [8].

By virtue of studies conducted in the recent years, the list of fungi whose sensitivity to strobilurins and triazoles can be multiplied with the help of chemosensitization by natural metabolites and their analogues includes commercially significant phytopathogens [61, 62, 74-76]. For some of them, the long-term benefits of using this strategy to amplify the protective effect of triazoles were demonstrated on plants, including field conditions [62, 76]. Furthermore, a possibility in principle of overcoming the resistance of natural strains of wheat Septoria spot pathogen to these fungicides [75] was demonstrated.

It was determined that in vitro azoxyastrobine (Quadris® KC 25 %, Syngenta AG, Switzerland) in combination with concentrations of thymol (monoterpene phenolic derivative of one of the aromatic compounds of thyme) not toxic for fungi and non-phytotoxic caused a much higher inhibition of growth of Bipolaris sorokiniana, Phoma glomerata, A. alternata and Parastagonospora nodorum (= Stagonospora nodorum) than in the case of its individual application. The effect of difenoconazole used together with thymol is also significantly amplified with regard to B. sorokiniana and P. nodorum, whereas fungitoxicity of DIVIDEND® KE 3 % (difenoconazole; Syngenta AG, Switzerland) for B. sorokiniana increased tenfold. The application of tebuconazole with 4-OBA, 2,3-BODA or thymol was accompanied by amplification of the inhibiting effect of fungicide on A. alternata. The introduction of tebuconazole alone in the growth medium of this fungus (in the form of fungicide Folicur® KE 25 % (Bayer AG, Germany) or only thymol in corresponding 0.5 and 10 ppm concentrations resulted in insignificant suppression of mycelium growth, whereas in case of their combination in the same concentrations the inhibition reached 50%, which exceeded the expectation cumulative effect almost twofold. The same tendency was observed for Fusarium culmorum during combination of the same fungicide with 4-OBA [61].

The compounds capable of increasing sensitivity of phytopathogenic fungi to fungicides were detected not only in plants. The examples of using metabolites of microorganisms as sensibilizers can be the experiments where they were used to amplify sensitivity of three wheat pathogens (P. nodorum, B. sorokiniana and F. graminearum) to four DMI-fungicides. For instance, recently we demonstrated the sensibilization of pathogen of common root rot and cereal mottle leaf (B. sorokiniana) to tebuconazole with the help of 6-demethylmevinolin (6-DMM), which P. citrinum produces. As the triazoles, 6-DMM inhibits strain biosynthesis; however, unlike DMI-fungicides it affects 3-oxy-3-methyl glutaryl-CoA-reductase responsible for one of the earlier stages of formation of steroid com-
pounds. In these experiments a range of combinations of fractions of Folicur® KE 25 % and 6-DMM was determined, which produced the synergism effect indicative of significant increase of sensitivity of pathogen to fungicide. In case of combined application of substances in the most effective combination of fractions of this range, complete suppression of growth of fungal colonies was achieved, whereas sensibilizer and fungicide, taken individually in the same concentrations gave rise only to 10-20% growth inhibition [74].

Furthermore, in exometabolites of F. sambucinum isolate, which is non-pathogenic for wheat, sensitizing activity was first identified with regard to P. nodorum during in vitro testing along with tebuconazole, which consisted not only in amplification but also in prolonging the fungicidal effect [61]. Subsequently, these metabolites were successfully used in greenhouse studies to amplify sensitivity of this pathogen to Folicur® BT KE 225 (active agents tebuconazole and its derivative triadimefon). The results of subsequent field tests showed that mutual treatment of plants with culture liquid filtrate F. sambucinum and this fungicide containing sensibilizers permits the reduction of its dosage as compared with the recommended fivefold without loss of protective potency and fungicidal effect against Septoria spot pathogen [76]. Whereas for wheat the condition of flag-leaf is critical for the crops, it is important that in tests with artificial inoculation of plants the fungicide treatment at the tillering stage along with the culture liquid filtrate better protected the flag-leaf during the entire vegetation period than fungicidal treatment. For plants sprayed with Folicur® BT KE 225 in usual dosage the average affected area of flag leaves was 12-15 smaller than for those that were not treated. If the fungicide dosage was one fifth of the norm, its individual application resulted in a 5-7-fold reduction of the total affected area, whereas after the usage of the same dosage of the preparation in combination with the culture liquid filtrate this figure decreased by almost 40 times. Based on sensibilizers from F. sambucinum a composite preparation was developed for plant protection [82].

It has been proven that the other active sensibilizers of microbial origin are cyclic lipopeptides (Iturin A, fengycin and surfactin) of one of the strains (JCK-12) of soil-inhabiting Bacillus amyloliquefaciens bacterium. The synergism between the essence containing them and triazoles (difenoconazole and tebuconazole), as well as fungicides with the other mechanisms of action (fludioxonil and benomyl) resulted in noticeable amplification of F. graminearum colony growth inhibition. The lipopeptides themselves did not display fungicidal activity until concentrations reached 30 ppm; however, the inhibiting effect of their mixtures with fungicides for fungal conidia germination was of synergetic nature. The greenhouse and field treatment of plants using the preparation based on cultural JCK-12 broth showed that its mutual usage with Almuri® fungicide (active agents difenoconazole and propiconazole; Syngenta, Korea) can significantly improve the efficiency of protecting wheat with this dual formulation [62]. The authors presume that increased sensitivity of the fungus to fungicides under the impact of JCK-12 can be the result of cell wall damage and change of permeability of cell membrane F. graminearum due to the mix of bacterial lipopeptides.

In terms of synthetic compounds, we have recently identified certain chemosensitizing activity in vitro in some phosphoanalogues of natural amino acids in tests with P. glomerata, A. alternata and F. culmorum [65]. For example, we have checked several structural analogues of amino acids inhibiting biosynthesis of polyketide mycotoxins, which in some fungi play an important role in pathogenicity. The chemosensibilization effect and synergism with tebuconazole (Folicur® KE 25 %) was identified both for nonfungicidal and for subfungicidal concentrations of these compounds. Whereas the structure of these analogues of
natural substances is known and is not very complex, its gradual modification with simultaneous definition of target activity could result in synthesis of relatively cheap and environmentally friendly preparations to increase fungicidal sensitivity of phytopathogenic fungi. The success of this concept would have become a major confirmation of an assumption about prospects of using synthetic compounds as chemosensitizers [8] commercial interest to which is still held back because of rather high production costs.

Finally, we managed to obtain new experimental evidence of efficiency of natural chemosensitizers against resistant forms of phytopathogens, and, specifically, against natural mutant of P. nodorum resistant to Dividend® (3% difenoconazole), sensitivity of which to this triazoles was successfully increased with the help of thymol [75]. When culturing the pathogen in media with sublethal concentrations of difenoconazole, a sector was identified in one of the colonies, which grew more actively than its other sectors. The inhibition of clone growth isolated from this sector in presence of the fungicide turned out almost twice weaker than for wild isolates. PNm1 strain resistant to Dividend® was selected on media with the increasing concentrations of fungicide, and it was determined that its resistance was attributable by the genetic mutation. The application the fungicide together with thymol resulted in statistically significant reduction of mutant strain resistance to the level corresponding to sensitivity of nonresistant parent isolate, whereas growth inhibition after adding difenoconazole together with the chemosensitizer to the medium exceeded the expected additive effect of these substances when used separately [75].

In conclusion of the discussion about resistance of phytopathogenic fungi to fungicides, we would like to point out that unlike medical antimycotic agents that should ensure complete destruction of the pathogen, the methods of plant protection are rather designed to attenuate the pathogen population development as long as possible at the level below the threshold of economic harmfulness. In this regard, the ability of certain chemosensitizers to prolong the fungicidal effect [61] is of particular interest.

Therefore, due to potentiation of fungicides, if they are used in combination with the sensibilizators, pathogens can be reliably controlled with a significant decrease of dosages and expenditure of preparations without loss of their fungicidal effect or even with an increased fungicidal effect. This suggests that using the chemosensitization approach in integrated crop protection could make protective measures more cost efficient and successful, inter alia, against resistant forms of phytopathogenic fungi. An important factor can be the affordability of many sensibilizators of natural origin. Analysis of the research in general shows prospects of development of efficient, environmentally compatible and biodegradable chemosensitizers for use in combination with fungicides.

References


32. Hayashi K., Schoonbeek H.J., de Waard M.A. Benfisol, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camp-


Ban R. Azoles have different strengths and perform diversely across Europe.


MOLECULAR MARKERS ASSOCIATED WITH HIGH EARLY GROWTH RATE OF RUSSIAN RICE (Oryza sativa L.) VARIETIES

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A b s t r a c t

The high rate during early growth is expected to be the main basis of next “green revolution”. Many researches take into account that this trait is mainly responsible for physiological advantage of heterotic hybrids in crops. The rapid development of the root system provides an advantage in absorption of mineral substances and the photosynthetic apparatus formation. Heterotic hybrids can quickly overpass the phase sensitive to stresses thereby improving the adaptability to different stress factors. Significant differences between varieties and subspecies in the growth rate of seedlings are widely used for effective breeding. The growth rate is a plant-height-related trait. Heritability of seedling height, weight, and the length of embryonic root is very high (87-90 %). This paper is the first report on loci that determine the growth rate during germination period in rice (Oryza sativa L.) varieties bred in Russia. The aim of our work was to reveal effective SSR markers for chromosome loci and regions which are involved in the control of traits responsible for high rate of early growth in the Russian rice (Oryza sativa L.) gene pool. Seeds of 32 varieties bred in Russia and hybrids were surface-sterilized for 40 min with a 20 % sodium hypochlorite solution (95.2 % active Cl; ООО Grinfild RUS, Russia). Thirty grains of each sample were germinated (in duplicate) for 7 days at 28-29 °C in a thermostat. Morphophysiological parameters of 20 seedlings of each variety or hybrid, i.e. the weight, the size of coleoptile and the embryonic root formation, were measures. As per the finding of this study, the loci that determine the seedling weight are located on four chromosomes near the markers RM261, RM405, RM463, RM242, RM6314, RM289, and RM126. The embryonic root growth rate is determined by two chromosomal regions on chromosomes 4 and 9, the seedling height — by only one locus located on the chromosome 5 near the marker RM289. One can simplify identification of chromosomal regions that determine target trait values in a set of unknown varieties or hybrids. Bulk breeding methods allows markers with the maximum phenotypic effect to be identified with no need to create a population recombinant inbred lines (RILs) or double haploid lines (DH) for mapping. In particular, exploiting cultivars which are contrast in the traits of interest is much more informative. Such phenotypic analysis reveals the most important loci for the entire group of varieties tested. Next, the markers can be found which are closely linked to the genes that determine the trait in the identified chromosomal region (http://www.gramene.org). Markers that are enough polymorphic to ensure reliable separation of varieties into contrasting groups may be further used in breeding work. Not always genes, differentiating varieties by a certain trait in one region of the world will be effective for another region. Therefore, such preliminary assessment is obligatory. Thus, in this study using original technique to simplifies identification of the loci of interest we have identified 8 chromosomal regions associated with early growth rate in Russian rice varieties.

Keywords: Oryza sativa L., rice, molecular markers, genetics, growth rate, early stages of ontogenesis, seedlings, weight, height, embryonic roots

One of the important agronomic features of rice intended for direct
seeding is the high growth rate of the shoots, which ensures the plants’ advantage both in case of flooding and in case of moisture deficiency. It has been found out that the growth rate can be the indicator of high productivity and presence of heterosis effect [1, 2]. However, in different populations the unequal results of mapping the genes responsible for the growth rate have been obtained.

The considerable polymorphism between varieties and between subspecies [3]. A high growth rate is related to the genes determining the plant’s height [4]. The heritability of the seedling height, weight and the length of the embryo root is 87-90% [5]. The traits characterizing the seedling are mutually related that is evidenced by the high correlation between them [6], but their genetics are mostly unstudied [7, 8]. The seedling height under different growing conditions is controlled by several genes, however, the correlation between the data obtained at contrasting temperatures has been noted to be low [9]. The field germination and growth rates of the varieties of *indica* subspecies in cold conditions are low, therefore the identification of the genes influencing on the seedling growth rate at low temperatures is important for the selection of varieties of this subspecies [10]. The relation of the genes determining the chlorosis of rice seedlings caused by cold with the loci responsible for growth rate has been shown. The loci which determine the seedling hormonal status including those involved in the biosynthesis of gibberellin participate in this process. The increased chlorophyll content also contributes to the increase in seedlings’ growth rate by accelerating the formation and accumulation of carbohydrates [11]. A high concentration of sucrose and glucose, as well as the amylase activity in germinating seeds are the prerequisites for the rapid growth of seedlings [12, 13]. Highly productive forms in most cases have a longer and branched root system at the initial stages of plant development [14, 15]. Five-day-old rice seedlings show maximum differences in this trait [16].

The rate of formation of the seedlings’ organs is determined by the effectiveness of photosynthesis and mineral nutrition at the early stages of development. In addition, the improvement of young plant nutrition also increases its resistance to biotic and abiotic stresses [17, 18].

A high growth rate in seedlings of hybrids with heterosis effect is due to gene overexpression. Note that overexpression of the genes determining the cells division rate, replication beginning, transcription and translation is deemed as one of the possible mechanisms which ensure a heterotic effect. When investigating almost 14 thousand genes, an overexpression was characteristic for more than 15%, wherein the expression of 9% of genes was 2 times higher than that of parents. An increased expression has been noted for regulatory and structural genes. A hormonal regulation, carbohydrate metabolism and mitochondrial activity are involved in this process [19, 20]. The obtained data make it possible to assume that during the hybridization, the starting advantage of F1 hybrids may be conditioned by the decrease in the methylation effects which increase the expression of heterosis promoting loci. Heterotic hybrids differ from their parental forms with a number of epigenetic changes of the genome including the methylation or removal of this effect. The optimal course of biochemical reactions caused by changing the functional state of genes, and cascade reactions in polygenic structures are responsible for the heterotic response of the hybrid genome [21].

High rate of seedling metabolism and growth also ensures yield stability of varieties due to the rapid passage of the phases which are most sensitive to stresses. Such traits are useful also in terms of the formation of even sprouts on flooded and unlevelled checks, because the high speed of the seedling passage through the water layer ensures the rice plant viability [22]. The genetics of these traits had been studied during seed germination for 5 days from under the water
layer (20 cm) in the dark. The hybrids between the samples of the \textit{indica} subspecies or intersubspecies hybrids were used in the work. When creating highly productive rice varieties, the control of traits participating in the obtaining of even sprouts can be ensured by the stacking of several positive QTLs (quantitative trait loci) which contribution to the trait formation and to an individual plant phenotype is insignificant.

The gene pool of the \textit{japonica} subspecies has not been used for a long time for the identification of the genes responsible for the resistance to germination in an oxygenless medium and for mapping QTLs which determine the formation of these traits [23]. For the first time such experiments were performed by Chinese scientists. Wang et al. [24] have studied 247 recombinant inbred lines (RILs) which were derived from the hybrid of two forms of the \textit{japonica} subspecies — Xiushui 79 variety and C Bao restorer line, as well as 98 backcross inbred lines (BILs) obtained from crossing Nipponbare (\textit{japonica})/Kasalath (\textit{indica})//Nipponbare (\textit{japonica}). Two relevant QTLs have been found on the 2nd and 7th chromosomes in the RIL population. The positive $qSAT-1-R$ allele obtained from C Bao variety determined 8.7% of the phenotypic dispersion by the coleoptile length. The $qSAT-2-R$ allele (the amplification product size 140 bps) was closely linked to the RM525 SSR marker to which 9.8% of the phenotypic dispersion is related. Its positive allele also belonged to C Bao variety. The $qSAT-7-R$ locus (the amplification product size 250 bps) was closely linked to the RM418 SSR marker [24]. Six QTLs on the coleoptile length in an oxygenless medium were found in the BIL population on the 2nd, 3rd, 5th, 8th, 9th and 12th chromosomes. These QTLs conditioned from 5.8 to 16.2% of the phenotypic manifestation of the trait. The Nipponbare variety carries the positive alleles of the $qSAT-2-B$, $qSAT-3-B$ and $qSAT-9-B$ genes. The Kasalath sample contains the positive alleles of the $qSAT-5-B$, $qSAT-8-B$ and $qSAT-12-B$ loci. In the Kasalath sample, the coleoptile was more than 11 mm longer on average than in the Nipponbare variety, in which the coleoptile length was 5.5 mm. The trait value in Kasalath/Nipponbare hybrids is $6.4 \pm 1.3$ mm, the variation coefficient is 20.2%. One QTL related to the seedling stem length was located between RM525 and RM2127. This $qCL-2-R$ locus determines 5.2% of phenotypic variability. Its positive allele belongs to Xiushui 79. The QTLs related to the seedling stem length have been found on four other chromosomes, but their contribution did not exceed 10%. The $qCL-1-B$ locus hybrids were obtained from the parent form of Nipponbare [24].

In other works with using recombinant inbred lines (RILs) for the molecular marking of populations, five QTLs which determine the coleoptile length under the stress (lack of oxygen) have been found. They are located on the 1st, 2nd, 5th, 5th and 7th chromosomes. The positive alleles of the $qAG-1$, $qAG-G2$ and $qAG-7$ genes which increase the rate of germination in oxygenless medium (anoxic growth, AG) are carried by the Kinmaze variety. The $qAG-5a$ and $qAG-5b$ loci have been inherited from the DV85 sample. The RILs have been obtained by crossing Kinmaze (\textit{japonica}) and DV85 (\textit{indica}). When phenotyping the trait for the subsequent statistical analysis, the average values of the coleoptile length were used [25-27].

From the point of view of the research technique, it is necessary to take into account that the germination time increases as the temperature decreases [11]. If seeds have been subjected to preliminary soaking (priming) for 24 or 48 hours at 20 °C and for 12 hours at 30 °C the germination time decreases. Thus seeds soaking for 12 hours at 30 °C reduce the germination time by almost 18 hours compared to the control. A shorter germination time is also characteristic of samples subjected to preliminary soaking followed by drying. In addition,
priming increases the uniformity in the growth rate of samples [28].

In the present work, we for the first time identified the loci which determine the growth rate during the germination period of domestic rice varieties.

Our goal was to find the loci and chromosomal regions which determine the rapid coming up and development of rice seedlings, as well as the study of the genetics of these traits.

**Techniques.** The experiments were carried out in 2013-2019. The collection samples (32 varieties of Russian selection) and hybrids of rice (*Oryza sativa* L.) had been studied using the generally accepted methods [16]. The hybridization between the varieties of domestic selection with contrast traits was carried out by the twell method (Borlaug’s method) according to the full diallel scheme [29].

The seeds were treated with the 20% sterilizing solution of Belizna, a household detergent sodium hypochlorite with the active chlorine content of 95.2% (Greenfield RUS LLC, Russia) for 40 minutes. 30 seeds from each sample (in 2 replicates) were grown in the thermostat during 7 days at 28-29 °C. The weight of seedling, sizes of coleoptile and embryo root of 20 plants of each cultivar or hybrid were measured.

DNAs were isolated from the etiolated seedlings and leaves using the STAB method in various modifications. The polymorphism was investigated with 60 neutral or linked to seedlings’ high growth rate SSR markers distributed over 12 chromosomes of rice. When marking the samples, the standard conditions of polymerase chain reaction (PCR) for the reaction mixture volume of 10 μl [30] and the following amplification mode were used: 5 min at 94°C (initial denaturation); 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing of primers), 2 min at 72 °C (synthesis) (35 cycles); 7 min at 72 °C (Multiback System DNA amplifier, Bio-Rad, USA). The PCR mixture included 40 ng DNA (2 μl), 1 μl of dNTPs (1 mM), 3.7 μl H2O, 1 μl buffer solution, 0.3 μl MgCl2; 1 μl of primers, 1 μl of Taq DNA polymerase (Evrogen CJSC, Russia). The PCR and visualization of amplification products were performed according to the technique of the International Rice Research Institute, Philippines [9]. The amplification products were separated by electrophoresis in polyacrylamide gel at 100V in the electrophoretic chamber designed for 204 samples (C.B.S. Scientific, USA).

The analysis of variance has been made basing on the relative size of the amplification product for the relevant marker. The amplification product size of a sample with its minimum value has been taken as 1 (one), the rest alleles were designated in accordance with the increase in the size of the amplification product. The means (M) and their standard errors (±SEM) for the morphophysiological traits have been calculated. The relationship between the data on genotyping and phenotyping of a trait have been analyzed after the statistical processing which has been performed using the Statistica 6.0 software (StatSoft, Inc., USA) with the analysis of variance and cluster analysis [31]. The validity of differences has been evaluated according to the Fisher’s F-test and p significance level.

**Results.** The markers we used are listed in Table 1.

The most of the hybrids were characterized by heterosis in terms of growth rate, therefore in the studied population the high growth rate was the dominant trait. The high growth rate ensured the high productivity of hybrids that is evidenced by the value of the correlation coefficient *r* > 0.9 between the traits which determine the productivity (total weight of grains per plant, weight of the main panicle, weight of the side panicles, number of filled spikelets) and which are related to the seedlings’ growth rate. The data analysis by the Hayman’s method showed that a trait value is determined by polygenes, as well as by the non-directedness of dominance, i.e. by the existence of both dominant and
recessive genes increasing the trait in the studied varieties [16, 32].

1. Distribution over the chromosomes of rice (Oryza sativa L.) of the neutral SSR markers and ones linked to the traits determining the seedlings’ size and formation rate.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of markers</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>RM104, RM239, RM260, RM3638, RM24</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>RM53, RM154, RM240, RM318, RM322, RM2770, RM5707</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>RM227, RM347, RM218</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>RM124, RM127, RM140, RM255, RM261, RM317, RM335, RM3276, RM6314</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>RM13, RM30, RM289, RM405, RM440, RM509, RM574, RM3561, RM6024</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>RM141, RM162, RM276, RM588, RM5371, RM6811</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>RM82, RM542, RM5508, RM7110, RM11</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>RM25, RM126, RM256, RM284, RM3155, RM8243</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>RM242, RM245, RM444, RM7048</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>RM258, RM590</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>RM286, RM3428</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>RM463, RM6410</td>
</tr>
</tbody>
</table>

Note. The information is available on the website http://www.gramene.org [32].

Characterization of the seedlings’ polymorphism has made it possible to divide the samples into the groups which significantly (p < 0.05) differ in morphophysiological traits (Table 2).

2. Polymorphism of Russian rice (Oryza sativa L.) varieties by morphophysiological traits (M±SEM, lab tests, 2013–2015)

<table>
<thead>
<tr>
<th>Variety</th>
<th>Seedling weight (SW), g</th>
<th>Group by SW</th>
<th>Embryo root length (ERL), cm</th>
<th>Group by ERL</th>
<th>Coleoptile length (CL), cm</th>
<th>Group by CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liman</td>
<td>1.08±0.0038e</td>
<td>5</td>
<td>0.9+0.0009e</td>
<td>2</td>
<td>5.5+0.018k</td>
<td>1</td>
</tr>
<tr>
<td>Garant</td>
<td>1.11+0.0021i</td>
<td>5</td>
<td>0.6+0.0013i</td>
<td>4</td>
<td>3.8+0.190m</td>
<td>3</td>
</tr>
<tr>
<td>Pavlovskiy</td>
<td>1.12+0.0011d</td>
<td>4</td>
<td>0.8+0.0008h</td>
<td>3</td>
<td>0.7+0.092o</td>
<td>5</td>
</tr>
<tr>
<td>Rapan</td>
<td>1.13+0.0007d</td>
<td>4</td>
<td>0.5+0.0010</td>
<td>5</td>
<td>2.5+0.057n</td>
<td>4</td>
</tr>
<tr>
<td>Szezhinka</td>
<td>1.14+0.0018i</td>
<td>4</td>
<td>1.0+0.0013s</td>
<td>2</td>
<td>4.0+0.126l</td>
<td>2</td>
</tr>
<tr>
<td>Primorsky</td>
<td>1.14+0.0045d</td>
<td>4</td>
<td>1.0+0.0020s</td>
<td>2</td>
<td>2.2+0.200n</td>
<td>4</td>
</tr>
<tr>
<td>Regul</td>
<td>1.25+0.0031d</td>
<td>4</td>
<td>0.8+0.0011h</td>
<td>3</td>
<td>2.6+0.067n</td>
<td>4</td>
</tr>
<tr>
<td>Atlant</td>
<td>1.25+0.0025d</td>
<td>3</td>
<td>0.8+0.0019h</td>
<td>3</td>
<td>3.5+0.155m</td>
<td>3</td>
</tr>
<tr>
<td>Lider</td>
<td>1.26+0.0033e</td>
<td>3</td>
<td>0.7+0.0006h</td>
<td>3</td>
<td>2.9+0.094p</td>
<td>4</td>
</tr>
<tr>
<td>Khazar</td>
<td>1.27+0.0041e</td>
<td>3</td>
<td>0.8+0.0015h</td>
<td>3</td>
<td>3.9+0.066m</td>
<td>3</td>
</tr>
<tr>
<td>Novator</td>
<td>1.27+0.0049c</td>
<td>3</td>
<td>0.7+0.0020</td>
<td>4</td>
<td>2.5+0.137n</td>
<td>4</td>
</tr>
<tr>
<td>Izumrud</td>
<td>1.29+0.0050c</td>
<td>3</td>
<td>0.9+0.0008c</td>
<td>2</td>
<td>3.0+0.093m</td>
<td>3</td>
</tr>
<tr>
<td>Kurchanka</td>
<td>1.33+0.0015s</td>
<td>3</td>
<td>1.0+0.0012s</td>
<td>2</td>
<td>5.1+0.095k</td>
<td>1</td>
</tr>
<tr>
<td>Serpantin</td>
<td>1.39+0.0017b</td>
<td>2</td>
<td>1.0+0.0009s</td>
<td>1</td>
<td>4.2+0.124l</td>
<td>2</td>
</tr>
<tr>
<td>Amerist</td>
<td>1.47+0.0049d</td>
<td>2</td>
<td>1.1+0.0007s</td>
<td>1</td>
<td>5.2+0.175k</td>
<td>1</td>
</tr>
<tr>
<td>Yantar</td>
<td>1.54+0.0029h</td>
<td>1</td>
<td>1.2+0.0006f</td>
<td>1</td>
<td>3.6+0.134m</td>
<td>3</td>
</tr>
</tbody>
</table>

Note. The analysis has been made on the samples which are the most contrasting by the trait. For each trait the differences between the values marked with different letters are statistically significant at p < 0.05.

We have done marking in the groups contrasting by the trait for the analysis of allelic diversity in loci. When investigating the Russian gene pool, most of the markers, which, according to other authors, are related to seedling growth rate, turned out to be monomorphic or their polymorphism was not related to the trait formation (Fig. 1).

In order to identify the chromosomal regions which determine the seedling traits in domestic rice varieties, the variance analysis of the relationship between the seedling size and weight with the allelic diversity of loci has been made. As the result, 10 microsatellite markers the polymorphism of which has the valid relationship (p < 0.05) with the changing of seedling size have been identified (Table 3).

We have also analyzed (Table 4) the available information on the relationship of the markers we identified with the loci responsible for any traits in rice plants (http://www.gramene.org). Previously, the genes associated with the root formation and germination energy have already been found in these chro-
mosomal loci. In the region of markers localization, the genes which determine the germination energy, resistance to drought, tolerance to low temperatures, root number, ratio of the root-to-stem length, relative weight of the roots and stability of the cell membranes under stress are located. According to our data, the stem formation was determined by loci located in the region of RM289 marker. In the zone of its localization, there are genes responsible for the germination rate and plant height [33]. The differences of the varieties’ clusters with the contrasting growth rates of seedling were valid for the RM261, RM405, RM463, RM242 and RM6314 markers located on the 4th, 5th, 12th, 9th and 4th chromosomes respectively (see Table 4). In the region where these markers are located, the loci related with the tolerance to moisture deficiency, number of roots, resistance to low temperatures, length of the root system and main panicle, differentiation of explants, relative root weight, rate of seedling formation and stability of the membrane complex of the sample have been described [33].

![Fig. 1. Molecular marking of Russian rice (Oryza sativa L.) varieties using SSR markers: A — RM7048 (monomorphic marker), B — RM213 (polymorphism is not related to the trait formation in Russian rice varieties), C — RM261 (polymorphism is related to the trait formation in Russian rice varieties). From the left to right: molecular weight marker, varieties Khankayskiy, Sadko, Primorskiy, Liman, Garant, Pavlovskiy, Rapan, Novator, Serpantin, Boyarin, Regul, Yantar, Zhemchug, Lider, Khazar, Nartsiss, Druzhnyy, Sprint, Viola, Dalnevostochnyy, Fontan, Kasun, Yupiter, Atlant, Kurchanka, Fakel, Snezhinka, Anait, Flagman, Izumrud, Nartsiss varieties, molecular weight marker (DNA length marker, 100 + 50 bps, Diaem, Russia).](image)

### Table 4

<table>
<thead>
<tr>
<th>SSR marker</th>
<th>SS Effect (intergroup sum of squares)</th>
<th>SS Error (intragroup sum of squares)</th>
<th>Fisher's F-test</th>
<th>Significance level p</th>
</tr>
</thead>
<tbody>
<tr>
<td>REM126</td>
<td>1.971</td>
<td>1.472</td>
<td>3.702</td>
<td>0.042</td>
</tr>
<tr>
<td>REM509</td>
<td>0.952</td>
<td>0.804</td>
<td>3.271</td>
<td>0.050</td>
</tr>
<tr>
<td>REM242</td>
<td>2.202</td>
<td>0.802</td>
<td>7.563</td>
<td>0.001</td>
</tr>
<tr>
<td>REM463</td>
<td>2.441</td>
<td>2.003</td>
<td>3.351</td>
<td>0.050</td>
</tr>
<tr>
<td>REM289</td>
<td>0.173</td>
<td>2.271</td>
<td>0.212</td>
<td>0.939</td>
</tr>
<tr>
<td>RM405</td>
<td>11.723</td>
<td>8.031</td>
<td>4.011</td>
<td>0.031</td>
</tr>
</tbody>
</table>

3. Analysis of the validity of differences in seedling growth rate in Russian rice (Oryza sativa L.) varieties using SSR markers
We have found the relationship between the seedling growth rates and many loci determining the adaptability to abiotic stresses. This result was expected, because the high rate of formation of the root and photosynthetic systems determines a lower dependence of a seedling on external factors through increasing its adaptability. The rapid passage by seedlings of the phases in which young plants are most sensitive to stress also reduces the likelihood of their damage by extreme temperatures or other factors reducing the viability [16].

The effectiveness of using the identified markers for clusterization of varieties was different, most of the markers allowed us to select only the most contrasting groups. For example, RM242, RM463 and RM6314 markers could only be used to identify the samples of the 1st group with the most intense manifestation of the trait. RM126 and RM242 allowed valid identification of two groups of samples, with the maximum and minimum value of the trait (Fig. 2).

The result of the researches can be considered natural, since the studied
traits are inherited polygenically. The loci with the multidirectional effects which compensate the effect of each other do not provide identification of the function of certain genes determining a trait. The chromosomal regions localized for the first time, which are related to the rapid formation of seedlings, subsequently will allow us to develop the marker-assisted selection techniques for Russian samples.

Fig. 2. Clusterization of the investigated rice (*Oryza sativa* L.) varieties of Russian selection basing on the root growth rate in the sprouting phase using the markers of two traits: A — RM126 (root length), B — RM242 (root length), C — RM242 (seedling weight); small squares are means (*M*), rectangles are *M*±SEM, “whiskers” are *M*±1.96 × SEM.

The formation of traits and effectiveness of functioning of many genes determining the growth rate depend on the plant photosynthetic activity and mineral nutrition. Therefore, the selection for growth rate requires the control not only of the loci and genes which are directly related to the seedling growth rate, but also of the complexes which determine the effectiveness of photosynthesis, mineral nutrition, activity of a number of enzymes and adaptability to stresses.

Due to the small sample size and the number of markers, we could identify only the loci with the maximum phenotypic effect. We have not found any markers to reliably separate the groups of varieties by the total and relative content of chlorophyll at the significance level recognized in biological studies (*p* ≤ 0.05). The decrease of the significance level to *p* ≤ 0.09 increased the sensitivity threshold of the method and nevertheless allows us to identify several markers which may be related to the said traits. The information on the genes localization collected on the website http://www.gramene.org makes it possible not only to use in the work well-known markers, but also to verify the obtained data, because the earlier found relationship of the identified chromosomal regions with the investigated traits has confirmed our results indirectly.

The RM245, RM162, RM154 and RM240 markers, which we identified as informative, are intragenic for the traits determining the effectiveness of photosynthesis [38]. The separation of the trait into its components made it possible
to establish that RM509 marker is related with the content of chlorophyll-a. We have not find in the literature any data on the association of the RM5361, RM5707, RM5508, RM347, RM509, RM600 and RM574 markers located on the 5th, 2nd, 7th, 3rd, 5th and 1st chromosomes and related to the separation of domestic varieties into groups with different photosynthesis efficiencies and with any traits of photosynthesis. The markers with four-digit numbers are relatively new, they were used in experimental works rarely. Perhaps, in the region of these markers there are genes which are specific for the Russian gene pool.

The RM574 and RM600 markers are known to be related to the effectiveness of mineral nutrition, which largely determines the traits we study, i.e. growth rate and photosynthesis [33]. With the deficiency of phosphorus, potassium and magnesium, the photochemical and dark reactions of photosynthesis are disturbed. It has been shown that the application of nitrogen fertilizers enhances the plant photosynthetic activity [16]. Many compounds functioning as electrons carriers contain iron (cytochromes, ferredoxin) or copper (plastocyanin). It is natural that when lack of these elements, the photosynthesis intensity decreases [33, 38].

Our results testify about the possibility of simplified identification of chromosomal regions which determine the trait parameters when investigating unknown samples. At the stage when thousands of genes are localized, a bulk breeding can be used to identify the markers related to the maximum desired phenotypic effect. For this, there is no need to create and perform phenotyping of populations as for recombinant inbred lines (RILs) or dihaploid lines (DH) that requires several years of work. Much more information can be obtained by using the varieties which are contrasting by the trait. This makes it possible within short time to determine the loci which are the most important for the trait formation in the whole investigated group of varieties. Such information will make it possible to put the markers closely linked to the genes determining the trait in the identified chromosome region. In further work, just these markers can be used if the study of their polymorphism will confirm the possibility of reliable separation of varieties into contrasting groups. This preliminary work is necessary because the genes which differentiate varieties by any trait in one region of the world are not always effective for other region, in which the trait can be determined by absolutely different loci.

The analysis of other authors’ papers showed that the loci related to the seedlings formation are localized on all rice chromosomes, and they are different in different samples. The dihaploid and recombinant or backcross inbred lines, hybrids of the second and third generations (from 80 to 2800 samples) have been used for marking [24, 34, 35]. The outcomes of the researches also were different. An increase in population volume does not guarantee the increase in marking efficiency. For example, when sampling amount of 2810 samples in the F2 population, the authors identified one locus, while in the population of 191 RILs, 15 loci have been identified [36, 37]. It is very important to select maximally contrasting samples as parent forms. In our work, the loci on the 4th, 5th, 9th and 12th chromosomes have been identified. The locus found on the 9th chromosome participated in the formation of traits responsible for the seedling weight and coleoptile length, two loci were found on the 5th chromosome, three loci on the 4th, and one — on the 12th chromosome. The information about qCTS-9 and qCTS-12 loci having the significant phenotypic effect (from 5.5 to 22.4%) found on the 9th and 12th chromosomes have been previously reported by Chinese and Indian scientists who worked mainly with populations based on interspecies hybrids [34, 35]. From one to three loci influencing the traits formation have also been found on the 5th and 4th chromosomes of the indica subspecies [25, 37]. The most often identified loci found on the 1st, 2nd, 8th chro-
mosomes were not related to polymorphism on the studied trait in the population of domestic rice samples, for which many loci linked to growth parameters were monomorphic that is explained by long-term task-oriented selection [33].

So, for the first time for the Russian rice gene pool, we have identified eight chromosomal regions related to the growth rate, and the technique which simplifies their identification when investigating unknown samples has been proposed. In order to detect the markers with the maximum phenotypic effect, a bulk breeding and varieties contrasting by a trait can be used. This will reveal the markers closely linked to the genes determining a trait in the identified chromosome region (http://www.gramene.org). If the study of polymorphism will confirm the possibility of reliable separation of varieties into contrasting groups using such markers, they can be used in the future work. As the result of our researches, it has been established that the weight of Russian varieties’ seedlings is determined by the loci located in the regions of location of the RM261, RM6314, RM405, RM242 and RM463 markers on the 4th, 5th, 9th and 12th rice chromosomes respectively. Two chromosome regions on the 4th and 9th chromosomes (RM126, RM242) determines the embryo root length, the locus on the 5th chromosome located in the RM289 marker region determines the seedling height. The technique developed in these researches is applicable for mapping the loci of quantitative traits of other crops.

REFERENCES


CREATION OF A SPRING SOFT WHEAT VARIETY GRENADE WITH THE USE OF INNOVATIVE BREEDING TECHNOLOGIES BASED ON THE ORIGINAL THEORY OF ECO-GENETIC ARRANGEMENT OF QUANTITATIVE TRAITS

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A b s t r a c t

Today, there is an excessive belief in the promise of molecular approaches to the problem of increasing yields, although so far there is not a single variety created by purely molecular methods. In addition, representatives of the new science, the epigenetics, rightly argue that in nature there are no specific genes for productivity and yield that could be molecularly marked or subjected to genomic editing. This article is the first to describe creation of a wheat variety Grenada using innovative breeding technologies emerged from the priority Russian Theory of Eco-Genetic Organization of Quantitative Traits (TEGOQT) which derived from the results of the Interdisciplinary DIAS Program (genetics of spring wheat productivity in Western Siberia) (1973-1984). The essence of these technologies are 1) a special selection of parental pairs on the basis of a deep analysis of the longest pedigrees of the old breeds of parents taken in crosses, 2) priority phenotyping of the group of the most productive varieties of the collection nursery for seven genetic-physiological systems (GPS) which positively or negatively contribute to the harvest, 3) selection of genotypes that have at least one GPS with the maximum plus contribution to the crop, 4) crossing of these genotypes to combine the plus contributions of all seven GPS in the future variety (with several saturations with the genome of one of the parents with the most valuable properties), 6) selection of elite plants after a number of stabilizing reproduction of the hybrid population under the conditions of typical dynamics of the environment lim-factors (in typical years for the breeding zone). Applying these technologies, we obtained a hybrid combination [F1 (Kazakhstanskaya rannespelaya × Tulunskaya 12) with five subsequent saturations with Tulunskaya 12 genome], from which the variety Grenada derived. Both parents of the maternal form Kazakhstanskaya rannespelaya, the Novosibirskaya 67 × Omskaya 9, having wide general adaptability, showed a much lower changes in GPS contribution to productivity as a response to changing environment and good combining ability. The Kazakhstanskaya rannespelaya variety created on their basis combines the best traits of the parents. As to the paternal and saturating form Tulunskaya 12, the improvement in quantitative traits is discrete-accumulative due to the genetic diversity of the East Siberian genotypes. The selection of elite plants under typical agro-climatic conditions resulted in higher yielding genotypes with a pronounced plasticity. A comprehensive assessment of the biotypes from this population in F5 according to seven GPS, positively contributing to productivity, showed their synergetic effect. This was well manifested in the early ripening line Lutescens 506-11 from which the Grenada variety derived. This variety successfully combines high productivity (26-39 % higher compared to the standard) with the resistance to lodging, drought, pre-harvest germination and the grain quality of valuable and strong varieties. A distinctive feature of the variety is the horizontal resistance to Septoria diseases, a dusty smut, powdery mildew, a red-breasted leech, and intra-stem pests. Grenada variety is much lower affected by rust fungi compared to the standard. From one hectare of arable land the Grenada variety gives 628 kg of protein (+119 kg to the standard variety). In 2019, the variety Grenada is zoned by State Commission on Variety Testing of the Ministry of Agriculture of the Russian Federation for the 9th (Ural) crop re-
gion including Bashkirtia (about 1 million hectares), Chelyabinsk (1 million hectares), Orenburg (4 million hectares), Kurgan (1 million hectares), and Tyumen (500 thousand hectares) regions. When Grenada occupies these areas (about 7 million hectares), an increase in grain yield will provide an annual economic effect of about 30 million rubles.

**Keywords:** Triticum aestivum L., soft wheat, breeding, variety, population, selection, biotype, geno-physiological system, grain, immunity

Increasing yields of various crops is currently not quite adequately associated with technologies based mainly on cell and tissue cultures [1, 2] and molecular genetics [3-6], i.e. different molecular markers [7, 8], marker-assisted selection (MAS), marker-assisted back-crossing (MABC), marker-assisted recurrent selection (MARS), genome-wide selection (GWS) or genomic selection (GS) [9, 10], and genomic editing [11].

It is commonly believed that key traits (corn yield, protein content, horizontal immunity) are controlled by a multiplicity of genes. Such traits were called quantitative (QT) or complex polygenic traits [12-15]. QT depends on combined activity of a set of genes and environment. Quantitative traits loci (QTL) may include a single gene or a group of genes contributing to trait value, and the effects normally change depending on conditions in which the trait is manifested [16]. Use of molecular markers enables examination of quantitative traits and identification of contributions to trait of the QTL, i.e. genes controlling complex traits of interest in breeding [17-19]).

In our opinion, however, the expectations that a key to a rapid breeding varieties required to meet the growing needs of the Earth population will be solely genetic-engineering together with molecular methods are excessive. There is still no literature mentioning the creation of a variety solely via molecular approach. In addition, epigenetics [20-22] rightly argue that in nature there are no specific genes for productivity and yield that could be molecularly marked or subjected to genomic editing. Even now in genetics and breeding, diallel analysis, despite it does not include any molecular characteristics of plant productivity traits, remains vastly effective in creating varieties of high yield and quality [23-25].

In 1973-1984 the USSR’s Interdisciplinary DIAS (diallel crossing) Program was aimed at studying genetics of spring wheat productivity in Western Siberia and Kazakhstan [26]. The experts of eight Siberian plant breeding centers and two research centers (Institute of Cytology and Genetics of the Siberian Branch of the USSR Academy of Sciences and Computing Center of the Siberian Branch of the USSR Academy of Sciences) participated in the study. The territory where the experiments took place spanned the area from Krasnovyansk (Ural) to Ivolginsk (Transbaikalia) in latitude and from Tyumen to Ust-Kamenogorsk in longitude. Fifteen parental varieties from Russia, USA, Japan, Sweden, Canada and India were diallel-crossed (each one with each other one). In winter, F₁ breed seeds were sowed in Krasnoyarsk phytotron to obtain F₂ seeds, and in summer parent varieties, hybrid breed F₁ and hybrid breed F₂ were sowed in a complete randomized block pattern with 4 replications in 8 geographical points. The experiments ran for 2 years. For each plant, 15 productivity traits were assessed, the estimates (about 5 million measurements) were entered in a DIAS databank and processed with the original programs developed in the Computing Center. For F₂ and further generations, the breeders applied individual selection in all 8 points, propagated the families and conducted the complete cycle of a standard breeding process with the assessment of the breeding material in a number of breeding nurseries. These experiments, in addition to new varieties, resulted in a discovered new system of productivity and yield regulation via a shift in a set of products of genes determining the productivity
trait when the limiting environmental factor changes. Based on this discovery, a priority Russian Theory of Eco-Genetic Organization of Quantitative Traits (TEGOQT, V.A. Dragavtsev Science School) gained momentum in 1984-2014 [27-29]. This theory generated 24 entirely new deductions that enabled 24 forecasts for any temporal dynamics of the environment lim-factors, and 10 know-hows to improve crop breeding for yield and quality.

Concurrently with development of TEGOQT, seven genetic-physiological systems (GPS) were discovered and described, which the breeders de facto use (often without realizing it) to enhance the productivity and yields of new varieties. An original method of phenotyping each of seven GPS was developed based on priority methods of quantitative identification of “plus” contributions of such systems to the productivity and yield of standard and new varieties. This distinguishes from conventional foreign phenotyping, where the resulting trait (yield) is consequentially broken down to smaller components, as it is not the traits that are handled, but GPS. The traits there serve as the axes of two-dimensional coordinate system where each GPS manifests its positive or negative input into productivity in free of all noises and genetically “stripped” quantitative value [31]. These GPS are as follows: attraction GPS that ensures during the ripening period an outflow of macronutrients from straw and leaves to the ear (in cereals), or from the stem and leaves to the calathidia (in sunflowers) (ATTR); GPS of microdistributions of attracted macronutrients between the grain and chaff in cereals, or between the kernel and husk in sunflowers (MIC); GPS of adaptability (frost-, cold-, drought-, heat-, salt-resistance, soil pH resistance, etc.) (AD); GPS of polygenic immunity (horizontal resistance) (IMM); GPS of phytocenosis densification tolerance (TOL); GPS of compensation with dry matter of effects of low dosages of soil nutrition (lim-factors of soil nutrition, i.e. N, P, and K) (EFF); GPS of genetic variability of ontogenesis phase length (ONT) [31].

This article, through the example of creation of a spring wheat variety Grenada, is the first to describe innovative technologies of breeding varieties surpassing the existing ones in yields and quality. These technologies became the practical implication of priority Theory of Eco-Genetic Organization of Quantitative Traits (TEGOQT) that has outstripped the global development of environmental plant genetics for 10-15 years. They are unrivaled throughout the world and an alternative to production of genetically modified plants, search for QTLs and attempts to mark productivity traits.

Our goal was to create a wheat variety using new breeding technologies we developed for enhancing the yields based on the theory of quantitative traits’ eco-genetic organization and on discovery of seven genetic-physiological systems determining enhancement of the main economically significant properties of plants.

Techniques. Experiments were run in 2001-2016 (an experimental field of the Agricultural Research Institute of Northern Transurals — Branch of Tyumen Scientific Centre of the Siberian Branch RAS) on bare fertilized dark gray soil fallow (N$_{30}$P$_{45}$K$_{30}$ per hectare), and in 2013-2014 at Ishim Agricultural Experimental Station (Tyumen province) on bare fertilized black soil fallow (N$_{30}$P$_{30}$ per hectare). In collection and breeding nurseries, 650 viable seeds per sq.m were sown (SKS-6-10 fractional seeder, Russia). Hybridization was carried out by twirl method (Borlaug method, a pollination method where the blooming father ear is rotated inside an individual insulator in the presence of neutered mother ear). The parents and F$_1$ and F$_2$ hybrids were sowed manually on 1 m$^2$ plots, 20 seeds per 1 running meter. Hybrids F$_3$-F$_5$ (stabilizing crops) without selection was sowed on 5 m$^2$ plots. The first selection was made from F$_5$ population, the
selected elite plants in the first-year breeding nursery (SP-1) were sowed manually in holes \((d = 25\, \text{cm})\). Care and observation of hybrid and breeding nurseries were as generally accepted. All crops were subjected to one treatment with herbicides Puma Super\textsuperscript{®} 7.5 oil-water emulsion (Bayer CropScience AG, Germany; 0.8 l/ha) + Granstar\textsuperscript{®} Pro water-dispersible beads (DuPont, USA; 0.015 kg/ha). In hybrid nursery (parents, \(F_1, F_2\)), the plants were harvested manually. Hybridologic analysis covered all productivity traits and morphological parameters. In competitive variety testing, bundle and structure analysis was held for sample bundles (for 25 plants with 4 replications).

For quantitative analysis of contributions of each of seven GPS in productivity, phenotyping was carried out in two-dimensional coordinate system of various productivity traits according to proprietary technology developed on the basis of TEGOQT [26, 30].

**Results.** In DIAS program [26], the following varieties were bred and zoned. DIAS 2 (developed by R.A. Tsilke) was recommended for Omsk and Novosibirsk provinces. Lutescens 70 (developed by V.V. Novokhatin) until lately was the main Transuars variety that occupied over 60% of wheat plantings. Altaiskaya 88 (developed by N.I. Korobeinikov) until now is grown in Altai, produces high-quality grain. Altaiskaya 92 (developed by N.I. Korobeinikov) is medium-early variety, spread in Western Siberia. Altaisky Prostor is a variety of hard wheat widely spread in Altai (developed by N.I. Korobeinikov). Kazakhstanskaya rannespelaya (developed by V.V. Novokhatin) is one of the main varieties in Northern Kazakhstan, produces high-quality hard grain. Kazakhstanskaya 17 (developed: V.V. Novokhatin) is hard wheat variety cultivated in Southern Kazakhstan. Rix (developed V.V. Novokhatin) is a grandchild variety of Kazakhstanskaya 17 × Karabalykskaya 92 producing yield in Northern Transuars (on fertilized fallow) up to 64 centners per hectare. Bagamskaya 93 (developed by V.P. Maksimenko) spread in Baraba Steppes of Western Siberia is distinguished by drought and cold resistance. Kantegeiyskaya 89 (developed by I.F. Demorenko and R.A. Tsilke) is zoned in Khakassia, Tyva and Mongolia. Gornouralskaya (developed by V.A. Vorobyov), a grandchild variety, was zoned in 2009 in the Middle Urals. Tyumenskaya 29 (developed by V.V. Novokhatin), a grandchild variety, was zoned in 10\textsuperscript{th} region of Russia in 2014; Grenada (developed by V.V. Novokhatin and V.A. Dragavtsev), is a grandchild variety, zoned throughout 9\textsuperscript{th} (Ural) region in 2019; the expected annual economic effect of Grenada variety, if growing on areas over 7 million hectares in this region, will make up to 30 billion rubles. Atlanta 1 (developed by V.V. Novokhatin and V.A. Dragavtsev) is a grandchild variety, pending state variety testing since 2018.

Conventional genetics since rediscovery of Mendel’s laws (1900) calls upon studying the genetics of quantitative trait. TEGOQT, however, has demonstrated that for a trait subjected to the phenomenon of genotype-environment interaction it is impossible to give the same genetic characteristic for all environments [27-29, 32]. It means that quantitative traits are determined by “vagrant spectra” (sets) of gene products changing for the same trait upon modification of environmental lim-factors and resulting in sudden vast changes in genotype dispersion (during day, weeks, months, years). For example, we have demonstrated that gene expression product sets determining transpiration intensity change twice a day [33].

In exploring theoretical bases for Grenada variety creation, we proceeded from the fact that analysis of the longest pedigree of varieties makes it possible to qualitatively substantiate how to select pairs for crossbreeding [34, 35] and to directionally configure genotypes via due selections [36]. Pedigree of Grenada
variety includes 69 varieties of various genetic and environmental geographical origins (Fig.). Our pedigree analysis was not conventional though. We were interested not only in parents of each ancestor, but dynamics of limiting environmental factors in a geographical point where the respective parent was created. Such approach enabled us to approximately identify which GPSs of adaptability for each of 12 stages of ontogenesis the parent possesses. In addition, it was found that the effectiveness of crossbreeding increases when the parent varieties and forms are close in their morphology but genetically heterogeneous.

This was particularly clear for winter branch that produced a renowned high-yield high-quality variety Bezostaya 1 (developed by P. P. Lukianenko), a mother form of spring intensive high-quality middle-late wheat Omskaya 9. Crossbreeding the latter with hard wheat variety Novosibirskaya 67 showing higher plasticity, in which the enhancement of quantitative productivity traits is caused by mutations due to discrete $\gamma$-irradiation (5000 R), created a plastic early-ripe hard variety Kazakhstanskaya rannespelaya. Genetics of quantitative traits of its parents, Novosibirskaya 67 ($\checkmark$) and Omskaya 9 ($\checkmark$), was studied under DIAS program [26]. Their combining ability was turned out to be well-defined [26]. Therefore, a hybrid combination Novosibirskaya 67 × Omskaya 9 was paid special attention. After multiple stabilizing cropping, an elite plant (Lutescens 1227-8-79 sample) was selected from F5 and propagated. It became a parent of hard wheat variety Kazakhstanskaya rannespelaya which has been widely cultivated in Northern Kazakhstan on 450 thousand to 1.2 million hectares for over 20 years. Early ripeness of this variety is caused by amalgamation of $Vrn$-1 and $Vrn$-3 gene products controlling accelerated organogenesis during the second half of vegetation [37, 38]. Kazakhstanskaya rannespelaya, just as its parents, belongs to West-Siberian ecotype. It is this variety that became a mother form of the new Grenada variety. As a father and saturation (B1) parent, early-ripe hard variety Tulunskaya 12 (East-Siberian ecotype) was used. The enhancement of its quantitative traits is of a discrete accumulative nature due to use of Canadian varieties (they accommodate old Russian Galician forms and Indian forms) and local East-Siberian genotypes originated in 16th century from Inner Mongolia and Mountainous China [39, 40]. Distinctive features of East-Siberian ecotype varieties are accelerated development, overall drought-resistance and high-quality grain production under chilly conditions. Seeds of these varieties are oblong with 1000-grain weight from 32 g [40], which is also typical for Tulunskaya 12 variety. Both parents of Grenada variety are distinguished by strong pubescence of leaves that have dark color and pronounced waxy coat.

Selection from early hybrid generations is ineffectual as the phenotypes of F2 plants do not correlate with yield in descendants. Phenotypic manifestation of individual productivity in F2–F3 may be due to heterozygous epistasis, dominance effects [41] and complicated by expression of heterozygous loci, i.e. superdominance (for a considerable number of traits) that disappears in three or four reproductions. Consistent decline of non-additive variance towards latter generations facilitates selection of productive genotypes [42, 43], as during reproduction (starting from F2 and on) the population is subjected to natural (evolutionary) selection that eliminates poorly adapting forms thus preserving genotypes that are more productive and better fit to local conditions. Such genotypes usually manifest a well-defined synergistic dynamics with typical environmental lim-factors in the breeding zone [36].

A hybrid population [F1 (Kazakhstanskaya rannespelaya × Tulunskaya 12)] once saturated with Tulunskaya 12 genome underwent stabilizing propagation up to F5 on fertilized ($N_{30}P_{45}K_{30}$) dark gray soil fallow in conditions typical
Pedigree of Grenada variety created by innovative breeding technology based on Theory of Eco-Genetic Organization of Quantitative Traits. Winter varieties are marked with asterisks.
for northern forest-steppe zone of North Transurals to select productive forms with genetically moderate development of ear, good ripening and filled grains. As per Kumakov [44], this is due to assimilates distribution (ATTR and MIC systems) and growth functions which differ from those in arid conditions. Potential productivity and resistance to drought are controlled by various GPS, wherefore they may be improved through selection independently from each other [45]. Thus, a single genotype may well combine these complex and, at first glance, contradicting properties [46].

In quantitative analysis of each GPS contributions to productivity, phenotyping was carried out in two-dimensional coordinate systems. For instance, in a system with “main ear culm weight” abscissa and “ear weight” ordinate, the dots of genotypes with plus- and minus-modifications (particularly, genotype that falls into dry soil on hubble will shift to the left and down, into a hollow with water and nitrogen — to the right and up) and with different adaptability (dots of more adaptive genotypes will go right and up, and those of less adaptive — left and down) will be distributed along the positive regression line. Genotypes with good GPS of attraction will shift along the negative regression line (the higher the attraction, the lighter the culm and the heavier the ear), so that the genotypes with better attraction GPS will congregate in the upper left area of the chart. The attraction deviations will not be masked by the noises of environmental variability of micro-niches under each genotype and variability of genotype adaptability (the effects of such noises will appear along the positive regression line). Thence, plus and minus genetic attraction shifts will be free of any noises and their values will be genetically clear (“stripped”, as per Serebrovsky). Use in breeding (phenotyping) of measurable quantitative values for seven GPS [30] each of which makes its plus contribution to the resulting trait (productivity) enabled estimation of plus contributions of different GPS to enhancement of yield resulting in identification in F₅ population of 506-11 Line that later became the Grenada common spring wheat variety. Seven GPS was also used in characterization of this variety.

Morphological features of Grenada variety are as follows: semi-erect bunch of upright tillers, slightly inclined cylindrical white ear of medium density (20.8 spikelets per 10 cm rachis) with waxy coating and asymmetrical awnlike appendices in the upper part. The stem is of average length (78-88 cm), lodged-resistant, with heavy waxy coating, short lower internodes (4.1-4.9 cm the fist, 8-9 cm the second), with of a 2.2-2.5 mm and 2.7-2.9 mm in diameter, respectively; leaves are short, wide, coarse, dark-green, with pubescence and waxy coating, non-inclined; kernels are large, egg-shaped, dark-red, with shallow groove and short pappus.

Grenada is an early-ripening variety (it ripens 3 days earlier than standard Novosibirskaya 31). During grain maturation, green biomass gradually desiccated thus ensuring better matter recycling (ATTR GPS function, attraction of macronutrients from straw and leaves to the ear). Therefore, the seeds of well-grained ear are weighty and well-filled both in the upper and lower parts. The grains are large with net weight of 39.7 g (+7.7 g to the standard) and high test weight of 798 g/l (+35 g/l to the standard). Technological indicators of grain and bread are at the level of hard and premium varieties. Protein yield per 1 ha is 628 kg, which is 119 kg more than the standard (Novosibirskaya 31). Even in provocative conditions this variety is resistant to grain pre-harvest sprouting.

New variety demonstrates well-defined GPS of microdistributions of attracted macronutrients between the grain and chaff in the ear, as ear chaff to total weight ratio indicates. It makes 23% and 19%, respectively, in parent varieties Kazakhstanskaya rannespelaya and Tulunskaya 12, 27% in Novosibirskaya
31, 30% in Iren, and 20% in new Grenada variety which convincingly proves the effectiveness of MIC GPS in this variety. Adaptability GPS (AD) ensures overall adaptability to particular combination of various lim-factors in the breeding zone. Their set changes depending on shifts in lim-factors. Such change results in the phenomenon of productivity rank shifts within the set of varieties from year to year, i.e. to the “genotype-environment interaction” (GEI) phenomenon [25, 47]. In North Transurals conditions, 25% phenotypic dispersion of yields is genotype-specific, while 80% genotypic dispersion is caused by GEI and only 20% by additive gene effects.

It is known [48] that while in the component productivity traits additive effects of gene often prevail, in the resulting productivity trait (weight of grains per plant) non-additive effects typically prevail. Therefore GEI control is the main reserve for increasing the yield [33, 49]. Recently, the nature of GEI phenomenon was completely decrypted [47]. AD GPS functioning in Grenada is manifested by tolerance to early summer drought due to well-developed root system, good overall drought-resistance due to dense cuticular waxy coating [50], good development on subacid soils and production of grain with high test weight in chilly months. These data result from by both 4-year competitive testing and state testing of Grenada variety in contrasting agroclimatic conditions on an extensive territory of 9th (Ural) plant-growing region.

GPS of polygenic immunity (horizontal resistance) in the new variety is due to dense cuticular waxy coating on the leaves and stems, which prevents germination of spores of pathogenic fungi. In provocative conditions (artificial contagion background) Grenada variety demonstrates high horizontal tolerance to Septoria disease, dust brand, mildew; as compared to standard, Grenada plants are much less affected by rust fungi, and is tolerant to cereal leaf beetle. Presence of dense pubescence of the leaves and stem also protects Grenada plants from intra-stem pests (Table).

Intensive breeding is accompanied by microevolutionary processes [51-54] due to recombinogenesis [55], effects of abiotic factors [56, 57], infections [58, 59], natural and artificial selection [46, 60], which enables the creation of productive, locally-adapted genotypes with well-defined synergism of their biotypes with the environment [61, 62]. This fosters the selection of forms containing densification tolerance GPS (TOL), which is well-demonstrated in Grenada variety. In North Transurals, due to limited vegetation period, wheat plants form yield mainly due to main stems [62]. At the standard seeding rate of 6.5 million seeds per 1 ha, a field germination rate within 70%, plant survivability of 93% and productive tilling capacity of 1.22, Grenada plants, according to long-term annual average data, form about 517 productive stems per 1 m². At the ear productivity of 1.1-1.2 g (28-30 grains of 0.04 g each), biological yield of the new variety is 5.7-6.2 t/ha.

GPS of dry matter production depending on soil nutrition (N, P, K lim-factors of soil; EFF) to a large extent determines the effectiveness of the variety at low doses of nitrogen, phosphorus, potassium. Fairly objective indicator of ATTR GPS and EFF GPS is the harvest index (plot grain weight to total dry biomass ratio). It makes 0.30-0.34 in the standard and compared varieties, and 0.42 in Grenada (i.e. grain percentage in total dry biomass of plants is 42%). This index enables the variety to form on dark gray soils (at low doses of mineral fertilizers N₃₀P₄₅K₃₀) the plants of average height (up to 88 cm) with limited tilling capacity (tilling factor of 1.22) and productively operating assimilation apparatus (due to short wide thick coarse non-drooping leaves that are not affected by pathogens, which is true for the stem as well). The plants vegetate long, effectively use photosynthetically active radiation (PAR) and also have
well-developed and active roots.

In the aggregate, the changes in GPS that were achieved as a result of hybridization and breeding ensured significant increase in grain yield. In competitive variety testing (Agricultural Research Institute of North Transurals), at high natural pathogenic impact on dark gray soil ($N_{30}P_{45}K_{30}$ fertilized fallow), the average yield of Grenada variety over 4 years (2013-2016) was 4.24 t/ha, i.e. 0.87 t/ha significantly higher (+26%) than in the standard Novosibirskaya 31 variety ($LSD_{05} = 0.24$ t/ha) (see Table). An increase was similar, although with lower yield (3.31 t/ha, +39% to the standard), on unfertilized fall-plowed fields. The increase of yield against the standard (+0.47 t/ha, or +17%) also occurred at Ishim Agricultural Experimental Station in south of Tyumen province during environmental tests in 2013-2014.

### Economic and biological characterization of Grenada common spring wheat variety as compared to the standard

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Units</th>
<th>Grenada</th>
<th>Novosibirskaya 31 (standard)</th>
<th>Deviation from standard</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agricultural Research Institute of North Transurals (Tyumen Province, 2013-2016):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>t/ha</td>
<td>4.24</td>
<td>3.37</td>
<td>+0.87 (+26)</td>
</tr>
<tr>
<td>LSD$_{05}$</td>
<td>t/ha</td>
<td>0.24</td>
<td>0.24</td>
<td>+0.00 (0)</td>
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<tr>
<td>Yield</td>
<td>t/ha</td>
<td>3.31</td>
<td>2.38</td>
<td>+0.93 (+39)</td>
</tr>
<tr>
<td>LSD$_{05}$</td>
<td>t/ha</td>
<td>0.26</td>
<td>0.26</td>
<td>+0.00 (0)</td>
</tr>
<tr>
<td><strong>Ishim Agricultural Experimental Station, (Tyumen Province, 2013-2014)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>t/ha</td>
<td>4.53</td>
<td>4.06</td>
<td>+0.47 (+17)</td>
</tr>
<tr>
<td>LSD$_{05}$</td>
<td>t/ha</td>
<td>0.18</td>
<td>0.18</td>
<td>+0.00 (0)</td>
</tr>
</tbody>
</table>

### Biological and technological parameters

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Units</th>
<th>Grenada</th>
<th>Novosibirskaya 31 (standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetation period</td>
<td>day</td>
<td>82</td>
<td>85</td>
</tr>
<tr>
<td>Plant height</td>
<td>cm</td>
<td>76</td>
<td>82</td>
</tr>
<tr>
<td>Resistance:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to lodging</td>
<td>points</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>to drought</td>
<td>points</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>to drooping</td>
<td>points</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Grain sprouting in ear (provocative conditions)</td>
<td>%</td>
<td>1.03</td>
<td>13.40</td>
</tr>
<tr>
<td>Weight of 1000 grains</td>
<td>g</td>
<td>39.7</td>
<td>32.0</td>
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<tr>
<td>Grain unit</td>
<td>g/l</td>
<td>798</td>
<td>763</td>
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<tr>
<td>Vitreousness</td>
<td>%</td>
<td>59</td>
<td>62</td>
</tr>
<tr>
<td>Content:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>of protein</td>
<td>%</td>
<td>14.8</td>
<td>15.1</td>
</tr>
<tr>
<td>of gluten</td>
<td>%</td>
<td>33.1</td>
<td>33.8</td>
</tr>
<tr>
<td>Flour strength</td>
<td>a.u.</td>
<td>386</td>
<td>311</td>
</tr>
<tr>
<td>P/L alveograph ratio</td>
<td></td>
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<tr>
<td>Valorimetric value</td>
<td>a.u.</td>
<td>52</td>
<td>70</td>
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<tr>
<td>Attenuation</td>
<td>f.u.</td>
<td>64</td>
<td>69</td>
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<tr>
<td>Gluten deformation index</td>
<td>gluten deformation units</td>
<td>80</td>
<td>78</td>
</tr>
<tr>
<td>Bread volume (no additives)</td>
<td>ml</td>
<td>720</td>
<td>697</td>
</tr>
<tr>
<td>Bread rating</td>
<td>points</td>
<td>4.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

### Resistance to pathogens

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Type/Infection load</th>
<th>Unit %</th>
<th>Grenada</th>
<th>Novosibirskaya 31</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Puccinia recondita</em> Dietl &amp; Holw., 1857 (artificial infection load)</td>
<td>type/%</td>
<td>1/10-2/20</td>
<td>2/20-3/60</td>
<td></td>
</tr>
<tr>
<td><em>Ustilago tritici</em> (Pers.) C.N. Jensen, Kellerm. &amp; Swingle (artificial infection load)</td>
<td>%</td>
<td>0.1-0.2</td>
<td>2.3-4.2</td>
<td></td>
</tr>
<tr>
<td><em>Septoria tritici</em> (high natural load)</td>
<td>type/%</td>
<td>Traces-1/5</td>
<td>2/20-3/60</td>
<td></td>
</tr>
</tbody>
</table>

Note. P/L — dough tenacity to extensibility ratio, units. Gluten deformation index indicates quality of gluten (in gluten deformation units); a.u. — alveograph units, f.u. — farinograph unit.

Stages of ontogenesis in Grenada plants in majority of years complied with the typical dynamics of lim-factors for Tyumen province. Therefore, no breeding-born shift in ONT GPS occurred. However, there are genetic bases for changing this trait as varieties with respective genotype are known [63]. The created variety is recommended for subtaiga, northern and southern
Thus, the scientific outcome of DIAS is the development of TEGOQT with its 24 deductions and 10 know-hows [7]. For the first time ever we have implemented a priority complex of innovative breeding technologies based on TEGOQT and discovered seven plant genetic-physiological systems (GPS) which in the end determine the yield of any varieties. Creation of the initial breeding material by these new technologies, including selection of parent pairs on the basis of GPS, crossbreeding, stabilizing reproduction, and final selection, was performed by a six-stage algorithm suggested and tested for the first time. The stage order is as follows: i) special selection of parental pairs based on deep analysis of the longest pedigrees of the old breeds with regard to typical dynamics of lim-factors in the areas of creation of such parent varieties; ii) phenotyping for seven GPS contributing to yield within the group of the most productive varieties of the breeding nursery (carried out according to the priority proprietary methodology); iii) selection of genotypes that have at least one GPS with the maximum plus contribution to the crop; iv) crossing of these genotypes to combine the plus contributions of all seven GPS in the expected variety; v) stabilizing reproduction of segregating generations to eliminate the effects of dominance, superdominance and heterozygous epistasis (with several saturations with the genome of one of the parents possessing the most valuable properties); vi) selection of elite plants after a number of stabilizing reproductions of the hybrid population under the typical dynamics of the environment lim-factors (in typical years).

So, a six-stage innovative technology that we suggest enables breeding varieties with the yield exceeding standard by up to 40% and more that guarantees considerable increase in grain harvesting in a particular territory. Of particular importance is the evaluation of selected genotypes by functional activity of seven genetic-physiological systems (GPS), i.e. by their plus contributions to yield, throughout breeding (from collection nursery, through nurseries for hybridization, selections, testing of families, to preliminary, environmental and competitive trials and trials in state variety testing network). Combining contributions of all seven GPS within the single designed variety allows breeders to create high-yield lodging- and pathogen-resistant adaptive and plastic varieties of common spring wheat with high-quality grain. The suggested innovative technology based on forecast of plus contributions of GPS to yield provided correct evaluation of breeding material and selection of early-ripe, high-yield, intensive plastic line Lutescens 506-11 from which the Grenada variety derived. Use of these technologies considerably reduces the number of breeding nurseries and, as a result, breeding costs.

REFERENCES

4. Pérez-de-Castro A.M., Vilanova S., Cacizares J., Pascual L., Blanca J.M., Díez M.J., Pro-


36. Novokhatin V.V. *Dostizheniya nauki i tekhniki APK*, 2016, 3: 42–45 (in Russ.).


42. Zhuchenko A.A. *Ekologicheskaya genetika kul’turnykh rastenii: adaptatsiya, rekombinogenez, agrobiotenos* [Ecological genetics of cultivated plants: adaptation, recombination, agrobiodiversity]. Kishinev, 1980 (in Russ.).

43. Gill K.S. *Karlikovy pshenity* [Dwarf wheats]. Moscow, 1984 (in Russ.).


45. Zhuchenko A.A. *Adaptivnyi potencial kul’turnykh rastenii (ekologicheskie osnovy)* [Adaptive potential of cultivated plants (ecological basis)]. Kishinev, 1988 (in Russ.).


**Effects of Dwarfing Wheat (Triticum aestivum L.) and Rye (Secale cereale L.) Genes in Spring Triticale Segregating Population as Studied in Pot Trials**

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**Abstract**

The urgent problem of triticale lodging may be reliably overcome by introgression of dwarfing genes into triticale cultivars. Notable, both wheat and rye dwarfing genes can reduce the height of triticale plants. Therefore, a single contribution of various dwarfing genes and their additive effects in triticale which is an intergeneric hybrid still remain intriguing in fundamental aspects and important for breeding practice. In our study, rye dwarfing gene \( Ddw1 \) has been transferred into spring triticale. Then we have hybridized winter triticale cv. Avanguard \( (Ddw1 Ddw1 Rht-B1a Rht-B1a) \) with spring triticale cv. Solovei Kharkovskii \( (ddw1 ddw1 \) \( Rht-B1b Rht-B1b) \) and used \( F_2 \) seeds to reveal the mechanism of inheritance of the studied dwarfing genes \( Ddw1 \) and \( Rht-B1b \) and to determine the effect of the dwarfing alleles on economically valuable traits in the segregating population of spring triticale. Under the greenhouse conditions, 273 plants of the spring type of the segregating population \( F_2 \) were grown to individually estimate plant height, the number and length of internodes, spikelet length and number per spike, spike density, grain weight, grain number and 1000-grain weight per the main spike. Each plant was also genotyped by PCR using the markers of the \( Ddw1 \) and \( Rht-B1 \) allelic state. To investigate inheritance patterns, the dominant and additive effects of genes were calculated. The second task was achieved by comparing plants homozygous for wild-type alleles \( (ddw1 \) and \( Rht-B1a) \) and short-stem alleles \( (Ddw1 \) and \( Rht-B1b) \) with estimation of both independent effect of each genes and their interlocus interaction. Using statistical methods (Fisher \( F \)-criterion, Mann-Whitney \( U \)-test, and Spearman rank correlation coefficient \( \rho \)), we found the significance of the differences and associations between phenotypic traits and genotype. Our studies have shown that the effects of the \( Ddw1 \) and \( Rht-B1b \) are somewhat different from those in wheat. The \( Ddw1 \) statistically significant affects plant height (by reducing up to 40 %, \( p = 0.05 \)), manifesting itself as a partially dominant allele. The \( Rht-B1b \) results in a decrease in the spring triticale plant height but less than the \( Ddw1 \) gene does (only up to 20 %, \( p = 0.05 \)). Hence, the \( Rht-B1b \) allele is proven to be partially recessive. In the presence of gene \( Rht-B1b \) a kernel weight increases from 1.4 g to 1.7 g (by 21.4 %) due to higher spike density and fertility. The \( Ddw1 \) gene introgression leads to a 16.7 % decrease (\( p = 0.05 \)) in the total grain weight per spike (from 1.8 g to 1.5 g) due to a 9.6 % decrease (\( p = 0.05 \)) in the 1000-grain weight (from 45.7 g to 41.3 g). In general, the \( Ddw1 \) and \( Rht-B1b \) genes affect the studied traits as antagonists. In summary, a combination of two dwarfing genes, \( Ddw1 \) from rye and \( Rht-B1b \) from wheat, makes it possible to maximize yield of dwarf spring triticale plants and is promising for breeding.

Keywords: spring triticale, \( Rht-B1b \), \( Ddw1 \), structural analysis, dwarfing genes, DNA markers, breeding

The main idea behind creation of triticale was to combine positive traits of...
rye *Secale cereale* L. (resistance to hostile conditions) and wheat *Triticum aestivum* L. (suitability for diversified use in food industry). The global production of triticale maintains stable growth and reached 15 million tons in 2016 with total crop acres equal to 4.2 million hectares [1], whereas the culture has both forage and food value [2, 3]. Lodging is among the drawbacks of triticale, limiting its wider spread during crop cultivation. Treatment with growth retardants is used to fight lodging; however, this makes the products more expensive and increases chemical load on the environment. The other approach to solving this problem is selective improvement of triticale and creation of varieties resistant to lodging. High correlation between resistance of triticale plants to lodging and plant height has been observed [4, 5]. Losert et al. [5] analyzed a collection consisting of 199 winter and 2 spring triticale crops and demonstrated that during the last 30 years a tendency of significant triticale plant height reduction has been observed (by 0.38 cm per year) and reduced lodging tendency as a result of selection, which is due to close interconnection of these two features (shorter plants, as a rule, are more resistant to lodging) [4, 6].

Plant height is a complex quantitative trait (7, 8). In hexaploid triticales (BBAARR) combining wheat (BBAA) and rye (RR) genomes the dwarfing can be ensured by wheat, rye genes and/or their combination genes. At this time, common wheat bears 24 dwarfing genes [9, 10]. *Rht-B1b* (=*Rht1*), *Rht-D1b* (=*Rht2*), *Rht-8c*, *Rht-B1e* (=*Rht11*) genes gained the biggest widespread in commercial wheat varieties. They are also effective at high dosages of fertilizers and possess pleiotropic effect on many agronomic characters [11-13]. In wheat, plant height is reduced by 10-15% on the average as compared to the height of *Rht-B1a* [14-16] wild type allele carrier when *Rht-B1b*, *Rht-D1b*, *Rht-B1e* dwarfing genes insensitive to gibberellin are present. In terms of impact on stalk growth these genes manifest themselves as recessive or partially recessive. The height of rye is also controlled by numerous genetic factors [17, 18]. A total of 14 different rye dwarfing genes [19-21] are already known, among which three are dominant genes, and *Ddw1* is of the highest selective value. In presence of *Ddw1* dominant gene the dwarfing of plants is up to 40%, for diploid rye and up to 55 % for tetraploid rye [22]. About 80% of rye varieties of Russian selection were created using *Ddw1* gene carriers and donors, which on the average allowed an increase in crop yield of winter rye by 12-15 % [23]. *Ddw1* gene was successfully transferred into winter triticale (Debo and Dalo varieties) [24, 25] and a number of dwarf varieties of winter triticale [26, 27] were created with this gene in Poland and Romania.

Various wheat dwarf genes and *Ddw1* rye dwarf gene are widely spread among commercial varieties of winter triticale. In spring triticale, dwarf genes are engaged not so actively. For instance, Korshunova et al. [28] by analyzing 86 samples of spring triticale identified *Rht-B1b* gene in 76 samples, whereas all of them belong to commercial varieties. However, *Ddw1* gene was not found in any samples of spring triticale. Therefore, at this time, the diversity of dwarf genes in spring triticale is very limited [28]. The 2R/2D chromosome substitution, which also dwarfs plants, is not encountered among commercial varieties of spring triticale [29, 30]. It is typical for wheat and winter triticale to have a large diversity of dwarfing genes, and in various varieties the reduction of plant height is ensured by various genes or their combinations. The spring triticale does not display such diversity, and no focused efforts to introduce other dwarfing genes in its genome have taken place. The introgressions of additional dwarfing genes from winter triticale and/or wheat in genomic pool of spring triticale can help solve the height problem of this culture and give a new impetus to its develop-
ment due to pleiotropic action of dwarfing genes to many agronomic characters. Furthermore, it has to be taken into account that mechanisms of action of dwarfing genes of plant height are complex, as a rule, it is implemented via involvement of response to phytohormones in various ways [31] and depends, inter alia, on gene dosage in the genome. The effects of different dwarfing genes in triticale genome, where genomes that are far apart are combined as a result of bi-generic crossing, arouse interest due to knowledge of specifics of interlocus interaction of genes and practical selection, and still have not been properly studied.

In this study, we showed that the effects of **Ddw1** dwarfing genes of rye origin and **Rht-B1b** dwarfing genes of wheat origin in spring triticale vary insignificantly from those in rye and wheat. The studied genes had different impact on height of triticale plants and acted as antagonists in terms of impact on productivity elements.

Our goal was to assess the impact of **Ddw1** and **Rht-B1b** genes and their effect of their interaction on plant height and other agronomic characters in spring triticale in the context of greenhouse studies in F\(_2\) segregating population.

**Techniques.** We selected those varieties that carry contrast combinations of dwarfing wheat and rye alleles as seed parents, i.e. winter triticale Avanguard variety (**Ddw1 Ddw1 Rht-B1a Rht-B1a** genotype; female parent) and Solovei Kharkovskii spring triticale variety (**ddw1 ddw1 Rht-B1b Rht-B1b** genotype; male parent). Hybridization of parent plants was performed by substitution method (a greenhouse of the Center of Molecular Biotechnology, Russian State Agrarian University—Timiryazev Moscow Agricultural Academy, 2014) to obtain F\(_1\) plants. The seeds of Avanguard parent variety were sown, 10 seeds per pot. During the tillering stage the plants were placed in vernalization chamber for 2 months at 5 °C. After vernalization (during the paniculation stage) the plants were again moved to the greenhouse; during the ligule stage the head was castrated and placed under an isolator made of parchment paper. A head of a cut male parent plant was placed under the isolator during blooming, whereas the stalk was placed in a vessel with water to maintain viability.

From the seeds of F\(_2\) generation sown in pots, 10 psc. per each, plants were grown at identical lighting conditions with dosed irrigation and equal fertilizer dosages (a greenhouse of the Center of Molecular Biotechnology, Russian State Agrarian University—Timiryazev Moscow Agricultural Academy). A total of 273 F\(_2\) plants were obtained. Because of a winter parent variety, F\(_2\) segregating population had several winter forms, which were rejected based on phenotype.

Height, number of joints, length of each joint, main head length (MHL), number of seeds per the main head (SNH), number of ears per the main head (ENH), main head seed weight (SWH) were analyzed individually in each plant. Furthermore, head density (HD) and 1000-seed weight (W\(_{1000}\)) were calculated as per [1] and [2], respectively:

\[
HD = \frac{\text{ENH}}{\text{MHL}},
\]

\[
W_{1000} = \frac{\text{SWH}}{\text{SNH}}
\]

Molecular markers were used to determine **Ddw1** and **Rht-B1** allele profiles in each F\(_2\) plant individually.

CTAB (cetyltrimethylammonium bromide) method was used for genome DNA extraction from each F\(_1\) and F\(_2\) plants to further evaluate the hybridity and the combinations of analyzed alleles [32].

The alleles of **Rht-B1** and **Ddw1** genes in F\(_1\) and F\(_2\) plants were determined in polymerase chain reaction (PCR) using molecular markers (primer synthesis was performed at Syntol LLC, Russia). Primers BF, MR1 and WR1In were used to identify **Rht-B1a** alleles (wild type) and **Rht-B1b**; PCR was per-
formed as recommended [33]. PCR products were separated in a 2% agarose gel electrophoresis (TBE buffer; molecular weight marker GeneRuler 100 bp DNA Ladder, Thermo Fisher Scientific, USA) and stained with ethidium bromide for UV-visualisation. Presence of Dw1 gene was determined with the primers for amplification of REMS1218 microsatellite loci sequence closely linked to this gene [34] as per the PCR protocol described by the authors of the molecular marker with fragment analysis (3130xl Genetic Analyzer, Applied Biosystems, USA). Dominant Dw1 Dw1 homozygote produces two fragments of 317 bps and 321 bps with identical peak height, whereas Dw1 ddw1 heterozygote has two fragments with different peak height, of 317 bps (high) and 321 bps (low). One 317 bps fragment is detected in ddw1 ddw1 recessive homozygote.

The pattern of allele inheritance was assessed based on genetic effects according to Smiryaev and Kilchevsky [35]. The additive (a) and dominant (d) effects were calculated regarding the average for the entire population. If d = 0 the inheritance is additive; if d = a, the allele is dominant, if d = -a, the allele is recessive; if 0 < |d| < |a|, the allele is partially dominant (for identical, positive or negative, sings of d and a) or partially recessive (for different sings of d and a); if |d| > |a|, heterozygote displays overdominance for the analyzed trait (heterosis).

The impact of dwarf alleles on traits in question was determined in two ways based on comparison of their manifestation in dwarf and wild type allele homozygotes. To evaluate the effects of each gene separately (independent analysis), the average population values were compared for homozygotes with wheat genes (Rht-B1a or Rht-B1b) only, i.e. without the impact of rye genotype, or for homozygotes with rye gene (Dw1 or dw1) only, i.e. without the impact of wheat genotype. To evaluate the combined effect of rye (Dw1) and wheat (Rht-B1) genotypes, the impact of wheat gene was estimated depending on the rye gene allele status, and vice versa (interlocus interaction).

Distribution of quantitative parameters was tested for normality by Shapiro-Wilk W-test. The arithmetical mean (M) and standard deviation (±SD) were determined. The significance of variance between homozygotes with wild type alleles (ddw1 or Rht-B1a) and dwarf alleles (Dw1 or Rht-B1b) was determined as follows. Nonparametric Mann-Whitney U-test was used to evaluate the differences between the two groups. Analysis of Spearman’s rank correlation coefficient (Spearman’s ρ) was used to summarize the strength and direction (negative or positive) of a relationship between compared values. Differences were considered statistically significant at p ≤ 0.05. To reveal reliable differences between the means, dispersion analysis and the least significant differences at the 5% level of significance (p = 0.05; LSD05) were used. Statistical analysis was performed with Statistica 10.0 software (StatSoft Inc., USA).

Results. In order to determine the mode of inheritance and impact of dwarfing genes on spring triticale agronomic characters in the greenhouse we used seeds of F2 generation from crossing Avanguard winter triticale variety (Dw1 Dw1 Rht-B1a Rht-B1a genotype) and Solovei Kharkovskii spring triticale variety (ddw1 ddw1 Rht-B1b Rht-B1b genotype). Avanguard combines high crop productivity with resistance to lodging, high winter- and cold resistance and good bread-baking qualities [36-38]. The second parent form, Solovei Kharkovskii, is grown for bread, technical and feed grain, possesses good bread-baking qualities, optimal height of culm (95-110 cm) which is robust and resistant to lodging [39].

The genotype of each F2 plant was determined in terms of dwarfing gene allele profiles (Fig.) and main agronomic characters.
Identification of alleles of Ddw1 genes (A and B) and Rht-B1 genes (C and D) in plants of spring triticale F₂ (Avanguard × Solovei Kharkovskii). Electropherogram of PCR products amplified with the primers to REMS1218 microsatellite locus (fragment analysis): A — homozygote for ddw1 allele, 317 bps; B — homozygote for Ddw1 allele, 317 bps and 321 bps. Electropherogram of PCR products amplified with BF + WR1 primers (C, Rht-B1a identification, 237 bps) and BF + MR1 (D, Rht-B1b identification, 237 bps): 1, 2, 5 — Rht-B1a Rht-B1a homozygotes; 6, 7, 8 — Rht-B1b Rht-B1b homozygotes; 3, 4 — Rht-B1a Rht-B1b heterozygotes; M — molecular weight markers (GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, USA).

Plant height. Statistical analysis revealed that Ddw1 shows incomplete dominance in triticale, Rht-B1b allele is a partially recessive, and both genes significantly affect plant height in greenhouse conditions (Table 1).

Identification of Ddw1 and Rht-B1b gene effects showed that in the resulting spring triticale segregating population of F₂ Ddw1 dwarf allele has additive effect in case of height reduction by 20.9 cm on average and dominant effect when height is reduced by 11.7 cm (d < a). The Ddw1 homozygotes and heterozygotes varied statistically significantly (p = 0.05). Thence, in terms of general impact on plant height this allele displays incomplete dominance. Rht-B1b allele turned out to be partially recessive, its additive effect amounted to 7.1 cm with dominant effect of +2.3 cm (0 < |d| < |a|) (homozygotes and heterozygotes Rht-B1b without Ddw1 vary significantly, p = 0.05). Other authors also described Rht-B1b allele as a partially recessive [40-43].

1. Statistical analysis of Rht-B1 and Ddw1 genes impact on plant height in F₂ segregating population of spring triticale hybrid (Avanguard × Solovei Kharkovskii; a greenhouse study)

<table>
<thead>
<tr>
<th>Genotype for Ddw1 alleles</th>
<th>Height, cm</th>
<th>Genotype for Rht-B1</th>
<th>Difference between homozygotes Rht-B1b and Rht-B1a, cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rht-B1b Rht-B1a</td>
<td>Rht-B1a Rht-B1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>height, cm</td>
<td>height, cm</td>
</tr>
<tr>
<td>Ddw1 Ddw1</td>
<td>76.3±13.8</td>
<td>88.4±22.2</td>
<td>92.6±23.6</td>
</tr>
<tr>
<td>Ddw1 ddw1</td>
<td>84.3±18.7</td>
<td>74.7±13.7</td>
<td>77.9±14.2</td>
</tr>
<tr>
<td>ddw1 ddw1</td>
<td>116.3±23.5</td>
<td>102.8±23.0</td>
<td>118.6±19.4</td>
</tr>
</tbody>
</table>

Note. The table shows mean values with standard deviation (M±SD); § — the results of independent analysis of allele effect; F(Ddw1) = 111.2 > F₀.₀₅ = 3.0; F(Rht-B1) = 5.0 > F₀.₀₅ = 3.0; F(Ddw1 × Rht-B1) = 5.2 > F₀.₀₅ = 2.4.

* The differences between homozygotes are essential at the 95% level of confidence probability (p = 0.05). Identical letters designate mean values identified in independent analysis for each gene, which did and differ statistically significantly at 95% level of confidence probability (p = 0.05).
When analyzing the effect of Rht-B1b allele without consideration of Ddw1 gene (see Table 1) the difference between homozygotes Rht-B1b Rht-B1b and Rht-B1a Rht-B1a was statistically significant and constituted 9 cm or 9.1 % (p = 0.05). Furthermore, independent analysis of Ddw1 effect identified that Ddw1 Ddw1 and ddw1 ddw1 homozygotes varied statistically significantly in terms of plant height (by an average of 40 cm or 34.4 %, p = 0.05).

When studying the interlocus interaction, the difference between Rht-B1b Rht-B1b and Rht-B1a Rht-B1a homozygotes in absence of Ddw1 allele also turned out significant and constituted 28 cm or 21.0 % (p = 0.05) (see Table 1). The reduction of soft wheat plant height with Rht-B1b allele as compared to carriers of wild type Rht-B1a allele can be up to 17 % [42, 44]. In presence of Ddw1 allele the differences between homozygotes and heterozygotes for Rht-B1b allele were statistically insignificant, which, possibly, can be explained by the masking effect of Ddw1 gene, i.e. the reduction of height on account of Ddw1 significantly exceeds such reduction due to presence of Rht-B1b.

The height difference between Ddw1 Ddw1 and ddw1 ddw1 homozygote plants that do not carry wheat Rht-B1b was significant (p = 0.05) and constituted −55.5 cm (−42.4 %), and with those carrying Rht-B1b allele the difference amounted to 28.1 cm (−27.3 %) (see Table 1). Lower difference between homozygotes for Ddw1 depending on presence of Rht-B1b can be explained by the fact that the Ddw1 gene reduces the height of plants for which the height has already been reduced due to Rht-B1b. The differences between Ddw1 Ddw1 homozygotes with and without Rht-B1b allele are statistically insignificant. Reported [22], the reduction of height of rye plants on account of Ddw1 allele amounts to 40-55%.

According to the data that we obtained in the greenhouse study, during statistical analysis of spring triticale for each of the analyzed dwarfing genes their effects are similar to those in wheat and rye. In spring triticale genome the effect of Ddw1 rye dwarf gene significantly exceeds the effect of Rht-B1b wheat dwarf gene. However, there is no cumulative effect of reducing plant height when both genes are present. In contrast [45, 46], when Rht-B1b and Rht-D1b or Rht-B1b/Rht-B1e/Rht-D1b and Rht-8 dwarfing genes are simultaneously present in wheat, plant height is reduced much more significantly than under the effect of only one gene. Therefore, if the goal of the selection process consists of reducing the height of triticale plants, the combination of two dwarfing genes (Ddw1 and Rht-B1b) will hardly prove more effective than introgression of one of the genes. Furthermore, it has to be taken into account that introduction of Ddw1 gene in spring triticale genome will allow reducing the height of plants to a larger extent than using Rht-B1b gene which is currently prevalent in commercial varieties of this culture.

Joint quantity and length. In the course of independent analysis we observed no impact of Rht-B1b wheat gene on the number and length of joints in spring triticale population. The presence of Ddw1 rye allele regardless of the Rht-B1 wheat gene alleles reduces the number of joints on the average by 0.2 joints (p = 0.05); the reliable reduction of length on account of Ddw1 was observed for all joints, whereas reduction was the highest for the upper 1st and 2nd joints (by 32.7 and 37.5 %, respectively, p = 0.05, Table 2).

During analysis of interlocus interaction in F2 segregating population we failed to observe Rht-B1b allele effect with regard to the number of joints both in the presence and absence of Ddw1 allele. Whereas Ddw1 allele masks the effect of Rht-B1b on plant height in spring triticale, we have studied the consequences of presence of Rht-B1b allele on change of interlocus length only in plants that
2. *Rht-B1* and *Ddw1* gene effects on plant joint length in F$_2$ spring triticale segregating population (Avanguard × Solovei Kharkovskii; a greenhouse study)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rht-B1 without Ddw1</th>
<th>Ddw1</th>
<th>Ddw1 without Rht-B1b</th>
<th>Ddw1 with Rht-B1b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1LJ</td>
<td>3UL</td>
<td>2UJ</td>
<td>1UJ</td>
</tr>
<tr>
<td>2</td>
<td>5.3±3.8*</td>
<td>17.6±4.2*</td>
<td>27.4±5.7*</td>
<td>34.7±8.7*</td>
</tr>
<tr>
<td>1</td>
<td>5.5±3.2a</td>
<td>19.2±4.6a</td>
<td>28.8±6.0a</td>
<td>40.6±9.3ab</td>
</tr>
<tr>
<td>0</td>
<td>7.0±3.8a</td>
<td>18.2±3.6a</td>
<td>32.6±6.5a</td>
<td>44.2±10.0a</td>
</tr>
</tbody>
</table>

Difference between homozygotes, cm (%):

-1.7 (−23.0 %) | −2.0 (−10.0 %) | −5.2 (−22.0 %) | −9.5* (−16.0 %) | 1.1* (18.6 %) | 5.3* (29.6 %) | 10.7* (37.5 %) | 13.0* (32.7 %) | −1.6 (−23.0 %) | 5.6* (18.6 %) | −13.0* (31.0 %) | −18.5* (42.0 %) | −1.3 (−25.0 %) | −3.7* (23.0 %) | −9.8* (38.0 %) | −10.6* (30.0 %)

Note. The table shows mean values with standard deviation (M±SD); 0 — homozygote for wild type allele (*Rht-B1a Rht-B1a* or *ddw1 ddw1*), 1 — heterozygote (*Rht-B1a Rht-B1b* or *Ddw1 ddw1*), 2 — homozygote for dwarf allele (*Rht-B1b Rht-B1b* or *Ddw1 Ddw1*), 1LJ — 1st lower joint, 1UJ — 1st upper joint, 2UL — 2nd lower joint, 3UJ — 3rd upper joint; § — the results of independent *Ddw1* effect analysis.

* The differences between homozygotes are statistically significant at 95% level of confidence probability (p = 0.05). Identical letters designate mean values identified in independent analysis for each gene (or gene combinations) which did not differ at 95% level of confidence probability (p = 0.05).
do not carry \textit{Ddw1} allele (see Table 2). The statistically significant reduction of length on account of \textit{Rht-B1b} occurred for the 1\textsuperscript{st} upper joint and amounted to 9.5 cm (−22.0%, \(p = 0.05\)). An overall dwarfing tendency was observed for all joints on account of \textit{Rht-B1b}. According to the published data, joint height is reduced unevenly in various wheat populations due to presence of \textit{Rht-B1b}. For instance, Liu et al. [4] report 17, 21 and 24\% distribution by joints (the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} joint from the top, respectively); whereas Hu [47] reports joint distribution of 23, 14 and 28\%. According to other papers [4, 47] and results we obtained, the biggest contribution to plant height reduction in the presence of \textit{Rht-B1b} is ensured specifically on account of length reduction of the 1\textsuperscript{st} joint.

The analysis of the first interlocus interaction showed that in presence of \textit{Rht-B1b} wheat dwarf allele the 1\textsuperscript{st} upper joint is shortened under the impact of \textit{Ddw1} allele on the average by 18.5 cm (42.0 \%), and due to \textit{Rht-B1b} allele by 10.6 cm (30.0 \%); in both cases the statistically significant height reduction (\(p = 0.05\)) was in three upper joints (see Table 2). The \textit{Ddw1} impact on joint length depending on presence/absence of \textit{Rht-B1b} is due to the fact that in absence of \textit{Rht-B1b} the 1\textsuperscript{st} joint is already dwarfed due to wheat dwarf gene effect.

Head length. \textit{Rht-B1b} allele did not display sufficient impact on head length. Statistically significant differences (at \(p = 0.04\)) were identified in independent analysis of impact of \textit{Ddw1} rye dwarf gene on head length using non-parametric Mann-Whitney \textit{U}-test (distribution deviated from normal). Furthermore, statistically significant correlation between head length and \textit{Ddw1} in plant genome (\(\rho = -0.15; p = 0.05\)) was identified using Spearman rank correlation test. Therefore, greenhouse study showed that \textit{Ddw1} rye gene statistically significantly reduced head length by 1 cm on the average.

We have not observed any statistically significant effect of interlocus interaction of two genes on main head length in spring triticale in F\textsubscript{2} segregating population in question. The average head length of plants was 10.3 cm without dwarfing genes, 9.3 cm with \textit{Ddw1} gene, 8.9 cm with \textit{Rht-B1b} gene and 9.5 cm with two genes (Table 3).

Number of ears. We have not observed statistically significant impact of two analyzed genes on the number of ears of main head in spring triticale in F\textsubscript{2} segregating population in question either in the course of independent analysis or during analysis of interlocus interaction (see Table 3); however, we can identify certain tendency in this trait change. For instance, in the presence of \textit{Ddw1} rye dwarf gene \textit{Rht-B1b} wheat dwarf gene increased the number of ears on the main head and a reverse effect was observed in absence of the rye gene (see Table 3).

Head density. In independent gene analysis (see Table 3) we identified statistically significant impact of \textit{Rht-B1b} gene (Mann-Whitney \textit{U}-test, \(p = 0.02\)) on head density increase. When using Spearman rank correlation analysis we also observed statistically significant correlation (\(\rho = 0.19, p = 0.05\)). The higher head density in this case was due to an increase in ears. In the course of independent analysis \textit{Ddw1} allele did not display any statistically reliable impact on head density of spring triticale. This is presumably connected with unidirectional nature of head length reduction tendencies and with the number of ears. Furthermore, we have not observed any statistically significant impact of genes in terms of this criterion in spring triticale in F\textsubscript{2} segregating population.

Number of grains in the main head. Analysis of the \textit{Rht-B1} gene inheritance showed its reliable additive impact on the number of grains in the main head (+3.3 grains or 9.2 \%, \(p = 0.05\)); furthermore, \textit{Rht-B1b} allele manifested itself as dominant, i.e. \textit{Rht-B1b} heterozygotes and homozygotes did not
vary significantly (see Table 3). These data are comparable with the impact of Rht-B1b gene on the number of grains in soft wheat. As reported [42, 44, 48, 49], this gene increases the number of ears, their fertility, and, consequently, the number of grains in soft wheat.

We have not identified the impact of Ddw1 gene on the number of grains per main head, although we observed the tendency to reduction of this parameter in absence of Rht-B1b dwarf allele. Furthermore, we have not observed any statistically significant impact of these two genes on the number of grains per main head in spring triticale in F2 segregating population in question (see Table 3).

Grain weight per main head. In independent analysis of each gene individual effect under greenhouse conditions we identified the following patterns (see Table 3). The presence of Rht-B1b wheat dwarf allele resulted in statistically significant increase of main head grain weight (Mann-Whitney U-test p = 0.04) from 1.4 to 1.7 g (by 21.4 %). Furthermore, these parameters reliable correlate (r = 0.15; р = 0.05). The presence of Ddw1 rye dwarf gene statistically significantly reduced the grain weight per main head (Mann-Whitney U-test p = 0.02) from 1.8 to 1.5 g (by 16.7 %) where r = −0.18 (p = 0.05).

3. Rht-B1 and Ddw1 gene effects on head and grain parameters in F2 spring triticale segregating population (M±SD; Avanguard × Solovei Kharkovskii; a greenhouse study)

<table>
<thead>
<tr>
<th>Genotype for Ddw1</th>
<th>Genotype for Rht-B1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rht-B1b</td>
</tr>
<tr>
<td></td>
<td>Main head length</td>
</tr>
<tr>
<td>F(Ddw1) = 0.8 &lt; F0.05 = 3.1; F(Rht-B1) = 0.1 &lt; F0.05 = 3.1; F(Ddw1 × Rht-B1) = 0.7 &lt; F0.05 = 2.4</td>
<td></td>
</tr>
<tr>
<td>Ddw1 Ddw1</td>
<td>9.5±3.4</td>
</tr>
<tr>
<td>dww1 dww1</td>
<td>8.9±2.1</td>
</tr>
</tbody>
</table>

| F(Ddw1) = 2.0 < F0.05 = 3.1; F(Rht-B1) = 2.0 < F0.05 = 3.1; F(Ddw1 × Rht-B1) = 1.8 < F0.05 = 2.4 |
| Ddw1 Ddw1 | 24.0±5.5 | 23.4±3.5 | 21.1±5.2 |
| dww1 dww1 | 25.4±4.2 | 23.7±3.4 | 22.1±4.1 |
| dww1 dww1 | 25.5±4.1 | 25.2±3.6 | 25.1±3.2 |

| F(Ddw1) = 2.0 < F0.05 = 3.1; F(Rht-B1) = 0.5 < F0.05 = 3.1; F(Ddw1 × Rht-B1) = 1.2 < F0.05 = 2.4 |
| Ddw1 Ddw1 | 25.1±7.0 | 26.6±3.2 | 23.2±4.5 |
| dww1 dww1 | 25.5±4.7 | 24.6±4.0 | 22.6±4.1 |
| dww1 dww1 | 27.4±5.3 | 25.5±5.6 | 25.4±6.1 |

| F(Ddw1) = 0.7 < F0.05 = 3.0; F(Rht-B1) = 2.5 < F0.05 = 3.0; F(Ddw1 × Rht-B1) = 0.9 < F0.05 = 2.4 |
| Ddw1 Ddw1 | 38.3±10.7 | 38.3±11.3 | 28.5±13.7 |
| dww1 dww1 | 36.4±17.8 | 36.2±12.5 | 32.2±15.8 |
| dww1 dww1 | 34.2±18.0 | 42.1±15.6 | 37.4±7.9 |

| F(Ddw1) = 1.9 < F0.05 = 3.1; F(Rht-B1) = 3.5 > F0.05 = 3.1; F(Ddw1 × Rht-B1) = 0.5 < F0.05 = 2.4 |
| Ddw1 Ddw1 | 1.70±0.45 | 1.67±0.66 | 1.27±0.69 |
| dww1 dww1 | 1.56±0.87 | 1.64±0.72 | 1.27±0.77 |
| dww1 dww1 | 1.61±0.92 | 2.01±0.80 | 1.66±0.40 |

When studying the interlocus interaction in the population, the statistically significant dominant effect of Rht-B1b allele with regard to main head grain weight was +0.28 g (16.0 %), i.e. this allele increases main head grain weight in heterozygotes (see Table 3). There were no statistically significant differences during interaction of two genes; however, a tendency for head grain weight increase was observed in presence of Rht-B1b and for head grain weight reduction in presence of Ddw1. Generally speaking, we can assume there is a tendency of mutual compensation of gene effects in the studied segregating population of spring triticale for the analyzed trait (reduction of head grain weight under the
influence of \( Ddw1 \) gene and increase of head grain weight under the influence of \( Rht-B1b \) gene).

1000-grain weight. When analyzing \( F_2 \) segregating population for each gene individually (see Table 3) it turned out that presence of \( Rht-B1b \) wheat dwarf allele does not have any statistically significant impact on the weight of 1000 grains. The presence of \( Ddw1 \) rye dwarf gene statistically significantly reduced the 1000-grain weight for main head (Mann-Whitney \( U \)-test \( p = 0.02 \)) from 45.7 to 41.3 g (by 9.6 \%) under greenhouse conditions. Furthermore, there was a statistically significant correlation between the analyzed parameters \( r = -0.17 \) (\( p = 0.05 \)). Consequently, the overall reduction of main head grain weight in presence of \( Ddw1 \) rye dwarf gene is primarily attributable to the reduction of caryopsis weight and not reduction of their quantity. When analyzing the mode of inheritance, we observed an insignificant dominant effect of \( Rht-B1b \) gene with regard to the 1000-grain weight, which is expressed in the increase of this characteristic in heterozygotes on the average by 4.2 g (10.1 \%). The insignificant additive effect of \( Ddw1 \) allele consisted in the 1000-grains weight reduction on the average by 2.0 g (4.5 \%), whereas \( Ddw1 \) manifested itself as dominant allele by revealing itself in homozygotes and heterozygotes.

This and the other patterns we identified require additional study on other populations of spring triticale and in field conditions.

To summarize, a greenhouse study we conducted on \( F_2 \) spring triticale hybrid segregating population showed that effects of \( Ddw1 \) dwarfing genes of rye origin and \( Rht-B1b \) of wheat origin are slightly different from the effects on rye and wheat. \( Ddw1 \) gene has statistically significant impact on plant height (its reduction reached 40 \%). However, potentially negative effects are observed in addition to that, which can backfire on crop yield of spring triticale. The presence of \( Ddw1 \) gene results in reduction of overall head grain weight due to a decrease in 1000-grain weight. Furthermore, \( Ddw1 \) gene, which in rye increases head size and grains per head, did not have this effect in our test on spring triticale. \( Rht-B1b \) gene also reduces plant height of spring triticale plant, but to a much smaller degree than \( Ddw1 \) gene (to 20 \%), and does not affect this trait at all in presence of \( Ddw1 \) gene. At the same time presence of \( Rht-B1b \) gene increased grain weight per head due to increase in head density, number of ears and fertility. In general, the dwarf genes in question (\( Ddw1 \) of rye origin and \( Rht-B1b \) of wheat origin) were antagonists in terms of their impact on grain productivity parameters. However, their combination in spring triticale, although it does not result in additional reduction of plant height relative to \( Ddw1 \) homozygotes, is promising for breeding due to potential of increasing crop yield on account of creation of dwarf forms.

REFERENCES


34. Smiryaev A.V., Kil'chevskii A.V. Genetika populyatsii i kolichestvennykh priznakov [Genetics of populations and quantitative traits]. Moscow, 2007 (in Russ.).
EFFECT OF ZEATIN ON in vitro EMBRYOGENESIS AND PLANT REGENERATION FROM ANther CULTURE OF HEXAPLOID TRITICALE (∗ Triticosecale Wittmack)

R.S. YERZHEBAYEVA, M.A. ABDURAKHMANOVA, Sh.O. BASTAUBAYEVA, D. TADJIBAYEV

A b s t r a c t

To achieve results sooner, cereal crop selection programs usually combine conventional methods, such as selection of parents and large-scale cross-breeding with haploid technology, a methodology which allows obtaining homozygous lines from the F1 hybrids. Methods of androgenesis (anther culture and isolated microspore culture techniques) have gained widespread use for selection of wheat and triticale. Currently, the main issue for the androgenesis in Triticale is the low efficiency of green plant regeneration. The present work, for the first time ever, utilizes cytokinin zeatin as an exogenic phytohormone in the induction medium, and determines its concentration optimal for improving embryo formation and green plant regeneration from the triticale anther culture. The aim of this research is to increase efficiency of the triticale anther culture, and study the effects of adding cytokinin zeatin to the nutrient medium on embryogenesis induction and regeneration. Two lines of spring triticale, YaTKh-327-11 and Zernokormovoye 5 (facultative), and two lines of winter triticale, T-968 and T-45, were used. Donor plants for the haploid technology were grown in the irrigated field of Kazakh Research Institute of Agriculture and Plant Growing LLP (Kazakhstan, Almaty Region). Cut spikes were subjected to low temperature (4 °C for 14 days), and then the anthers, after they were isolated, to high temperature (32 °C for 3 days). The spikes were sterilized with 0.1 % solution of mercuric chloride. Modified mW14 medium was used as the basic nutrient medium for embryogenesis induction. Five variants of nutrient medium were studied, with concentration of phytohormone zeatin gradually increasing in each subsequent variant (0.2 mg/l, 0.4 mg/l, 0.6 mg/l, 0.8 mg/l, 1.0 mg/l), and medium without zeatin served as control. The study conducted on 4 genotypes of triticale has shown that addition of zeatin to the nutrient mediums in concentrations of 0.2-0.8 mg/l increased the rate of androgenic structure formation by 42.3-65.2 %. Maximal effect on the androgenic structure formation was achieved at 0.4 mg/l concentration of zeatin, with 112 androgenic structures (AS) per 100 anthers on average compared to 67.8 AS per 100 anthers in control group. In the embryogenesis inducing nutrient mediums with 0.4-0.6 mg/l zeatin concentrations the rate of embryogenesis was 16.9-24.1 % higher compared to the control, with embryos having bipolar structure, and producing stem and roots during the regeneration, which indicates positive effect of zeatin on differentiation and organogenesis of the dividing microspore cells. All the variants in the experiment showed a significant increase in the rate of regeneration compared to the control with no zeatin added. In embryos transplanted from the medium containing 0.6 mg/l zeatin the rate of green plant regeneration was the highest reaching 6.3 pcs/100 anthers. It has been established that addition of zeatin and the effect of genotype were the statistically significant factors for androgenic structure formation and regeneration. Efficiency of spontaneous chromosome doubling in triticale amounted to 26.5 %, which has allowed producing 97 double haploid lines from the promising lines of triticale without colchicination.
Keywords: triticale, anther culture, zeatin, embryo, regeneration, albino plants, green plants, spontaneous doubling

Triticale (× *Triticosecale* Wittmack) is a species designed by crossbreeding the wheat (*Triticum* spp.) and rye (*Secale cereale* L.). Combination of alleles of both ancestors enables the plants to adapt to the environment that is less favorable for the wheat but ensures better production of biomass and fodder quality. Triticale possesses considerable potential for production of grain and fodder, although the research for improvement of yield of this species remains behind the similar works in respect of other cereals. It also becomes popular as a cover crop for improvement of soil and reduction of depletion of nutrients. Triticale, just as rye, is suitable for both linear and hybrid breeding methods. Achievements in molecular biology and diversity of genetic resources of wheat and rye may be used to improve triticale [1].

Spiked cereals acreage in Kazakhstan was 14209.3 thousand hectares in 2018 [2]. For Kazakhstan, cultivation of spring and winter triticale forms is important, although the relevant breeding programs are just developing. Cereal crop selection programs usually combine conventional methods, i.e. selection of parent pairs, large-scale cross-breeding and haploid technology (anther and isolated microspore cultures), a methodology which allows obtaining homozygous lines from the F₁ hybrids. The effectiveness of the main methods of triticale anther culture and isolated microspore culture depends on genotype, growing conditions, time of harvesting of donor plants, preliminary treatment (cold, warmth, carbohydrate deficiency), composition of nutrient medium for induction of embryogenesis and plant regeneration. Most progress in studying triticale anthers was achieved due to development of wheat haploid technology [3].

For now, fairly high performance was achieved through use of two androgenesis methods [4-7]. Isolated microspore culture is more effective as compared to the anther culture although this method is more labor-intensive and requires fine manipulations in density-gradient fission of microspores, which may be difficult when handling a large number of hybrid combinations.

Growth regulators are one of the important factors in androgenesis. Embryogenesis induction can be manipulated through use of various types and concentrations of exogenic phytohormones and regulating their presence in the nutrient medium [8]. Auxins are introduced to the anther and isolated microspore cultures of cereals for the purpose of initiation of microspore fission. Addition of 2,4-dichlorophenoxyacetic acid (2,4-D) allows for high results in obtaining androgenic structures and green plants (9). Use of 2,4-D auxin is described in many reports of success [4, 10, 11]. As growth regulators acting in combination with auxin, 6-benzylaminopurine (6-B) and kinetin are used the most [12-14]. However, in some studies where zeatin was used, fairly high results have been achieved in the frequency of embryoids (53-68%) and green plant formation (20-22 plants/100 anthers) [15, 16].

Zeatin is a cytokinin class phytohormone extracted from unripe corn seeds. In cultural plant media, it is a widely use alternative to kinetin, 6-benzylaminopurine or isopenetyl-adenosine [17]. Zeatin participates in in vitro differentiation of callus tissue and in organogenesis [18-21], successfully used for experimental androgenesis of pepper and eggplant [22, 23].

Despite effective protocols and continuous optimization of nutrient media, culture and pre-treatment conditions and other factors increasing the dihaploid line production, there is still an issue of reproducibility of results, low production of green plants and dependence of the result on the genotype. For large-scale application and production of double haploid, triticale needs optimization of existing tested protocols.
In our experiments involving triticale anther cultures, cytokinin zeatin was for the first time used as exogenic phytohormone of induction medium. Its optimal concentration was established and the process of embryoids and regeneration of green plants was improved.

The purpose of this research was the improvement of efficiency of the triticale anther culture technology and studying the effect of adding cytokinin zeatin to nutrient medium for embryogenesis induction and green plant regeneration.

Techniques. Spring (YaTKh-327-11 and Zernokormovoye 5) and winter (T-968 and T-45) triticale varieties and lines responsive to embryogenesis induction and anther culture regeneration were used in the experiment [24, 25].

Donor plants for haploid technology were grown in 2017 (irrigated field, Kazakh Research Institute of Agriculture and Plant Growing LLP, Republic of Kazakhstan, Almaty Region, Karasarai district). Unripe inflorescences were harvested from donor plants during the phase of flag leaf still in leaf sheath with microspores at medium and late uninuclear development stages. Microspore development stage was assessed according to the generally accepted methodology (light microscopy of temporary squash preparations) [26].

All cut donor plants were cured in the refrigerator at 4 °C for 14 days [27]. Cold-treated spikes were sterilized with 0.1% mercury dichloride for 6 minutes on the shaker, than flushed for three minutes thrice with sterile distilled water.

As a basic medium for embryogenesis induction in studying the effect of zeatin phytohormone, modified mW14 medium [28] was used with addition of 90 g/l of maltose (TM Media, India), 1 000 mg of glutamine-L (AppliChem GmbH, Germany) [12], 2 mg/l of synthetic auxin 2,4-D (Aldrich Chemistry, USA), 50 g/l of ficoll 400 (Sigma Life Science, Sweden). For removal of inhibition of embryogenesis with phenolic compounds egested from obsolescent anthers, nutrient medium was supplemented with ascorbic acid (4 mg/l) (24). Experiment variants differed in zeatin (Sigma-Aldrich, India) concentration in nutrient medium (I, II, III, IV, V — 0.2; 0.4; 0.6; 0.8 and 1.0 mg/l, respectively), the control was zeatin-free medium.

Anthers were extracted from spikes under aseptic conditions and placed on plastic Petri dishes with a diameter of 60 mm (100 anthers per dish containing 6 ml of liquid nutrient medium for embryogenesis induction) [29]. In each variant, 500 anthers were used. To prevent contamination, an antibiotic (cefatoxime) in a concentration of 200 mg/l was added to the nutrient medium. Anthers were incubated in the dark at 32 °C for the first 72 hours, whereafter they were moved to the incubator with a temperature of 25-28 °C until new formations appeared.

In the process of extraction and after inoculation to culture medium, the state of microspores was monitored using MT4000 microscope (Meiji Techno, Japan; ×40-×1000 magnification).

Androgenic structures (AS) that reached the size of 2.0-2.5 mm, were re-inoculated on nutrient medium for regeneration on Petri dishes with a diameter of 90 mm (20-30 androgenic structures per dish). Smaller AS were left on the medium for further growth. After every such inoculation, a medium was added 1 ml of similar fresh medium.

Material inoculated on nutrient medium for plant regeneration was incubated at 16-hour photoperiod, 10 000 lux illumination and temperature of 24-26°C. For regeneration, a premix of Murashige and Skoog (MS) nutrient medium components (Sigma Life Science, USA) with addition of 2 mg/l of zeatin (Sigma-Aldrich, India), 30 g/k of sucrose (AppliChem GmbH, Germany) and 6 g/l of agar (B&V srl, Italy) was used. For root formation, a premix of MS nutrient medium...
with addition of 0.5 g/l of casein hydrolysate (Fluka Analytical, USA), 20 g/l of sucrose, 2 mg/l of indolebutyric acid-3 (IBA) (Sigma Life Science, China), 6 g/l of agar was used.

Ploidy of plants obtained was measured on Cy Flow Ploidy Analyser (Sysmex Partek GmbH, Germany). Samples for analysis were prepared using CyStain® UV Precise P kit (Sysmex Partek GmbH, Germany).

Adaptation of regenerant plants to soil was carried out in a climatic chamber KBWF 720 (Binder GmbH, Germany) where the temperature of 23-24 °C, illumination of 8 000-10 000 lux and humidity of 80% were maintained. During the first 2 weeks (adaptation period), the regenerant plants were sprinkled with a phytohormone solution (0.5 mg/l kinetin, 2 mg/l gibberellic acid, 3 mg/l nicotinamide). Regenerant plants of winter lines of triticale were vernalized in a refrigerated chamber for 6 weeks at 3-4 °C and constant illumination.

Statistical processing was carried out in open-source R programming language, R version 3.2.3 (2015-12-10) (Wooden Christmas-Tree) (https://www.r-project.org/alt-home/). Mean (M) and standard deviations (±SD) were calculated. Standard parametric tests were run using integrated libraries and extra suites (dplyr, ggplot, pisch and others): regression analysis, analysis of variance (ANOVA) and pairwise comparison of means by Tukey test.

Results. Microspore development and androgenic structure formation was monitored throughout the period of anthers cultivation. Escape of microspores from the anther sac into liquid nutrient medium happened very fast and made 70-80%. Emergence of the first androgenic structures was registered 18-25 days after commencement of cultivation depending on triticale genotype. The majority of androgenic structures was forming of separate microspores in the process of direct embryogenesis and possessed all structures typical for a normal embryo. In YaTKh-327-11 genotype, AS emergence was registered in the middle of 3rd week of culture in all experiment variants. In three other samples, visible AS started to emerge on the 4th week of cultivation.

1. Embryogenesis and plant regeneration in spring and winter lines of triticale (× Triticosecale Wittmack) with various zetazin concentrations in the induction medium of (M±SD)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group</th>
<th>control</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A n d r e n i c s t r u c t u r e s p e r 100 a n t h e r s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YaTKh-327-11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zernokormovoye 5 (variety)</td>
<td></td>
<td>74.0±15.2</td>
<td>143.0±8.4</td>
<td>160.0±15.8</td>
<td>122.0±41.4</td>
<td>126.0±25.1</td>
<td>76.0±35.0</td>
</tr>
<tr>
<td>Winter lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-968</td>
<td></td>
<td>64.0±16.7</td>
<td>93.0±8.3</td>
<td>98.0±11.4</td>
<td>98.0±19.2</td>
<td>100.0±11.4</td>
<td>110.0±29.1</td>
</tr>
<tr>
<td>T-45</td>
<td></td>
<td>83.0±12.0</td>
<td>108.0±14.8</td>
<td>130.0±15.8</td>
<td>110.0±23.8</td>
<td>110.0±16.4</td>
<td>84.0±35.1</td>
</tr>
<tr>
<td>M±SD</td>
<td></td>
<td>67.8±12.2</td>
<td>99.8±31.5</td>
<td>112.0±37.6</td>
<td>96.5±24.8</td>
<td>97.5±26.7</td>
<td>79.0±22.8</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Spring lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YaTKh-327-11</td>
<td></td>
<td>1.4±1.4</td>
<td>2.2±0.8</td>
<td>2.6±1.9</td>
<td>3.6±2.1</td>
<td>2.4±1.3</td>
<td>2.8±1.1</td>
</tr>
<tr>
<td>Zernokormovoye 5 (variety)</td>
<td></td>
<td>2.2±0.8</td>
<td>4.4±1.5</td>
<td>7.0±1.6</td>
<td>7.8±2.2</td>
<td>7.4±1.5</td>
<td>8.6±2.9</td>
</tr>
<tr>
<td>Winter lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-968</td>
<td></td>
<td>2.2±0.8</td>
<td>4.0±0.7</td>
<td>5.4±1.1</td>
<td>7.0±1.0</td>
<td>4.4±7.7</td>
<td>5.8±2.1</td>
</tr>
<tr>
<td>T-45</td>
<td></td>
<td>2.6±1.1</td>
<td>4.2±1.4</td>
<td>6.4±2.1</td>
<td>6.8±0.4</td>
<td>5.0±2.3</td>
<td>5.8±2.1</td>
</tr>
<tr>
<td>M±SD</td>
<td></td>
<td>2.1±0.4</td>
<td>3.7±0.9</td>
<td>5.4±1.7</td>
<td>6.3±1.6</td>
<td>4.8±1.7</td>
<td>5.7±2.1</td>
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<td></td>
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<td></td>
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<tr>
<td>Spring lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YaTKh-327-11</td>
<td></td>
<td>33.4±13.0</td>
<td>50.2±7.5</td>
<td>25.8±14.1</td>
<td>18.6±6.1</td>
<td>33.4±4.7</td>
<td>28.±8.3</td>
</tr>
<tr>
<td>Zernokormovoye 5 (variety)</td>
<td></td>
<td>34.6±7.5</td>
<td>26.8±2.9</td>
<td>23.6±4.0</td>
<td>28.2±4.9</td>
<td>24.2±3.7</td>
<td>21.0±3.8</td>
</tr>
<tr>
<td>Winter lines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-968</td>
<td></td>
<td>34.2±4.0</td>
<td>26.7±2.9</td>
<td>22.3±4.0</td>
<td>29.0±6.5</td>
<td>23.8±7.4</td>
<td>20.4±5.0</td>
</tr>
<tr>
<td>T-45</td>
<td></td>
<td>31.6±7.7</td>
<td>26.9±2.9</td>
<td>32.2±13.9</td>
<td>26.0±8.1</td>
<td>21.0±5.6</td>
<td>19.4±4.8</td>
</tr>
<tr>
<td>M±SD</td>
<td></td>
<td>33.4±11.2</td>
<td>32.6±10.1</td>
<td>25.9±3.8</td>
<td>25.4±4.1</td>
<td>25.6±2.7</td>
<td>22.4±1.7</td>
</tr>
</tbody>
</table>

Note. For groups, see Techniques section.
In control group, the average of 67.8 AS per 100 anthers formed in all samples, in experimental group it was 96.5-112 AS per 100 anthers (Table 1). The largest formation of androgenic structures was registered in the variant II of experiment (zeatin concentration of 0.4 mg/l), where the average value was 112 AS per 100 anthers. On some Petri dishes, up to 300 AS has formed. The results substantially exceeded the values obtained in anther culture by other authors: over 50 embryo-like structures per 100 anthers on CHB-3 and NPB99 media [30], 5.8-20.7 embryo-like structures per 100 anthers on 190-PAA and 190-D/K media [13], and 47.2-55.5 embryo-like structures per 100 anthers on mW14 medium [4]. It should be noted, however, that in some studies where the isolated microspore cultures were used, a fairly high degree of formation of androgenic structures, up to 500 embryo-like structures per 100 anthers, was observed in responsive model genotypes [4, 7].

To determine the dependence of embryogenesis on genotype and zeatin phytohormone, the analysis of variance was conducted (Table 2). Zeatin and genotype turned out to be statistically significant factors in formation of androgenic structures and regeneration.

### 2. Statistical analysis (ANOVA) of effect of genotype and zeatin in the induction medium on embryogenesis and plant regeneration in spring and winter triticale (× Triticosecale Wittmack) lines

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Androgenic structure formation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>3</td>
<td>22328.0</td>
<td>&lt; 2.2e−16**</td>
<td></td>
</tr>
<tr>
<td>Zeatin</td>
<td>5</td>
<td>4885.7</td>
<td>6.76e−07**</td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>111</td>
<td>570.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Green plant regeneration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>3</td>
<td>73.8</td>
<td>4.87e−13**</td>
<td></td>
</tr>
<tr>
<td>Zeatin</td>
<td>5</td>
<td>47.4</td>
<td>1.70e−12**</td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>111</td>
<td>2.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Albino plant regeneration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>3</td>
<td>219.1</td>
<td>0.027067*</td>
<td></td>
</tr>
<tr>
<td>Zeatin</td>
<td>5</td>
<td>387.5</td>
<td>0.000119**</td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>111</td>
<td>69.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Note. df — number of degrees of freedom, MS — mean square, F-value — F-test statistics, P-value — significance.

* and ** Effect is statistically significant at p ≤ 0.01 and p ≤ 0.0001.

### 3. Regression analysis of effect of genotype and zeatin in the induction medium on embryogenesis and plant regeneration in spring and winter triticale (× Triticosecale Wittmack) lines

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Zeatin concentration, mg/l</th>
<th>green plant regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 (Intercept)</td>
<td>29.4***</td>
<td>3.6***</td>
</tr>
<tr>
<td>0.2</td>
<td>32.0***</td>
<td>1.6***</td>
</tr>
<tr>
<td>0.4</td>
<td>43.3***</td>
<td>3.2***</td>
</tr>
<tr>
<td>0.6</td>
<td>28.3***</td>
<td>4.2***</td>
</tr>
<tr>
<td>0.8</td>
<td>29.5***</td>
<td>2.7***</td>
</tr>
<tr>
<td>1.0</td>
<td>11.2</td>
<td>3.7***</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zernokormovoye 5 (variety) (Intercept)</td>
<td>29.4***</td>
<td>3.6***</td>
</tr>
<tr>
<td>YaTKKh-327-11</td>
<td>63.3***</td>
<td>−3.7***</td>
</tr>
<tr>
<td>T-968</td>
<td>39.8***</td>
<td>−1.4***</td>
</tr>
<tr>
<td>T-45</td>
<td>50.0***</td>
<td>−1.1*</td>
</tr>
</tbody>
</table>

* and ** and *** Factor value is statistically significant at p ≤ 0.01; p ≤ 0.001 and p ≤ 0.0001.

After determining a substantial difference between the groups as a whole, we have applied regression analysis for determining a quantitative correlation between the indicators and factors (zeatin, genotype). For formation of androgenic structures, the highest β-regression factor (43.3) was detected in the variant II where zeatin concentration was 0.4 mg/l (Table 3). Genotypes
demonstrated distinct response to androgenic technology (see Table 3): highest β-regression factors were typical for YaTKh-327-11 and T-45, 63.3 and 50.0, respectively.

Tukey test for pairwise comparison of the average count of formed androgenic structures at different concentrations of zeatin and in control variant demonstrated statistically significant difference ($p_{adj} < 0.01$) for concentrations of 0.2-0.8 mg/l. The largest difference between group means was observed between control group and variant II, 43.2 (Tukey test results). In general, the results obtained correlate with the visual assessment of differences presented in the box plot (Fig. 1).

Androgenic structures that reached the size of 2.0–2.5 mm were reinoculated on nutrient medium for regeneration (Fig. 2, D). Plant regeneration took place for 3-14 days after passage. In YaTKh-327-11 and T-968 genotypes, plant regeneration was partially observed on embryogenesis induction medium in the dark. From among the androgenic structures inoculated in the control variant, the average of 2.1 green plants per 100 anthers regenerated for different genotypes. The largest frequency of regeneration of green plants was registered in the variant III. Maximum green plant regeneration frequency was registered for Zernokormovoye 5 genotype (8.6/100 anthers) (see Table 1). The results of our research correlate with the data obtained by Eudes et al. [30] and Tuvesson [31] (6 or more green plants per 100 anthers). Higher values (10.8–16.8 green plants per 100 anthers) were obtained by Hungarian scientists [4, 13].

The regression analysis has shown positive impact of increase in zeatin concentration on green plant regeneration (see Table 3). The highest regression factor ($\beta = 4.2$) was obtained in variant III (see Table 3). Presence of statistically significant difference ($p_{adj} < 0.05$) between the experimental and control groups was identified in all zeatin concentrations. The largest difference (4.2) was established between control group and experience variant III.

Regeneration of albino (chlorophyll-free) seedlings in control variant made 33.4/100 anthers on the average for the lines. As zeatin concentration increased, the number of albino seedlings decreased, and their least number was registered in experiment version V (22.4/100 anthers).

Haploid lines may be obtained from isolated anthers through direct regeneration of somatic embryos (embryoidogenesis) and through callusogenesis. Effectiveness of embryoid formation depends on the genotype and may vary considerably from 0 to 95% [32]. Only few researchers pay attention to differentiating the formed androgenic structures as calluses and embryoids [30].

**Fig. 1.** Formation of androgenic structures in spring and winter triticale (× *Triticoscale* Wittmack) lines at different concentrations of zeatin in the induction medium and depending on genotype: — Zernokormovoye 5 (variety), — T-45, — T-968, — YaTKh-327-11.
Upon re-inoculation on agarized medium for regeneration, all androgenic structures in our experiments were differentiated as embryoid (Fig. 3) with bipolar structure and calluses. From the control zeatin-free medium, 949 AS were re-inoculated, 57.2% of which were embryoids (Table 4). In all variants where cytokinin was present in the medium, higher percentage of embryoid formation (60.8-71.0%) was registered, except for variant I (45.0%). Maximum percentage of embryoids has formed in variant III (71.0%). Embryoid regeneration of plants occurred in 92% of cases, resulting in formation of sprouts and roots.
4. Formation of embryoids, green plants and dihaploid lines in spring and winter triticale (\textit{x} Triticosecale Wittmack) depending on zeatin concentration in the induction medium

<table>
<thead>
<tr>
<th>Variant</th>
<th>Reinoculated androgenic structures</th>
<th>Embryoids, total (%)</th>
<th>Calluses, total (%)</th>
<th>Green plants</th>
<th>Plants, total (%)</th>
<th>Spontaneously doubled dihaploid lines, total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>949</td>
<td>543 (57.2)</td>
<td>406 (42.8)</td>
<td>45</td>
<td>36 (80.0)</td>
<td>29 (80.5)</td>
</tr>
<tr>
<td>I</td>
<td>1397</td>
<td>630 (45.0)*</td>
<td>767 (54.0)</td>
<td>79*</td>
<td>48 (60.7)</td>
<td>42 (87.5)</td>
</tr>
<tr>
<td>II</td>
<td>1554</td>
<td>1040 (66.9)**</td>
<td>514 (33.1)</td>
<td>110**</td>
<td>87 (79.1)</td>
<td>68 (78.2)</td>
</tr>
<tr>
<td>III</td>
<td>1366</td>
<td>970 (71.0)**</td>
<td>396 (29.0)</td>
<td>114**</td>
<td>95 (83.3)</td>
<td>86 (90.5)</td>
</tr>
<tr>
<td>IV</td>
<td>1344</td>
<td>860 (64.0)**</td>
<td>484 (36.0)</td>
<td>93**</td>
<td>68 (73.1)</td>
<td>60 (88.2)</td>
</tr>
<tr>
<td>V</td>
<td>1185</td>
<td>720 (60.8)**</td>
<td>465 (39.2)</td>
<td>117**</td>
<td>89 (76.1)</td>
<td>81 (91.0)</td>
</tr>
<tr>
<td>Bcero</td>
<td>7795</td>
<td>4793 (61.0)</td>
<td>3002 (39.0)</td>
<td>558</td>
<td>423 (75.8)</td>
<td>366 (86.5)</td>
</tr>
</tbody>
</table>

N o t e. For variant description, see Techniques section.

* and ** Effect is statistically significant at p \leq 0.001 and p \leq 0.0001 (ANOVA); \text{-} impact is statistically insignificant.

For adaptation to the ground, green plants that had well-formed root system and leaves were selected. In 24% of plants, lack of roots, poor development, leaf curl due to insufficient formation of mechanical tissue were found. During the first 2 weeks of transplanting (adaptation period), regenerant plants were sprinkled with phytohormone solution and irrigated with water solution of macro- and microelement salts, MS iron chelate. Soil acclimatization was carried out in the climatic chamber at high humidity (80%). Adaptation to ground was passed by 366 plants, which made 86.5% (see Table 4).

The final result of the described triticale anther culture technology is the obtention of dihaploid plants. According to the tested protocols, the microspores are exposed to high and low temperatures promoting spontaneous doubling of chromosomes during the early stages of cultivation. Spontaneous doubling allows us to omit the colchicination process which the regenerant plants withstand poorly. In our experiment, spontaneous doubling was registered in 26.5% of 366 regenerant plants. This is the average for triticale which correlates with the data of other researchers [4, 7, 9], in some experiments it reached 57% [33]. Haploid plants in which spontaneous dihaploidization did not occur were subjected to colchicination during the tillering stage after sufficient rooting and development. All dihaploid plants obtained were raised to seeds under greenhouse conditions (see Fig. 2, H, I).

Thus, adding zeatin phytohormone in concentrations of 0.2–0.8 mg/l to the liquid nutrient medium MW14 for embryogenesis induction in spring and summer triticale results in 42.3–65.2% higher formation of androgenic structures (AS). The strongest effect on emergence of androgenic structures was achieved by adding 0.4 mg/l of zeatin (average formation of 112 AS per 100 anthers). In nutrient medium where zeatin concentration was 0.4–0.6 mg/l, higher frequency, as compared to control group, of formation of embryoids (by 16.9–24.1%) with
bipolar structure and producing the sprout and roots during organogenesis was established. That is, zeatin in the induction medium improves the differentiation of fissile microspore cells and organogenesis and promotes formation of embryos. We have also registered significant increase in formation of green plants in all experiment variants (3.7-6.3/100 anthers) against control (2.1/100 anthers). The highest green plant regeneration frequency (6.3/100 anthers) was observed in embryos replanted from nutrient medium that contained zeatin in 0.6 mg/l concentration. Effectiveness of spontaneous dihaploidization in triticale was 26.5%, which enabled us to obtain 91 dihaploid triticale lines without injurious colchicination process.

REFERENCES


GROWTH OF BUCKWHEAT (Fagopyrum esculentum Moench) SEEDLINGS AND THE ACCUMULATION OF PRIMARY AND SECONDARY METABOLITES UNDER VARIOUS MINERAL NUTRITION CONDITIONS

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A b s t r a c t

Buckwheat (Fagopyrum esculentum Moench) is an important agricultural crop; Russia, China and Ukraine are the world leaders of its production. In addition to the unique nutritional characteristics, it is characterized by the formation of various phenolic compounds including rutin widely used in medicine. The study of the various metabolites formation at the initial growth stages as well as those under the different conditions of mineral nutrition is important for estimation of plant potential productivity and adaptation to environmental conditions. In this paper, we showed the regulatory effect of macro- and microelements on the growth and accumulation of primary and secondary metabolites in buckwheat plants. For the first time, the formation of primary and secondary metabolites in the aerial parts of a new and promising Russian buckwheat variety Dasha (approved by the State Register of the Russian Federation in 2018) has been characterized. The aim of this work was to study the initial stages of F. esculentum ontogenesis, including the assessment of the morphophysiological characteristics of seedlings under various mineral nutrition conditions, as well as the accumulation of photosynthetic pigments, sugars, and phenolic compounds in cotyledon leaves. Studies were conducted using two varieties of this culture included in the State Register of the Russian Federation in 2004 and 2018 (Devyatka and Dasha, respectively). Plant cultivation was carried out by a roll method in water (control) and Hoagland-Arnon nutrient medium (sample) at 24 °C and 16-hour illumination in laboratory conditions. In the seedlings, the height of the hypocotyls, the length of the roots, and the mass of cotyledon leaves was determined. The water content of the plant material was analyzed after it was dried to constant weight at 70 °C. The spectrophotometric method was used to determine the amount of chlorophyll a and b (λ = 665 nm and λ = 649 nm), carotenoids (λ = 440 nm), sugars (λ = 490 nm), the total amount of soluble phenolic compounds (λ = 725 nm), flavonoids (λ = 415 nm) and phenylpropanoids (λ = 330 nm) in ethanol extracts from cotyledon leaves of seedlings of different ages. The cultivation of buckwheat on Hoagland-Arnon nutrient medium contributed to faster growth of aboveground organs compared to control; in contrast, the growth of underground organs was the same in both cases. In most cases, in the experimental samples, the differences in the accumulation of photosynthetic pigments (chlorophyll a and b, carotenoids) and soluble sugars in the cotyledons of two buckwheat varieties were revealed to be higher than in control. As for the accumulation of phenolic compounds, it was not obviously dependent on the level of mineral nutrition. As an exception, in cotyledons of seedlings cultivated on a nutrient medium, the content of phenylpropanoids changed to a greater extent compared to control and reached high values at the end of the investigation period. It should also be noted that on a nutrient medium at the late ontogeny stages cotyledons of Dasha seedlings significantly accumulate pigments, sugars and phenolic compounds in comparison with Devyatka. Thus, the obtained data indicate that the amount of mineral elements is important for the initial stages of F. esculentum ontogenesis. Faster growth of seedlings and the accumulation of primary and secondary metabolites in their leaves is characteristic of the experimental samples, compared to the control. Therefore, in plant cultivation, the different levels of mineral nutrition make it possible to regulate the plant growth and development, as well as the accumulation of various metabolites.
Forming and development of seedlings is an important stage of ontogenetic development of plants that depends on endogenous stockpile of seed metabolites and their conversions, as well as impact of exogenous environmental factors, including soil humidity, temperature, light, mineral nutrition [1, 2]. For this period, transition from heterotrophic to autotrophic nutrition type, changes in structural arrangement of cells and tissues, energy processes (respiration, photosynthesis), state of hormonal, antioxidative and other systems are typical [3-5].

Some of important regulators of plant viability are phenolic compounds, one of the most widespread secondary metabolites present in all cells and tissues [6, 7]. Their content depends on plant species, ontogenesis phase, conditions of growing and mineral nutrition [8-10]. Functionality of phenolic compounds is extremely diverse and connected with the processes of photosynthesis, respiration, growth and development of plants, as well as stress-resistance [6, 11, 12].

Buckwheat (*Fagopyrum esculentum* Moench) is an important agricultural crop; it is cultivated in many countries of the world and is successfully used in various industries. Buckwheat is characterized by considerable accumulation of phenolic compounds, including rutin, a substance with high capillary-restorative effect [13, 14]. The largest concentration of these secondary metabolites was found in aboveground plant organs, especially in leaves and flowers [15, 16]. There were reports of formation of phenolic compounds in seedlings, where their amount was less and the composition was less diverse as compared to adult plants [17, 18]. Since phenolic compounds possess high biological and antioxidative effects, including as potential functional nutrition components [13], studying their accumulation during the initial plant ontogenesis phases is of practical interest.

In this paper, having compared a number of morphological, physiological and biochemical indicators of two buckwheat varieties (Devyatka and Dasha) under different mineral nutrition, we have discovered the regulatory effect of macro- and microelements on the early ontogenesis processes and accumulation of primary and secondary metabolites in aboveground organs at certain variety specificity of plant responses. For the new promising Dasha variety that had been entered in the State Register of Selection Achievements Authorized for Use in the Russian Federation in 2018, these processes are characterized for the first time.

Our goal was to evaluate the features of initial ontogenesis stages, morphophysiological characteristics and accumulation in cotyledon leaves of photosynthetic pigments, sugars, and phenolic compounds in buckwheat seedlings depending on the provision with mineral nutrition elements.

**Techniques.** Devyatka and Dasha variety buckwheat studied [19, 20] were obtained in Russian National Research Institute of Leguminous Crops and were entered in State Register of Selection Achievements Authorized for Use in the Russian Federation in 2004 and 2018, respectively.

The seedlings were grown by a roll method [10]. The seeds were placed in Petri dishes on a watered filter paper (control) and Hoagland-Arnon nutrient medium (sample) [21]. After curing in the dark for 24 hours, they were moved to the filter paper rolls (15 per roll) that were placed in the plastic tumblers (7 rolls per tumbler) containing water or nutritious medium, and were grown in phytotron chamber of the Institute of Plant Physiology of the Russian Academy of Sciences at 24 °C and 16-hour photoperiod (5 000 lux). The seedlings that were at the same phase of ontogenetic development were taken for study: for those grown on water, it were the 11th, 14th and 18th days of growth, and for the seedlings grown...
on nutrient medium the 6th, 11th and 14th day (respective phases 1st, 2nd and 3rd). The criteria were the form and size of cotyledon leaves that were used for biochemical study.

Morphophysiological parameters of seedlings, i.e. height of aboveground part and root length, as well as cotyledon leaves weight, were assessed. Tissue water content was determined after dehumidification of vegetation material at 70 °C in a thermostat to constant weight [10].

In order to extract the pigments, seedling leaves were homogenized in a 96% ethanol in the dark. Homogenate was centrifuged (CM-50, ELMI Ltd., Latvia) for 5 minutes at 13 000 rpm. Spectrophotometric method (SF-46, LOMO, Russia) was used to define a and b chlorophylls ($\lambda = 665$ nm and $\lambda = 649$ nm, respectively) and carotenoids ($\lambda = 440$ nm) in the supernatant liquid. They were measured by standard method [22].

Sugars were extracted by 96% ethanol extraction [23]. In the supernatant liquid resulting from centrifuging homogenate (2 minutes, 16 000 rpm), total sugar content was defined by spectrophotometric method through reaction with phenol and sulfuric acid (absorption at $\lambda = 490$ nm) [24]. Sucrose was used to construct calibration curve.

In order to extract phenolic compounds, vegetation material was homogenized in 96% ethanol and cured at 45 °C for 45 minutes [10, 24]. Homogenate was then centrifuged (2 minutes, 16 000 rpm). Supernatant liquid was used to identify different classes of phenolic compounds by spectrophotometric method.

Analysis of variance (ANOVA) was carried out with SigmaPlot 12.3 (http://www.sigmaplot.co.uk) and Microsoft Excel software. Tables and charts contain arithmetic means ($M$) and standard errors of mean (±SEM). Superscripts represent the significance of differences of mean values determined by Tukey test at $p \leq 0.05$.

**Results.** An important indicators of plant growth and development are their morphophysiological properties that depend on the ontogenesis phase, species and variety peculiarities and impact of external factors, including mineral nutrition [2, 4, 8].

Devyatka and Dasha buckwheat varieties are mid-season, high-yield and lodge-resistant [25, 26]. During the breeding of Dasha variety, the selection criteria were high grain content and photosynthetic activity [20]. This variety is characterized by a more pronounced resistance to drought, Ascochyta stem blight and mildew as compared to Devyatka variety [26]. Hence it can be assumed that there are certain differences in morphophysiological characteristics of the said varieties, including at the early phases of ontogenesis, starting from forming and development of cotyledon leaves.

When grown in water culture (control), root length of seedlings of both varieties was almost the same and increased throughout the period of study (Table 1). By phase 3, it increased by 57–60% vs. phase 1. It indicates the considerable similarity in the initial stages of growth of underground organs of the seedling of both buckwheat varieties [1].

Forming and development of aboveground organs is supported by both endogenous stockpile of metabolites and forming of new metabolites through
photosynthesis [2, 27]. In our experiments, the height of hypocotyls in the Devyatka seedlings throughout all ontogenesis phases significantly exceeded the same in Dasha variety (by 16% on the average, \( p \leq 0.05 \)). In both varieties, their increase was observed on transition to the 2\(^{nd}\) phase (by 40% vs. 1\(^{st}\) phase), whereafter the hypocotyl height did not change.

The data obtained correlates with hypocotyl weight which in Devyatka variety seedlings was significantly (\( p \leq 0.05 \)) higher than in Dasha variety. At the same time, in Devyatka variety it remained unchanged during the first two phases of ontogenesis, and by the 3\(^{rd}\) phase it increased by 19% (\( p \leq 0.05 \)). In Dasha seedlings, hypocotyl weight did not change throughout the growth period. These results are the evidence of the faster growth and gaining of biomass in overground organs of traditional variety (Devyatka) seedlings as compared to the next-generation variety (Dasha).

Forming and development of leaves enables the plants to switch to autotrophic nutrition type [1, 28]. At all ontogenesis stages, the weight of cotyledon leaves of seedlings of both varieties was small and virtually the same. The only exceptions were the Devyatka seedlings in which at the 3\(^{rd}\) phase the cotyledon leaf weight was 33% higher (\( p \leq 0.05 \)) (see Table 1).

### 1. Age-dependent morphophysiological characterization of seedlings of two buckwheat (Fagopyrum esculentum Moench) varieties under different growth conditions \((M \pm SEM, \text{lab experiment})\)

<table>
<thead>
<tr>
<th>Ontogenetic phase</th>
<th>Root length, cm</th>
<th>Hypocotyl height, cm</th>
<th>Cotyledon leaf weight, g</th>
<th>Cotyledon leaf water content, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control (water)</strong></td>
<td>Devyatka variety</td>
<td>Dasha variety</td>
<td>Hoagland-Arnon nutrient medium</td>
<td>Hoagland-Arnon nutrient medium (test)</td>
</tr>
<tr>
<td>1(^{st})</td>
<td>7.08±0.30(^{bc})</td>
<td>9.85±0.41(^{cd})</td>
<td>0.13±0.02(^{b})</td>
<td>89.53±1.62(^{a})</td>
</tr>
<tr>
<td>2(^{nd})</td>
<td>11.67±0.44(^{b})</td>
<td>14.07±0.31(^{b})</td>
<td>0.14±0.02(^{b})</td>
<td>91.07±0.37(^{a})</td>
</tr>
<tr>
<td>3(^{rd})</td>
<td>12.04±0.80(^{a})</td>
<td>14.32±1.12(^{a})</td>
<td>0.16±0.02(^{c})</td>
<td>91.05±0.16(^{a})</td>
</tr>
<tr>
<td>Hoagland-Arnon nutrient medium</td>
<td>Devyatka variety</td>
<td>Dasha variety</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1(^{st})</td>
<td>7.42±0.32(^{c})</td>
<td>8.27±0.46(^{c})</td>
<td>0.11±0.01(^{c})</td>
<td>88.77±0.69(^{a})</td>
</tr>
<tr>
<td>2(^{nd})</td>
<td>11.85±0.44(^{b})</td>
<td>11.74±0.31(^{b})</td>
<td>0.11±0.01(^{b})</td>
<td>91.24±0.17(^{a})</td>
</tr>
<tr>
<td>3(^{rd})</td>
<td>13.00±1.00(^{a})</td>
<td>11.97±1.07(^{a})</td>
<td>0.12±0.01(^{a})</td>
<td>91.43±1.45(^{a})</td>
</tr>
</tbody>
</table>

**Note.** Statistically significant differences of mean values at \( p \leq 0.05 \) are marked with different Latin characters.

On Hoagland-Arnon nutrient medium (experiment), i.e. under the conditions of provision with macro- and microelements, development of seedlings speeded up against control group. In morphophysiological characteristics, 6-day old seedlings in test group corresponded to 11-day old seedlings in control group, 11-day olds corresponded to 14-day olds, 14-day olds corresponded to 18- day olds.

Root length of the seedlings of the two varieties did not differ at any stages of study. However, during the 2\(^{nd}\) and 3\(^{rd}\) phases, it was lesser than in control group (see Table 1). Root growth throughout the period made 30%, i.e. availability of macro- and microelements in the environment slowed down the development of underground organs.

Hypocotyl height in Devyatka variety seedlings during the 1\(^{st}\) and 2\(^{nd}\) phases of ontogenesis was the same in test and control variants, and during the 3\(^{rd}\) phase it was larger in the experimental samples. In Dasha variety, the experimental variant always significantly (\( p \leq 0.05 \)) differed from control group; during the 1\(^{st}\) phase, the values were lower, during the 2\(^{nd}\) and 3\(^{rd}\) phases they were
higher. It should be also noted that hypocotyl height in Devyatka variety seedlings was 50% smaller than in Dasha seedlings during the 1\textsuperscript{st} phase, and later it became almost equal. Its total increase throughout the study period made 50% in Devyatka variety and 72% in Dasha variety. As for the hypocotyl weight, it was almost the same in both varieties, and over the seedling growth period increased by 44%. Its values throughout all phases in the experimental variant exceeded the control.

Measuring the weight of cotyledon leaves of the seedlings of the two buckwheat varieties showed no significant differences between them. In the process of ontogenesis, it increased by 25% in Devyatka variety and by 28% in Dasha variety. In general, almost all indicators of aboveground organs in the experimental variant seedlings, especially at the final stage of study (the 3\textsuperscript{rd} phase), were significantly (p \leq 0.05) higher than those of control group, which evidences the stimulating effect of nutrient solution.

Tissue water content is an important indicator for evaluation of physiological state of plants [1]. Water content in cotyledon leaves of seedlings of the two buckwheat varieties was the same and was increasing during ontogenetic development (see Table 1). The highest values were registered during the final growth phase. Provision of buckwheat seedlings with mineral nutrients did not affect this value.

### 2. Age-dependent pigment content in cotyledon leaves of the seedlings of two buckwheat (Fagopyrum esculentum Moench) varieties under different growth conditions (M±SEM, lab experiment)

<table>
<thead>
<tr>
<th>Ontogenesis phase</th>
<th>Chlorophylls, mg/g dry weight</th>
<th>Chlorophylls, a/b</th>
<th>Carotenoids, mg/g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a + b</td>
</tr>
<tr>
<td>Control (water)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Devyatka variety</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st}</td>
<td>5.29±0.28\textsuperscript{d}</td>
<td>1.24±0.12\textsuperscript{e}</td>
<td>6.53±0.40\textsuperscript{d}</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>6.19±0.05\textsuperscript{e}</td>
<td>1.51±0.04\textsuperscript{d}</td>
<td>7.70±0.09\textsuperscript{c}</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>5.67±0.87\textsuperscript{c}</td>
<td>1.48±0.26\textsuperscript{d}</td>
<td>7.15±1.13\textsuperscript{d}</td>
</tr>
<tr>
<td>Dasha variety</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st}</td>
<td>5.14±0.18\textsuperscript{d}</td>
<td>1.30±0.11\textsuperscript{c}</td>
<td>6.44±0.29\textsuperscript{d}</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>5.25±0.42\textsuperscript{d}</td>
<td>1.38±0.12\textsuperscript{b}</td>
<td>6.63±0.54\textsuperscript{d}</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>5.14±0.68\textsuperscript{d}</td>
<td>1.39±0.04\textsuperscript{d}</td>
<td>6.53±0.72\textsuperscript{d}</td>
</tr>
<tr>
<td>Hoagland-Arnon nutrient medium (test))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Devyatka variety</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st}</td>
<td>2.28±0.02\textsuperscript{f}</td>
<td>3.51±0.05\textsuperscript{f}</td>
<td>5.79±0.07\textsuperscript{f}</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>5.44±0.18\textsuperscript{e}</td>
<td>1.49±0.16\textsuperscript{c}</td>
<td>6.93±0.34\textsuperscript{d}</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>7.25±0.03\textsuperscript{b}</td>
<td>1.90±0.15\textsuperscript{b}</td>
<td>9.15±0.18\textsuperscript{a}</td>
</tr>
<tr>
<td>Dasha variety</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st}</td>
<td>3.19±0.09\textsuperscript{f}</td>
<td>3.78±0.26\textsuperscript{f}</td>
<td>6.97±0.35\textsuperscript{d}</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>6.22±0.53\textsuperscript{c}</td>
<td>1.65±0.12\textsuperscript{c}</td>
<td>7.87±0.65\textsuperscript{c}</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>9.09±0.61\textsuperscript{a}</td>
<td>2.39±0.17\textsuperscript{a}</td>
<td>11.48±0.70\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textbf{Note.} Statistically significant differences of mean values at p \leq 0.05 are marked with different Latin characters. Dashes mean that carotenoids were found starting from the 2\textsuperscript{nd} phase of ontogenesis.

Plant photosynthesis is the main biological process supporting the life of all organisms on the planet [27]. Its effectiveness is evaluated by content of a and b chlorophylls and their ratio in leaves [28]. Under control conditions of our experiments, we have found differences in accumulation of a and b chlorophylls in cotyledon leaves as the seedlings of both buckwheat grew (Table 2). In Devyatka variety, a chlorophyll content during the 1\textsuperscript{st} phase was the least, during the 2\textsuperscript{nd} phase significantly increased by 17% (p \leq 0.05), and by the 3\textsuperscript{rd} phase reduced by 10% but still exceeded that of the 1\textsuperscript{st} phase. The similar, but less pronounce tendency was found in respect of b chlorophyll. In cotyledon leaves of Dasha variety seedlings, content of a and b chlorophylls throughout the study period did not change and was almost the equal to the same in Devyatka variety seedling in the 1\textsuperscript{st} phase.

In assessment of photosynthetic productivity of plant tissues, it is im-
important to take into account the $a$ to $b$ chlorophyll ratio which, in optimal growing conditions, approaches 3 [27, 28]. For cotyledon leaves of seedlings of both buckwheat varieties in the control group, higher $a$ to $b$ chlorophyll ratios were registered, and to the larger extent it was typical for 1st and 2nd phases (see Table 2).

Plant pigment system, in addition to chlorophylls, contains carotenoids which participate in functioning of reaction centers and light-harvesting complexes of chloroplast photosystems, absorb light in blue spectrum, protect photosynthetic apparatus from photodestruction and perform other protective functions [29, 30]. In both buckwheat varieties, similar tendencies in accumulation of carotenoids were observed, i.e. high content during the 1st phase, further increase during the 2nd phase (approximately by 20%) and considerable decrease during the 3rd phase (almost double). Hence the initial stages of forming and development of cotyledon leaves in buckwheat seedlings at low level of mineral nutrition are characterized by considerable accumulation of carotenoids, which may be the evidence of their important role during this period of ontogenetic development [31].

Study of pigment accumulation in cotyledon leaves of buckwheat seedlings grown on nutrient medium has revealed somewhat different tendencies. The content of $a$ and $b$ chlorophylls in them significantly ($p < 0.05$) grew throughout the observation period, which was not typical for control group (see Table 2). During the 1st phase, the amount of $a$ chlorophyll in cotyledon leaves of Devyatka and Dasha seedlings was minimal (2.3 and 1.6 times, respectively, lower than in control group, $p \leq 0.05$). $b$ chlorophyll content during this phase was the largest, and was almost 3 times the control value ($p \leq 0.05$). Further development of cotyledon leaves (2nd and 3rd phases) were accompanied by significant ($p \leq 0.05$) increase of $a$ and $b$ chlorophylls in them, which was to the larger extent manifested in Dasha variety. In its breeding, the selection for photosynthetic productivity of plants was conducted [27], and this feature has manifested even at the earliest stages of their development. It should also be noted that the total content of $a$ and $b$ chlorophylls in cotyledon leaves in experimental variants throughout the study period increased significantly ($p \leq 0.05$), i.e. by 58% in Devyatka variety and by 64% in Dasha variety.

As for $a$ to $b$ chlorophyll ratio in cotyledon leaves, during the 1st phase it was low (0.06 and 0.80 in Devyatka and Dasha varieties, respectively), and during the 2nd and 3rd phases increased considerably and became almost equal for both varieties (3.76 on the average). These values corresponded to control group, i.e. there was a considerable similarity in forming photosynthetic apparatus in buckwheat cotyledon leaves during the later ontogenesis phases which did not depend on mineral nutrition of seedlings (see Table 2).

In the experimental variants, accumulation of carotenoids in cotyledon leaves was almost the same in the seedlings of the both buckwheat varieties (see Table 2). It was registered starting from phase 2, but it was lower than in control group, and by phase 3 it increased almost 2.5 times ($p \leq 0.05$) and considerably exceeded the control.

In general, availability of nutrients promoted the effective formation of chlorophylls and carotenoids, which was the result of rapid growth of plants, development of cotyledon leaves and forming of chloroplasts, the important sources of energy and metabolites [27, 28].

It is well-known that during the initial ontogenesis phases, the plants need considerable energy and metabolites for growing and building up biomass [1, 2]. Soluble sugars become the main transport form for assimilates and may serve as initial substrates for many metabolic processes and formation of structural ele-
ments of cells and tissues, which is necessary for seedling development [32].

Accumulation of soluble sugars in cotyledon leaves in control groups was almost the same in the seedlings of the two buckwheat varieties over three phases of ontogenesis (Fig. 1, A). For the 1st phase, the amount of sugars was high, for the 2nd phase it significantly decreased (by 51% at p ≤ 0.05), during the 3rd phase it increased (p ≤ 0.05) and reached the initial values. The differences were caused by provision with initial substrates for formation of soluble sugars, namely spare substances in buckwheat seeds (1st phase), their subsequent depletion and active growth of seedlings (2nd phase) and finally by photosynthesis (3rd phase) resulting in increase in metabolite concentration and accumulation of soluble sugars [23, 32].

For accumulation of soluble sugars in cotyledon leaves of seedlings grown on nutrient medium, another tendency was observed (see Fig. 1, A). In Devyatka variety, in all stages of the study the content of soluble sugars was the same and sufficiently close to the control group during 1st and 3rd phases. In Dasha variety, it was equal to that in Devyatka variety during the 1st phase, decreased by 25% (p ≤ 0.05) during the 2nd phase, and increased by 47% (p ≤ 0.05) during the 3rd phase. Such pattern is only typical for Dasha variety which had been created by breeders through selection for photosynthetic activity [20, 26].

Phenolic compounds are ones of the most important plant metabolites, whose roles, just as chemical structure, are extremely diverse [6, 7]. When growing under control conditions, during the 1st phase the amount of phenolic compounds in Devyatka variety cotyledon leaves exceeded that of Dasha variety by 13% (see Fig. 1, B). During the 2nd and 3rd phases, the accumulation of phenolic compounds significantly (p ≤ 0.05) increased in Devyatka and Dasha varieties by 22% and 28%, respectively, and became the same. To a certain extent it could be the cause of equal photosynthetic activity of cotyledon leaves during the said ontogenesis period, which is evidenced by content of photosynthetic pigments in them (see Table 2). It is known that chloroplasts are one of the main spots of biosynthesis of phenolic compounds in green plant cells [33].

When grown on nutrient medium, total content of phenolic compounds in cotyledon leaves of the seedlings of both buckwheat varieties in the majority of cases was significantly (p ≤ 0.05) lower than in control group (see Fig. 1, B). In Devyatka variety, the least value was registered during the 1st phase, by the 2nd phase it increased by 32% (p ≤ 0.05) and remained at that level until the 3rd

![Fig. 1. Content of sugars (A) and phenolic compounds (B) in cotyledon leaves of buckwheat (Fagopyrum esculentum Moench) Devyatka (white bars) and Dasha (gray bars) variety seedlings grown on water (left) and Hoagland-Arnon nutrient medium (right) during ontogenesis. Significant differences of mean values at p ≤ 0.05 are denoted by different Latin characters above the bars.](image_url)
phase, just as in control group. In Dasha variety, the amount of phenolic compounds during the 1st phase also was the least and did not differ from that of Devyatka variety. By the 2nd phase it significantly increased by 15% (p ≤ 0.05), and by the 3rd phase it doubled and reached its maximum value.

As it was mentioned before, phenolic compounds in the plants are extremely diverse in their structure and are represented by different classes [7]. The simplest of them are phenylpropanoids [6]. In cotyledon leaves of buckwheat seedlings grown under control conditions, the content of phenylpropanoids during the 1st phase was the least, which to the large extent was manifested in Dasha variety (Fig. 2, A). By the 2nd phase, it became significantly (p ≤ 0.05) larger (in Devyatka and Dasha varieties by 50% and 60%, respectively), and in future (3rd phase) decreased in Devyatka variety by 14% (p ≤ 0.05) and remained unchanged in Dasha variety. The result was the same content of phenylpropanoids in cotyledon leaves of the seedlings of both buckwheat varieties by the end of study period.

With sufficient mineral nutrition, accumulation of phenylpropanoids in cotyledon leaves during the 1st phase and especially during the 2nd phase was lower as compared to control, and during the 3rd phase exceeded it (see Fig. 2, A). During the 1st phase, this value was the least, which to the larger extent manifested in Devyatka variety. By the 2nd phase, it significantly (p ≤ 0.05) increased (in Devyatka and Dasha varieties by 53% and 22%, respectively). The largest changes in phenylpropanoid content were registered during the 3rd phase when their values increased sharp (by 50% on the average, p ≤ 0.05) and became sufficiently close to those of the control group.

It is known that it is typical for the buckwheat to form flavonoids, the most widespread phenolic compounds in overground organs of the plants [6, 16]. When grown on water, the flavonoid content in cotyledon leaves of Devyatka variety was almost twice that value in Dasha variety during the 1st phase of ontogenesis. During the 2nd phase it did not change, and during the 3rd phase it decreased by 22% (p ≤ 0.05). Another trend was observed in Dasha variety: flavonoid content increased by the 2nd phase by 41% (p ≤ 0.05) and did not change further.

When grown on nutrient medium, flavonoid content in buckwheat seedling cotyledon leaves differed from control values in both varieties (see Fig. 2, B). In Devyatka variety it increased by 25% (p ≤ 0.05) during the 1st and 2nd phases whereafter it remained the same, while in Dasha variety the increase was observed throughout the study period (by 32% for the 1st and the 2nd phase, and by 43% for the 3rd phase; p ≤ 0.05).

Fig. 2. Phenylpropanoid (A) and flavonoid (B) content in cotyledon leaves of buckwheat (*Fagopyrum esculentum* Moench) Devyatka (white bars) and Dasha (gray bars) variety seedlings grown on water (left) and Hoagland-Arnon nutrient medium (right) during ontogenesis. Statistically significant differences of mean values at p ≤ 0.05 are denoted by different Latin characters above the bars.
All of the above demonstrates that the mineral nutrition conditions considerably affect the initial ontogenesis phases of buckwheat plant. When grown on Hoagland-Arnon nutrient medium, the rate of growth of overground organs and accumulation of pigments ($a$ and $b$ chlorophylls and carotenoids) in cotyledon leaves was higher as compared to control group, being to the larger extent manifested by the end of the study period (3rd ontogenesis phase). There are reports on the positive effects of mineral nutrition on building up of biomass of plants and content of different forms of chlorophyll in plant leaves [22, 34, 35]. As for $a$ to $b$ chlorophylls ratio, this indicator did not depend on the mineral nutrition conditions.

Sugars are the intermediate products of photosynthesis [28]. Positive correlation was fairly often registered between their accumulation and chlorophyll content [28, 32]. However in case of seedlings of two buckwheat varieties, no clear tendency was observed. Total content of phenolic compounds in cotyledon leaves of control group was higher than in the test group. Thus, under the conditions of better provision of buckwheat seedlings with mineral elements, the amount of secondary phenolic metabolites in the aboveground organs decrease, which may the result of their intensive growth. There were reports of decrease in accumulation of polyphenols as the plant growth activated [6, 9]. The content of their certain classes (phenylpropanoids and flavonoids) in cotyledon leaves of Dasha seedlings grown on nutrient medium normally increased, while in Devyatka seedlings it decreased, except for the 3rd growth phase, when an opposite tendency occurred. Changes in biosynthesis of certain classes of phenolic compounds in buckwheat seedlings under different mineral nutrition conditions require further research.

Thus, the initial stages of ontogenetic development of the two buckwheat varieties seedlings are defined by the stockpile of nutrients in the seeds and availability of micro- and macroelements. Introduction of the latter speeds up the growth of overground organs (by 20-30% on the average), development of cotyledon leaves (by 25% for Devyatka variety and by 42% for Dasha variety) and enhances photosynthetic activity as compared to the similar indicators of seedlings grown on water instead of nutrient medium. This affects the pigment content, accumulation of primary (sugars) and secondary (various classes of phenolic compounds) metabolites. Therefore, changes in provision with mineral nutrition elements during plant cultivation enable the regulation of their growth and development and metabolite accumulation.

**REFERENCES**

15. Vysochina G.I. Feno'nye soedineniya v sistematike i filogenii semeistva grechishnykh [Phenolic compounds in the taxonomy and phylogeny of the buckwheat family]. Novosibirsk, 2004 (in Russ.).
SCREENING OF RUSSIAN POTATO CULTIVARS (Solanum tuberosum L.) WITH DNA MARKERS LINKED TO THE R-GENES CONFERRING EXTREME RESISTANCE TO POTATO VIRUS Y

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Abstract

Common potato Solanum tuberosum L. is infected by about 40 viruses, of which one of the most harmful is the potato virus Y (PVY). Crop losses of PVY susceptible cultivars can reach 80 %. At present, marker-assisted selection (MAS) using DNA markers linked to the Ry genes is widely used to create varieties highly resistant to PVY. MAS increases breeding efficiency and also allows one to assess the genetic protection or genetic vulnerability of the varieties’ gene pool. A number of genes conferring different types of PVY resistance have been identified in potato, of which Ry genes, conferring extreme resistance (the absence of accumulation of viruses in infected plants regardless of the virus strain), are most often involved in breeding programs in different countries. The search for effective markers of target genes remains relevant due to MAS prospects. This paper is the first report on the use of STM0003 marker associated with Rysto gene for screening Russian potato varieties; another marker, Ry364, was previously used once on a small sample of Russian varieties. The objective of this work was to screen 178 domestic varieties with three markers, STM0003, Ry364, and RYSC3 linked to Rysto, Rychc, and Ryadg genes which were introgressed into the breeding gene pool from wild species S. stoloniferum, S. chacoense, and from the Andean native varieties of S. tuberosum ssp. andigenum, respectively. As a result of the molecular screening, 39 (21.8 %) of 178 varieties were selected for which diagnostic fragments of at least one of the three markers were revealed, including 7.3 % varieties with STM0003 marker (Rysto), 11.7 % varieties with Ry364 (Rychc), and 4.5 % varieties with RYSC3 (Ryadg) marker. The obtained results indicate a low level of genetic defense against PVY of the analyzed cultivars’ subset among which 78.2 % varieties have none of the markers linked to the Ry genes. We compared molecular screening results with published PVY resistance/susceptibility data. The marker STM0003 linked to the Rysto gene had the highest diagnostic value, as almost all varieties with this marker are highly resistant (immune) to PVY, while the Ry364 and RYSC3 markers are not so efficient.

Keywords: potato, PVY, DNA markers, MAS, varieties

Potato plants are infected by about 40 viruses belonging to 13 families [1, 2]. Potato virus Y (PVY) of Poytvirus genus, Potyviridae family is one of the most harmful viruses affecting common potato (Solanum tuberosum L.) [1]. Representatives of 9 plant families can be PVY host plants in nature, and in experimental conditions — representatives of 31 plant families can be infected by PVY [3]. About 60 aphid species can be the natural vectors of this virus [4, 5]. Interac-
tions between numerous host plants and vectors as well as tolerance of many varieties (symptomless of infected plants) result in increase in viral load and lead to significant yield losses which can rich up to 80% in susceptible to PVY cultivars [1, 5-7, 8]. The cultivation of virus resistant varieties is the most efficient and environmentally safe method for plant protection; therefore, breeding of highly resistant to viral diseases varieties is of high priority.

A number of genes conferring different types of PVY resistance have been mapped in potato. The most studied resistance genes include the Ny-genes determining a strain-specific hypersensitive response (HR) of infected plants and the Ry-genes which prevent virus multiplication (no virus accumulation in infected plants regardless of virus strain — extreme resistance — ER) [8-12]. Therefore, potato cultivars with Ry genes are completely immune to PVY. The resistance of cultivars carrying Ny genes might be overcome by actively mutating virus [13, 14]; besides, hypersensitive response depends on temperature [15, 16] that is critical in the context of climate change.

Immunity to infection by PVY has been identified in accessions of many potato species [17], however the ER genes were introgressed into the breeding gene pool mainly from the three species: tightly linked genes Ry_sto/Ry-f_sto — from wild Mexican species S. stoloniferum [18-22]; Ry_chc — from Argentinean wild species S. chacoense [11, 23] and Ry_adg — from resistant to PVY Andean landraces of S. tuberosum ssp. andigenum [10, 19, 24]. Many varieties which are immune to PVY infection were developed based on involvement of S. stoloniferum accessions into the breeding programs of a number of Western European countries (Germany, Hungary, the Netherlands, Poland, Scotland) [18, 21, 22, 25]. The hybrids with S. stoloniferum were also actively used by Russian breeders [26-28]. Highly resistant to PVY accessions of S. tuberosum ssp. andigenum were most often used in the USA breeding programs [29, 30], in Spain [31] and Peru [32, 33]. Japanese breeders most frequently used PVY resistant accessions of S. chacoense to create immune potato varieties [11, 12, 34, 35]. The Russian breeding programs also actively used resistant samples of S. chacoense [26-28].

1. DNA markers of Ry-genes conferring extreme resistance to PVY which were most frequently used in molecular screening

<table>
<thead>
<tr>
<th>Gene (chromosome)</th>
<th>Marker</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ry_sto (12)</td>
<td>STM0003 (SSR)</td>
<td>[20*, 31, 36, 38, 43-45]</td>
</tr>
<tr>
<td></td>
<td>YES3-3A (SCAR), YES3-3B (SCAR)</td>
<td>[22*, 46-49]</td>
</tr>
<tr>
<td></td>
<td>YES3-3A (SCAR)</td>
<td>[21*, 45]</td>
</tr>
<tr>
<td></td>
<td>YES3-3B (SCAR)</td>
<td>[39, 42, 50-56]</td>
</tr>
<tr>
<td>Ry-f_sto (12)</td>
<td>GP81, GP122, GP204, GP269 (nuc CAPS)</td>
<td>[19*, 43]</td>
</tr>
<tr>
<td></td>
<td>GP122-718 (CAPS)</td>
<td>[21*, 36, 44, 48, 53, 59, 60]</td>
</tr>
<tr>
<td></td>
<td>GP122-614 (CAPS)</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>GP122-564 (CAPS)</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>GP122-406 (CAPS)</td>
<td>[36*, 39, 42, 44, 64-67]</td>
</tr>
<tr>
<td>Ry_adg (11)</td>
<td>RYSC3 (SCAR)</td>
<td>[30, 31, 38, 40, 42, 44, 45, 48-58, 62, 64-66, 68*-77]</td>
</tr>
<tr>
<td>Ry_chc (9)</td>
<td>Ry186 (SCAR)</td>
<td>[12, 40, 51, 54-56, 78, 79]</td>
</tr>
<tr>
<td></td>
<td>38–530 (RAPD)</td>
<td>[11*, 34, 35, 51, 52, 80]</td>
</tr>
<tr>
<td></td>
<td>Ry364 (SCAR)</td>
<td>[12*, 42]</td>
</tr>
</tbody>
</table>

Note. SSR — simple sequence repeat, SCAR — sequence-characterized amplified region, CAPS — cleaved amplified polymorphic sequence, RAPD — random amplification of polymorphic DNA; letter (*) indicates the references to studies. in which corresponding DNA markers were developed.

The marker-assisted selection (MAS) with the use of DNA markers linked to the Ry-genes (intragenic markers have not yet been developed) increases breeding efficiency and is commonly used now to create immune to PVY potato varieties [31, 34-36]. Furthermore, the application of DNA markers associated with Ry_sto/Ry-f_sto [22, 37-39], Ry_adg [38, 40], Ry_chc [11, 40] genes can provide the data about diversity, genetic protection from viral diseases and genetic vulnerability to viruses of the breeding gene pool [11, 22, 37-40]. The list of the DNA markers of...
Ry genes most commonly used in MAS is shown in Table 1. Note that the distances between the certain Ry gene and different markers of this gene can vary significantly. For instance, the RAPD marker 38-530 linked to the gene Ry\textsubscript{chc} (recombination frequency is 16.3%) has been reported as efficient marker for MAS [11], and SCAR markers Ry364 and Ry186 developed later flank Ry\textsubscript{chc} at a distance of 0.085 and 0.203 cM [12, 41].

DNA markers of Ry gene have a different diagnostic value. For instance, Witek et al. [61] detected three CAPS markers of the Ry-f\textsubscript{sto} gene (GP122-718, GP122-614, GP122-564) in all 24 Polish varieties which are immune to PVY, whereas 31 susceptible cultivars had negative MAS results. However, German researchers who analyzed the segregating population of androgenic dihaploids from immune to PVY variety Assia (having in pedigree S. stoloniferum) failed to detect linkage between the resistance gene and the marker GP122-718; relative linkage (34.9 cM) was shown only for one marker of the GP series — GP81 [20]. Cernák et al. [43] indicated high diagnostic value of the SSR marker STM0003 (linkage 2.95 cM with Ry\textsubscript{sto}), whereas all markers of the GP series [21] did not segregate in the mapping population analyzed in this study. It may be assumed that Cernák et al. [43] analyzed recombinant genotypes and their progeny, in which GP markers were linked with recessive allele of Ry\textsubscript{sto} gene.

The search for efficient markers of the target Ry genes remains relevant due to prospects of marker-assisted selection.

Our objective was to screen a large subset of domestic potato cultivars using three markers, STM0003, Ry364, RYSC3 linked with the Ry\textsubscript{sto}/Ry-f\textsubscript{sto}, Ry\textsubscript{chc}, Ry\textsubscript{adg} genes correspondingly, which confer extreme resistance to potato virus Y. The STM0003 marker was involved in molecular screening of Russian varieties for the first time, and the Ry364 marker had been previously used once in molecular screening of a small set of domestic varieties [42].

**Techniques.** The subset included 178 domestic potato cultivars (138 were bred in Russia, 22 — in Republic of Belarus, 11 — in Ukraine, and 4 — in other adjacent countries) from collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR). The cultivars were qualified as resistant or susceptible to PVY based on data from scientific publications and catalogues of potato varieties bred in Russia and in Republic of Belarus [27, 81-84].

DNA was extracted from frozen leaves by a modified CTAB extraction method [85]. Trueness to type of varieties grown in the VIR’ field gene bank was previously verified based on plant morphological characters.

Polymerase chain reaction (PCR) mixture (20 µl) contained 10 ng DNA, 1× reaction buffer (Dialat Ltd, Russia), 2.5 mM MgCl\textsubscript{2}, 0.6 mM of each dNTP, 0.2 mM of each (forward/reverse) primer and Taq DNA-polymerase (1 U) (Dialat Ltd, Russia). PCR was conducted using Mastercycler® Nexus Gradient thermal cycler (Eppendorf, Germany). For RYSC3 and Ry364 primers PCR conditions were followed to the literature [12, 68] and were modified by the use of the touchdown option for the microsatellite marker STM0003 as following: 3 min 15 s at 94 °C; 45 s at 94 °C, 1 min 30 s at 54 °C with a 0.5 °C gradual reduce in each next cycle, 1 min at 72 °C (8 cycles); 45 s at 94 °C, 45 s at 50 °C, 1 min at 72 °C (30 cycles); final elongation for 5 min at 72 °C. The reactions were performed at least three times for each sample.

The highly PVY resistant cultivars with the diagnostic markers of the Ry\textsubscript{sto}/Ry-f\textsubscript{sto}, Ry\textsubscript{adg}, Ry\textsubscript{chc} genes were used as positive controls: cv. Ania for STM0003 [36], cv. Saikai 35 for Ry364 [12], cv. Effect for RYSC3 [64]; deionized water was used as negative control.

Amplicons were separated in 2% agarose gel with ethidium bromide for UV visualization (Gel Doc XR+ gel documentation system, Bio-Rad, USA). Size of the
fragments was determined with the molecular weight marker 100 bp + 1500 + 3000, SibEnzim, Russia.

The t-test (Student's t-test) was used for statistical analysis with a significance level at $p \leq 0.05$.

**Results.** The PCR screening of $Ry$-genes was performed with known from literature primers (table 2).

### 2. Markers of PVY resistance genes $Ry$ used in screening of Russian potato cultivars

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Primer sequence (5'→ 3')</th>
<th>Tm, °C</th>
<th>Diagnostic fragment</th>
<th>References</th>
</tr>
</thead>
</table>
| $Ry_{sto}$ | STM0003 | F: GGAGAATCATACAAACCCAG  
R: AATTGTAACCTCTGTTGTGGTG | 50 | 111 bps | [20], [86] |
| $Ry_{adg}$ | RYSC3 | 3.3:3.3: ATACACTCATCTAAATTGGGG  
ADG23R: AGGATATACGGCATATTTCGGA | 60 | 321 bps | [68] |
| $Ry_{chc}$ | Ry364 | Ry364-14: CTATTTAAGTCTGTTGACTAAGCAG  
RY364-19: GGCTATATGTTCAATGAATTCATGCTAA | 55 | 298 bps | [12], [41] |

**Screening of Russian potato (Solanum tuberosum L.) varieties using markers of PVY resistance genes:**

A, B, C: 1 — Korona, 2 — Babushka, 3 — Olimp, 4 — Sintez, 5 — Resurs, 6 — Severyanin, 7 — Avrora, 8 — Veselovskyi 2-4.

D: 1 — Aksamit, 2 — Moskovetskiy, 3 — Skarb, 4 — Effect, 5 — Golubizna, 6 — Temp, 7 — Barin, 8 — Kristall.

Results of molecular screening revealed diagnostic fragments of at least one of the three markers in 39 (21.9%) of 178 cultivars (Fig., Table 3). We detected diagnostic fragments of the Ry364 marker of $Ry_{chc}$ gene in 21 (11.8%) cultivars, of the STM0003 marker of $Ry_{sto}$ — in 13 (7.3%) cultivars, and of the RYSC3 marker of $Ry_{adg}$ gene — in 8 (4.5%) cultivars. Three cultivars (Brianskiy ranniy, Buket, Zhivitsa) had the two markers, Ry364 and RYSC3, simultaneously. The overwhelming majority of the analyzed cultivars (78.0%) didn’t have any of the markers of $Ry$ genes (see table 3). According to the Student’s t-test, differences in the frequency of cultivars with markers to different $Ry$-genes were not significant with the exception of two groups with the Ry364 ($Ry_{chc}$) and RYSC3 ($Ry_{adg}$) markers, where frequency of cultivars with the Ry364 marker was significantly higher ($p < 0.05$).

Among 39 cultivars in which at least one of the three markers of $Ry$-genes was detected (see Table 3), for 18 cultivars (Brianskiy ranniy, Brianskiy krasny, Vektar, Golubizna, Zhivitsa, Kolobok, Korona, Loshitskiy, Meteor, Moskovetskiy, Nakra, Olimp, Pogarskiy, Rezerv, Resurs, Sokolskiy, Effect, Yubiley Zhukova) a high and very high resistance to PVY has been reported, and 6 cultivars (Barin, Zoltskiy, Ilinskiy, Kristall, Skarb, Temp) were described as moderately resistant [26, 27, 81-84]. We have not found any available information...
about phenotypic resistance to PVY for the remaining 15 cultivars. In sum, almost all tested cultivars with STM0003 marker of \( R_y^{sto} \) gene were highly resistant (immune) to PVY, whereas some cultivars with the \( R_y^{364} \) and \( R_y^{SC3} \) markers were moderately resistant.

3. Molecular screening of 178 potato (\( \textit{Solanum tuberosum} \) L.) Russian cultivars using three markers of PVY resistance genes

<table>
<thead>
<tr>
<th>Haplotype grouping</th>
<th>Gene/marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R_y^{sto} )</td>
</tr>
<tr>
<td>Group I, ( n = 13 ) (7.3 %)</td>
<td>1</td>
</tr>
<tr>
<td>Ilnisky; highly resistant and extremely resistant cultivars: Bryanskiy krasny, Vektor beloruskiy, Kolobok, Korona, Meteor, Moskvoretkiy, Nakra, Olimp, Pogarskiy, Resurs, Sokolskiy, Yubilei Zhukova</td>
<td></td>
</tr>
<tr>
<td>Group II, ( n = 3 ) (1.7 %)</td>
<td>0</td>
</tr>
<tr>
<td>Buket; highly resistant and extremely resistant cultivars: Bryanskiy ranni, Zhivitsa</td>
<td></td>
</tr>
<tr>
<td>Group III, ( n = 18 ) (10.1 %)</td>
<td>0</td>
</tr>
<tr>
<td>Imandra, Krasnaya gorka, Lider, Maugli, Oktyabrenok, Primonskiy (= Pri-12), Rasvet, Severyanin, Sineva, Sintez, Khibinskiy ranni, Bezhitiski(^a), Bronnicki(^a), Kristall(^a), Skarb(^a), Temp(^a); highly resistant and extremely resistant cultivars: Rezerv, Loshitskiy(^a)</td>
<td></td>
</tr>
<tr>
<td>Group IV, ( n = 5 ) (2.8 %)</td>
<td>0</td>
</tr>
<tr>
<td>Barin, Zolkiy, Yubileyno Osseti; highly resistant and extremely resistant cultivars: Golubizna, Effekt</td>
<td></td>
</tr>
</tbody>
</table>

| Group V, \( n = 139 \) (78.1 %) | 0 | 0 | 0 |

The number (%) of varieties with a diagnostic fragment of marker: 13 (7.3 %) \( \times \) 8 (4.5 %) \( \times \) 21 (11.8 %)

Note: “1” — presence and “0” — absence of the diagnostic fragment.
Letter (\( a \)) marks cultivars with unstable amplification of \( R_y^{364} \) diagnostic fragments.

Among 139 cultivars for which we have not identified none of the three markers of the three \( R_y \) genes, clear and consistent data about PVY resistance were found only for 43 varieties. Thus, high PVY resistance was reported for 17 cultivars (Bryanskii delikates, Bryanskii nadezhny, Garant, Druzhny, Zhigulevski, Kartni, Kuznechanka, Lasunak, Lyubava, Manifest, Nikulinskiy, Odisey, Prizer, Skoroplodny, Smena, Falenskiy, Chayka) and moderate and low PVY resistance was reported for 21 cultivars [26, 27, 81, 82, 84, 87]. For the rest 101 cultivars we have not found such information.

Unfortunately, most cited above sources did not provide data on whether the detected type of PVY resistance was due to the absence of virus accumulation in infected plants (ER), or to the hypersensitive response (HR), field resistance or to the resistance to virus vectors. It is also not specified, which methods were used to evaluate resistance (field tests under natural viral load...
during wide spread of diseases or artificial inoculation, i.e. mechanical inoculation or grafting). Therefore, the available information is not enough for exact matching of the results of molecular screening with data on PVY resistance characters phenotyping.

As was mentioned above, two of the three markers, Ry364 (R_{y, chc} gene) and STM0003 (R_{y, sto} gene), were used in molecular screening of all 178 cultivars for the first time. However, for a number of cultivars the information about potential presence/absence of these genes had been obtained earlier, though with the use of other markers, i.e. RAPD marker 38-530 (R_{y, chc} gene) [11], as well as SCAR marker YES3-3A [22] and CAPS marker GP122-406/EcoRV [21] for the R_{y, sto}/R_{y, f, sto} genes. For these cultivars we can compare the result of molecular screening performed with different markers of the same gene. For instance, we identified diagnostic fragment 111 bps of the STM0003 marker of R_{y, sto} gene in 13 cultivars (see Table 3) in which we had earlier detected another marker of this gene — YES3-3A [39, 51], as well as the GP122-406 [39, 64] and GP122-564 markers [37] of R_{y, f, sto} gene closely linked with R_{y, sto}. Therefore, the results obtained with all three markers (STM0003, YES3-3A and GP122) of the Ry-genes introgressed from S. stoloniferum completely matched for these 13 domestic cultivars. Ten of these 13 cultivars (Bryanskiy krasny, Vektar, Kolobok, Korona, Moskvoretskiy, Nakra, Pogarskiy, Resurs, Sokolskiy, Yubiley Zhukova) have interspecific hybrids with S. stoloniferum in their pedigree [17, 28, 88, 89]. The diagnostic fragment of the RYSC3_{321} marker is rare: it was found in 8 out of 178 screened cultivars (see Table 3, Fig.). It has to be pointed out that presence of the SCAR marker RYSC3 of the R_{y, adg} gene was reported earlier for four cultivars — Effect [64], Bryanskiy ranniy and Golubizna [51], and Zhivitsa [49]. Furthermore, it was also mentioned that RYSC3_{321} marker is not present in cultivars Oktobernok [58] and Resurs [72]. However, there are only a few data about negative MAS results with this marker which were obtained for small subsets [42, 58, 72].

It has to be pointed out that the RYSC3 marker of the R_{y, adg} gene was identified in several immune to PVY cultivars (e.g. Bryanskiy ranniy, Golubizna, Effect), which have in their pedigrees hybrids with S. stoloniferum (as the Ry gene donors) [26]. However, the markers of R_{y, sto}/R_{y, f, sto} genes expected in these cultivars were not identified. In this connection we would like to emphasize the following. RYSC3 marker is considered as specific for R_{y, adg} gene which was mapped on chromosome 11. However, initially there was a report about mapping of the R_{y, sto} gene on the same chromosome [90]. Later, an opinion about wrong mapping of the R_{y, sto} gene on chromosome 11 was accepted. The other researchers [68] identified PVY resistance gene located on chromosome 11 as R_{y, adg}. This was due to the absence of co-segregation of the markers of R_{y, sto} gene with the RYSC3 marker [36]; as well as to the fact that in resistant potato cultivars RYSC3 marker and M45 marker described by Brigneti et al. [90] were always identified together [70]. It seems, however, quite likely that the one ortholog of Ry gene conferring extreme resistance to PVY and linked with the RYSC3 marker is located on chromosome 11 both in S. stoloniferum and in S. tuberosum ssp. andigenum. Indeed, we earlier detected RYSC3 marker in 5 out of 8 (62.5%) accessions of S. stoloniferum and only in 2 out of 95 (2.1%) accessions of S. tuberosum ssp. andigenum from the VIR potato collection [65].

Based on the literature data, interspecific hybrids with S. chacoense were involved in breeding of a number of Russian cultivars: Alena, Alisa, Borodyanskiy rozovy, Bryanskiy delikates, Bryanskiy krasny, Bryanskiy nadezhny, Goryanka, Krepysh, Lugovskoy, Meteor, Nakra, Nikulinskiy, Pobeda, Saprykinskiy, Sentyabr, Utenok [28, 88, 89]. However, in our study we did not detect the Ry364 marker in these cultivars. It must be pointed out that in four highly re-
sistant cultivars, Bryanskij delikates, Bryanskij nadezhny, Meteor, and Nikuliniskiy, another marker of $R_{Ychc}$ gene — RAPD marker 38-530 — was detected earlier [51]. At the same time, $S. chacoense$ was specified in pedigree of only the two cultivars — Bezhitiskiy and Bronnickiy [89], out of 21 those had the $Ry364_{298}$ diagnostic fragment of the $R_{Ychc}$ gene. Hence it cannot be ruled out that in the initial interspecific hybrids with $S. chacoense$ involved in breeding of mentioned above PVY resistant domestic varieties there were recombination events in the region between $R_{Ychc}$ gene and the $Ry364$ marker. Intragenic markers required for selection of genotypes with functional allele of $R_{Ychc}$ gene have not been developed yet.

To summarize, the obtained results allow us to draw the following conclusions. In our study, the STM0003 marker associated with $Ry_{stot}$ gene has the highest diagnostic value. Almost all cultivars with this marker are highly resistant (immune) to potato virus Y (PVY), whereas $Ry364$ and RYSC3 markers turned out be not so effective. The results of MAS indicate a low genetic protection against PVY in the studied subset of domestic cultivars of which 78.2% had none of the markers of $Ry$-genes.

REFERENCES


13. Valkonen J.P.T. Elucidation of virus-host interactions to enhance resistance breeding for control.


34. Fujimatsu M., Hashizume H., Fudan T., Koma Y., Sanetomo R., Ono S., Hosaka K. Harima-


57. Lopez M., Riegel R., Lizana C., Behn A. Identification of virus and nematode resistance genes


59. Luksha V.I., Voronkova E.V., Guakasyan O.N., Ermishin A.P. V sbornike: *Molekulyarnaya i prikladnaya genetika* [In: Molecular and applied genetics]. Minsk, 2012: 82-87 (in Russ.).


77. Herrera M. del R., Vidalon I.J., Montenegro J.D., Riccio C., Guzman F., Bartolini I., Ghislain M. Molecular and genetic characterization of the *Ryadv* locus on chromosome XI from Andigena potatoes conferring extreme resistance to potato virus *Y*. *Theoretical and Applied Ge-


84. *Sorta kartofelya rossiiskoi selektssii /Pod redaktsiei E.A. Simakova* [Russian potato varieties. E.A. Simakov (ed.)]. Moscow, 2018 (in Russ.).


89. *Katalog mirovoi kollektii VIR. Vyp. 829. Selektissionnye sorta kartofelya Rossii i SNG* [Catalog of the VIR World Collection. Iss. 829. Selection potato varieties of Russia and the CIS]. St. Petersburg, 2015 (in Russ.).

ALLELE VARIABILITY OF AMYLASE INHIBITOR GENE AI IN POTATO VARIETIES AND LINES

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Abstract

The economic efficiency of potato varieties includes not only yield characteristics, but also taste preservation during storage. Storing potato tubers at low temperatures leads to the degradation of starch and the accumulation of reducing sugars; the latter during heat treatment contribute to the deterioration of taste and participate in acrylamide synthesis. Starch degradation to simpler compounds is achieved in two pathways: hydrolytic and phosphorolytic. In the hydrolytic pathway, hydrolases, including α- and β-amylase, are responsible for cleavage of starch, and exhibit different activities depending on the tissue, organ type, cell localization, and plant species. Amylase activity is regulated at the post-translational level by an amylase inhibitor (AI), which binds amylase and blocks the active site of the enzyme, thereby reducing the catalytic activity. Although AI role in plant is very important, present data on the AI genes and encoded proteins in representatives of the genus Solanum are extremely limited. In this study, AI sequences were obtained and analyzed in 36 potato varieties and lines of domestic and foreign selection. Two types of A1 coding sequence were identified, 621 and 630 bp, depending on presence of 9-bp insert GGTGCAWTT at the 3´-end of the cDNA. The analyzed gene was characterized by an extremely high polymorphism level: exonic sequences contained 134 SNPs (single nucleotide polymorphisms) (21.3 %), which resulted in 69 amino acid substitutions (33.0 %) in the encoded proteins. Detected GAI/F202 insertion in the C-terminal region of some AI proteins resulted from the 9-bp 3´-gene insertion. Among the 69 amino acid substitutions identified, only 11 are radical and may lead to a change in the protein conformation. All of the analyzed potato accessions were heterozygous and possessed several allelic variants of the gene. In total, 70 allelic variants of the gene and 69 associated protein variants are identified. The largest number of single nucleotide polymorphisms is among the allelic variants of the gene in the varieties Lux (18 substitutions), Irbitskii (17 substitutions) and Gala (16 substitutions). The largest number of amino acid substitutions is in the AI proteins in the Gala (9 substitutions) and Gornyak (8 substitutions) varieties.

Keywords: Solanum tuberosum, potato varieties, amylase inhibitor, AI gene, allelic variants

Potato (Solanum tuberosum) holds one of the leading positions in agriculture of many countries as food crop, commercial crop and forage crop [1, 2]. In the Russian Federation the area of potato cultivation covers different climatic zones from North of the Arctic Circle to southern borders, and gross yield of this culture amounts to approximately 30 million tons, i.e., almost 10% of global potato production (380 million tons; data of FAO-STAT for 2017). Potato is one of four most important nutrient carbohydrate sources after wheat, rice and corn, and contains a large amount of vitamins and minerals [3-5].

The main nutrition value of potato is determined by the contents and qualitative composition of starch in tuber. Starch consists of a mix of two homo-
polysaccharides, line amylose and branched amylopectine, which differ in structure and biosynthesis pathways. Both amylose and amylopectine are $\alpha$-1,4-glucane chains, where amylopectine consists of short chains linked between each other with $\alpha$-1,6-glucosidic bonds in branching points [6-8]. Starch metabolism is well-studied. At least 40 enzymes are known that participate in carbohydrate metabolism in potato tubers and determine the contents and composition of starch and other carbohydrates [9-13].

Starch content in potato tubers can reach 25% [14]). After crop harvesting the tubers are stored up to several months at low temperature (2-4 °C). Exposure to low temperature can induce cold-induced sweetening (CIS) of tubers, which manifests itself in strengthened starch hydrolysis and, consequently, in accumulation of reducing sugars [15-18]. During high temperature treatment reducing sugars interact with $\alpha$-amino acids and carcinogenic acrilamide is formed, and taste deteriorates [19-21]. The cold-induced sweetening is also affected by tuber ripeness, mechanical damage, biotic and abiotic stress etc. [20, 22].

Starch cleavage into simpler compounds is achieved in two ways (hydrolytically and phospholitically). First, hydrolases, including $\alpha$-amyloses (AMY, EC 3.2.1.1) and $\beta$-amyloses (BAM, EC 3.2.1.2) [23, 24], are responsible for starch cleavage. The amyloses are endoamylolytic ($\alpha$-amyloses) and exoamylolytic ($\beta$-amyloses) enzymes, which specifically hydrolyze $\alpha$-1,4-glucosidic bonds and form linear and branched maltooligosaccharides [25, 26].

Presently, 5 isoforms of $\alpha$-amylose and 10 isoforms of $\beta$-amylose are known in plants, which can exhibit different activity depending on tissue, organ type, cellular localization and plant type [26-28]. Gene expression for nine amylases ($StAmy1$, $StAmy23$, $StBAM1$, $StBAM3$, $StBAM4$, $StBAM5$, $StBAM7$, $StBAM8$ and $StBAM9$) was identified in potato tubers, where only three ($StAmy23$, $StBAM1$ and $StBAM9$) have high expression under low temperature storage [26, 29].

The amylase activity is regulated post-translationally by amylase inhibitor (AI), which binds to amylase and blocks active enzyme site or changes its conformation, thus reducing catalytic activity [30, 31]. The potato gene encoding amylase inhibitor was first identified in $S. berthaultii$ ($SbAI$) [32]; however, its sequence is not provided in NCBI database.

In a number of studies Zhang et al. [29, 32, 33] showed that during low temperature storage potato tubers resistant to cold-induced sweetening as compared to CIS-sensitive tubers are characterized by higher $SbAI$ gene expression; furthermore, negative correlation was identified between the number of $SbAI$ gene transcripts and content of reducing sugars. Subsequent studies on transgenic plants showed that at low temperatures $SbAI$ gene suppression in CIS-resistant potato lines results in increase in $StAmy23$, $StBAM1$ and $StBAM9$ amylase activity and increased volume of reducing sugars in tubers. At the same time, $SbAI$ gene overexpression in CIS-sensitive potato lines at low temperatures caused suppression of such amylases [32].

In spite of essential significance of amylase inhibitors in potato cold-induced sweetening process, the information about variability of the aforementioned gene and its possible allele variants is lacking. For instance, GenBank NCBI (https://www.ncbi.nlm.nih.gov/) database contains information about full size gene $AI$ (JX523608.1) and its mRNA (JX523606.1) only for anonymous sample of $S. tuberosum$. Furthermore, this database contains $AI$ sequences of other representatives of $Solanum$ genus, $S. lycopersicum$ (XM_004233967.3, CP023759.1, HG975515.1) and $S. pennellii$ (HG975442.1, XM_015211800.2).

In this study, we identified amylase inhibitor $AI$ gene sequence in 36 domestic and foreign potato varieties and lines, and determined possible allele
variants of this gene and the protein it encodes for the first time.

The purpose of this study is to determine the sequences of amylase inhibitor (AI) gene and proteins encoded in cultivated potato varieties and lines, to evaluate their genetic variability, and to determine the allele variants of AI gene.

Techniques. The plants were collected in Lorkh All-Russian Research Institute of Potato Farming (Moscow Province, Russia). Gene sequence (JX523608.1) and mRNA sequence (JX523606.1) of S. tuberosum available in the GenBank NCBI database were used in a comparative evaluation of AI gene polymorphism.

The DNA was isolated from young leaves by a modified potassium acetate method [34].

Primers were developed based on AI gene sequences of Solanum genus members (JX523608.1, JX523606.1, XM_004233967.3, CP023759.1, HG975515.1, HG975442.1, XM_015211800.2) available in GenBank NCBI, which allows amplifying full-sized AI (SbaI_F 5´-ACTATGGGCTTTTCATTACTCTA-3´; SbaI_R 5´-TTACATCAAAGAATAGTTGTATAAC-3´) and overlapping internal gene region (SbaI_in1R 5´-TCGTGAGAATAGTCTCTTGC-3´; SbaI_ex1F 5´-GTAACATGGCTCGCGTTC-3´; SbaI_ex3F 5´-AACAGAGGCTCCAACTGC-3´; SbaI_in3R 5´-GGATAGTTTGGACACATAACTT-3´). The amplification was performed with a reagent kit (Dialat LTD, Russia). The reaction mixture contained in 15 µl 10× buffer, 1.5 µM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of SbaI_F and SbaI_R primers; 0.2 U of BioTaq DNA of polymerase (Dialat LTD, (Russia) and 100 ng of genomic DNA. The reaction was performed as follows: denaturation for 40 s at 95 °C; primer annealing for 30 s at 54 °C, DNA elongation for 2 min at 72 °C (35 cycles); final elongation for 7 min at 72 °C (BioRad C1000 thermocycler, Bio-Rad, USA).

The resultant amplicons approximately 2 kbps long visualized in 1% agarose gel were cut out and purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). During cloning of full-size AI gene sequences of analyzed potato varieties and lines Quick-TA Kit (Eurogen, Russia) was used. The nucleotide sequences of fragments were determined on ABI 310 Capillary DNA Analyzer automatic sequencer (Applied Biosystems, USA) (Center for Collective Usage Bioinzheneriya RAS).

The resulting sequences were aligned and analyzed with MEGA 7.0 software [35]. Potential impact of amino acid substitutes on the structure and functions of proteins were evaluated using web-service PROVEAN (http://provean.jcvi.org/index.php) [36].

Results. Nucleotide polymorphism of amylase inhibitor gene. We amplified and later cloned amylase inhibitor (AI) genes in 36 potato lines and varieties of domestic and foreign origin (Table 1). Because modern potato varieties are tetraploid, we sequenced five clones of each analyzed sample to identify AI gene allele variants.

Complete nucleotide sequence of AI gene was determined for all analyzed potato samples as a result. The comparison of the obtained nucleotide sequences with data available in GenBank NCBI database for AI genes and mRNA identified their high homology (> 90%) in samples we studied and in other representatives of Solanum genus.

Analysis of exon-intron organization determined that, along with the other known plant amylase inhibitor genes, all AI gene sequences of S. tuberosum contain four exons. The length of AI gene of analyzed potato varieties varied from 1781 bps (in Meteor 1 variety) (hereinafter the numbers in variety name represent allele variant number) to 1872 bps (in Red Scarlett 2 variety). Very high polymorphism was identified in AI nucleotide sequences, i.e. it contained 530 var-

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ialle sites (single nucleotide polymorphisms, SNPs), and total degree of polymorphism amounted to 27.0%.

1. Allele variants of Al gene nucleotide sequence in studied potato lines and varieties

We identified two types of exon sequences with different length, 621 and 630 bps due to presence of a large number of samples of 9-nucleotide insertion (GGTGCAWTT) in 3’-end region. It has to be pointed out that exon sequence variability in samples that we studied turned out unexpectedly high. We identified 134 variable sites in encoding sequences, with a 21.3% polymorphism which is much higher than for other known carbohydrate metabolism genes. For instance, studies of specially selected polymorphic fragment of acid vacuolar invertase gene Pain-1 (exon V-terminating codon) in S. tuberosum cultivars showed the variability of this region not more than 9% [37, 38]. When analyzing genes associated with starch phosphorylation in 192 potato lines from New Zealand, α-glycan-Н2О-dikinase (GWD) gene turned out to be the most polymorphic, and its variability was < 5%, whereas polymorphism of isoform starch-synthase gene (SS I-III) did not exceed 3.4% [39].

The introns in studied sequences differed in length and variability significantly and, apart from a large number of nucleotide substitutes (396 in total), contained insertions and deletions. The size of Al gene intron sequences varied within range of 1151 to 1251 bps. The biggest differences were identified in intron I, which included extended insertions (up to 47 bps). In some cultivars in gene position 347-400 bps we identified a region containing nonhomologous insertions varying in length. The sequences of introns II and III had deletions not exceeding 18 nt (e.g. GATATATTCTCTY_{1406}, GTAT_{1452}), and TATACC_{1298} in-
As has been mentioned earlier, five *AI* gene clones were sequenced and analyzed for each sample, which allowed us to characterize homozygous/heterozygous status of this gene. All potato samples analyzed turned out heterozygous for the specified gene and several of its allele variants corresponded to them (see Table 1).

While analyzing exon sequences we identified 70 variants for 36 cultivars and lines. Earlier, only 11 allele variants for full-scale encoding sequence of acid vacuolar invertase gene had been reported for 19 cultivars [37]. It is noteworthy that as a result of the analysis we failed to identify an allele variant typical for the group of cultivars. All analyzed samples were characterized by specific allele *AI* gene variant. It has to be pointed out, however, that a number of allele variants differed from each other only by 1-2 nucleotide substitutions. The highest number of difference in terms of allele variants were displayed by the following cultivars: Luxe (18 SNPs), Irbitrskii (17 SNPs) and Gala (16 SNPs). This high gene variability and a large number of allele variants are not quite typical for plant genes and, in particular, for potato.

**Amino acid polymorphism of amylase inhibitor.** The resulting *AI* gene nucleotide sequences were translated. The corresponding amino acid sequences amounted to 206 and 209 amino-acid residue. The identified protein length differences were attributable to the presence of GAI/F202 insertion in terminal region due to GGTGCAWTT insert at 3´-end region. Out of 134 exon-specific SNPs, 69 resulted in amino acid substitution, whereas amino acid polymorphism amounted to 33.0%. We conducted PROVEAN-analysis and determined that 11 out of 69 substitutions are radical and can affect protein conformation. Therefore, the study identified 69 variants of amino acid *AI* sequence whose characteristics are shown in Table 2. *AI* sequences of Gala (9 substitutes) and Gornyak (8 substitutes) contained the highest amount of amino-acid residue substitutions.

| Note. The numbers in variety name indicate allele variant number. The variable amino acid sites are highlighted with green, whereas radical ones are highlighted with dark green (see http://www.agrobiology.ru). To summarize, this study for the first time describes amylase inhibitor *AI* gene sequence in 36 cultivated potato lines and varieties, potential allele variants of this gene and encoded proteins. Furthermore, we identified a very high nucleotide (21.3%) and amino acid polymorphism (33.0%). It has to be pointed out, |
|---|---|

2. Allele variants of amylase inhibitor amino acid sequence in studied potato lines and varieties
however, that identified amino acid residue substitutions are in most cases (58 out of 69) neutral and, theoretically, should not result in conformational protein change. The findings allow us to continue searching for correlation between allele At gene variants and sensibility of potato lines or cultivars to cold-induced sweetening.

REFERENCES


DONORS OF POTATO (*Solanum* L.) PLASTICITY AND YIELD STABILITY TRAITS IN THE ENVIRONMENTAL CONDITIONS OF NORTH FOREST STEPPE OF WESTERN SIBERIA

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**Abstract**

Potato (*Solanum tuberosum* L.) varieties possessing sustainable high yield under varying environmental conditions and other valuable properties, e.g. resistance to diseases and pests, are much appreciated by practitioners. Seeking for donor plants with high environmental plasticity and stability in specific cultivation zone is a key point, especially in creating highly productive adaptive varieties for regions with severe agro-climatic conditions. This paper reports the first assessment of new potato hybrids created in the soil and climatic conditions of Western Siberia, as donors of high yielding and complex relative resistance to fungal diseases, potato Y-virus, and golden potato nematode. The best of them are already involved in practical selection for productivity and high adaptability. Our goal was to assess the parameters of adaptability in created potato hybrids under the conditions of the northern forest-steppe of Western Siberia (Kuznetsk Basin, Kemerovo Region, Kemerovo District, 2014-2018; 70 m² plots with 20 m² test area arranged randomly in four repetitions). Planting was carried out in the third decade of May at 35.0 thousand bushes per 1 ha (70½35 cm; a Cramer pot-a-planter, CRAMER Technik, Germany). The samples (n = 170) including collection potato hybrids created in Kemerovo Research Institute of Agriculture were examined in a collection nursery. The varieties Lyubava (early season), Nevskii (medium-early ripening) and Tuleevskii (medium-ripening) were the standard. According to our research data, the Lyubava, Nevskii, Tuleevskii varieties and hybrids 6-4-11 and 22103-10 are extensive type potato genotypes with low environmental plasticity (b_i = 0.28-0.91 < 1). Hybrid 3-21s-11 (b_i = 1.53) with medium yield stability (S_i² = 14.6) shows the greatest response to external conditions. Hybrids 22103-10 and 3-21c-11 are donors of resistance to potato virus Y (gene *Ry*<sub>chc</sub>), golden potato nematode *Globodera rostochiensis* (Woll.) (gene *H1*) and pale nematode *G. pallida* (Stone) Behrens. (gene *Gpa2*). According to a complex of the traits, three hybrids of the intensive type (17-5/6-11, 1-5-12 and 1615-10) possess high adaptiveness, i.e. an increased environmental plasticity (b_i = 1.38, 1.20, and 1.17) and high stability (S_i² = 1.1, 9.4, 5.2), and are of particular value for breeding. Moreover, the hybrid 17-5/6-11 is a donor of resistance genes to potato virus Y (PVY) (*Ry*<sub>chc</sub>) and golden potato nematode (*H1*), with three markers — TG689, 57R, and N195). Hybrid 1-5-12 contains a combination of the *H1* genes (for all three markers) and *Gro1-4* gene of resistance to *G. rostochiensis*, *Gpa2* gene of resistance to *G. pallida*, and genes *Ry*<sub>chc</sub> and *Ry*<sub:>60</sub> conferring resistance to PVY. Long-term field surveys of resistance to fungal pathogens, *Phytophthora infestans* (Mont.) De Bary, *Alternaria solani* (Ell. Et Matr) Sor., *Fusarium oxysporum* Schlt., *Rhizoctonia solani* J.G. Kühn and *Actinomyces scabies* Gussow showed a 7-9 point relative stability in all tested hybrids.

Keywords: Solanum tuberosum L., potato, yields, adaptability, genotype×environment interaction, plasticity, stability

Potato (*Solanum tuberosum* L.) is one of cornerstone food crops cultivated in more than one hundred countries; it is the fourth ranking production crop in the world and the first ranking non-corn crop [1, 2]. The advantage of potato...
is its capacity to form crop yield in a wide range of agrosystems and high specific production of dry weight of food product per unit of crop acreage [3]. Potato gets increasingly greater attention as the source of not only carbohydrates but also of vitamins, minerals, dietary fibers [4]. This is the reason of ongoing interest in studies to improve nutrient properties of potato and to increase its resistance to biotic and abiotic factors of the environment [5].

The availability of warmth and humidity has significant impact on potato plants during their active growth and during tuber formation [6, 7]. Drought is one of the main factors preventing the growth of plants and reducing productivity of land ecosystems in many regions of the world [8]. Due to global warming, there are steps that need to be taken to ensure adaptation of cultures in these conditions, including creation of new genotypes with mechanisms of protection against stresses [9].

It is no less relevant to increase potato resistance to the most harmful and ubiquitous diseases, such as phytophthora rot, viruses, potato cyst nematode, Alternaria blight, rhizoctonia disease and bacterial rot [10]. Success in breeding varieties which provide complex protection against pathogens depends in many aspects on selection and systemization of donors, and on mobilization of *Solanum* wild forms and varieties to create on their basis the effective sources of resistance [11]. Molecular markers, closely associated with resistance genes, significantly intensify the search for valuable forms due to broader testing and simultaneous selection of genotypes with a complex of oligogens [12], notably reducing the time to create new varieties.

Genetic diversity of plant collection facilitates assessment of primary agronomic characters manifestation in specific edaphoclimatic conditions and identification of most valuable donors [13, 14]. Indeed, creating a variety implies not only obtaining and selecting new genotypes, but also identifying an ecological niche in which these genotypes will provide high productivity, ecological sustainability and product quality [15].

Harsh agroclimatic conditions, late blight epidemics of 1800s, and spreading viral diseases during plant reproduction reduced genetic diversity of potato varieties [16, 17]. Therefore, researchers and breeders around the world are actively seeking for economically valuable properties among the tuber forming *Solanum* L. species of *Petota* section [18].

In this study, we identified samples with high crop yield, resistance to unfavorable environmental conditions and complex relative resistance to fungal diseases, potato virus Y and golden potato nematode under edaphoclimatic conditions of Siberia among potato hybrids that we obtained. These samples have already been involved in breeding adaptive and high yielding potato varieties. The purpose of this study was to evaluate donor properties of potato forms in terms of adaptability in the environmental and geographical conditions of Western Siberia.

**Techniques.** The experiments were performed in the northern forest-steppe of Western Siberia (Kuznetsk Basin, Kemerovo Province, Kemerovo District) in the years which differed in meteorological condition. Particularly, 2014 and 2016 were the most unfavorable, with insufficient precipitations at the beginning of the vegetation and high temperatures in combination with overwetting during the tuber formation; 2015, 2017 and 2018 were the most favorable in the hydrothermal regime during the vegetation period with sufficient wetting and moderate temperatures.

A total of 170 samples, including collection potato hybrids (*Solanum tuberosum* L.) of Kemerovo Research Institute of Agriculture, were examined in a collection nursery. The varieties Lyubava (early season), Nevskii (medium-early
ripening) and Tuleevskii (medium-ripening) were standards. Planting was carried out in the III decade of May (35.0 thousand plants per hectare, 70×35 cm scheme; a Cramer potato planter, CRAMER Technik, Germany) in four replications. The total plot area was 70 m², 20 m² test plots were randomly distributed. Complete fallow (heavy loamy leached chernozem soil with 8.52% humus, 25.0 mg/kg N-NO₃, 140 mg/kg P₂O₅, and 80 mg/kg K₂O, pH 6.1) was a predecessor.

Morphological and economic traits were described and assessed according to methodological guidelines [19]. Scores of plant resistance to fungal disease was assessed visually at a 9-point scale (1 — very low, 3 — low, 5 — moderate, 7 — high, 9 — very high) in field conditions under natural infection as per the Council for Mutual Economic Assistance (CMEA) International Classification of potato species of *Tuberarium* (Dun.) Buk. section, genus *Solanum* L. [20] and methodological guidelines [21].

Eberchart and Russel [22] parameters of environmental plasticity (b), stability (S²), and index of environmental conditions (I_j) were calculated based on evaluation of positive response of the genotype to the improved cultivation environment.

DNA markers linked to potato virus Y (PVY) resistance genes (Ry_adg, Ry_che, Ry_sto, markers RYSC3, Ry186, YES3-3A) [23-25], *Globodera rostochiensis* (Woll.) golden potato nematode Rol, Ro4 subtypes resistance genes (H1 and Gro1-4, markers TG689, 57R, N195, Gro1-4-1) [11, 26, 27] and *Globodera pallida* (Stone) Behrens pale nematode Pa2 subtype resistance genes (Gpa2, Gpa2-2 marker) [11] were identified by multiplex PCR. DNA was extracted from 200 mg of tuber tissue with GMO-MagnoSorb Kit (Syntol, Russia). Tissue samples were homogenized (Precellys 24, Bertin Corp., USA) with 800 µl lysing buffer from the kit and transferred to a 2 ml tube for DNA isolation using Savarska 02 robot work station (Syntol, Russia) as per the manufacturer’s protocol. The amplification was performed in multiplex using 2.5× PCR mixture M428 (Syntol, Russia). The reaction mixture (25 µl) contained a 10× buffer for Taq DNA-polymerase (Syntol, Russia), a 2.5 mM mixture of dNTPs (Evrogen, Russia), 25 mM magnesium chloride (Fermentas, Latvia), 5-10 pmol of each primer (Syntol, Russia), 0.5 U Taq DNA polymerase (Syntol, Russia), 10 ng of analyzed DNA and 10-13 µl autoclaved double-distilled water. The described primers [28] were used and protocols were optimized during validation tests in a single cyclogram [12] as follows: 10 min at 94 °C; 30 s at 94 °C, 30 s at 68 °C, 30c at 72 °C (5 cycles); 30 s at 94°C, 30 s at 58 °C, 30 s at 72 °C (35 cycles); 30 s at 94 °C, 5 min at 72 °C. For sequencing, DNA fragments were pre-amplified as follows: 10 min at 94 °C; 30 s at 94 °C, 30 s at 68 °C, 1.5 min s at 72 °C (5 cycles); 30 s at 94 °C, 30 s at 58 °C, 1.5 min at 72 °C (35 cycles); 30 s at 94 °C, 30 s at 58 °C, 1.5 min at 72 °C (35 cycles); 30 s at 94 °C, 5 min at 72 °C. Multiplex PCR for eight markers was performed using an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA). The amplification products were analyzed using a genetic analyzer Nanofor® 05 (Institute for Analytical Instrumentation RAS, Russia). The forward primers were marked with fluorescent dyes 6FAM or 5R6G (Syntol, Russia). The data were processed with DNA Fragment Analysis software (Institute for Analytical Instrumentation RAS, Russia) [29].

The statistical processing was performed with Sne-decor program (developed by O.D. Sorokin, Russia) using variation and dispersion analysis methods [30, 31]. Means (M), standard deviations (±SD) and coefficients of variation (CV, %) of yield values were calculated. In dispersion analysis, F-Fischer test were used at a 5% level of significance, and mean (M) and their standard errors (±SEM) were determined along with contribution of each factor to the total dis-
persion ($q^2$, squared factor loading). The least significant difference was calculated at a 5% level of significance (LSD$_{0.05}$).

**Results.** Long study of wild tuber producing plants of *Tuberarium* section of *Solanum* genus has revealed numerous sources of resistance to a wide range of pathogens (fungi and oomycetes, bacteria, viruses and nematodes) and unfavorable environment factors (ground frosts, temperature increase, drought), which appeared due to natural selection pressure [32, 33].

In view to creation of valuable sources of resistance to pathogens, in our work we used in hybridization potato varieties and interspecific hybrids from the VIR collection (St. Petersburg, Russia). These were Mors (277. 1/24 × III.74.562/3 N), resistant to wart disease, golden potato nematode, relatively resistant to viral diseases; Post 86 (I177196/190 × Polesye pink), resistant to wart disease, moderately resistant to scab, bacterial rot, phytophthora rot; hybrid 89-1-12 {Preikulskii ranii × [Wilja × (S. andigenum US-W 1793 × S. rubinii k-2890-4)]) × {[Primero-sa × (S. andigenum US-W 1793 × S. rubinii k-2890-4)] × (Suna × S. stoloniferum k-2490-5)] × (S. demissum k-1539 × S. vernei D 459), resistant to phytophthora rot, nematode, viruses X and Y; Sagitta [Schwalbe × (S.adg. 54/3/14 × Oberar-mbacher Frahe)], resistant to wart disease, potato nematode (Rol), virus X; Baszta (PW 31 × Granola), resistant to wart disease, golden potato nematode, relatively field resistant to phytophthora rot, is poorly affected by potato scab, resistant to virus Y. All in all, 19 hybrids obtained in Kemerovo Agricultural Research Institute in 2014-2018 tested. These hybrids are deposited to the collection of the institute as 27-7c-11, 12-7c-11 (Lyubava × Mors); 22103-10 (Lazar × 89-1-12); 175-10 (Alpinist × Adretta); 5-20c-12, 9-20c-12 (Nikulinskii × Belorussskii 3); 1-5-12 (Lazar × Karlena); 15-13c-11 (Udalets × Garant); 9-14-12, 141-13 (Nakra × pollen mix Udacha variety + 180-1 + 89-1-12); 11-13 (Nikulinskii × Karlena); 81-13 (Lyubava × Sagitta); 161-13 (Zarevo × Karlena); 84-13 (Lyuba- vava × Sagitta); 6-14-11 (Tuleevskii × Post 86); 3-21c-11 (Tuleevskii × Mors); 3-11-11 (Bora valley × Avrora); 1615-10 (Nevskii × Zhukovskii ranii); 17-5/6-11 (Baszta × 89-1-12). The use of interspecific hybrids allowed us to produce potato forms and varieties resistant to bacterial disease, phytophthora rot, nematodes and viruses [34].

**Field resistance of potato hybrids to diseases.** As per CMEA International Classification of potato species of *Tuberarium* (Dun.) Buk. section of *Solanum* L. genus [20], the scale we used is applicable to generalized estimation of perennial data of field resistance of breeding material. We annually recorded plant diseases in field conditions (breeding nurseries) due to natural infection; whereas 2014 and 2016 were the years of epiphytoty furthered by high temperatures in combination with overwetting during the second period of vegetation.

Based on the perennial visual survey of plants for fungal diseases caused by *Phytophthora infestans* (Mont.) de Bary (phytophthora rot), *Alternaria solani* (Ell. et Mart.) Sor. (Alternaria blight), *Fusarium oxysporum* Schlecht. (Fusarium blight), *Rhizoctonia solani* J.G. Kühn (rhizoctonia disease) and *Actinomycyes sace-bies* Gussow (potato scab) [18], we can say that samples with complex resistance of 7 points and more are of selective value (Table 1). Potato hybrids 3-21c-11, 1615-10, 6-14-11, 22103-10, 17-5/6-11, 1-5-12, 27-7c-11, 12-7c-11, 175-10, 5-20c-12, 9-20c-1, 15-13c-11, 9-14-12, 141-13, 81-13, 161-13, 84-13, 3-11-11 and standard varieties Lyubava, Tuleevskii and Nevskii displayed high and extremely high resistance to fungal diseases (7-9 points ) on the average during years of testing in field conditions. All samples listed in Table 1 with the exception of 11-13 hybrid (Nikulinskii × Karlena) with moderate resistance to rhizoctonia disease showed high and very high tuber resistance to phytophthora rot, rhizoctonia disease and potato scab.
1. Scores of potato (*Solanum L.*) hybrid resistance to diseases as compared to standard varieties (st) (Kemerovo Province, 2014-2018, natural infection load)

<table>
<thead>
<tr>
<th>Variety, hybrid</th>
<th>Origin</th>
<th>Resistance to diseases, points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fusarium blight</td>
</tr>
<tr>
<td>Lyubava (st)</td>
<td>9 9 9</td>
<td>7 7 9 7</td>
</tr>
<tr>
<td>Nevskii (st)</td>
<td>9 9 9</td>
<td>8 7 9 9</td>
</tr>
<tr>
<td>Tuleevskii (st)</td>
<td>9 9 8</td>
<td>7 9 8 9</td>
</tr>
<tr>
<td>3-21c-11</td>
<td>Tuleevskii × Mors</td>
<td>9 9 9</td>
</tr>
<tr>
<td>1615-10</td>
<td>Nevskii × Zhukovskii ranii</td>
<td>9 9 9</td>
</tr>
<tr>
<td>6-14-11</td>
<td>Tuleevskii × Post 86</td>
<td>9 8 9</td>
</tr>
<tr>
<td>22103-10</td>
<td>Lazar × 89-1-12</td>
<td>9 9 8</td>
</tr>
<tr>
<td>17-5/6-11</td>
<td>Baszta × 89-1-12</td>
<td>9 9 8</td>
</tr>
<tr>
<td>1-5-12</td>
<td>Lazar × Karlena</td>
<td>9 8 8</td>
</tr>
<tr>
<td>27-7c-11</td>
<td>Lyubava × Mors</td>
<td>8 8 9</td>
</tr>
<tr>
<td>12-7c-11</td>
<td>Lyubava × Mors</td>
<td>9 9 8</td>
</tr>
<tr>
<td>175-10</td>
<td>Alpinist × Adretta</td>
<td>8 9 9</td>
</tr>
<tr>
<td>5-20c-12</td>
<td>Nikulenskii × Belorusskii 3</td>
<td>9 9 9</td>
</tr>
<tr>
<td>15-13c-11</td>
<td>Udalez × Granat</td>
<td>8 9 7</td>
</tr>
<tr>
<td>9-20c-12</td>
<td>Nikulenskii × Belorusskii 3</td>
<td>9 9 9</td>
</tr>
<tr>
<td>9-14-12</td>
<td>Nacra × pollen mix (Udacha + 180-1 + 89-1-12)</td>
<td>9 9 9</td>
</tr>
<tr>
<td>141-13</td>
<td>Nacra × pollen mix (Udacha + 180-1 + 89-1-12)</td>
<td>9 9 9</td>
</tr>
<tr>
<td>11-13</td>
<td>Nikulenskii × Karlena</td>
<td>9 9 9</td>
</tr>
<tr>
<td>81-13</td>
<td>Lyubava × Sagitta</td>
<td>9 9 8</td>
</tr>
<tr>
<td>161-13</td>
<td>Zarevo × Karlena</td>
<td>9 9 8</td>
</tr>
<tr>
<td>84-13</td>
<td>Lyubava × Sagitta</td>
<td>8 8 7</td>
</tr>
<tr>
<td>3-11-11</td>
<td>Bora valley × Avrora</td>
<td>9 9 7</td>
</tr>
</tbody>
</table>


Susceptibility of potato hybrids to viral infections and nematodes. More than 40 viruses were described, which affect potato in natural conditions [35]; however, potato virus Y (PVY) is deemed the most dangerous and widespread [36, 37]; potato virus X (PVX) also causes significant damage [38, 39]. The DNA markers associated with genes of resistance to diseases and pests significantly improve the efficiency of selection of valuable genotypes during early stages [25] and intensify search for such genotypes [40-42]. Multiplex PCR is a new methodology of simultaneous testing varieties and lines for several genes controlling resistance to viruses and nematodes based on DNA markers [11, 43]. Table 2 shows markers and the corresponding diagnostic fragment sizes used in the study.

2. R-genes and associated DNA markers used for molecular screening of potato (*Solanum L.*) samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Trait</th>
<th>DNA marker (diagnostic fragment size, bp)</th>
<th>Ссылка</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ry1adg</td>
<td>11</td>
<td>Immunity to potato virus Y (PVY)</td>
<td>RYSC3 (321)</td>
<td>[22]</td>
</tr>
<tr>
<td>Ry1chc</td>
<td>7</td>
<td>Immunity to PVY</td>
<td>Ry186 (587)</td>
<td>[23]</td>
</tr>
<tr>
<td>Ry1sto</td>
<td>12</td>
<td>Immunity to PVY</td>
<td>YES3-3A (341)</td>
<td>[24]</td>
</tr>
<tr>
<td>H1</td>
<td>5</td>
<td>Resistance to <em>Globodera rostochiensis</em> pathotypes Ro1, Ro4</td>
<td>TG689 (141)</td>
<td>[25]</td>
</tr>
<tr>
<td>H1</td>
<td>5</td>
<td>Resistance to <em>G. rostochiensis</em> pathotypes Ro1, Ro4</td>
<td>57R (452)</td>
<td>[26]</td>
</tr>
<tr>
<td>H1</td>
<td>5</td>
<td>Resistance to <em>G. rostochiensis</em> pathotypes Ro1, Ro4</td>
<td>N195 (337)</td>
<td>[11]</td>
</tr>
<tr>
<td>Gro1-4</td>
<td>7</td>
<td>Resistance to <em>G. rostochiensis</em> pathotypes Ro1, Ro4</td>
<td>Gro1-4-1 (602)</td>
<td>[11]</td>
</tr>
</tbody>
</table>

The resolving power of capillary electrophoresis is sufficient for identification of fragments similar in length, inter alia due to fluorophores varying in spectrum. The genetic analyzer used in the study (Nanofor 05) with high sensitivity allows us to determine the amplicon size at single nucleotide accuracy. The molecular and genetic analysis that we performed identified the markers of dom-
inant alleles of genes for PVY resistance \( R_y \) and golden potato nematode resistance \( H1 \) (for three markers, TG689, 57R and N195) in 17-5/6-11 hybrid (Table 3). Furthermore, in 22103-10 and 3-21c-11 samples we revealed YES3-3A marker linked to \( R_y \) gene and TG689, 57R, and N195 markers identifying \( H1 \) gene, as well as Gpa2-2 marker of \( Gpa2 \) gene (see Table 3). The 1-5-12 hybrid carried the combination of dominant allele genes \( H1 \) (for all three markers), \( Gro1-4 \) which controls resistance to \( G. \) rostochiensis, \( Gpa2 \) encoding resistance to \( G. \) pallida, and also \( R_y \) and \( R_y \) genes for PVY resistance. In 6-14-11 hybrid genotype we detected two markers, 57 R and N 195, for \( H1 \) gene controlling resistance to \( G. \) rostochiensis.

3. Detection of \( R \)-genes controlling resistance to pathogens and nematode pests in potato (\( S. \)olanum L.) standard varieties (st) and obtained hybrids by DNA markers

<table>
<thead>
<tr>
<th>Diseases agent</th>
<th>( R )-gene</th>
<th>DNA marker</th>
<th>Lyubava (st)</th>
<th>Nevskii (st)</th>
<th>Nuleevskii (st)</th>
<th>17-5/6-11</th>
<th>16-15-10</th>
<th>3-21c-11</th>
<th>22103-10</th>
<th>6-14-11</th>
<th>1-5-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato virus Y (PVY)</td>
<td>( R_y )_adg</td>
<td>RYS/C3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>( R_y )hc</td>
<td>Ry186</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( R_y )sto</td>
<td>YES3-3A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>( G. ) rostochiensis</td>
<td>( H1 )</td>
<td>TG689</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>57R</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N195</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>( G. ) pallida</td>
<td>( Gro1-4 )</td>
<td>Grol-4-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( Gpa2 )</td>
<td>Gpa2-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note. 0/+ means presence or absence of the gene marker.

Crop yield, environmental plasticity and stability of potato hybrids. We evaluated the crop yield and resistance to unfavorable factors in 3-21c-11, 1615-10, 6-14-11, 22103-10, 17-5/6-11 and 1-5-12 hybrids which stood out in terms of complex resistance to PVY, golden potato nematode and fungal diseases (Table 4). The results show that the impact of the environment on potato crop yield amounts to 55.9 %, and genotype contribute 11.5 %.

4. Crop yield (t/ha) stability and plasticity in potato (\( S. \)olanum L.) hybrids depending on environmental conditions in different year as compared to standard varieties (st) (Kemerovo Province)

<table>
<thead>
<tr>
<th>Variety, hybrid</th>
<th>2014</th>
<th>2015</th>
<th>2016</th>
<th>2017</th>
<th>2018</th>
<th>Early maturity group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyubava (st)</td>
<td>16.7</td>
<td>12.4</td>
<td>17.7</td>
<td>20.0</td>
<td>24.2</td>
<td>Late maturity group</td>
</tr>
<tr>
<td>3-21c-11</td>
<td>25.2</td>
<td>12.7</td>
<td>20.7</td>
<td>22.3</td>
<td>24.2</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>1615-10</td>
<td>22.4</td>
<td>12.8</td>
<td>21.6</td>
<td>21.2</td>
<td>27.6</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>6-14-11</td>
<td>30.4</td>
<td>15.9</td>
<td>24.7</td>
<td>20.0</td>
<td>22.3</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>Early maturity group</td>
<td>23.9</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>Nevskii (st)</td>
<td>26.3</td>
<td>15.0</td>
<td>22.8</td>
<td>21.7</td>
<td>22.8</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>17-5/6-11</td>
<td>28.3</td>
<td>15.3</td>
<td>26.1</td>
<td>26.1</td>
<td>31.1</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>Tuleevskii (st)</td>
<td>13.8</td>
<td>15.3</td>
<td>19.7</td>
<td>20.3</td>
<td>21.1</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>22103-10</td>
<td>30.1</td>
<td>16.4</td>
<td>24.7</td>
<td>26.6</td>
<td>20.8</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>1-5-12</td>
<td>27.8</td>
<td>13.9</td>
<td>18.5</td>
<td>23.1</td>
<td>25.8</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>( \Sigma x_j )</td>
<td>221.0</td>
<td>129.7</td>
<td>196.5</td>
<td>201.3</td>
<td>228.0</td>
<td>Mid-season group</td>
</tr>
<tr>
<td>( x_i )</td>
<td>24.6</td>
<td>14.4</td>
<td>21.8</td>
<td>22.4</td>
<td>25.3</td>
<td>Mid-season group</td>
</tr>
</tbody>
</table>

Note. \( Cv \) — variation coefficient, \( Y_j \) — averaged variety crop yield on the j-the test year, \( b_i \) — regression coefficient, \( S_i^2 \) — stability coefficient, \( \Sigma x_j \) — sum of crop yields of all varieties on the j-th test year, \( x_i \) — averaged crop yield of all varieties on the j-th test year.

Potato (\( S. \) tuberosum) is believed to be rather sensitive to the combination of high temperatures and precipitation deficit. The droughts that become
more frequent threaten stable production of the crop, therefore, a possibility of complex phenotypic response of potato plants to drought [44, 45] and genetic foundations of potato tolerance to such conditions [46] are being studied globally. Plant response to water availability is determined by biologic properties of plants and by other factors. Among other things, it depends on genotype × environment interaction resulting in protective response at the level of functions of leaf stomatal apparatus [47]. We compared annual environmental indices (I_j) in order to characterize environmental factors in places where the genotypes were assessed. In 2014, 2015, 2016, 2017 and 2018, I_j value was 2.9; -73; 0.1; 07 and 3.6, respectively.

Based on the calculations the conditions that can be deemed most favorable were in 2014 and 2018. During these years the average crop yield for the test group was the highest, 24.6 and 25.3 t/ha, respectively. In 2014, the crop yield was the highest in hybrids 22103-10 of mid-season group (30.1 t/ha which is 16.4 t/ha higher than 13.8 t/ha for Tuleevskii standard variety), and 6-14-11 of early maturity group (30.4 t/ha which exceeded the 13.7 t/ha yield of Lyubava standard variety). In the early-season group reliable increase of crop yield compared to the standard was demonstrated by hybrids 1615-10 (by 5.7 t/ha) and 3-21c-11 (by 8.5 t/ha) (LSD_{0.05} = 0.77 t/ha). The mid-season 1-5-12 hybrid in 2014 showed almost 2 times higher yield (27.8 t/ha) than Tuleevskii standard variety. In 2018, at LSD_{0.05} = 1.0 t/ha, the most productive hybrids were 1-5-12 (25.8 t/ha vs. 21.1 t/ha for Tuleevskii standard), 17-5/6-11 (31.1 t/ha vs. 22.8 t/ha for Nevskii standard) and 3-21c-11 (32.3 t/ha vs. 24.2 t/ha for Lyubava standard). In years with favorable weather conditions, all of these hybrids exceeded standards by 16.0 to 31.5% in terms of average crop yield for 5 years. Yield variability (Cv) for varieties and hybrids was from 18.1 to 31.4%, where Tuleevskii variety showed the lowest value in general for the test group, which proves its plasticity and stability, whereas 3-21c-11 hybrid showed the highest variability.

Potato plants interact both with abiotic and biotic factors. As a result of effect of a combination of factors the metabolism changes differently than under the effect of each factor individually (which additionally depends both on the nature of the effect and on biological peculiarities of the genotype) because molecular signaling pathways that control abiotic and biotic tension can manifest both synergism and antagonism. Abiotic tensions intensify plant stress and can cause cell damage, which negatively affects potato crop yield, quality and market value of tubers [7, 48].

We evaluated the dependence of crop yield in potato varieties and hybrids on external factors using regression coefficient b_i for plasticity and stability coefficient S_i^2 for stability as related to the dispersion of character deviations of each sample from regression line. The calculations showed low plasticity (b_i < 1) of Lyubava (b_i = 0.81), Nevskii (b_i = 0.88) and Tuleevskii varieties (b_i = 0.28). They poorly respond to the cultivation conditions, which is due to extensive genotypes. Among extensive varieties Nevskii displayed high stability (S_i^2 = 4.43), which guarantees high annual crop yields. The extensive hybrid group also included 6-4-11 (b_i = 0.91) and 22103-10 (b_i = 0.84) hybrids. 3-21c-11 hybrid (b_i = 1.53 > 1) stood out in terms of plasticity. It belongs to the intensive type due to high response to cultivation conditions. We qualified 3-21c-11 hybrid as belonging to genotypes with average crop yield stability (S_i^2 = 14.6).

Hybrids with b_i > 1, whereas S_i^2 approaches zero, are of the highest value. Among the analyzed genotypes, 17-5/6-11 produced the most stable crop yield (S_i^2 = 1.1) although its responsiveness to cultivation environment
was high \((b_1 = 1.38)\). The hybrids 1615-10 \((b_1 = 1.17, S_1^2 = 5.2)\) and 1-5-12 \((b_1 = 1.20, S_1^2 = 9.4)\) with high crop yield stability were recognized in the intensive genotype group.

So we revealed the following extensive type genotypes: Lyubava \((b_1 = 0.81)\), Nevskii \((b_1 = 0.88)\), Tuleevskii \((b_1 = 0.28)\) varieties, 6-4-11 \((b_1 = 0.91)\) and 22103-10 \((b_1 = 0.84)\) hybrids. Hybrid 3-21c-11 \((b_1 = 1.53; S_1^2 = 14.6)\) is a genotype of intensive type with average crop yield stability. Intensive potato hybrids with increased environmental plasticity and crop yield stability, i.e. 17-5/6-11 \((b_1 = 1.38; S_1^2 = 1.1)\), 1615-10 \((b_1 = 1.17; S_1^2 = 5.2)\) and 1-5-12 \((b_1 = 1.20; S_1^2 = 9.4)\), are of the highest value. All hybrids of extensive and intensive types with improved environmental plasticity and stability are relatively resistant \((7-9)\) points to fungal diseases (phytophthora rot, Alternaria blight, Fusarium blight, rhizoctonia disease and potato scab). Hybrid 17-5/6-11 is the donor of resistance to potato virus Y \((R_y\_chc\) gene) and golden potato nematode \((H_1\) gene revealed with three markers, TG689, 57R and N195). Hybrids 22103-10 and 3-21c-11 can be donors of resistance to potato virus Y \((R_y\_chc\) gene), golden potato nematode \((H_1\) gene) and pale nematode \((Gpa\_2\) gene). Hybrid 1-5-12 combines dominant alleles of \(H_1\) and \(Gro\_1\_4\) genes controlling resistance to golden potato nematode, \(Gpa\_2\) gene for resistance to pale nematode, and genes for PVY resistance \(R_y\_chc\) and \(R_y\_sto\).

REFERENCES

12. Rogozina E.V., Terent'eva E.V., Potokina E.K., Yurkina E.N., Nikulin A.V., Alekseev Ya.I. Multiplex PCR-based identification of potato genotypes as donors in breeding for resistance to


ANALYSIS OF MYCOBIOME IN DAMAGED POTATO (Solanum tuberosum L.) LEAVES BY USING METAGENOMIC APPROACHES

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A b s t r a c t

The problem of potato diseases caused by fungi and fungi-like organisms is relevant for all regions of the world cultivating this crop, since it is mycoses that cause the most significant damage to plants (A. Bernreiter, 2017). The traditional approaches for identification of potato pathogens are aimed at identifying a specific pathogen and do not take into account neither other, often unknown pathogens, nor the other most important component — the beneficial microbiota of the phyllosphere community whose alterations can also become one of the causes of diseases. The novelty of this work lies in the fact that the high-throughput sequencing methods we used here is free of this disadvantage and makes it possible to identify virtually all plant microorganisms, including the phyllosphere and endosphere. The purpose of this study was to use metagenomic approaches to analyze the total fungal and fungi-like community in potato leaves that have morphological markers of damage by pathogens of the genus Alternaria and Phytophthora which are the causative agents of early blight and late blight. For fungal and fungi-like communities analysis, DNA samples was extracted from leaves of potato (Solanum tuberosum L.) cultivar Nikulinsky, affected by “alternaria” and “phytophthora” diseases types, which were later used to create amplicon libraries of ITS1 and ITS2 fragments and high-throughput sequencing on the Illumina MiSeq platform (Illumina, Inc., USA). During the bioinformatic data processing with the Illumina software and the PIPITS software package (H.S. Gweon et al, 2015), 187 OTU, 113 phylotypes for the ITS1 library and 249 phylotypes for ITS2 were identified. Subsequent annotation of OTU and taxonomic analysis of the resulting communities were carried out with the QIME program (J.G. Caporaso et al., 2010), the diversity coefficients within the community were calculated using the PAST software package (O. Hammer et al., 2001). Comparison of the fungal communities obtained for both types of lesion using different universal primers for the ITS1 regions (M. Usyk et al., 2017) and ITS2 (T.J. White et al., 1990) showed that only the first pair is suitable for the detection of phytophthora, and in general gives a more even community structure. The tools of automatic annotation turned out to be insufficient for objective identification of alternaria in samples, as a result we had to use the methods of manual search with the BLASTn program (S.F. Altschul et al., 1990). Since the primer pair ITS2 does not allow identification of Phytophthora in the samples, the further comparative analysis of the fungal communities of the two types of lesion was carried out using data only from the ITS1 library. The data of taxonomic analysis showed that in the affected areas for both types of mycoses a rich fungal community is formed, and, in the case of “late blight”, the fraction of the pathogen is about 30 % in the community, and in the variant with “early blight”, only 2.07 % with a significant part (about 15 %) accounted for by Phytophthora, which does not exclude the case of secondary lesion. Thus, it was shown that in the fungal and fungi-like communities formed in the areas affected by disease, the proportion of pathogens is no more than 30 %, which indicates a pronounced dynamics of the taxonomic composition of fungi in the affected area. It is obvious that high-throughput sequencing methods have a very high po-
Potential in fundamental and applied research on plant diseases of a microbiological nature.

Keywords: fungi, fungi-like organisms, pathogens, potato, Phytophthora infestans, Alternaria sp., high throughput sequencing

Potato is an essential agricultural crop actively cultivated in many regions of the world; therefore, the problem of losing potato crops due to various diseases is relevantly high both in Russia and abroad. Plant diseases can be caused by bacteria, viruses, fungi and fungus-like organisms. Mycoses and lesions are characterized by the most severe course of the disease where representatives of fungus-like microbial flora, and oomycetes in particular [1], act as pathogens. The pathologies caused by them consist in manifestation of mildew, spotting and molds of fruit and seeds [2].

Plant leaf is an ecological niche populated by a community of microorganisms, including fungi and fungus-like organisms of phylloplane, endophytes and phytopathogens. Any imbalance in the community due to changing weather conditions, chemicals in the soil or for other reasons can result in pathogen domination and disease development [3, 4]. The useful inhabitants of phyllosphere which increase resistance to harmful representatives of microbial flora are diazotrophs, antagonists and bacteria ensuring plant growth [3]. The pathogenic organisms inhibiting the phylloplane represent potential threat for the host and can belong both to biotrophs and necrotrophic pathogens [3, 4]. The saprophytes affecting weakened plants, e.g. Fusarium fungi, can develop as the secondary infection masking the primary pathogen.

Phytophthora rot is one of the most dangerous potato diseases in the regions with humid and moderate climate, which is caused by Phytophthora infestans (Mont.) de Bary oomycete [5]. This pathogen affects the leaves reducing the assimilatory activity of the plant during tuber formation and provokes their further decay during storage [6]. The spread of phytophthora rot at the end of 1840s in Europe, especially in Ireland, resulted in loss of potato crops, which caused the Great Irish famine (Irish potato famine) [7]. In Russia and Europe the damage caused by phytophthora rot (depending on edaphoclimatic conditions of the region) can be from 10-12% to 50% of the entire potato crop yield [8, 9]. Alternaria imperfect fungi cause potato blight, which poses a serious threat for the regions with more arid climate characterized by presence of short-term precipitations and abundant night dew [4, 5]. Alternaria also affects leaf surface reducing assimilatory activity of the plant and resulting in crop yield loss of up to 40%. Furthermore, alternaria causes reduction of potato starch content, increases the share of non-saleable tubers [5, 6, 10] and causes accumulation of mycotoxins and allergens [2].

Saprophytic fungi of Fusarium genus cause fusarial potato wilt. Smirnov et al. [11] showed that pathogenic Fusarium-Alternaria complex becomes one of the reasons behind potato lodging in different regions of Russia and can emerge as primary and secondary lesion after rhizoctonia solani, phytophthora rot or bacteriosis. Earlier fusarial rot and early blight were widespread in southern regions, but now these diseases became typical for the European part of the country [4, 5, 11].

Early blight and phytophthora rot are widespread in all potato farming regions, and scientists in Russia and abroad analyze how they can be countered. In particular, the studies of phylloplane [3, 12], endophytic [13, 14] and edaphic fungal communities are known [15, 16], as well as studies of plant sections directly affected by mycosis [17, 18]. As a result, the pathogens of phytophthora rot, early blight and other mycological diseases have been identified. The effect of various agricultural methods [15] and fungicides [8, 10, 19], which can be
used in combination with the other means [20], e.g. with culture liquid of *Klebsiella planticola* [21], on pathogens is actively researched. The resistance of a number of potato varieties to pathogens of early blight and phytophthora rot has been studied [9, 22].

It is critical to properly identify pathogens in order to effectively combat them [23]. It is known that early blight pathogens are a complex of alternaria fungi where fungal forms with small and large spores [10] are distinguished by ecological properties, host specificity, pathogenicity, toxicogenity, sensitivity to fungicides, and geographic distribution [2, 4]. For identification, cytological methods are traditionally used, along with microscopy and isolation of pure cultures [1, 24], as well as specific symptoms on affected plants [1, 25], moist chamber method to stimulate formation of infected structures, and checking pathogenicity on susceptible plants. Furthermore, spectroscopy [25-27], mass spectroscopy [1], biosensor diagnostics [25], molecular methods based on polymerase chain reaction [28-30], cloning followed by Sanger sequencing with universal primers [17, 31] and enzyme-linked immunosorbent assay [32] are modern methods.

However, as has been noted above, it is important to analyze the community in general, including imbalance detection in phylloplane communities. This task is beyond the scope of methods focused on identification of a specific pathogen and not taking into account the other pathogenic organisms and the condition of normal nonpathogenic microbial flora, whose plant protection function is often underestimated. High throughput sequencing [3, 16, 33] provides an opportunity to evaluate not only known and unknown pathogens, for which no diagnosticums have been created to date, but also the normal flora. We selected two pairs of universal primers to ITS1 and ITS2 regions [35, 36] among primers developed for the creation of fungal amplicon libraries [23, 33, 34], which enable taxonomic identification of most fungal groups and fungus-like organisms to genus and even species [16, 37, 38].

In this study, for the first time we analyzed pathogenic and nonpathogenic fungal and fungus-like microbial flora using metagenomic approach on potato plants affected by diseases of early blight type and phytophthora rot type. We have assessed the presence and absence of the main pathogen and taxonomic structure of the entire community in the affected leaf area. It was shown for the first time that the actual pattern of the community is of complex and not completely definitive nature (e.g. the primary pathogen can be a minor component of the entire community). Taxonomic analysis of communities with both primers revealed pathogenic *Alternaria* fungus in a sample with signs of early blight type. Primers to ITS1 region helped identify oomycete *Phytophthora* for both lesion types.

Our subjective was to fully identify species composition of fungi and fungus-like organisms involved in potato leaf phytopathogenesis by high throughput sequencing and two universal primers.

**Techniques.** Leaf samples of potato (*Solanum tuberosum* L.) Nikulinsky cultivar with visible symptoms were collected in experimental field of Vavilov Russian National Plant Genetics Resources Institute (St.Petersburg—Pushkin, 59°42′37.78″C; 30°25′41.26″B) in 2017. Two types of lesions were identified based on morphological characters.

DNA was extracted directly from the affected leaf section using AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer’s instruction.

Taxonomic composition of fungal community and fungus-like organisms was identified in each sample with amplicon libraries of intergenic transcribed
spacers of ribosomal operons (ITS1 and ITS2). The diagnostic fragment was amplified in PCR (T100 Thermal Cycler, BIO-RAD Laboratories, Inc., USA) using following primer pairs: ITS1_30F-GTCCCTGCCCTTTGTACACA/ITS1_217R-TTTCGCTGCGTTCTTCATCG for ITS1 [35], ITS3-GCATCGATGAAGAACGCAGC/ITS4-TCCTCCGCTTATTGATATGC for ITS2 [36] with addition of service sequences as per the protocol of Illumina, Inc. (USA) containing linkers and barcodes. Phusion Hot Start II High-Fidelity polymerase (Thermo Fisher Scientific, USA) was used in PCR according to the manufacturer’s protocol.

PCR products were purified with AMPure XP (Beckman Coulter, USA) as per Illumina, Inc. recommendation. The libraries were further prepared in compliance with the manufacturer’s instructions (MiSeq® Reagent Kit Preparation Guide, USA). The libraries were sequenced using Illumina MiSeq and MiSeq® Reagent Kit v3 (600-cycles) (Illumina, Inc., USA) with bilateral reading (2×300 nt).

The identified sequences were processed with Illumina software (Illumina, Inc.), QIIME software [39] and PIPITS software [40].

Data of sequencing were used for taxonomic profiling and comparing variants for ITS1 and ITS2 primers. During the analysis the representation of different taxa as well as abundance of communities were analyzed. Parameter reflecting the number of taxa (richness, i.e. the expected number of phylotypes), Simpson index (evenness, i.e. even distribution by phylotypes) and Shannon index were calculated using PAST software [41].

**Results.** Based on the symptoms on potato leaves lesions were classified as diseases of early blight or phytophthora rot types (Fig. 1).

Common primers to ITS2 region [36] and wide-range primers to ITS1 region [35] were used (Fig. 2) in high throughput sequencing to identify pathogen species in the affected zone. Both lesion types were evaluated for each region in terms of composition and fungal and fungus-like community.

The number of reads (sequences) for samples obtained with a pair of primers for ITS1 with early blight and phytophthora rot signs amounted to 55000 and 54000 respectively, for ITS2 — 36000 and 21000. Processing of ITS1 and ITS2 libraries was performed separately, because OUT (operational taxonomic units) isolation can be performed only for homolog libraries. Bioinformatic processing for ITS1 resulted in 187 OUT and 113 phylotypes, for ITS2 — in 249 OUT and 127 phylotypes.

After automatic OTU annotation (UNITE databank, https://unite.ut.ee/, PIPITS software) a large group of OTU was taxonomically attributed only at kingdom level. Furthermore, a significant amount of *Alternaria* fungus (0.01 and 0.08% for ITS1, and 0.07 and 0.04% for ITS2 in variants with early blight and phytophthora rot signs, respectively) were not found in libraries for both primer pairs; moreover, phytophthora was not detected using ITS2 library. For this reason,
unannotated OTU were checked manually for taxonomic affiliation with BLASTn program [42]. The issue of incorrect OTU taxonomic diagnosis is mentioned by Halwachs et al. [43] who further recommend to manually adjust data with BLAST-based algorithm to clarify the results of automatic annotation. Some of the reasons why proper OTU are unannotated may be insufficient number of reference sequences in databases or different taxonomic resolution by ITS for different groups of fungi at genus and species levels [37].

The communities of fungi and fungus-like organisms were generally evaluated based on automatic taxonomic diagnosis. When comparing the results obtained for two regions we observed differences in taxonomic composition both among communities characteristic of two lesion types and among libraries obtained using primers to ITS1 and ITS2 (Table 1). For further comparison we used only the taxa, the share of which at least in one of the libraries amounted to more than 1% (see Table 1).

The differences in specificity of the primers we chose were observed as early as at the large taxon level (Fig. 3). For instance, broadly specific primers to ITS1 captured representatives of Oomycota phylum, which includes phytophthora rot; however, a significant amount of reads corresponded to plant organisms. This coincides with features of ITS1 primers described by Xu [16]. In turn, the primers to ITS2 that we selected captured only representatives of the fungal kingdom without identifying plant homologs. However, they demonstrated a more narrow specificity as compared to the ITS1 pair, resulting in lack of oomycetes in libraries, which makes this primer pair not suitable for phytophthora rot identification in affected samples.

To evaluate the percent of pathogens in the resulting communities, the results of automatic annotation for UNITE database and sequence check in BLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were summarized (Table 2). The table was used to evaluate the efficiency of selected primers to identify the pathogens in question.
1. Taxonomic diagnosis of main operational taxonomic units (OTU) of fungal and fungus-like organisms in potato leaves (Solanum tuberosum L. cultivar Nikulinsky) with lesions of phytophthora rot and early blight types (experimental fields of Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg—Pushkin, 59°42’37,78”N; 30°25’41,26”E, 2017)

<table>
<thead>
<tr>
<th>Taxonomic assignment</th>
<th>OUT percent in the community</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Altern_Its1</td>
</tr>
<tr>
<td>k_Fungi; Other; Other; Other; Other</td>
<td>29.45</td>
</tr>
<tr>
<td>o_Capnodiales; f_Cladosporiaceae; g_Cladospore</td>
<td>0.03</td>
</tr>
<tr>
<td>k_Fungi; p_Ascomycota; e_Dotheideomycetes; o_Capnodiales; Other; Other</td>
<td>0.02</td>
</tr>
<tr>
<td>o_Pleosporales; f_Didymellaceae</td>
<td>1.02</td>
</tr>
<tr>
<td>k_Fungi; p_Ascomycota; e_Dotheideomycetes; o_Pleosporales; f_Phaeosphaeriaceae</td>
<td>1.59</td>
</tr>
<tr>
<td>k_Fungi; p_Ascomycota; e_Dotheideomycetes; o_Pleosporales; Other; Other</td>
<td>0.04</td>
</tr>
<tr>
<td>k_Fungi; p_Ascomycota; e_Sordariomycetes; o_Hypocreales; f_unidentified; g_unidentified</td>
<td>0.00</td>
</tr>
<tr>
<td>k_Fungi; p_Ascomycota; Other; Other; Other; Other</td>
<td>1.26</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Microbotryomycetes; o_Sporidioboliales; f_Sporidiobolaceae; g_Sporobolomyces</td>
<td>0.82</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Cystofilobasidiales; f_Cystofilobasidiaeae; g_Cystofilobasidium</td>
<td>2.70</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Cystofilobasidiales; f_Mrakiaceae; g_Mrakia</td>
<td>0.00</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Cystofilobasidiales; f_Mrakiaceae; g_Itersonilila</td>
<td>18.40</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Cystofilobasidiales; f_Mrakiaceae; g_Udenomyces</td>
<td>0.20</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Filobasidiales; f_Filobasidiaeae; g_Filobasidium</td>
<td>0.22</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Tremellales; f_Bulleraceae</td>
<td>0.03</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Tremellales; f_Bulleraceae; g_Bullera</td>
<td>4.41</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Tremellales; f_Bulleribasidiaeae</td>
<td>0.10</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; o_Tremellales; f_Bulleribasidiaeae; g_Dioszegia</td>
<td>1.18</td>
</tr>
<tr>
<td>k_Fungi; p_Basidiomyctota; e_Tremellomycetes; Other; Other; Other; Other</td>
<td>1.51</td>
</tr>
<tr>
<td>k_Plantae; p_unidentified; c_unidentified; o_unidentified; f_unidentified; g_unidentified</td>
<td>16.68</td>
</tr>
<tr>
<td>k_Stramenopila; p_Oomycota; e_Oomycetes; o_Peronosporales; f_Peronosporales_fam_Incertae_sedis; g_Phytophthora</td>
<td>15.47</td>
</tr>
</tbody>
</table>

Note. The data of high throughput sequencing with MiSeq platform (Illumina, Inc., USA) and an automatic annotation using UNITE database (https://unite.ut.ee/); k — kingdom, p — phylum, c — class, o — order, f — family, g — genus. Altern and Phyty, respectively, are samples with symptoms of early blight and phytophthora rot types; Its1 and Its2 are primers we used to create amplicon libraries.

2. Percentage of pathogens in fungal and fungus-like communities of potato leaves (Solanum tuberosum L. cultivar Nikulinsky) with lesions of phytophthora rot and early blight types as resulted from high throughput sequencing libraries of ITS1 и ITS2 fragments (experimental fields of Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg—Pushkin, 59°42’37,78”N; 30°25’41,26”E, 2017)

<table>
<thead>
<tr>
<th>Community</th>
<th>Percent in the community</th>
</tr>
</thead>
<tbody>
<tr>
<td>by primer type</td>
<td>by lesion type</td>
</tr>
<tr>
<td>ITS1</td>
<td>early blight</td>
</tr>
<tr>
<td>phytophthora rot</td>
<td>0.11</td>
</tr>
<tr>
<td>ITS2</td>
<td>early blight</td>
</tr>
<tr>
<td>phytophthora rot</td>
<td>0.006</td>
</tr>
</tbody>
</table>

3. Diversity in fungal and fungus-like communities of potato leaves (*Solanum tuberosum* L. cultivar Nikulinsky) with lesions of phytophthora rot and early blight types (experimental fields of Vavilov Russian National Plant Genetics Resources Institute, St. Petersburg—Pushkin, 59°42′37″N; 30°25′41″E, 2017)

<table>
<thead>
<tr>
<th>Parameter, index</th>
<th>Altern Its1</th>
<th>Phyth Its1</th>
<th>Altern Its2</th>
<th>Phyth Its2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of taxa</td>
<td>19</td>
<td>18</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Simpson index</td>
<td>0.81</td>
<td>0.77</td>
<td>0.54</td>
<td>0.43</td>
</tr>
<tr>
<td>Shannon index</td>
<td>1.90</td>
<td>1.87</td>
<td>1.31</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Note. Altern and Phyt are leaves with lesion of early blight and phytophthora rot types, respectively; Its1 and Its2 are primers used to create libraries.

It must be pointed out that major irregularity was observed in ITS2 libraries in presenting taxa, for instance, there was a noticeable bias in the direction of representatives of *Capnodiales* (*Ascomycota*) order (see Table 1), which were predominant in two liaison variants resulting in significant reduction of variety parameter of the resulting community. This can be clearly seen when comparing the Simpson index characterizing the eveness of taxon distribution (Table 3). The same ratios were identified during analysis of the number of taxa or species richness and Shannon index. It is interesting that in reports of other authors [37] who studied the same community using ITS1 and ITS2, the libraries showed rather identical results during annotation of resulting OTE. The degree of result reproducibility was higher for basidiomycetes and lower for ascomycetes, particularly, a large number of clusters were allocated for them in a variant with ITS1. On the contrary, our data show oversaturation of ITS2 libraries with representatives of *Ascomycota* phylum due to *Capnodiales* order members.

When comparing communities at family level, the representatives of *Cladosporiaceae*, *Didymellaceae*, *Phaeosphaeriaceae*, *Cystofilobasidiaceae*, *Mrakiaceae*, *Bulleraceae* and *Bulleribasidiaceae* turned out common for both primer variants. The representatives of *Sporidiobolaceae*, *Filobasidiaceae* and *Peronosporales*, to which *Phytophthora infestans* belongs (see Table 1), were typical only for the ITS1 variant.

Both primer pairs were suitable for identification of alternaria; however, for no explicable reason the automatic identification system did not attribute the fungi of this type and attributed them to the group of unidentified organisms. Nevertheless, by using manual adjustment based on BLASTn data the *Alternaria* genus fungi were identified and, apparently, they account for a noticeable part of the community.

Whereas comparison of ITS1 and ITS2 libraries, and more precisely, of the primers used, demonstrated apparent predominance of the former, we subsequently worked with ITS1 library only.

The data of taxonomic analysis for the sample affected with early blight type showed that unidentified representatives of *Fungi* kingdom were predominant (27.38%). The representatives of *Itersonilia* (18.4 %) and *Phytophthora* (15.47%) genera were rather numerous. The plant ITS accounted for 16.68%. Fungi of *Bullera* (4.41%), *Cystofilobasidium* (2.7%) and *Alternaria* (2.07 %) genera (see Table 3) along with non-attributed representatives of *Tremellomycetes* order (1.51%) and ascomycetes (1.26%), as well as *Dioszegia* (1.18%) genus fungi were observed at family level. Representatives of *Bulleraceae* and *Bulleribasidiaceae* families, as well as *Udeniomyces* and *Filobasidium* genera (see Table 1) were observed in insignificant quantities (below 1% of the community). In the event of phytophthora rot type, two groups of organisms were predominant, i.e. unidentified representatives of *Fungi* kingdom (32.85 %) and oomycetes of *Phytophthora* genus (29.85%). The representatives of *Itersonilia* genus and plant ITS in the community accounted for 8.86 and 8.00%, respectively. They were followed, in
descending order, by representatives of Hypocreales order (3.23%), Sporobolomyces (2.64%), Bullera (2.01%), Udeniomyces (1.59%), Cystofilobasidium (1.46%) and Filobasidium (1.40%) genera. Also, a small number of taxa were observed in the community, whose share did not exceed 1% (see Table 1).

The ITS1 data showed presence of phytophthora rot pathogen in both samples, 15.47% and 29.85% of community, respectively, for early blight and phytophthora rot damage types. The representatives of Alternaria genus were also found, their share was 2.1 and 0.1%, respectively, for early blight and phytophthora rot types (see Table 3).

Regardless of lesion type, significant number of reads fell upon unidentified fungal organisms. The structure of the community obtained for sample with early blight showed presence of two subdominant taxa, Itersonilia and Phytophthora genera. In the phytophthora rot variant the Phytophthora genus acted as the second dominant. Also, both communities showed taxa either not identified in the other community or identified in insignificant quantities. For early blight damage type these were unidentified representatives of Tremellomycetes class and Phaeosphaeriaceae family fungi; for phytophthora rot these were unidentified representatives of Hypocreales order and Sporobolomyces, Udeniomyces and Filobasidium genera.

In studies on pathogens in fungal communities of phyllospheres and potato tubers, the Alternaria genus fungi are observed on affected plants in significant quantities; however, it has to be pointed out that the authors mostly use isolates grown on special nutrient media [12, 17, 18]. The studies of endophytic mycobiota of a healthy plant based on this approach also confirm significant presence of Alternaria genus fungus [13]. At the same time, detailed analysis of high throughput sequencing shows a small share of pathogen in the community (0.35%) [14], and in weakened plant phyllosphere affected by Podosphaera fungus it shows a more significant (0.35–4.6 %) portion. These data demonstrate the advantages of metagenomic approach in similar studies.

Therefore, we have identified serious differences in taxonomic specificity of two primer pairs, ITS1 and ITS2. The ITS1 primers help identify not only more fungal taxa and fungus-like organisms, but also demonstrate higher levels of evenness in distributing the sequences by taxa. However, due to broader specificity, primers to ITS1 capture plant sequences. The automatic taxonomic database diagnosis not always reveal several taxa in the community. This can strongly distort the results of analysis, specifically in cases when certain process or absence of a specific pathogenic organism should be diagnosed. For this reason, combination of several tools to diagnose a target organism can have a positive impact on the results of the research. The community of affected potato leaves of Nikulinsky cultivar turned out quite rich. Interestingly, in case of phytophthora rot the share of pathogen did not exceed 29.9%, which is indicative of the dynamics of taxonomic composition of affected tissues. Apparently, during the first stage of infection there is almost always a major pathogen, and subsequently damaged zones are populated by opportunist microbial flora. Apparently, this explains low numbers of alternaria in the sample corresponding to early blight. There is a good chance that in the described case the presence of phytophthora rot can also be the secondary lesion.

To summarize, the results of taxonomic analysis showed a rather rich community of fungi and fungus-like organisms for lesions of both types, whereas a share of primary pathogen in the community when affected by phytophthora rot type accounts for about 30%, and only 27% when affected by early blight type whereas a significant part (about 15%) can be attributed to phytophthora rot (possibly, due to secondary damage). It is certain that high throughput sequencing
methods show a lot of promise in identifying plant pathogens; however, these methods require significant methodic work both at choosing primers and during analysis of libraries, when certain important taxa can be lost.

REFERENCES


NOVEL SOLID-PHASE MULTIBIORECYCLED BIOLOGICS
BASED ON Bacillus subtilis AND Trichoderma asperellum AS EFFECTIVE
POTATO PROTECTANTS AGAINST Phytophthora DISEASE

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A b s t r a c t

A total of 17 biologics based on the producer strains Bacillus subtilis and Trichoderma asperellum (= T. harzianum) are currently approved in Russia to protect potatoes from diseases. Great world experience has been gained in producing and use of traditional dry and liquid biologics. However, multirecycled industrial wastes as substrates for biologics are still not used anywhere in the world, and there is little information on effectiveness of formulations produced by industrial wastes’ multistage bio Recycling. This paper reports a successful experience of the sequential use of plant wastes as substrates for mushrooms and then for microbial strains to produce granular antifungal biologics. This is a relevant approach to biotechnologies for safer utilization of wastes as resources of cheap and affordable raw materials and their transformation into useful products. Our objective was to develop brand new multirecycled biologics based on plant pathogen antagonists and to estimate their efficacy. Plant wastes were converted to substrates for B. subtilis B-10 and T. asperellum T-36 producer strains by shiitake Lentinula edodes (Berk.) Pegler and oyster mushroom Pleurotus ostreatus (Jacq.: Fr.) P. Kummer HK-35 serial cultivation. The nutritional value of the obtained double biorecycled substrate, due to decomposition of cellulose and lignin of sawdust and wheat bran mixture by shiitake and oyster mushroom, was higher as compared to that of the initial substrate used for shiitake growing or of peat, a common solid-phase fermentation substrate. In particular, the protein content was higher (9.4±0.3 % vs. 2.7±0.3 % and 4.3±0.1 %, respectively), the nitrogen level was higher (1.5±0.3 % vs. 0.4±0.1 % and 0.6±0.1 %), and the C:N ratio reduced (38.3 vs. 81.2 and 92.9). Liquid microbial inoculums were cultured in standard Czapek (Biocompas -С Ltd., Russia) and corn-molasses (Carguil Ltd., Agroresource Ltd., Russia) nutrient media. Solid-phase fermentation of the double biorecycled lignin- and cellulose-containing substrate inoculated with 0.9½10^9 spores/ml B. subtilis B-10 and 2.8½10^10 CFU/ml T. asperellum T-36 to produce the biologics took 10 days at 25-28 °C. The obtained biologics were tested on potato cv. Elizaveta in plot trials in the Leningrad Province (Producers’ Cooperative Shushary, 2011). A reciprocally orthogonal scheme was used, and the plots were arranged in 4 replicates over 0.5 ha, with 10 m² test plot size and 482 plants sampled in total. A single application was performed at planting on May 12, 2011. The tubers were mixed with the biologics in the bunker of the potato-planting unit at a rate of 1 kg per 1.5 ton tubers (2 kg/ha). The basic potato growing technology included i) post-planting application of Sen -cor® herbicide (800 g/l, Bayer Crop Science, Germany); ii) post-germination double application (with one-week interval) of Terraflex® 17/17/17 inoculant (2.8 and 1.6 kg/ha, Nu3 N.V., Belgium); iii) post-germination single application of Aquadon micro inoculant (2.0 l/ha, Orgpolymersyntes, Russia), Extrasil® microbe fertilizer agent (2.0 l/ha, BisolbyInter Ltd., Russia), Zircon inoculant (10 g/ha, ANO Nest-M, Russia), herbicides Lazurite (0.5 l/ha, AO Avgust, Russia) and Titus™ (20 g/ha, DuPont, USA); and iv) treatments with fungicides after row closure as follows: Bravo® (1.5 l/ha, Syngenta AG, Switzerland) and Ridomil gold® (1.5 l/ha, Syngenta AG, Switzerland) in 2 weeks; Revus® (250 g/ha, Syngenta AG, Switzerland) in 4 weeks, and Shirlan® (0.4 l/ha, Syngen-
ta AG, Switzerland) in 6 weeks. The final fertilization with Terraflex® (2.8 kg/ha) combined with Shirlan® treatment (0.4 l/ha) were carried out 2 weeks before harvesting. The basic agrotechnology without biologics served as the control. Standard biometric and phytopathological indicators were used. The disease signs and biometric parameters were assessed in 3-week seedlings (1-2 leaf layer phase) and at row closure. Then two disease surveys were performed at the beginning and at the end of blooming, and final indicators for tubers were estimated at harvesting. Data processing by ANOVA and Student’s t-test for pairwise comparison revealed that the biologics caused a significant increase in plant growth rate and the leaf area growth at the beginning of vegetation. The healthy tuber yield was 240 and 690 g/m² higher for *B. subtilis* B-10 and *T. asperellum* T-36 biologics, respectively, as compared to the control (p ≤ 0.10). Due to the biologics, the late blight intensity was 7.2 times lower and 11.6 times lower, respectively (p ≤ 0.01). The number of affected tubers, including those with signs of secondary bacterial infection, decreased almost 2 times, by 140 and 130 g/m², respectively (p ≤ 0.01). Thus, solid plant waste multirecycling is a prospective way to produce granular environmentally safe biologics for plant protection against diseases. In the developed three-step technology, the wastes from edible mushroom double cultivation on sawdust mixed with wheat bran possess high nutritional value as a substrate for solid microbial cultures.

Keywords: multirecycled biologics, efficacy, potato, diseases, protection, microbial antagonists, multirecycling, *Bacillus subtilis, Trichoderma asperel lum*

In 2019, 17 biologicals were permitted in Russia for potato protection against disease (the State Catalogue of Pesticides and Agrochemicals Permitted for Application in the Territory of the Russian Federation. Moscow, 2019). Most of them (13 preparations) are bacteria-based, 11 are products of liquid-phase culture of *Bacillus subtilis* (Ehren.) Cohn, and 3 products were obtained in liquid-phase or liquid-phase-solid-phase culture of *Trichoderma asperel lum* Samuels, Lieckf. & Nirenberg (= *Trichoderma harzianum*) strains [1]. These species and strains are characterized by a large variety of metabolic processes, are hardy when cultured and well-suited for intensive technologies, and also possess environmental plasticity [2-5]. Complex composition of their bioactive compounds with different action provides their bactericidal and fungicidal properties or hyperparasite activity (for *Trichoderma* spp.) [6-8]. A number of microbial metabolites promote plant growth and development, and some are capable of increasing nonspecific plant resistance to diseases [9, 10]. The peculiarities of secondary metabolism of *B. subtilis* and *T. asperel lum* strains determine multiple functionality of preparative form on their basis [11, 12].

Biological preparations are produced by different methods. Submerged culture technology is deemed more appropriate as controlled fermentation conditions provide a standardized final product. These technologies include concentration and drying steps which increase the costs, and moreover, the biological effects of these preparations require more time. The spore-forming bacteria are not picky in terms of cultivation conditions, and their spores sustain drying without loss of viability and biological activity. The micromycete strains that produce prolific biomass in submerged culture poorly form conidia in a liquid medium [18]. When fermented of on a solid substrate, micromycetes realize their potential of conidogenesis to the fullest extent possible [13].

During production of biological preparations, solid-phase fermentation helps solve the problem of cheap and available raw materials for commercial biotechnologies via using plant waste of different related industries [14]. In biologicals obtained on plant substrates, producer strains for a long time remain viable both in the preparation and in soil after application [14]. The anthropogenic plant wastes of wood and timber industries, public utilities, forestry and agriculture containing lignocellulosic complex which is hard to recycle by most microorganisms are most efficiently used only in commercial edible mushroom growing [15-17]. Only xylotrophic basidial macromycetes are capable of decomposing lignocellulosic substrates and enriching low-value rough plant waste with fungal protein, easily digested carbohydrates, vitamins and mineral components,
thus providing an opportunity for usage of such substrates in various biotechnological processes [18-21]. After commercial mushroom growing completed and fruiting bodies are harvested, the substrate with penetrated mycelium can be used as feed additives, fertilizers or a substrate to grow other edible mushrooms and microorganisms with different target activity [22-24].

The usage of edible mushroom cultivation waste as a substrate containing cheap, accessible sources of nutrition and a mix of microelements required for fast growth and development of microorganism cultures is viewed as a promising way to produce biologicals [25, 26]. In more recent time the development of brand new multiple stage, non-waste, resource-saving and environmentally safe technologies of anthropogenic waste bioconversion based on higher basidial macromycetes and producer strains is of increasing interest (27). A wealth of experience has been accumulated globally in terms of production of liquid- and solid-phase fermentation on plant substrates (peat) and increased efficiency of commercial biological preparations based on production strains B. subtilis and T. asperellum [28, 29], including those used against potato diseases [30, 31]. However, it should be emphasized that multiconverted anthropogenic waste is not used anywhere in the world as a substrate for the production of biological products. There is lack of information about obtaining and efficiency of preparative forms developed based on multi-step (multi-)bioconversion of anthropogenic and agricultural waste [32].

This paper provides results of successful application of a brand new guided multiconversion of waste into useful products, and, specifically, of multiple usage of plant waste in commercial bioculture of edible mushrooms and production of granulated antifungal biological preparations. The multibioconversion of plant anthropogenic and agricultural waste was performed during sequential cultivation of Lentinula edodes (Berk.) Pegler (shiitake mushroom) and Pleurotus ostreatus (Jacq.: Fr.) P. Kummer НК-35 (oyster mushroom) mushrooms, and Bacillus subtilis B-10 and Trichoderma asperellum T-36 strains. The study shows increased nutrient value of converted waster compared to traditional substrates. Experimental samples of biological preparations against potato phytophthora rot improved plant protection in field test by 70-75% compared to the basic agrotechnical measures.

The purpose of the research is to develop a method of obtaining multiconversion biological preparations based on antagonists of plant pathogens and to evaluate the protective action of such biological preparations in potato farming.

**Techniques.** The collection strains of Bacillus subtilis B-10 and Trichoderma asperellum T-36 (National Collection of Microorganisms Pathogenic for Plants and their Pests; Innovation Technologies of Plant Protection Center for Collective Usage of Scientific Equipment, All-Russian Research Institute of Plant Protection; the collection was registered on January 28, 1998, No. 760 at the World Federation for Culture Collections, World Data Centre for Microorganisms — WFCC WDCM, Japan). Shiitake Lentinula edodes (Berk.) Pegler (summer hybrid) mycelium and oyster mushroom Pleurotus ostreatus (Jacq.: Fr.) P. Kummer HK-35 (shock-less hybrid) mycelium (Sylvan Hungaria Zrt., Hungary) were used for mushroom growing.

The KLePo (C3) substrate for subsequent solid-phase cultivation of microorganisms according to multibioconversion technology were obtained via double conversion of commercial substrate for shiitake ПLe (C1) based on plant waste, the oak wood shavings (88.9%) and wheat siftings (10%) with addition of CaCO₃ (0.1%) and CaSO₄•2H₂O (1%) (by weight at 70% humidity). First, shiitake mushrooms were cultivated on sterilized C1 (ПLe) for 3 months at 18-23 °C and 85-95% air humidity by semi-commercial small-volume submerged solid-phase method, resulting in fruiting bodies and spent substrate (C2). Then,
Oyster mushrooms were cultivated on С2 for 2 months at 20-22 °C and 85-95% air humidity until the formation of fruiting bodies and С3 substrate completely penetrated by mycelia of these mushrooms. The composition of С3 (edible basidiomycete cultivation waste) was characterized based on its nutrition value (contents of polysaccharides, protein, total and amino nitrogen, amino acids, vitamins and microelements) by comparing with industrial substrate С1 (ILLe) for shiitake cultivation and with lowland peat used in production of peat-based biologicals. The analyses were performed in Agrophysical Institute (St. Petersburg) in compliance with GOST GOST 26177-84 (Fibertec 8000 system), GOST 51417-99 (Digestor 2520 system), GOST 31675-2012 (Fibertec system) (all systems by Foss Tecator, Sweden); GOST 32903-2014 (Steyer liquid chromatograph, Aquilon, Russia); GOST 15962-2014 (atomic absorption spectrometer AA 240, Varian Techtron Pty Ltd, Australia); GOST 15607-2015 and GOST 34230-2017 (Steyer liquid chromatograph, Aquilon, Russia).

For inoculum (the first stage of biologicals production), T. asperellum Т-36 was grown 5 days in liquid Czapek standard synthetic medium (2 g/l NaNO3, 1 g/l KH2PO4, 0.5 g/l MgSO4, 0.5 g/l KCl, 0.01 g/l FeSO4, 20 g/l glucose; pH 7.0) (Biocompass-C, Russia) at 24-26 °C (250 rpm, New Brunswick™ Innova® 44 incubator shaker, Eppendorf, Germany). B. subtilis B-10 strain was cultured 3 days in optimized corn-molasses medium (30 g/l corn-steep extract, 15 g/l molasses; pH 7.8) (Cargill, Agroresurs LLC, Russia) at 27-28 °C (150 rpm, New Brunswick™ Innova® 44 incubator shaker). The titres of inoculums were determined by serial dilution procedure with plating onto agar-based media (Czapek agar, HiMedia Laboratories, India; dry nutrient agar SPA, Scientific and Production Association Microgen, Russia). The titers were 2.5×10^9 CFU/ml for T. asperellum Т-36 and 3.8×10^11 CFU/ml for B. subtilis B-10. In the second stage (solid-phase culture) С3 was inoculated with T. asperellum Т-36 (2.8×10^10 CFU/ml) and B. subtilis B-10 (0.9×10^9 spores per ml), and the strains were grown for 10 days at 25-28 °C in a thermostatically controlled chamber (laboratory thermostat PRO TC 30/120-500, Scientific and Production Association Prooborudivaniye, Russia).

The nutrient media and substrates were sterilized by autoclaving (5075ELVPV D, Tuttnauer Europe B.V., Netherlands).

The experimental samples of multiconversion biological preparations were tested in plot tests (Producers’ Cooperative Shushary, Leningrad Province) on table purpose potato variety Elizaveta (medium early, high-yield, moderate resistant to phytophthora rot, cultivated in the Central, North-Caucasian, North-Western, Northern, Volga-Vyatka and Far Eastern region of Russia, released in the North-Western Region; originated by North-Western Agricultural Research Institute and Vsevolozhsky Plant Breeding Station).

The preparations were applied once during planting (May 12, 2011, in the morning at 17 °C and 72% relative air humidity). The tubers were mixed with biological preparations in a potato planting device bunker (1 kg per 1.5 tons of tubers, 2 kg per ha). The preparations were combined with basic agrotechnical and protective measures (in control group, the biologicals were excluded). The plot areas in test and control groups were 0.5 hectares each. The basic potato growing technology included i) post-planting application of Sencor® herbicide (800 g/l, Bayer Crop Science, Germany); ii) post-germination double application (with one-week interval) of Terraflex® 17/17/17 inoculant (2.8 and 1.6 kg/ha, Nu3 N.V., Belgium); iii) post-germination single application of Aquadon micro inoculant (2.0 l/ha, Orgpolymersyntes, Russia), Extrasol® microbe fertilizer agent (2.0 l/ha, BisolbyInter Ltd., Russia), Zircon inoculant (10 g/ha, ANO Nest-M, Russia), herbicides Lazurite (0.5 l/ha, AO Avgust, Russia) and Titus™ (20 g/ha, DuPont, USA); and iv) treatments with fungicides...
after row closure as follows: Bravo® (1.5 l/ha, Syngenta AG, Switzerland) and Ridomil gold® (1.5 l/ha, Syngenta AG, Switzerland) in 2 weeks; Revus® (250 g/ha, Syngenta AG, Switzerland) in 4 weeks, and Shirlan® (0.4 l/ha, Syngenta AG, Switzerland) in 6 weeks. The final fertilization with Terraflex® (2.8 kg/ha) combined with Shirlan® treatment (0.4 l/ha) were carried out 2 weeks before harvesting (September 5, 2011).

A reciprocally orthogonal scheme was used; the plots were arranged in 4 replicates over 0.5 ha, with 10 m² test plot size; 482 plants were sampled in total [33]. Standard plant biometric and phytopathologic parameters (plant growth rate, foliage, plant disease incidence and development, crop loss, absolute biological efficiency of the preparation and biological efficiency compared to control) [34] were used. Two biometric estimations and registration of onset of symptoms of the disease were performed on 3-4-week-old potato seedlings at the phase of leaf layers 1-2 and with closing of the rows on 6-7-week-old plants at the phase of leaf layers 9-10; two phytopathological records of onset of symptoms of the disease were performed at the start and end of blossoming (July 19 and August 16, 2011, respectively); one registration was performed during harvesting of tubers on September 5, 2011 [35]. Potato harvest was established using five randomly distributed 1 m² plots per variant in tests and control [33, 35].

The statistical processing with Microsoft Excel 2010 and Statistica 6.0 software (StatSoft, Inc., USA) included dispersion analysis (ANOVA), calculation of mean values (M), standard error of the mean (±SEM). In pair-wise comparison of variants the statistical significance of differences was analyzed based on Student’s t-test.

**Results.** By comparing the composition of multiconversion waste after cultivation of edible basidiomycetes (C3 — КLePo) with the composition of multiconversion waste of commercial substrate for *L. edodes* cultivation (C1 — ΠLe), and of lowland peat (table 1) showed that substrate base of lab samples after multibioconversion can be characterized as organic fertilizers containing available carbohydrates from decomposition of cellulose and lignin of C1 by basidiomycetes with the increased content of nitrogen and protein and a reduced C:N ratio (see Table 1).

### 1. Converted shiitake mushroom—oyster mushroom substrate (KLePo) composition after sequential cultivation of *Lentinula edodes* and *Pleurotus ostreatus* on plant waste as compared to commercial substrate for shiitake mushrooms (ΠLe) and lowland peat (M±SEM, semi-commercial cultivation)

<table>
<thead>
<tr>
<th>Component</th>
<th>ΠLe (a)</th>
<th>KLePo (c)</th>
<th>Lowland peat (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of absolute dry weight:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellulose</td>
<td>36.5±1.2*** (a/c)</td>
<td>16.7±0.3** (c/b)</td>
<td>15.6±0.3</td>
</tr>
<tr>
<td>lignin</td>
<td>24.3±3.4* (a/c)</td>
<td>17.3±0.5</td>
<td>22.1±0.4*** (b/c)</td>
</tr>
<tr>
<td>crude protein</td>
<td>2.7±0.3</td>
<td>9.4±0.3*** (c/b; c/a)</td>
<td>4.3±0.1</td>
</tr>
<tr>
<td>total nitrogen</td>
<td>0.4±0.1</td>
<td>1.5±0.3*** (c/b; c/a)</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>amino nitrogen</td>
<td>0.3±0.1</td>
<td>1.2±0.1*** (c/b; c/a)</td>
<td>0.1±0.02</td>
</tr>
<tr>
<td>ash</td>
<td>13.5±3.2*** (a/c)</td>
<td>2.5±1.6</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>Amount per absolute dry weight, ng/kg:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>essential amino acid</td>
<td>14.1±2.3</td>
<td>14.3±9.9</td>
<td>9.2±0.1</td>
</tr>
<tr>
<td>amino acid pool</td>
<td>32.4±10.3</td>
<td>31.4±16.7</td>
<td>22.9±0.1</td>
</tr>
<tr>
<td>Ca</td>
<td>1343.1±228.7</td>
<td>1465.4±351.7</td>
<td>2625.3±52.8*** (b/c)</td>
</tr>
<tr>
<td>Na</td>
<td>157.4±10.2</td>
<td>189.3±57.2</td>
<td>380.3±28.2*** (b/c)</td>
</tr>
<tr>
<td>K</td>
<td>3089.3±32.4</td>
<td>2643.5±321.2*** (c/b)</td>
<td>1250.1±28.9</td>
</tr>
<tr>
<td>Mn</td>
<td>454.1±89.6</td>
<td>518.8±185.6</td>
<td>360.8±85.4</td>
</tr>
<tr>
<td>Fe</td>
<td>329.6±59.8</td>
<td>278.3±87.2</td>
<td>208.8±21.1</td>
</tr>
<tr>
<td>biotin</td>
<td>0.03±0.01</td>
<td>0.04±0.01*** (c/b; c/a)</td>
<td>0.02±0.00</td>
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<tr>
<td>thiamine</td>
<td>0.3±0.1</td>
<td>0.5±0.1</td>
<td>0.62±0.0</td>
</tr>
<tr>
<td>riboflavin</td>
<td>1.7±0.3</td>
<td>4.3±0.1*** (c/b; c/a)</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>C:N</td>
<td>81.2</td>
<td>38.3</td>
<td>92.9</td>
</tr>
</tbody>
</table>

**Note.** Substrate description (ΠLe, KLePo, lowland peat) see in Techniques section.
* * * The differences between KLePo and ΠLe (c/a; a/c) and between KLePo and lowland peat (b/c; c/b) are statistically significant at p ≤ 0.10; p ≤ 0.05 and p ≤ 0.01, respectively.
Concentrations of nutrients for *T. asperellum* T-36 and *B. subtilis* B-10 growth on the shiitake mushroom—oyster mushroom converted substrate significantly (p ≤ 0.01) exceeded the values for lowland peat which is commonly used as a solid-phase substrate in manufacturing biologicals. The total nitrogen and protein content was 2.0 times higher, microelements and vitamins were 1.5–2.0 times higher, and concentration of easily accessible carbohydrates was more than 1.5 times higher (see Table 1).

The titre of the multiconversion biologicals based on *T. asperellum* T-36 and *B. subtilis* B-10 in solid-phase culture on C3 (KLePo) was 10^{10} CFU/g.

During testing field efficiency of these preparations against potato phytophthora rot, an average monthly temperature and relative air humidity were as follows: 13.1 °C and 67.2% in May; 19.8 °C and 70.1% in June; 24.4 °C and 68.5% in July; and 19.1 °C and 69.2% in August. In 2011, 42 sunny days were registered during plant vegetation. In 2011, field tests revealed significant growth acceleration (p ≤ 0.01) and an increase in plant foliage by 1.2 times under the effect of both multiconversion biologicals as compared to the basic technology only (without use of biologicals) at the beginning of plant vegetation. The yield of healthy tubers as influenced by the preparations based on *B. subtilis* B-10 and *T. asperellum* T-36 strains significantly (p ≤ 0.10) exceeded control, by 240 and 690 g/m^2, respectively (Table 2).

### 2. Development of potato plants (Elizaveta variety) as influenced by lab samples of multiconversion biologicals based on *Trichoderma asperellum* T-36 and *Bacillus subtilis* B-10 (M±SEM, Producers Cooperative Shushary, Leningrad Province, 2011)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Average plant growth rate, mm per day</th>
<th>Rate of foliage growth, leaf layers per day</th>
<th>Yield of healthy tubers, kg/m^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>layer 1-2</td>
<td>layer 9-10</td>
<td>layer 1-2</td>
</tr>
<tr>
<td>BAPM + LO Т-36 SHV, G</td>
<td>1.65±0.05***</td>
<td>10.61±0.32***</td>
<td>0.17±0.01***</td>
</tr>
<tr>
<td>BAPM+ LO B-10 SHV, G</td>
<td>1.51±0.04</td>
<td>12.71±0.27***</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>BAPM (control)</td>
<td>1.49±0.04</td>
<td>12.09±0.36</td>
<td>0.15±0.01</td>
</tr>
</tbody>
</table>

**Note.** BAPM means basic agrotechnical and protective measures; LO Т-36 SHV, G — laboratory samples of multiconversion biological preparations based on *T. asperellum* T-36, LO B-10 SHV, G — laboratory samples of multiconversion granulated biological preparation based on *B. subtilis* B-10 (samples were obtained using conversion shiitake—oyster mushroom substrate).

*, **, *** Differences with the control are statistically significant at p ≤ 0.10; p ≤ 0.05 and p ≤ 0.01, respectively.

### 3. Phytophthora rot damage during blossom and weight of affected tubers in potato variety Elizaveta as influenced by lab samples of multiconversion biologicals based on *Trichoderma asperellum* T-36 and *Bacillus subtilis* B-10 (M±SEM, Producers Cooperative Shushary, Leningrad Province, 2011)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Phytophthora rot incidence, %</th>
<th>Phytophthora rot intensity, %</th>
<th>Weight of affected tubers, kg/m^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>E</td>
<td>S</td>
</tr>
<tr>
<td>BAPM + LO Т-36 SHV, G</td>
<td>13.8±0.7*</td>
<td>16.1±0.6*</td>
<td>1.7±0.7*</td>
</tr>
<tr>
<td>BAPM+ LO B-10 SHV, G</td>
<td>14.9±0.4*</td>
<td>18.6±0.2*</td>
<td>2.1±0.3*</td>
</tr>
<tr>
<td>BAPM (control)</td>
<td>57.5±1.8</td>
<td>62.7±3.2</td>
<td>10.1±3.6</td>
</tr>
</tbody>
</table>

**Note.** S — start of blossoming, E — end of blossoming; BAPM means basic agrotechnical and protective measures; LO Т-36 SHV, G — laboratory samples of multiconversion biological preparations based on *T. asperellum* T-36, LO B-10 SHV, G — laboratory samples of multiconversion granulated biological preparation based on *B. subtilis* B-10 (samples were obtained using conversion shiitake—oyster mushroom substrate).

* Differences with the control are statistically significant at p ≤ 0.01.

The multiconversion biologicals based on *B. subtilis* B-10 and *T. asperellum* T-36 reduced the incidence of phytophthora rot 3.4- and 3.9-fold, respectively, and its development 7.2- and 11.6-fold, respectively (p ≤ 0.01). In comparison with the control group the weight of affected tubers, including those showing signs of secondary bacterial infection, significantly (p ≤ 0.01) decreased almost 2.0 times (by 140 and 130 g/m^2, respectively) (Table 3).
4. The efficiency of a single use of novel multiconversion biologicals based on *Trichoderma asperellum* T-36 and *Bacillus subtilis* B-10 against phytophthora rot (Elizaveta variety, Producers Cooperative Shushary, Leningrad Province, 2011)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Biological effectiveness, %</th>
<th>Biological effectiveness from control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAPM + LO T-36 SHV, G</td>
<td>84.4</td>
<td>74.7</td>
</tr>
<tr>
<td>BAPM+ LO B-10 SHV, G</td>
<td>81.4</td>
<td>69.8</td>
</tr>
<tr>
<td>BAPM (control)</td>
<td>38.3</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** BAPM means basic agrotechnical and protective measures; LO T-36 SHV, G — laboratory samples of multiconversion biological preparations based on *T. asperellum* T-36, LO B-10 SHV, G — laboratory samples of multiconversion granulated biological preparation based on *B. subtilis* B-10 (samples were obtained using conversion shiitake—oyster mushroom substrate).

In our tests, the biological effectiveness of potato plant protection scheme which includes fertilizers and growth promoters, herbicides, and multiple applications of chemical fungicides did not reach 40% (Table 4). Low efficiency of conventional measures is due to the increasing resistance of pathogens to chemical pesticides during the recent years as a result of anthropogenic transformation of agricultural ecosystems and their phytosanitary deterioration [1, 26, 31].

The multiconversion biologicals that we suggest increased total biological efficiency of basic protective measures by more than 2.2 times (see Table 4). Given the agrotechnical measures and protective chemical treatments used, the efficiency of multiconversion preparations based on *T. asperellum* T-36 and *B. subtilis* B-10 was rather high, 74.7 and 69.8%, respectively (see Table 4).

Most biological preparations based on *B. subtilis* and *T. asperellum* recommended in Russia for potato plant protection against disease during vegetation have titres $10^9$-$10^{11}$ CFU/g and $10^8$-$10^{10}$ CFU/ml respectively depending on their preparative forms [1, 12]. In the suggested novel multiconversion biologicals the titre is $10^{10}$ CFU/g which corresponds to analogs, e.g. Alirin B, TAB; Alirin B, SP; Alirin B, Zh; Gliocladin, TAB; Gliocladin, SK; Gliocladin, SP; Gliocladin, Zh; Trihozin, SP (all produced by Management Company ABT-Group and All-Russian Institute of Plant Protection, Russia) [30, 32]. It is a known fact that waste substrates resulting from commercial cultivation of edible mushrooms are successfully used in agriculture as organic fertilizers [36, 37]. Among other things, such waste show good results when used as fertilizer and growth promoter during potato farming [38]. In our report, the substrate resulted from a two-stage biological conversion of cellulose- and lignin-containing industrial and agricultural wastes by edible basidiomycetes (shiitake mushroom and oyster mushrooms) is an actual organic fertilizer enriched with microelements and vitamins [25, 36]. An increase in biometric parameters of potato plants and tuber yield emphasized in this study, as well as improvement of traditional integrated potato protection offer prospects for development of protective biologicals which additionally possess the properties of biofertilizers and biostimulants [39-43]. The biologicals we propose in this paper, when used in field conditions at a suggested dosage, ensured reliable protective effect corresponding to that described by global developers for similar formulations [44-47].

Overall, our findings show a possibility to produce most suitable for soil application and environmentally safe granulated biologicals using multibioconversion of plant industrial waste by edible mushrooms and then by microorganisms producing bioactive metabolites. The new data have been obtained about nutrient value of the substrates, resulted from cultivation of edible mushrooms, when these substrates are used in solid-phase culture as growth media for strains producing biological preparations. One application of novel multiconversion biologicals based on *Bacillus subtilis* B-10 and *Trichoderma asperellum* T-36 is proven to increase the efficiency of generally accepted basic potato protection measures against phytophthora rot by 70 and 75%, respectively.
REFERENCES

1. Zeiruk V.N., Kuz’michev A.A., Glez V.M., Derevyagina M.K., Vasi’leva S.V., Abashkin O.V. *Fitosanitarnoe sostoyanie i meropriyatiya po bor’be s osnovnymi boleznyami i vreditelyami v period vegetatsii i khraneniya kartofelya* [Phytosanitary condition and measures to combat the main diseases and pests during the growing season and storage of potatoes]. Moscow, 2014 (in Russ.).


Future farming systems

Symbiotic interactions

INFLUENCE OF MUTATION IN THE GENE Sym26 OF THE GARDEN PEA (Pisum sativum L.) ON THE ORGANIZATION OF TUBULIN CYTOSKELETON IN NODULES

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A b s t r a c t

Symbiotic nodule is a unique organ forming on legume roots. Indeterminate nodules (with prolonged meristem activity) (F. Guinel, 2009) are characterized by differentiation of both the nodule cells and the bacteria that infect nodule and are converted into a form specialized for nitrogen fixation — bacteroids. Bacteroids surrounded by a membrane of plant origin, form organelle-like symbiosomes (A. Tsyganova et al., 2018; T. Coba de la Peña et al., 2018). Cell differentiation leads to appearance of uninfected (free of bacteria) and infected cells filled with many thousands of symbiosomes formed in the central part of nodule (A. Tsyganova et al., 2018). A prolonged activity of the meristem results in histological zonation of the indeterminate nodule. A meristem, an infection zone, a nitrogen fixation zone are distinguished, and a senescence zone appears in the basal part of a mature nodule (F. Guinel, 2009). Obviously, the tubulin cytoskeleton plays an important role in the development of a nodule, but until now researchers had a focus on the early stages of nodule development (A. Timmers, 2008). Only recently it was revealed that the tubulin cytoskeleton plays a key role in the differentiation of nodule cells (A. Kitaeva et al., 2016). It was shown that in nodules of garden pea (Pisum sativum L.) and barrel medic (Medicago truncatula Gaertn.) the release of bacteria into the cytoplasm of a plant cell prevents the formation of a regular pattern of cortical microtubules, oriented parallel to each other and perpendicular to the longitudinal axis of the cell, typical for uninfected cells. This leads to an irregular pattern of cortical microtubules, the appearance of which contributes to the transition of infected cells to isodiametric growth (A. Kitaeva et al., 2016). Endoplasmic microtubules build a mold for the growth of infection threads, and support the location of infection droplets and symbiosomes in infected cells (A. Kitaeva et al., 2016). However, changes in the organization of the tubulin cytoskeleton during senescence of nodule cells have not been studied. In this study, using immunocytochemical analysis and confocal laser scanning microscopy, the organization of the tubulin cytoskeleton in the nodules of the pea mutant SGEFix⁻3 (sym26) (V. Tsyganov et al., 2000) was studied. This mutant is characterized by the formation of ineffective nodules with premature degradation of symbiotic structures (T. Serova et al., 2018). It was shown that in the mutant line, the formed patterns of cortical and endoplasmic microtubules did not differ from those of the initial line SGE. Cortical microtubules formed an irregular pattern in meristematic and infected cells and regular pattern in uninfected and colonized cells. Endoplasmic microtubules surrounded the nucleus in interphase cells, formed spindles and preprophase bands during mitosis, and also surrounded infection threads. At the same time, in the senescence zone in degrading cells, complete depolymerization of the tubulin cytoskeleton occurred in both infected and uninfected cells. In the initial line, senescence was induced only in four-week-old nodules, and microtubule depolymerization was also observed in senescent cells. Thus, the complete depolymerization of microtubules in various types of nodule cells can be a cytological marker of its senescence.

Keywords: legume-rhizobial symbiosis, microtubules, symbiosome, bacteroid, infection thread, nodule senescence, immunolocalization, Pisum sativum
A characteristic feature of legumes is their interaction with nodule bacteria, rhizobia, which results in formation of symbiotic root nodules. A symbiotic nodule is a unique organ, in which a plant creates ecological niches for rhizobia, which gain the ability to fix atmospheric nitrogen [1]. In various species of Fabaceae symbiotic nodules differ in structure. There are two types of nodules, determinate and indeterminate [2]. Determinate nodules have a meristem that is active during a short period, resulting in a spherical shape of mature nodules. Meristem of indeterminate nodules remains active for a long time leading to appearance of histological zonation in the central part of nodule having elongated shape. As a result there are nodule meristem, the infection zone, where rhizobia are released into the cell cytoplasm, and the nitrogen fixation zone, in which they acquire nitrogen fixation ability by differentiating into the specialized forms, called bacteroids [2, 3]. After four weeks post inoculation, the senescence zone appears proximal to the nitrogen fixation zone [4]. Along with bacteroid differentiation, a pronounced differentiation of nodule cells is observed [5]. A number of cells remain uninfected while infected cells grow in size significantly and become filled with numerous symbiosomes. Symbiosome contains a bacteroid separated from plant cell cytoplasm by a symbiosome membrane of plant origin [5, 6]. Apparently, plant cell differentiation during symbiotic nodule development is accompanied by significant cytoskeleton reorganization [7].

The reorganization of actin microfilaments was described as one of early responses triggered by Nod factors [8-10]. Actin microfilaments are also required for the formation of infection thread [8-13]. Recently, it was shown that actin microfilaments are involved in release of rhizobia into the cytoplasm of the plant cells from infection droplets and facilitate symbiosome accommodation [14].

Numerous microtubules were identified in infection sites in curled root hairs as early as in the mid-1980s [15, 16]. Later on, the engagement of microtubules in root hair curling, initiation and growth of the infection thread was discovered [17-22]. In mature nodules the organization of microtubules was studied in alfalfa (Medicago sativa L.) [23], soybean (Glycine max L.) [24], garden pea (Pisum sativum L.) [25] and white lupin (Lupinus albus L.) [26]; however, the three-dimensional organization of tubulin cytoskeleton was not described in detail, specifically around infection threads and infection droplets [7]. Only recently we showed changes in the pattern of cortical microtubules during cell differentiation in nodules of pea and Medicago truncatula Gaertn. [27]. For instance, in uninfected cells the cortical microtubules are parallel to each other and perpendicular to longitudinal axis of the cell [27]; a similar pattern is characteristic of pea root cells in the transition zone [28, 29]. In infected cells of pea and M. truncatula nodules the cortical microtubules form irregular pattern promoting an isodiametric growth of these cells [27]. In nodules of both analyzed species the endoplasmic microtubules formed a dense network around infection structures, i.e. infection threads and droplets, creating a matrix for their development [27]. Significant differences were observed in the pattern of endoplasmic microtubules forming the network between symbiosomes. For instance, in pea the microtubules were randomly located between symbiosomes, which coincided with the lack of order in the location of symbiosomes themselves. At the same time, in infected cells of M. truncatula nodules microtubules were parallel to the symbiosomes, which, in turn, were perpendicular to the cell wall [27].

In this report we show for the first time that both natural and induced nodule senescence is accompanied by depolymerization of microtubules.

The aim of the study was to evaluate the impact of natural senescence and senescence induced by mutation in the pea Sym26 symbiotic gene leading to premature degradation of symbiotic structures on organization of tubulin cyto-
Techniques. In our work we used the initial line SGE of garden pea (*Pisum sativum* L.) [30] and its mutant SGEFix−3 (sym26) [31] forming ineffective nodules with premature degradation of symbiotic structures (the senescence zone is formed as early as 2 weeks after inoculation), i.e., with early senescence phenotype [32]. The plants were inoculated with *Rhizobium leguminosarum* bv. *viciae* 3841 strain [33].

The seeds were surface sterilized for 15 min with concentrated sulphuric acid and rinsed 10 times with sterile water. The plants were grown in plastic pots, filled with 100 g of sterile vermiculite, at 21 °C, 75% relative humidity, illumination of 280 μM photons m⁻² c⁻¹ and day/night mode of 16/8 h (a growth chamber MLR-352H, Sanyo Electric Co., Ltd, Japan). Nitrogen-free nutrient solution was used for watering [34]. The nodules for analysis were collected from 10 plants 2 weeks after inoculation, for SGE line also 4 weeks after inoculation. Three independent experiments were performed.

Method of nodule fixation and tubulin immunolocalization was described earlier [27]. Monoclonal mouse antibody to tubulin (DM1A clone, Sigma-Aldrich, USA) was used for visualization of microtubules (1:1000 dilution, incubation during the night at 4 °C). Goat antibody conjugated with Alexa Fluor 488 (Life Technologies, USA) was used as secondary antibody to mouse γ-globulin (1:500 dilution, incubation during 90 min at 28 °C). To identify nuclei and bacteria sections were stained for 7 min with propidium iodide (0.5 µg/ml). After rinsing the sections were embedded into ProLong Gold® antifade reagent (Thermo Fisher Scientific, USA) under cover glasses.

The microtubule pattern in nodule cells was analyzed using laser confocal scanning microscope LSM780 and ZEN2012 software (Carl Zeiss, Germany).

Results. The histological organization of 2-week-old nodules of SGE line did not differ from that described earlier [35], the meristem, the infection zone and the nitrogen fixation zone were identified (Fig. 1, A). In 2-week-old ineffective nodules of SGEFix−3 mutant (sym26) we observed the meristem, the infection zone and a zone corresponding to the nitrogen fixation zone, along with the senescence zone (Fig. 2, B), as it was described earlier [32].

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**Fig. 1.** Histological organization of 2-week-old nodules of pea (*Pisum sativum* L.) initial line SGE (A) and mutant line SGEFix−3 (sym26) (B): I — meristem, II — infection zone, III — nitrogen fixation zone, III’ — zone corresponding to the nitrogen fixation zone in the initial line, IV — senescence zone (longitudinal sections). Confocal laser scanning microscopy (microscope LSM780, ZEN2012 software, Carl Zeiss, Germany). Merge of single optical sections of differential interference contrast
and red channel. Scale bar 100 µm.

Fig. 2. Organization of tubulin cytoskeleton in meristematic cells (A, B), and in cells of the early (C, D) and the late (E, F) infection zone of 2-week-old nodules of pea (Pisum sativum L.) mutant SGEFix-3 (sym20): n — nucleus, ic — infected cell, uic — uninfected cell, cc — colonized cell, arrows indicate infection threads, asterisk marks mitotic structures (longitudinal sections). Confocal laser scanning microscopy (microscope LSM780, ZEN2012 software, Carl Zeiss, Germany). Scale bar 10 µm.
A-F: immunolocalization of tubulin (microtubules) — green channel, DNA staining with propidium iodide (nuclei and bacteria) — red channel.

A, C, E: merge of single optical sections of differential interference contrast, green and red channels.

B: maximum intensity projections of green and red channels based on z-stacks from 50 optical sections.

D, F: maximum intensity projections of green channel based on z-stacks from 30 (D) and 70 (F) optical sections.

Previously, we have described the tubulin cytoskeleton organization in 2-week-old nodules of SGE line; therefore, in this study the tubulin cytoskeleton organization was analyzed only in SGEFix−3 mutant (sym26). To compare microtubules organization in senescent wild type cells, 4-week-old nodules of SGE line were also analyzed, in which such cells could have been identified.

The meristem cells in SGEFix−3 mutant nodules (sym26) were small in size and had centrally located nucleus. The cortical microtubules lying at different angles to each other and often criss-crossing formed irregular pattern (see Fig. 2, A, B). The endoplasmic microtubules in interphase cells enveloped the nucleus connecting it with the cell periphery (see Fig. 2, A, B). In mitotic cells, endoplasmic microtubules formed a mitotic spindle and a preprophase band (see Fig. 2, A, B). The observed patterns of cortical and endoplasmic microtubules of meristematic cells in SGEFix−3 (sym26) nodules were similar to those in SGE [27].

Three cell types observed in the early infection zone were i) uninfected cells, ii) colonized cells where infection structures (infection threads and infection droplets) were present but no bacterial release into the cytoplasm occurred, and iii) infected cells (see Fig. 2, C, D). The uninfected cells were lack of endoplasmic microtubules, whereas cortical microtubules formed a regular pattern, i.e. they were parallel to each other and perpendicular to the longitudinal axis of the cell (see Fig. 2, C, D). In colonized cells the pattern of cortical microtubules was identical to that of uninfected cells (see Fig. 2, C, D). Furthermore, endoplasmic microtubules located along the infection thread were observed (see Fig. 2, C, D). In infected cells cortical microtubules formed an irregular pattern (see Fig. 2, C, D). Previously, for pea wild type line SGE and M. truncatula wild type line A17 it was demonstrated that microtubules serve as a matrix for infection thread growth and surround infection droplets thereby preparing them for bacterial release into the nodule cell cytoplasm [27].

In the late infection zone, the uninfected and especially infected cells increased in size; moreover, they retained the microtubular patterns described above (see Fig. 2, E, F). Furthermore, a well-defined network of randomly positioned endoplasmic microtubules was observed between symbiosomes (see Fig. 2, E, F).

In the zone corresponding to the wild type nitrogen fixation zone the infected cells further increase in size; moreover, they continued to support irregular patterns of cortical and endoplasmic microtubules passing among symbiosomes (Fig. 3, A, B). The involvement of tubulin cytoskeleton in the positioning of symbiosomes in nitrogen-fixing cells of pea and M. truncatula was previously identified; it was shown that the positioning of endoplasmic microtubules between symbiosomes varied for the analyzed species [27]. The observed pattern of endoplasmic microtubules around symbiosomes in the infected nodule cells of SGEFix−3 (sym26) mutant did not differ from that of SGE nodules.

The similarity of patterns in nodules of initial line and its mutant is indicative of the fact that up to a point the development of infected cells in both genotypes is identical. Indeed, it was reported earlier that morphologically differentiated bacteroids that undergo premature degradation are typical for SGEFix−3
Fig. 3. Organization of tubulin cytoskeleton in cells of a zone corresponding to the nitrogen fixation zone in the initial line (A, B), in cells of the senescence zone (C, D) of 2-week-old nodules in SGEFix-3 (sym26) mutant, and in senescent cells (E, F) of 4-week-old nodules of pea (Pisum sativum L.) initial line SGE: n — nucleus, ic — infected cell, ic’ — infected cell with early signs of degradation, uic — uninfected cell, cc — colonized cell, dic — degrading infected cell, duic — degrading uninfected cell, arrows indicate infection threads, asterisk marks mitotic structures (longitudinal sections). Confocal laser scanning microscopy (microscope LSM780, ZEN2012 software, Carl
Zeiss, Germany). Scale bars 10 µm (A, B) and 20 µm (C-F).

A-F: immunolocalization of tubulin (microtubules) — green channel, DNA staining with propidium iodide (nuclei and bacteria) — red channel.

A, C, E: merge of single optical sections of differential interference contrast, green and red channels.

B, D: maximum intensity projections of green channel based on z-stacks from 50 optical sections.

F: maximum intensity projections of green and red channels based on z-stacks from 50 (D) и 45 (F) optical sections.

The senescence zone was observed in the distal part of the nodule (see Fig. 1, B). In this zone degradation of symbiotic structures in cells was observed, which was accompanied by complete depolymerization of both cortical and endoplasmic microtubules (see Fig. 3, C, D). As reported earlier, the pattern of endoplasmic microtubules located between symbiosomes in Sprint-2Fix (sym3I) pea mutant with undifferentiated bacteroids was similar to that of wild type [36], whereas in M. truncatula dnf1-1 mutant [37], which also formed undifferentiated bacteroids, fast depolymerization of microtubules occurred [27]. Apparently, depolymerization of microtubules in SGEFix-3 (sym26) mutant in cells of the senescence zone is related to degradation of symbiotic structures [32] and activation of nutrient reutilization. It is possible that the fast depolymerization of microtubules in M. truncatula dnf1-1 mutant is due not to lack of bacteroid differentiation in this mutant, but rather to activation of degradation of symbiotic structures accompanied by a depolymerization of tubulin and actin cytoskeleton [38].

The senescent infected and uninfected cells with signs of degradation of symbiotic structures (see Fig. 3, E, F) were identified in SGE line in the basal part of 4-week-old nodules, and in such cells we observed depolymerization of microtubules (see Fig. 3, E, F). In SGE line, the senescence only starts in 4-week-old nodules and peaks in 6-week-nodules [32], whereas 4-week-old nodules of SGEFix-3 (sym26) mutant display almost complete degradation of symbiotic structures and expansion of the senescence zone that often fills the entire nodule [32].

To summarize, we have studied the impact of natural senescence on microtubule pattern in nodules of the initial line SGE. We have also investigated the influence of a mutation in the pea Sym26 symbiotic gene on tubulin cytoskeleton. The mutation sym26 leads to formation of ineffective nodules and induced premature degradation of symbiotic structures. It was shown that in SGEFix-3 (sym26) mutant the patterns of microtubules in cells of meristem, the infection zone and zone, which corresponds to the nitrogen fixation zone of wild type do not differ from the initial line SGE. This is indicative of normal development of nodule cells in the mutant line until induction of premature degradation of symbiotic structures followed by complete destruction of tubulin cytoskeleton both in infected and uninfected cells. Identical depolymerization of microtubules occurs during natural senescence of nodule cells in SGE line. Therefore, complete depolymerization of microtubules in different nodule cells is observed both in case of natural and induced senescence, and can be a cytological marker of senescence.

REFERENCES


MULTIFUNCTIONAL BIOLOGICS WHICH COMBINE MICROBIAL ANTI-FUNGAL STRAINS WITH CHITOSAN IMPROVE SOFT WHEAT (Triticum aestivum L.) YIELD AND GRAIN QUALITY


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A b s t r a c t

The developing of effective and high-tech preparations for microbiological plant protection is a crucial problem of agricultural biotechnology. In this paper, we revealed differences in the crop structure, grain introspective characteristics and resistance of soft wheat plants to root rot when using novel multifunctional bioactive preparations. Our objective was to investigate effects of developed bioactive compositions based on microbial antagonists of plant pathogenic and chitosan complexes on spring soft wheat (Triticum aestivum L.) variety Leningradskaya 6. Plant protection against root rot, productivity and grain quality assessed by the methods of microfocus X-ray radiography and gas-discharge visualization were estimated in two-year field tests (Leningrad Province, 2016-2017). The weather conditions during growing season of 2016 were more favorable for wheat plants compared to 2017 due to a slight temperature fluctuation and a significant amount of precipitation. The number of spikelets per spike, flag and pre-flag leaf area, the weight of spike and vegetative parts were the indicators for wheat productivity, germination energy, seedling length, dynamics of plant growth phase and height, the number, length and weight of roots. According to significant positive influence of the studied compositions on yield structure, the biopreparations rank as follows: in 2016 — Vita-plan, Zh (ООО AgroBioTekhnologiya, Russia) > Vitaplan, Zh + Chitosan II (test preparation containing 50 and 100 kDa chitosans with the addition of 0.1 % vanillin, FSBSI VIZR) > Gamair, SP (ООО AgroBioTekhnologiya, Russia) > Chitosan I (test preparation containing 50 and 100 kDa chitosans with the addition of 0.05 % salicylic acid, FSBSI VIZR) > Chitosan II; in 2017 — Vita-plan, Zh > Vitaplan, SP > Vitaplan, Zh + Chitosan II > Gamair, SP > Chitosan II. In 2016, a combined use of Vitaplan, Zh and Chitosan II changed significantly not only the plant vegetative part weight, but also the spike weight, while separate use of Chitosan II significantly increased the vegetative biomass only. In 2017, the same combination of the biologicals made the flag leaf 86.84 % larger and the root weight 83.33 % higher compared to the control. In 2016, Chitosan I led to reliable 19.0 % increase (t = 3.0; p < 0.05) in potential grain yield compared to the control, however, there were no significant differences for Vitaplan, Zh, Chitosan II and their combination Vitaplan, Zh + Chitosan II. On the contrary, in 2017 Vitaplan, Zh + Chitosan II caused the maximum reliable (t = 7.2; p < 0.05) increase in yield (by 82.6 %). Vitaplan, Zh and Vitaplan, Zh + Chi-
tosan II possess maximum efficiency against Helminthosporium root rot. Due to Vitaplan, Zh + Chitosan II, in 2016 root rot disease frequency was 80% lower compared to the control, and in 2017 no symptoms were observed which may be due to less favorable weather conditions for root rot disease in 2017 compared to 2016. According to our findings, the potential grain yield in wheat correlates significantly and positively with grain X-radiographic projection area, integrated grain brightness and total intensity of the gas-discharge fluorescence. Chitosan I, Chitosan II and Vitaplan, F + Chitosan II have the greatest impact on grain structure and quality parameters assessed by X-ray and gas-discharge visualization. Perhaps the effectiveness of the studied drugs depended on weather conditions, but was generally positive in terms of the main assessed indicators. Thus, our data convincingly indicate the effectiveness of multifunctional biologics which combine microbial antagonists of fungal plant pathogens with chitosan, an activator of plant diseases resistance, to protect wheat against root rot, to increase grain yield with better quality.

Keywords: *Triticum aestivum* L., spring soft wheat, biological preparations, chitosan composition, yield structure, root rot, grain quality, microsofus X-ray, gas discharge visualization

Spring wheat is the main food crop and an important item of Russian export. Obtainment of high stable yield of quality spring wheat grain is only possible subject to a number of measures that include the use of general soil-protective technologies and methods of enhancement of soil fertility, correct crop rotation with sufficient saturation of bare fallows optimal for the conditions and objectives of variety cultivation as well as compliance with agrotechnical requirements meeting biological peculiarities of the crop variety. Currently, however, the potential productivity of crops is frequently achieved only by a third, which supports the necessity to improve cultivation technologies [1, 2].

Obviously, optimization of conditions for growing and development of plants throughout all ontogenesis stages is one of the most important objectives in crop production. Its achievement is to the large extent connected with development of production technologies and application of environmentally-friendly multifunctional preparative forms capable of effectively reducing the spread and development of dangerous diseases and improving the disease resistance of plants as well as stimulating their growth and development.

Creation of effectiveness and high-technology preparative forms for microbiological protection of plants is the key issue of agricultural biotechnology. Such forms include such biological preparations registered in Russia as Vitaplan, Alirin-B and Gamair which are manufactured in the form of wettable powders, suspension concentrates, tablet and liquid forms (joint development of OOO AgroBioTekhnologiya, Moscow, and All-Russian Institute of Plant Protection) [3, 4]. Biological preparations have demonstrated high effectiveness in control of diseases of principal agricultural crops, promoted the increase in yield and quality of plant products. In addition, in some instances it was found that introduced strains of antagonist microbes considerably affect the variety composition of a complex of soil-inhabiting plant pathogenic fungi and suppressive properties of soils in agrocoenosis [5].

Over the past years, new-generation preparations have been developed, e.g. the disease-resistance inducers based on chitosan, the results of application of which are widely discussed [6-8]. Chitosan is a natural polymer with β-D-glucosamine and N-acetyl-β-D-glucosamine units [9] obtained through deacetylation of chitin of crustaceans, insects, and fungi [10-13]. Positive effects of chitosan on plant growth and development were confirmed [14]. Treatment of wheat leaves with chitosan resulted in increase in concentration of phenolic acids, particularly ferulic acid. Chitosan stimulates generation of precursors of lignin (p-coumaric, ferulic, sinapic acids), and synthesis of phenolic acids possessing antimicrobial properties, i.e. benzoic, p-coumaric, caffeic, protocatechuic, chlorogenic, ferulic and gallus acids [15, 16]. Wheat plants treated with chitosan and exposed to drought stress, as compared to control set, demonstrated
considerable increase in growth, germination rate, grain moist content, length and activity of roots, as well as changes in physiological indicators (i.e. activity of superoxide dismutase, peroxidase, catalase, malondialdehyde and chlorophyll contents) [17]. Treatment with chitosan, through increasing the chlorophyll content in wheat leaves, caused a 13.6% yield increase as compared to control group [17].

In the recent years, the research is carried out in Russia to create the compositions based on immobilization of antagonist microbes *Bacillus subtilis* M-22 and *Trichoderma viride* T-36 in chitin-chitosan carriers for effective protection of vegetable crops from Fusarium infection and nematodes [18, 19]. High protective effect (up to 70%) of such complex biopreparations is caused by combination of properties of antagonist microbe with the ability of chitosan to enhance, in conjunction with biologically active substances, the mechanisms of natural resistance of plants to pathogens. It also demonstrates the synergetic effect of composition components.

Seeds of required quality are the condition the high wheat yield [20]. In this regard, there is a number of standard tests regulated by ISTA (International Seed Testing Association) and of promising seed quality control tests based on imaging technologies. The method of microfocus X-ray radiography is for many years used both in Russia [21-22] and abroad (23-26). It is used to detect various structural seed defects (stress cracks, enzyme-mycotic attrition, internal germination, latent pest colonization, Snn pest contamination, physical damage and defectiveness of grain kernel, blind-seed disease). Computer microtomography allows researchers to obtain a 3D image of the internal caryopsis structure [27] and visualize some structural defects [28].

Over the past 10 years, the data was obtained regarding the possibility to use the method of terahertz imaging to determine the seed variety purity [29], seed quality [30] and ultra-early forecasting of laboratory germination rate of seeds [31]. Seed imaging in terahertz range enables detection of changes occurring during germination just 6 hours after seed soaking [31].

The presented work for the first time demonstrates the effectiveness of multifunctional preparations based on microorganism strains, the antagonists of infection agents, and activators of plant disease resistance, the chitosan compounds, for increasing the yield and protection of spring soft wheat from diseases. The results define the differences in the wheat yield structure and resistance to root rot upon application of multifunctional preparations and identify their impact on introscopic characteristics of the grain.

The purpose of our study is to justify the feasibility of use of multifunctional preparations based on antagonists of infection agents and chitosan compounds for spring soft wheat protection from root rot and increase of grain yield, as well as to evaluate the quality of grain through microfocus X-ray radiography and gas-discharge imaging.

**Techniques.** The experiments were run on spring soft wheat plants (*Triticum aestivum* L.) variety Leningradskaya 6 (k-6490; provided by the department of genetic wheat resources of Vavilov All-Russian Institute of Plant Genetic Resources, VIR) in 2016-2017 (VIR experimental field). The seeding was performed on May 7 on a 1.0 m$^2$ plot through row cropping with 15-cm row spacing and spacing in the row of 1-2 cm (400 seeds/m$^2$). Depth of seeding 5-6 cm.

In 2016 experiment pattern provided for the following scenarios: no treatment (control); Gamair, SP (OOO AgroBioTehknologiya, Russia) as a standard; Vitaplan, Zh (OOO AgroBioTehknologiya, Russia); chitosan complex Chitosan I (experimental sample, All-Russian Institute of Plant Protection, FSBSI VIZR); Vitaplan + Chitosan II complex. Gamair, SP is a fungicide based
on *Bacillus subtilis* M-22 strain (wetting powder, viable cell titer defined in CFU/g). Powder (5 g) was dissolved in 10 l of water and used to treat 1 t of seeds by semidyrry method. Vitaplan, Zh is a culture liquid of *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D strains (1:1 ratio, live cells and *B. subtilis* spores titer of $10^{10}$ CFU/ml). The seeds (50 g) were soaked in 100 ml of culture liquid for 1 hour. Chitosan I complex contains 100 kDa and 50 kDa chitosans (1:1 in weight parts), a mix of succinic and glutamic acid (organic acids) at the ratio of chitosan:organic acids 1:1 and 0.05 % salicylic acid. Chitosan II complex includes 100 kDa and 50 kDa chitosans (1:1), a mix of succinic and glutamic acid (organic acids) of chitosan:organic acids 1:1 and 0.1% of vanillin. 50 kDa and 100 kDa chitosans were obtained by us through oxidative breakdown of 150 kDa chitosan (85% deacetylation) with sodium nitrite in acidic conditions (Bioprogress, Russia). The seeds were treated with the both complexes by semidyrry method, 80 g per 10 l of water per 1 t of seeds. When treating with Vitaplan, Zh + Chitosan II complex, Chitosan II was added to culture liquid of Vitaplan Zh biopreparation until the Chitosan II concentration reached 0.1% (50 g of seeds were soaked in 100 ml of culture liquid for 1 hour). Vegetating plant in 2016 were treated on June 24, July 9 and 19. The standard, Gamair, SP, was used as 10 g of preparation per 300 l of water. Vitaplan, Zh was dissolved in water to one-tenth, liquid consumption was 100 ml/m². When spraying the plants with aqueous solutions of Chitosan I and Chitosan II preparations, the concentration (0.1%) was measured by the main component (chitosan; liquid consumption was 100 ml/m²). In a scenario that included Chitosan II complex, indoleacetic acid (0.0015%) was added as the main plant growth hormone instead of vanillin. When using Vitaplan, Zh + Chitosan II complex, culture liquid with the titer of $10^{10}$ CFU/ml was water-dissolved to one-tenth; liquid consumption was 100 ml/m².

In 2017 experiment included five scenarios: no treatment (control); Vitaplan, SP as a standard, 10 g of preparation per 300 l of water; Vitaplan, Zh; Chitosan II chitosan complex; Vitaplan, Zh + Chitosan II complex. Wheat seeds prior to seeding were treated and vegetative plants were sprayed according to the pattern applied in 2016. During wheat tillering, the number, length and weight of roots (primary radicle root, radicle and coleoptile roots) were measured. The number and length of nodal roots were also defined. In each scenario, each 20 plants were evaluated twice. Wheat ontogenesis phases were registered by Eucarpia (EC) scale (Zadoks scale).

In studying the yield structure, the data of productive and overall tillering capacity, plant height, ear length, number of spikelets per ear, ear weight were evaluated. Weight of vegetating parts of plants, area of flag and pre-flag leaves were measured in accordance with methodological guidelines [32].

Potential wheat yield $\mathbf{Y_p}(t/ha)$ was measured by productive tilling capacity and number of plants per 1 m²: $\mathbf{Y_p} = \mathbf{M_E T_P P_D} \times 10000$, where $\mathbf{M_E}$ is the grain weight per ear of a single plant, t; $\mathbf{T_P}$ is a productive tilling capacity of a sample (the number of stems with ears per a single plant); $\mathbf{P_D}$ is plant density (the number of plants per 1 m²).

The plant affection by root rot was defined in field conditions during wheat tillering phase (on July 15, 2016) by the generally accepted scale: 0 – epicotyl unaffected, 1 – isolated stains on epicotyl, 2 – major lesions, 3 – severe lesions, the plant died. In each experiment scenario, each 20 plants were evaluated twice.

Development of root rot was estimated by average weighted extent of plant affection $\mathbf{R_e}$ [33]:
Re = \sum(\frac{a_b}{A_K})\times100,
where \(a\) is the number of plants with similar symptoms, \(b\) is the corresponding score, \(A\) is a number of plants studied (healthy and diseased), \(K\) is the maximum scale score.

In 2016, a laboratory experiment was also held to define the grain germination energy (%) upon treatment with the aforesaid biological preparations and chitosan compounds (control group remained untreated) (commenced on May 30). In each experiment version, Petri dishes were used to analyze 100 grains (June 1), the length of seedlings was measured 1 day after transferring to moist chamber (June 2) and on the next day (June 3).

Microfocus X-ray radiography and gas-discharge visualization (GDV) methods were applied to evaluate introspective characteristics of the grain. X-ray radiography of wheat grains was performed with a serial mobile X-ray unit PRDU-02 (ZAO ELTEKH-Med, Russia), \(\times3,0\) zoom coefficient. Analysis of digital X-ray images of wheat grains was carried out with Agrus-Bio software (OOO ArgusSoft, Russia). On an X-ray projection of caryopsis, the area (cm\(^2\)), perimeter (cm), length (cm), width (cm), circularity (relative units), elongation (relative units), irregularity (relative units), average brightness (brightness units), standard brightness deviation (brightness units), optical density (relative units) and integrated optical density (relative units) were measured. Gas-discharge visualization (electrophotography with registration and quantitation of characteristics of corona effect emerging upon seeds exposure to high-energy electromagnetic field) was carried out on a serial GRV-Kamera apparatus equipped with analytical software GRV-Nauchnaya Laboratoriya (OOO Biotekhprogress, Russia). The following parameters of digital gas-discharge images of grain were analyzed: luminescence area (pixels), total luminescence intensity (relative units), form factor (relative units), average isoline radius (pixels), normalized standard deviation of isoline radius (pixels), isoline length (pixels), isoline-measured entropy (relative units), isoline-measured fractality (relative units).

Statistical analysis was carried out with SPSS 21.0, Statistica 6.0, MS Excel 2016 software [34]. In calculations, the methods of parametric statistics (based on mean \(M\) and standard error of mean \(\pm SEM\), 95 % confidence intervals and Student’s \(t\)-test) and multivariate statistics (cluster and factor analysis) were used.

Results. Weather conditions in 2016 in Leningrad Province were characterized by higher average monthly temperature (the standard was exceeded by 3.4 °C in May, and the excess was within 1°C in June through August; the precipitation in May was 64 % of the standard, however, during the summer months it exceeded the standard considerably (137% in June, 191% in July, 227% in August). In May-July 2017, reduction in average monthly temperature was within 2.5 °C vs. the standard; precipitation in May reached only 29%, which is considerably below the standard; in summer, that indicator increased and reached 115% of the standard in June, 115% in July and 175% in August. Thus, vegetation period of 2016 was characterized by more favorable weather conditions for plant growth, given insignificant temperature fluctuations and considerable amount of precipitation.

Table summarizes the effects of biological preparations and chitosan complexes on spring soft wheat productivity indicators and potential yield. In 2016 (under increased average monthly temperatures and considerable excess of precipitation during summer month) in a scenario where the seeds were treated with Gamair, SP, the germination energy was 25% higher than in control group, and reached the maximum value of 96.5%. Significant (\(p < 0.05\)) increase in the length of the sprout occurred in the scenarios with Gamair, SP and Vitaplan, Zh, by 73.7% and 69.5%, respectively.
Leningradskaya 6 variety spring soft wheat (*Triticum aestivum* L.) productivity upon application of multifunctional preparations based on antagonist microorganisms and chitosan compounds (M±SEM, St. Petersburg—Pushkin, a test field, 2016)

<table>
<thead>
<tr>
<th>Experiment scenario</th>
<th>E₉₅, %</th>
<th>Lₛ₉, mm</th>
<th>P, score</th>
<th>h, cm</th>
<th>Nᵣ, pcs.</th>
<th>Lᵣ, mm</th>
<th>Mᵣ, h</th>
<th>Nₙᵣ, pcs.</th>
<th>Lₙᵣ, mm</th>
<th>Nₛₑ, pcs.</th>
<th>Sᵣₑ, cm²</th>
<th>Sₚᵣₑ, cm²</th>
<th>Mₑ, g</th>
<th>Mᵥₑ, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (water)</td>
<td>71.4</td>
<td>9.6±2.0</td>
<td>62.5±1.6</td>
<td>81.3±1.8</td>
<td>4.4±0.6</td>
<td>61.0±5.4</td>
<td>0.3±0.1</td>
<td>6.5±0.7</td>
<td>56.4±4.1</td>
<td>12.8±0.6</td>
<td>7.1±0.4</td>
<td>7.6±0.5</td>
<td>0.4±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Gamair, SP</td>
<td>96.5</td>
<td>16.6±1.9*</td>
<td>64.5±0.6</td>
<td>86.6±3.0</td>
<td>6.4±0.4*</td>
<td>75.6±4.7</td>
<td>0.3±0.05</td>
<td>5.9±0.7</td>
<td>41.2±4.7*</td>
<td>13.5±0.6</td>
<td>7.9±0.6</td>
<td>9.2±0.4*</td>
<td>0.5±0.1</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Vitaplan, Zh</td>
<td>86.8</td>
<td>16.2±1.9*</td>
<td>67.8±1.1*</td>
<td>90.1±2.5*</td>
<td>6.5±0.5*</td>
<td>76.8±4.8*</td>
<td>0.3±0.04</td>
<td>7.0±0.5</td>
<td>54.6±3.9</td>
<td>14.2±0.5</td>
<td>6.4±0.6</td>
<td>8.4±0.8</td>
<td>0.5±0.03</td>
<td>2.3±0.1</td>
</tr>
<tr>
<td>Vitaplan, Zh + Chitozan II</td>
<td>74.4</td>
<td>19.6±2.0</td>
<td>69.4±0.8*</td>
<td>98.6±2.8</td>
<td>5.9±0.5*</td>
<td>62.1±3.8</td>
<td>0.4±0.02</td>
<td>7.4±0.7</td>
<td>48.0±4.8</td>
<td>13.9±0.6</td>
<td>8.1±0.4*</td>
<td>7.6±0.5</td>
<td>0.6±0.03*</td>
<td>2.5±0.2*</td>
</tr>
<tr>
<td>Chitosan I</td>
<td>82.4</td>
<td>9.1±1.6</td>
<td>67.9±0.9*</td>
<td>87.9±2.6</td>
<td>6.2±0.5*</td>
<td>69.1±3.4</td>
<td>0.3±0.05</td>
<td>6.9±0.7</td>
<td>53.3±3.9</td>
<td>14.3±0.5</td>
<td>7.5±0.7</td>
<td>8.0±0.5</td>
<td>0.6±0.1</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Chitosan II</td>
<td>79.8</td>
<td>8.9±1.2</td>
<td>60.7±2.2</td>
<td>83.6±3.6</td>
<td>4.6±0.5</td>
<td>70.4±5.0</td>
<td>0.4±0.1</td>
<td>7.7±0.7</td>
<td>66.1±5.1</td>
<td>12.2±0.8</td>
<td>7.9±0.7</td>
<td>6.6±1.1</td>
<td>0.6±0.1</td>
<td>2.7±0.3</td>
</tr>
</tbody>
</table>

Nota. E₉₅ — grain germination energy, Lₛ₉ — sprout length, P — plant phase, h — plant height, Nᵣ — number of roots, Lᵣ — root length, Mᵣ — root weight, Nₙᵣ — number of nodal roots, Lₙᵣ — nodal root length, Nₛₑ — number of spikelets per ear, Sᵣₑ — flag leaf area, Sₚᵣₑ — pre-flag leaf area, Mₑ — ear weight, Mᵥₑ — vegetation part weight.

* Differences with control group are statistically significant at p < 0.05.
Fig. 1. Leningradskaya 6 variety spring soft wheat (*Triticum aestivum* L.) productivity indicators upon application of multifunctional preparations based on antagonist microorganisms and chitosan compounds: A — Eucarpia (EC)-scale ontogenesis phase, B — ear weight, C — vegetation part weight, D — complex of indicators (a — number of positive changes, b — number of significant positive changes); 1 — control (water), 2 — Gamair, SP, 3 — Vitaplan, Zh, 4 — Vitaplan, Zh + Chitosan II, 5 — Chitosan I, 6 — Chitosan II (St. Petersburg—Pushkin, a test field, 2016).

The samples treated with Vitaplan, Zh preparation together with Chitosan II complex showed the highest growth rate by Eucarpia (EC) scale ontogenesis phases during the earing stage, significant increase of score by 11%, $t = 7.8$, $p < 0.05$ as compared to the control group. Maximum intensive plant development was ensured through treatment with Vitaplan, Zh and Chitosan I preparations (Fig. 1, A). Significant ($p < 0.05$) increase in plant height, by 10.8%, during the earing stage was observed for the scenario where the seeds were treated with Vitaplan, SP.

In all experiment scenarios that provided for the use of preparations, the number of roots from epicotyl (primary radicle root, radicle and coleoptile roots) has increased vs. the control group. The number of roots significantly increased upon application of Gamair, SP (by 44.7%, $t = 2.7$, $p < 0.05$), Vitaplan, Zh (by 46.3%, $t = 2.6$, $p < 0.05$), Vitaplan, Zh together with Chitosan II (by 32.3%, $t = 2.7$, $p < 0.05$), and Chitosan I (by 32.3%, $t = 2.3$, $p < 0.05$). Chitosan II chitin complex did not actually affect this indicator (see Table).

Virtually in all experiment scenarios (Vitaplan, Zh, Vitaplan, Zh + Chitosan II, Chitosan I), there was a significant ($p < 0.05$) increase in the number of wheat roots as compared to the control group. The preparations did not produce any significant effect on the number and length of nodal roots, number of spikelets per ear, area of flag and pre-flag leaves. At the same time, Vitaplan, Zh preparation when combined with Chitosan II complex has a considerable positive effect on wheat ear weight (a 65.2% increase vs. the control group, $t = 7.2$, $p < 0.05$) (see Fig., B), and also on the weight of green parts of the plant (by 28.4%, $t = 2.9$, $p < 0.05$) (see Fig., C). When Chitosan I was used, significant growth of only the weight of green parts occurred (by 39.8%, $p < 0.05$).

Figure 1 (D) shows the normalized stacked column chart reflecting variation in number of positive and statistically significant positive changes for 14 indicators of wheat productivity due to use of biopreparations and chitosan complexes as compared to the control. At first, the indicators with positive change in value of certain productivity indicators as compared to control val-
ues (as per scenarios) were selected. Then they were ranked using Student's t-test at p = 0.05, which gives a decrease in biological efficiency as follows: Vitaplan, Zh > Vitaplan, Zh + Chitosan II > Gamair, SP > Chitosan I > Chitosan II.

Cluster analysis (k-means method) [34] divided all biological preparations and chitosan compounds into two groups of effectiveness based on changes of the average values of the set of indicators vs. control. The first group comprised Gamair, SP, Vitaplan, Zh, and Chitosan I, and Vitaplan, Zh + Chitosan II and Chitosan II formed the second group. Preparations of the second group, as compared to the first group, showed a more express effect on the root weight (by 12.7%, \( t = 5.8; p < 0.05 \)), number of nodal roots (by 14.7%, \( t = 6.7; p < 0.05 \)), length of nodal roots (by 13.0%, \( t = 2.6; p < 0.05 \)), flag leaf area (by 10.4%, \( t = 4.4; p < 0.05 \)), ear weight (by 27.1%, \( t = 7.4; p < 0.05 \)), green part weight (by 11.7%, \( t = 4.7; p < 0.05 \)). Use of Vitaplan, Zh + Chitosan II and Chitosan II resulted in insignificant drop in plant development (by 2.8% as per ontogenesis phase), significantly smaller number of roots and their length (by 25.7%, %; \( t = 5.7; p < 0.05 \) and by 11.3 %; \( t = 4.4; p < 0.05 \), respectively).

Principal factor analysis [34] using Varimax normalized axis rotation procedure (factor impacts in the procedure are normalized by dividing by square root of relevant dispersion) allowed evaluation of the interrelations between relative changes in wheat productivity indicators caused by biological preparations and chitosan compounds (Fig. 2, A). The highest effect on productivity was characteristic of Vitaplan, Zh + Chitosan II complex, while Chitosan II caused the slightest effect.

Chitosan I complex promoted a 19.0% increase in potential wheat yield (\( t = 2.8, p < 0.05 \)) vs. the control (see Fig. 2, B). We have found no considerable differences in potential yield between scenarios for Vitaplan, Zh, Vitaplan, Zh + Chitosan II complex and Chitosan II. Upon Gamair, SP application, the potential wheat yield was 25.0% lower (\( t = 3.5, p < 0.05 \)) compared to control.

In 2017, at reduced average monthly temperature vs. the standard, insignificant precipitation (29% of May standard) and excess of precipitation during the summer month, maximum significant increase in yield (82.6 %, \( p < 0.05 \)) occurred in Vitaplan, Zh + Chitosan II scenario (Fig. 3). As compared to 2016, in control group this indicator was significantly higher, by 77.1%. In Vitaplan, Zh and Vitaplan, Zh + Chitosan II scenarios, the yield has increased considerably, by 2.3 t/ha and 4.3 t/ha, respectively. Insignificant differences over years
were observed for Chitosan II complex.

In 2017, all preparations other than Vitaplan, Zh significantly and positively speeded up the plant development over phases (Vitaplan, Zh + Chitosan II by 25.04%, Chitosan II by 33.59%, Vitaplan, SP by 25.58%) and increased their height (Vitaplan, Zh + Chitosan II by 32.57%, Chitosan II by 45.22%, Vitaplan, SP by 49.44%) as compared to control (average increase by 10.0% and 16.2%, respectively). Vitaplan, Zh + Chitosan II complex increased the number of spikelets in an ear (by 7.66% vs. control), productive and overall tilling capacity (by 116.00% and 22.19%, respectively). In this scenario, the plants also distinguished by larger flag leaf area (by 86.84%) and root weight (by 83.33%).

Figure 4 shows the number of positive (negative) and significantly positive (negative) changes in indicator values of wheat productivity caused by preparations as compared to the control group. By their biologic effectiveness, the preparations could be ranked as follows: Vitaplan, Zh > Vitaplan, SP > Vitaplan, Zh + Chitosan II > Chitosan II. Vitaplan, Zh + Chitosan II complex also caused the growth of the maximum number of wheat productivity indicators as compared to control group (90%, with 35% significant changes).

Damage to plants caused by root rot was evaluated during the stem elongation phase. As the studies showed, the principal infection agent was Bipolaris sorokiniana (Sacc.) Shoem. Vitaplan, Zh and Vitaplan, Zh + Chitosan II complex demonstrated maximum effectiveness against Helminthosporium root rot. In 2016 Vitaplan, Zh + Chitosan II scenario provided the reduction in root rot occurrence by 80% as compared to the control group, and in 2017 no disease symptoms were found (Fig. 5).

Spearman’s non-parametric correlation analysis of introscopic data obtained by radiography and gas-discharge visualization methods for the harvested grains has shown that potential yield of wheat $Y_g$ positively and significantly ($p < 0.05$) correlates with the radiograph projection area $S_p$ ($r = 0.9$), integrated brightness of grains $I_{g, int.}$ ($r = 0.8$) and total intensity of gas-discharge fluorescence $I_{gdf, total.}$ ($r = 0.8$). Dependencies among these indicators may be described by regression equations: $Y_g = 29.36 - 5.04S_p^2 + 0.23S_p^3$ ($r^2 = 0.8$); $Y_g = -10.46 + 0.000038I_{g, int.}^2 - 0.00000000029I_{gdf, total.}^3$ ($r^2 = 0.83$) and
Y_g = -17.68 + 37.82 I_{gdf, total}^2 + 18.09 I_{gdf, total}^3 (r^2 = 0.89). Values of 1000-grain weight of M_{1000} positively correlate with integrated grain brightness I_{g, int}:
M_{1000} = -1.99 + 0.000042 I_{g, int} (r^2 = 0.9).

Fig. 5. Development of Helminthosporium root rot in Leningradskaya 6 variety spring soft wheat (Triticum aestivum L.) in 2016 (A) and 2017 (B) upon application of multifunctional preparations as compared to control: 1 — Chitosan II, 2 — Chitosan I, 3 — Vitaplan, Zh + Chitosan II, 4 — Vitaplan, Zh, 5 — Gamair, SP, 6 — Vitaplan, SP (St. Petersburg—Pushkin, a test field).

According to microfocus X-ray radiography, in case of Chitosan I compound application wheat grains had better morphometric and densitometric parameters as compared to control group, i.e. considerably larger radiograph projection area (by 9.26%, t = 2.5), length (by 3.90%, t = 2.5) and width (by 5.84%, t = 2.3), increased perimeter (by 4.00%, t = 2.4) and average size (by 4.50%, t = 2.7), higher average fluorescence brightness (by 5.76%, t = 3.5) and significantly lower optical density (by 3.8%, t = 3.5). That is, treatment of wheat plants with Chitosan I compound resulted not only in increase of grain size but improved their endosperm density. Larger average brightness of radiographs was found in grains after use of Vitaplan, Zh + Chitosan II (by 6.00%, t = 4.3), Chitosan I compound (by 5.76%, t = 3.5) and Chitosan II (by 9.93%, t = 5.4) as compared to the control. The largest maximum brightness was observed in grains obtained in scenario with Chitosan I (6.4% increase, t = 2.5) and Chitosan II (10.2% increase, t = 3.6). Lower average radiograph brightness (by 7.6%, t = 2.7) vs. the control was discovered in grains obtained through use of Gamair, SP preparation. In the Chitosan II scenario, the grains had lower circle factor values (by 3.70%, t = 3.0), circularity (by 7.22%, t = 2.9) and larger elongation (by 4.90%, t = 2.2) vs. the control.

When Chitosan II was used, gas-discharge characteristics of wheat grains differed drastically from control group in form factor characterizing the irregularity of gas-discharge image and related to grain weight (23.1% higher, t = 2.4). Due to use of Chitosan I compound, the grains had larger isoline fractality values and larger gas-discharge isoline length as compared to control group (by 2.8%. t = 3.8 and by 20.9%, t = 2.2, respectively). These parameters are presumably also connected with size characteristics of grains. In Vitaplan, Zh + Chitosan II and Chitosan II scenarios, the total intensity of gas-discharge image of the grain was considerably less as compared to control (by 19.3%, t = 4.1 and by 15.9%, t = 3.1, respectively). Reduction in intensity of gas-discharge fluorescence is typical for the grains that have better growth indicators during germination.

Microfocus X-ray radiography and gas-discharge visualization demonstrated that, compared to control group, introscopic characteristics of grain were to the largest extent changed in Chitosan I, Vitaplan, Zh + Chitosan I and Chitosan II scenarios. Treatment of wheat with only Vitaplan, Zh biological preparation rendered no effect on introscopic characteristics of grains. With the use of Gamair, SP biological preparation (standard), introscopic characteristics of
grains changed insignificantly.

As mentioned above, due to transition to biological farming, the researchers lately pay special attention to development of alternative plant protection methods. There are several antagonistic microorganisms effective against the wide range of infecting agents, e.g. *Pseudomonas fluorescens* PCL1751 and *P. putida* PCL1760 [35], *Bacillus* spp. [5, 36, 37], as well as *Trichoderma* species [5, 38]. In general, they are expedient to be used in practice as a sound alternative to synthetic chemical fungicides. However, the effectiveness of biological preparations based on antagonistic microbes is often insufficient.

Another way to control diseases may be the enhancement of natural resistance of plants to pathogen. Compounds that launch own protective mechanisms in plants are called resistance inducers. Among them, special role is played by chitosan and its derivatives. Biological activity of chitosan is connected with its ability to induce protective plant immunity responses [39, 40]. Presence of chitosan in cell walls of some microorganisms, particularly plant pathogenic fungi, determines the most important property of this polymer, the pathogen-associated molecular pattern (PAMPs) that is recognized by plant pattern recognition receptors (PRR). This results in activation of a set of nonspecific plant protective responses (pattern-triggered immunity, PTI), including synthesis of phytoalexins, lignification of cell walls, deposition of callose, synthesis of PR proteins, generation of reactive oxygen species (ROS) and nitrogen (NO), etc. [41].

To enhance potential effect of microbe antagonists, many scientists research joint application of biological agent and resistance inducer. Rajkumar et al. [42] have demonstrated that chitin may stimulate the effectiveness of *Pseudomonas fluorescens* (SE21 and RD41 strains) in controlling pepper bare patch. Adding chitin improves the plant protection by stimulating the production of affiliated metabolites that promote antagonistic activity and/or stimulate plant protective properties. Co-treatment of vegetating pepper, cucumber, tomato plants with *Saccharomyces cerevisiae* conjointly and chitosan has reduced the development of mildew twice or more [43]. Niranjan et al. [44] reported the results of testing compositions containing two *Bacillus* strains and chitosan as a carrier, and established their capability of growth promoting and enhancing resistance of millet to mildew. The most effective method was based on combining the introduction of chitosan to soil along with treatment of seeds with antagonist microbe strains [45]. Thus, resistance inducers in combination with bioactive substances are very promising for future use of antagonistic microorganisms in controlling plant diseases, especially in greenhouses [46]. Of interest are the compositions of antagonist microbes, e.g. of *Bacillus* genus, with chitosan and its derivatives.

The main issue in assessing the effectiveness of plant treatment for the quality of seed and bread grain is quantitative objectification of stimulating activity. One of solutions may be the development and application of modern imaging facilities and information facilities for express diagnostics of latent grain heterogeneity. Germination parameters are closely related to morphometric indicators of seeds that can be defined through radiographic study, particularly, in lab tests larger seeds germinate earlier and better than smaller ones [47]. It was stated [48] that optical characteristics of radiographs are important for ensuring seed quality. Relative optical density parameter allows us to make conclusions regarding the density of internal seed tissues and hence their physiological quality [49].

It should be noted that the effectiveness of multifunctional preparation Vitaplan, Zh + Chitosan II in respect of potential wheat yield was determined to the largest extent by its effect on productive tilling capacity, which may be
connected with the more developed root system (more roots and longer roots) and marked reduction in affection by Helminthosporium root rot when this preparation was applied, as compared to other scenarios we tested. In addition, the use of Vitaplan, Zh + Chitosan II complex demonstrated a more rapid plant passing through ontogenesis phases.

Thus, the research undertaken has convincingly demonstrated the prospects of multifunctional preparations combining useful traits of antagonistic microorganisms and chitosan, a plant disease-resistance activator, for protection of wheat from root rot, yield gain and improvement of grain quality. According to significant positive changes in productivity, the biopreparations rank as follows: in 2016 — Vitaplan, Zh > Vitaplan, Zh + Chitosan II > GamaIr, SP > Chitosan I > Chitosan II; in 2017 — Vitaplan, Zh > Vitaplan, SP > Vitaplan, Zh + Chitosan II > Chitosan II. In 2016, a combination of Vitaplan, Zh and Chitosan II in the weather conditions more favorable for plant growth (higher temperature and precipitation), ensured a significant changes not only in the plant green part weight, but also in the spike weight, while separate use of Chitosan II significantly increased the green biomass only. In 2017 (at lower average monthly temperature and considerable amount of precipitation), in this scenario the plants distinguished by their flag leaf area and root weight (86.84 % and 83.33 % increase against control group). In 2016, Chitosan I led to reliable 19.0 % increase ($t = 3.0; p < 0.05$) in potential grain yield compared to the control, however, there were no significant differences for Vitaplan, Zh, Vitaplan, Zh + Chitosan II complex and Chitosan II. On the contrary, in 2017 Vitaplan, Zh + Chitosan II caused the maximum reliable ($t = 7.2; p < 0.05$) increase in yield, by 82.6 %. Vitaplan, Zh and Vitaplan, Zh + Chitosan II complex possess maximum efficiency against Helminthosporium root rot. In Vitaplan, Zh + Chitosan II scenario, in 2016 root rot occurrence was 80 % lower compared to the control, and in 2017 no symptoms were observed, which may be due to less favorable weather conditions for root rot disease in 2017 compared to 2016 (particularly, lower average monthly temperatures and considerable amount of precipitation over the summer period). Potential grain yield in wheat correlates significantly and positively with grain X-radiographic projection area, integrated grain brightness and total intensity of the gas-discharge fluorescence. Chitosan I, Chitosan II and Vitaplan, F + Chitosan II have the greatest impact on grain structure and functional characteristics.

**REFERENCES**


33. Popov Yu.V. *Zashchita i karantin rastenii*, 2011, 8: 45-47 (in Russ.).
34. Nasledov A.D. *IBM SPSS Statistics 20 i AMOS: professional'nyi statisticheskii analiz dannykh* [IBM SPSS Statistics 20 and AMOS: Professional statistical data analysis]. St. Petersburg, 2013 (in Russ.).
CELLULOLYTIC BACTERIA AND ASSOCIATION OF EFFECTIVE MICROORGANISMS FOR BIOCONTROL OF ROOT ROT INFECTIONS IN SUGAR BEET (Beta vulgaris L.)

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Abstract

At the present time, many farmers growing sugar beet (Beta vulgaris L.) reduce application of fertilizers and crop rotations, which leads to accumulation of phytopathogens. The main pathogens of sugar beet causing root rot are fungi from genera Fusarium Lk.:Fr. and Alternaria (Fr.) Keissi. Chemical fungicides are used worldwide to protect crops but plant pathogenic fungi acquire resistance against conventional chemicals. Therefore, the biological methods of plant protection are relevant. In our previous works, we have designed the association of effective microorganisms (EM Association) which includes nitrogen-fixing Azotobacter chroococcum and phosphate-mobilizing Bacillus megaterium bacteria. This association can increase the productivity of sugar beet plants but does not possess antifungal activity against sugar beet root rot. Cellulolytic bacteria are an important component of microbiocenoses. They play a significant role in soil processes; their number is an indicator of soil fertility and ecological quality. Colonizing rhizosphere of plants, they synthesize bioactive substances, including antifungal metabolites. Among cellulolytic bacteria there are active antagonists of fungal root rot causative agents. In this paper we present our research findings on the antifungal properties of a new cellulolytic strain Bacillus sp. C-82/3 and the first effective association of this strain with nitrogen-fixing and phosphate-mobilizing bacteria which promotes sugar beet growth and yield. The goal of the research was to evaluate antifungal activity of the novel strain of cellulolytic bacteria Bacillus sp. C-82/3 isolated from soil rhizosphere of healthy sugar beet plants in the South-East of Kazakhstan (Zhambyl region), to enrich the EM association developed with this strain, and to assess the plant growth promoting activity of the improved EM Association and its ability to biocontrol root rot infections under field condition. Antifungal activity was determined in agar block diffusion tests against Alternaria alternata (Fr.) Keissi, Fusarium solani (Mart.) Sacc. and F. oxysporum Schlecht. The strain was grown on the Hutchinson’s medium (1.0 g/l K2HPO4, 0.1 g/l CaCl2, 0.3 g/l MgSO4, 2.5 g/l NaNO3, 1.0 g/l NaCl, 0.01 g/l FeCl3, 20 g/l wheat straw, 5.0 g/l yeast extract; pH 7.0). Blocks with growing culture were cut out, and put on Petri dishes with potato-dextrose agar earlier inoculated with fungi, and cultured at 28 °C for 3 days. Antifungal activity was assessed by the diameter of growth inhibition zone. To study the plant growth promoting activity of the EM association with Bacillus sp. C-82/3, the cv. Aisultan seeds were treated with the bacterial suspension (10⁷ cells/ml) at 23 °C for 2 hours. The stem and root length measured in the inoculated seedling after 30-day growing in a climatic chamber (Constant Climate Chamber HPP750, Memmert GmbH + Co. KG, Germany) were compared to the control. Field tests were conducted in the South-East of Kazakhstan (Zhambyl region, Kaiyndy farm) in 2017-2018. The results of lab screening showed high antifungal activity of the novel strain Bacillus sp. C-82/3 with the mean inhibition halos of 28.9±0.2 mm for F. oxysporum, 38.2±0.3 mm for F. solani, and 46.6±0.9 mm for A. alternata. The improved EM Association which includes three strains (Bacillus megaterium, Azotobacter chroococcum and Bacillus sp. C-82/3) was characterized by high growth-promoting activity. Germination of the inoculated seeds was 7-16 % higher, and stem and root length increased 1.2-1.5-fold and 1.1-2.0-fold, respectively, as compared to control (p ≤ 0.05). We also revealed the high ability of the EM Association containing Bacillus sp. C-82/3 strain to suppress sugar beet root rot pathogens in soil bioecosion. Seed inoculation with the microbial association decreased the damage to seedlings 2.3 times, to roots 3.0 times. The yield of sugar beet was 34.2±2.3 c/ha higher compared to control (p ≤ 0.05). Thus, our data are the first evidence that the EM Association with Bacillus sp.
C-82/3, a new cellulolytic strain with high antifungal activity that we have detected, is effective against root rot infection and promotes an increase in sugar beet yield under field condition.

Keywords: Beta vulgaris L., sugar beet, biological control, cellulolytic bacteria, effective microorganisms association, antifungal activity, growth-promoting activity, phytopathogenic fungi, root rot

Sugar beet (Beta vulgaris L.) is one of the main industrial crops. In global agriculture it occupies 7.913 million hectares. The largest quantities of sugar beet are produced in Russia, followed by France and USA [1, 2]. At the same time, growing of sugar beet severely damages the soils as harvesting remove a considerable amount of nutrients, which results in reduction of soil microbial mass and its biodiversity [3-5]. In addition, many beet-seeding farms neglect crop rotation and seed sugar beet on the same fields for 5-7 years [6, 7]. All this results in accumulation of plant pathogens causing diseases, typically root rots [8-10]. Losses in crop yield due to root roots in Russia run up to 30%, with 20-40% in Kazakhstan and 50% in Kyrgyzstan [11-14]. In other countries, root rots also inflict considerable losses (30-35%) [15-17].

Modern agriculture is characterized by an extensive use of fungicides. Plant protection chemicals cause certain issues, and negative effects of fungicides eventually step up. The fungi develop resistance to fungicides, which requires increase in dosage of preparations [18-20]. Due to environmental damage, the matter of chemical pesticide reduction becomes more urgent. An alternative is biological methods of plant protection. Topical is the search for microorganisms and development on their basis of biopreparations to control plant pathogens [21-24].

Recently, associations of effective microorganisms (EM associations) with a wider range of biological activity as compared to monocultures are more and more preferable [25-27]. EM associations include nitrogen fixing, phosphate mobilizing, cellulolytic, silicate solubilising and other microorganisms that possess protective and stimulating effects, they synthesize and provide the plants with required substances (ferments, vitamins, amino acids), improve nitrogen and phosphorus nutrition, which results in increased yield and better product quality [28, 29]. This reduces a dosage of mineral fertilizers and chemical plant protectors, so the final product becomes more ecologically friendly and safe for humans [30-32].

In our previous works, we have designed the association of effective microorganisms which includes nitrogen fixing Azotobacter chroococcum and phosphate mobilizing Bacillus megaterium bacteria that positively affected the productivity of sugar beet through improvement of nitrogen and phosphorus plant nutrition but possessed no antifungal effects.

Cellulose comprises the main plant residues in soil, wherefore cellulolytic microorganisms are important members of microbiocenosis and play a significant role in soil processes [33, 34]. Their soil count is an indicator of soil fertility and ecological status [35]. Ability of cellulolytic bacteria to grow in plant rhizosphere of, synthesizing B vitamins, amino acids and ferments ensures their high effectiveness for biological control of plant pathogens [36, 37]. Among cellulolytic bacteria there are active antagonists of pathogenic fungi causing root rots of crop species [38, 39]. Therefore, it seemed promising to include cellulolytic bacteria in EM associations.

This paper contains the study of the antifungal properties of a new cellulolytic strain Bacillus sp. C-82/3 and discusses the designing of the first EM association of nitrogen fixing and phosphate mobilizing bacteria with this strain. It is shown that EM association improves nitrogen and phosphorus nutrition of plants, possesses antifungal activity and produces bioactive agents, which posi-
tively influences the development and productivity of sugar beet plants.

Our goal was to evaluate antifungal activity of cellulolytic bacteria Bacillus sp. C-82/3, to introduce this strain into EM association of effective microorganisms, to assess plant growth promoting activity of this EM association and its ability to biocontrol root rot infections in order to improve field productivity of sugar beet.

Techniques. Bacillus sp. C-82/3 cellulolytic bacteria strain was isolated from rhizosphere of healthy sugar beet plants in South-East Kazakhstan (Zhambyl region). The strain was grown on the Hutchinson’s medium, 1.0 g/l K$_2$HPO$_4$, 0.1 g/l CaCl$_2$, 0.3 g/l MgSO$_4$, 2.5 g/l NaNO$_3$, 1.0 g/l NaCl, 0.01 g/l FeCl$_3$, 20 g/l wheat straw, 5.0 g/l yeast extract, tap water (1 l). Three strains of plant pathogenic fungi, Alternaria alternata (Fr.) Keissl, Fusarium solani (Mart.) Sacc. and Fusarium oxysporum Schlecht causing root rots in sugar beet, were testers in determination of Bacillus sp. C-82 antifungal activity (the strains were kindly provided by LP Scientific Production Center of Microbiology and Virology). These fungi were tested according to Koch’s triad postulates and were characterized by high pathogenicity.

The fungi were grown on potato dextrose agar medium (PDA) (Himedia, India) for 10 days at 25 °C and stored at 4 °C. Antifungal activity was determined in agar block diffusion tests. PDA medium melted and cooled to 40 °C was inoculated with conidia suspension (10$^8$ CFU/ml, 1 ml per 100 ml of medium), and poured into Petri dishes. Cellulolytic bacteria were grown on the Hutchinson’s medium for 5 days at 28 °C, whereafter the blocks (7 mm in diameter) with growing culture were cut out, placed into Petri dishes on agar earlier inoculated with fungi, and cultured for 72 hours at 28 °C. Control group was the blocks cut out of pure media. Antifungal activity was assessed by the diameter of growth inhibition zone.

Biocompatibility of EM association strains was studied by perpendicular streak method [41].

To evaluate the growth promoting effects of designed EM association, the bacteria strains forming the association were grown separately in elective media, cellulolytic bacteria C-82/3 in Hutchinson’s medium, nitrogen fixing bacteria in Ashby medium, phosphate mobilizing bacteria in Muromtsev medium (28 °C in shaker at 180 rpm). Then, the bacteria suspensions with the titer of 1×10$^7$ cells per ml were mixed (1:1:1). Seeds of Aisultan sugar beet were inoculated with C-82/3 strain or EM association containing all three strains. Strain suspension and association titer was 1×10$^7$ cells per ml, the seeds were treated for 2 hours at 23 °C. Treated seeds were sown in pots filled with 300 g of the common gray soil (collected from fields of Kaiyndy farmstead in Zhambyl region, Republic of Kazakhstan) with 1.2% humus, 36.6 mg/kg of easy hydrolysable nitrogen, 14.0 mg/kg of labile phosphorus, and 342.5 mg/kg of labile potassium. Negative control was the seeds treated with sterile water [42].

In 30 days, plant stem and root lengths were measured. Experiments were run in a growth chamber (Constant Climate Chamber HPP750, Memmert GmbH + Co. KG, Germany). Daytime was 12 hours at 24-26 °C under cold white light (6 500 K) and warm light (2 700 K); nighttime was 11 hours at 17-19 °C; 60 to 75% humidity were adjusted automatically. Tests were arranged with three repeats, 10 plants each.

Under field conditions, EM association of three bacterial strains, Bacillus megaterium, Azotobacter chroococcum and Bacillus sp. C-82/3, was tested for 2 years (Kaiyndy farmstead, 2017-2018) on common gray soil with 1.2-1.5% humus, 34.6 mg/kg of easy hydrolysable nitrogen, 14.0 mg/kg of labile phosphorus, and 350 mg/kg of labile potassium; sugar beet as a predecessor. Tests were thrice
repeated, plots were arranged systematically. The EM association (bacterial suspension titer of $1 \times 10^7$ cells per ml) was applied to the seeds (50 ml of suspension per 1 kg of seeds moistened with 1 l/kg water). Seeds were treated for 2 hours. Positive control group was the seeds treated with chemical preparations TMTD 80% (Avgust, Russia) and Score® (Syngenta AG, Switzerland) at the rate of 6.0 kg/t and 0.4 l/hectare, respectively. These fungicides are allowed for application in Kazakhstan to protect sugar beet from seed and soil-born fungal infections. Negative control group was untreated seeds. The agrotechnology of beet growing was as generally accepted for the aforesaid farming area. All experiments were repeated 3 to 5 times. Germination, root infections, yield, sugar content and sugar yield were evaluated according to generally accepted methods.

Statistical processing was performed with STATISTICA 10.0 software (StatSoft Inc., USA). The tables and figures present mean values ($M$) and their standard deviations ($\pm SD$) at $p \leq 0.05$.

**Results.** *Bacillus* bacteria inhabit plant rhizosphere [44] and are known as producers of various metabolites. The most important biologically active substances synthesized by these bacteria are peptides, lipopeptides, polyketide compounds, bacteriocins and siderophores [45]. Also, *Bacillus* genus species possess antagonistic activity against plant pathogens [46, 47] and promote growth of agricultural plants [38, 48]. The C-82/3 bacteria strain being discussed was attributed to *Bacillus* genus and it was assumed that it has the properties intrinsic to this genus. In addition, cellulolytic bacteria demonstrate high antifungal activity towards plant pathogenic fungi causing crop diseases [49, 50]. In our experiments, during lab evaluation of C-28/3 strain antifungal activity, the diameter of zone of growth inhibition was 28.9±0.2 mm for *F. oxysporum*, 38.2±0.3 mm for *F. solani*, and 46.6±0.9 mm for *A. alternata*. Such species specific effect of bacteria on plant pathogenic fungi correlates with other reports [22, 24].

The association of nitrogen fixing bacteria *Az. chroococcum* and phosphate mobilizing bacteria *B. megaterium* that we created earlier affected the sugar beet yield under field conditions. This is due to improved nitrogen and phosphorus nutrition of plants (data not shown). The association, however, possessed no antifungal effects on root rot causative agents. Thereby, antagonistic bacteria must be included in association to control plant pathogens.

The effectiveness of association is determined by a combination of biological traits of comprising strains [51, 52]. In development of EM association, it is particularly important to take into account the possibility to combine the strains. The main indicator in association designing is biological compatibility, i.e. presence or lack of antagonism among partner strains.

The perpendicular streak method [41] we used to evaluate biocompatibility of growth-promoting strains of the association and C-82/3 strain confirmed that these strains do not inhibit each other’s growth and development (Fig. 1), i.e. they show lack of antagonism. The result showed that C-82/3 strain could be introduced into the EM association. Biocompatibility of the studied bacteria strains may be explained by
the fact that in soil these physiological groups do not compete for nutrition sources [53]. In nature, they do not become antagonists, i.e. do not synthesize secondary metabolites to adversely affect each other.

It is well known that *Bacillus* genus species dominate in plant rhizosphere [44]. They are highly resistant to environmental factors and capable of synthesizing biologically active substances [28, 29]. A number of works state that due to these bioactive products *Bacillus* bacteria can stimulate plant growth [55, 56].

In a climate camera, during the cultivation of beet plants from seeds treated with C-82/3 strain and EM association that includes three bacteria strains it was found that they had growth promoting properties (Fig. 2). Thus, upon treatment with C-82/3 strain an average stem length and root length have increased 1.2±0.1 times and 1.1±0.1 times, respectively, as compared to control group (p ≤ 0.05). For the EM association, the indicators have increased 1.5±0.1 and 2.0±0.3 times (p ≤ 0.05). Pre-seeding treatment with C-82/3 strain and the EM association also enhanced seed germinating capacity to 86±1.5% and 93±1.7% (p ≤ 0.05), respectively, vs. 80±1.3% (p ≤ 0.05) in control group. In lab testing, we used the Aisultan variety sugar beet seeds recommended for cultivation in South-East Kazakhstan.

As was mentioned above, the growth-promoting capability of the EM association was caused by nitrogen fixing *Az. chroococcum* and phosphate mobilizing *B. megaterium* bacteria improving nitrogen and phosphorus nutrition of plants; *Bacillus* sp. C-82/3 strain, potentially, synthesizes vitamins, amino acids and phytohormones. Capability of C-82/3 to synthesize secondary metabolites promoting plant growth correlates with data found [28, 55, 56]). In addition, C-82/3 strain produces cellulases that can partially destroy the hard episperm thus enhancing germinating capacity [39].

Field tests demonstrated high effectiveness of the EM association consisting of *B. megaterium*, *Az. chroococcum* and *Bacillus* sp. C-82/3 strains as an agent of biocontrol of *Fusarium* and *Alternaria* fungi causing sugar beet root rot. Pathogens affected the plants throughout vegetation, starting from the 1st leaf pair to root-crop harvesting. First signs of root rot appeared in late May to early June, and reached maximum by harvesting (from late September to early October). The EM association reduced the incidence of root diseases in sugar beet seedlings 2.3-fold, in root-crop 3.0-fold as compared to control group (Table 1).

The microorganisms also positively influenced yield and quality of root-crop (Table 2). After application of the EM association, the sugar beet yield has increased by 34±2.3 centners per hectare or by 12.6±1.1% (p ≤ 0.05) vs. control group, sugar content in roots increased by 4.0%, sugar harvesting by 9.0 centners per hectare vs. control. It should be noted that treating seeds with the association did not considerably affect the aboveground biomass of sugar beet plants.
(data not shown), which is particularly important for the plants grown to use their root-crops [21].

1. Field germinating capacity and incidence of root infections in Aisultan variety sugar beet (*Beta vulgaris* L.) seedlings as depends on pre-seeding treatment (*M±SD*, Kaiyndy farm, Zhambyl region, Republic of Kazakhstan, 2017-2018)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Sprouts per m²</th>
<th>Root infections incidence, %</th>
<th>Sugar yield, centners/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>seedling black stem</td>
<td>Fusarium root rots</td>
</tr>
<tr>
<td>EM association</td>
<td>74.3±2.4*</td>
<td>17.2±1.1*</td>
<td>7.1±0.9*</td>
</tr>
<tr>
<td>TMTD + Score® (reference)</td>
<td>72.7±2.1*</td>
<td>18.5±1.4*</td>
<td>10.0±0.8</td>
</tr>
<tr>
<td>Control group (no treatment)</td>
<td>68.2±1.9</td>
<td>40.3±2.3</td>
<td>21.6±1.3</td>
</tr>
</tbody>
</table>

Note: EM association incorporates *Bacillus megaterium*, *Azotobacter chroococcum* и *Bacillus sp. C-82/3* bacteria. In a chemical treatment variant, TMTD 80% (Avgust, Russia) and Score® (Syngenta AG, Switzerland) at the rate of 6.0 kg/t and 0.4 l/ha, respectively, were used.

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

2. Yield and qualitative indicators of root-crops in Aisultan variety sugar beet (*Beta vulgaris* L.) under field conditions depending on pre-seeding treatment (*M±SD*, Kaiyndy farm, Zhambyl region, Republic of Kazakhstan, 2017-2018)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Yield, centners/ha</th>
<th>Control group excess, centners/ha</th>
<th>Sugar content, %</th>
<th>Sugar yield, centners/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM association</td>
<td>304.0±10.4*</td>
<td>34.0±2.3*</td>
<td>12.6±1.1*</td>
<td>14.9±1.1</td>
</tr>
<tr>
<td>TMTD + Score® (reference)</td>
<td>282.0±9.8*</td>
<td>12.0±0.9*</td>
<td>7.1±0.2</td>
<td>13.5±0.8*</td>
</tr>
<tr>
<td>Control group (no treatment)</td>
<td>270.0±7.9</td>
<td>11.2±0.5</td>
<td>4.8±0.2</td>
<td>48±1.5</td>
</tr>
</tbody>
</table>

Note: EM association incorporates *Bacillus megaterium*, *Azotobacter chroococcum* и *Bacillus sp. C-82/3* bacteria. In a chemical treatment variant, TMTD 80% (Avgust, Russia) and Score® (Syngenta AG, Switzerland) at the rate of 6.0 kg/t and 0.4 l/ha, respectively, were used.

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

Our findings are in line with the studies evidencing the increase in productivity of agricultural crops through use of effective microorganisms [57, 58]. Many works have established their growth-promoting activity [27, 48, 59]. Previously, however, effective microorganisms were used only for biological control of pathogenic fungi [46, 60] or for crop growth promotion [42, 55]. The ability of such microorganisms to synthesize bioactive metabolites was also employed [47, 61]. The EM association we designed is characterized by a complex action, i.e. it improves nitrogen and phosphorus nutrition of plants, possesses antifungal effect, produces biologically active agents, promotes sugar beet plant growth and development, which altogether contributes to enhancement of yield production and sugar content in root-crops.

Thus, our findings indicate high antifungal activity of new strain of cellulolytic bacteria *Bacillus* sp. C-82/3 against plant pathogenic fungi causing sugar beet root rot. The biocompatibility of this strain with nitrogen fixing and phosphate mobilizing bacteria strains is demonstrated, which enabled the creation of new association of effective microorganisms (EM association) possessing antifungal effect and positively affecting the productivity and quality of sugar beet. Two-year field tests of this EM association in South-East of Kazakhstan showed the potential for its introduction in soil biocenosis to control root rots caused by *Fusarium* and *Alternaria* fungi. The suggested EM association provides a 2.3 times reduce in incidence of fungal infection in seedlings and decreases the diseases of root-crop 3.0-fold while increasing the yield by 12.6% and sugar harvest by 9.0%. In addition to positive effect on the sugar beet yield, the EM association promoted increased sugar content in root-crops. The proven high effectiveness of the EM association supports the expediency of its application as biological control agent in in Russia, Kazakhstan and other neighboring countries with similar natural and climatic conditions.


Mau A.A., Izmukhambetov Zh.D. Kompleksnaya sistema zashchity posevov sakharoi svekly ot vreditelei, boleznei i sornyakov dlya uslovii yuga i yugo-vostoka Kazakhstana [A comprehensive system for the protection of sugar beet crops from pests, diseases and weeds in the south and southeast Kazakhstan]. Almaty, 2012 (in Russ.).

Selivanova G.A. Zemledelie, 2013, 4: 31-37 (in Russ.).

Stognienko O.I., Shamin A.A. Zashchita i karantin rastenii, 2014, 8: 12-15 (in Russ.).


Shabae V.P. Sel'skokhozyaistvennaya biologiya [Agricultural Biology], 2005, 3: 55-61 (in Russ.).


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Shabae V.P. Sel'skokhozyaistvennaya biologiya [Agricultural Biology], 2005, 3: 55-61 (in Russ.).


Bzdýk R.M., Olchowick J., Studnicki M., Oszako T., Sikora K., Szmidla H., Hilszczaińska D. The impact of effective microorganisms (EM) and organic and mineral fertilizers on the growth


36. Filetova N.N. *Metabolity aerobnykh tsellulyozoliticheskikh mikroorganizmov i ikh rol' v pochvakh* [Metabolites of aerobic cellulolytic microorganisms and their role in soils]. Novosibirsk, 2010 (in Russ.).


40. Egorov N.S. *Osnovy ucheniya ob antibiotikakh* [Basics of the antibiotics doctrine]. Moscow, 2004 (in Russ.).


43. Borovikov V.P. *Polyarnoe vvedenie i sovremennyi analiz dannykh v sisteme STATISTICA* [A popular introduction to modern data analysis in the STATISTICA system]. Moscow, 2013 (in Russ.).


PRE-SOWING PROTECTION OF INOCULATED SOYBEAN
Glycine max (L.) Merr. SEEDS BY WATER-SOLUBLE POLYMER COMPOSITIONS AND THEIR SOLID-PHASE MODIFICATION

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A b s t r a c t

The effectiveness of some water-soluble polymers as film-forming agents that provide better adhesion of bacteria to seeds (like multicomponent formulations in modern chemical dressings) remains practically relevant. The likely candidate adhesives are low and high molecular weight sodium alginate (FMC polymer), hydroxypropyl methylcellulose (HPMC) (Colorcon®, Colorcon, Inc., USA), polyethylene glycol (PEG), carbomer-carbopol 940 (Necardis SA), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (povidone, PVP) (K15). Film-forming polymers can also improve the shelflife of biologicals, their compatibility with chemical protective agents and resistance to UV radiation, temperature extremes and drying, thus increasing survival of bacteria on the surface of inoculated seeds. These allow practitioners to carry out seed pre-sowing inoculation beforehand. Developed polymer compositions should be more effective than single-component, provided that they remain cost-effective and convenient for practical use. This paper is the first to report the effects of various polymer combinations on inoculated seeds and the improvement of protective properties of the water-soluble polymers by activated charcoal, a solid-phase component. Among the polymers tested, polyvinylpyrrolidone is revealed to be the most effective for rhizobial survival due to longer allowable interval between seed inoculation and sowing. Our objective was to compare survival rate of<br>Bradyrhizobium japonicum 634b inoculum for soybean cv. Belgorodskaya 7 seeds as influenced by water-soluble polymers polyvinylpyrrolidone, polyvinyl alcohol, sodium alginate and carboxymethylcellulose as additives. Our findings indicate that 10 % polyvinylpyrrolidone solution is the most effective among the studied polymers. Its use increases more than 10-fold the survival of nodule bacteria on seeds 10 days after inoculation of seed material. Variants with different concentrations of carboxymethyl cellulose and sodium alginate do not ensure bacterial survival on seeds for more than 3 days. It is possible to create an effective polymer-carbon composition with a lower concentration of polyvinylpyrrolidone (7.5 % polyvinylpyrrolidone and 5.0 % activated charcoal). This composition is more effective than polyvinylpyrrolidone without coal, and provides a 20-30 % reduction in bacterial death on inoculated seeds after the first 5-7 days of seed storage.

Keywords: symbiotic nitrogen, Bradyrhizobium japonicum, inoculation, soybean, polyvinylpyrrolidone, polyvinyl alcohol, sodium alginate, carboxymethylcellulose

Soybean is a valuable leguminous crop and is of vast food, fodder and agricultural importance [1–3]. Soybean seeds are rich in easily digestible protein (up to 39–42%) and valuable oil (up to 18–23%), and its herbage harvested not later than the bean plumpness phase is a nutritious (22-23 fodder units per 100 g herbage) and vitamin-rich (50–60 mg of carotene per 1 kg herbage) fodder [4, 5]. Due to intensive nitrogen fixation and high crop management practices, soy-
bean plays a positive environment-forming role in crop rotation and considerably increases the companion crop yield [6, 7]. Soybean is considered a good predecessor for cereals, tilled and fodder crops. From the agrotechnological point of view, soybean is very plastic, and, depending on agricultural, soil and climatic conditions, can be cultivated both as cereal and as tilled crop by varying within the extensive range the fertilization and protector application rates [8, 9]. A wide diversity of varieties with different earliness and demands on growth factors allows the soybean plant to easily adapt to growing conditions. All of the above allows us to view soya as a multipurpose crop [10].

An important agrobiological feature of soybean is its capability to form nitrogen fixing legume-rhizobia symbiosis [11, 12]. Such symbiosis completely provides the needs of plant in nitrogen [13], however such an intensive nitrogen fixation is possible only in optimal conditions, particularly if active virulent symbiont bacteria are present in soil in an amount sufficient for effective symbiosis [14, 15]. Normally, it can only be achieved through artificial pre-seeding inoculation of soybean with nodule bacteria [16, 17]. It is important not only to choose the right strain of bacteria, which will be most effective in appropriate soil and climatic conditions, but also to properly inoculate the seeds with such a preparation, which, for inoculants used today, means, among other things, mandatory seeding-down of treated seeds on the treatment day [18].

In practice, it is this requirements that is the most difficult for major farms and often neglected. Typically, it results in considerable reduction of effect of inoculation, which is manifested in poor nodulation and further nitrogen deficiency and hence in considerable underharvesting. Therefore, the researchers lately pay their attention to development of methods enabling the increase in the number of nodule bacteria on inoculated seed at the time of its seeding. One of the most promising ways is to combine polymer solutions acting as adhesives, film-formers and rhizobia protectors on treated seeds with inoculant [19, 20]. Polymer solutions must, first, fix the bacteria in polymer films, thus preserving the largest possible number of bacteria on treated seeds, and second, enhance bacterial resistance to adverse environmental factors, such as desiccation, sunlight, rapid temperature changes and seed exudates toxic for rhizobia. In combination, such properties contribute to increase of period between seed inoculation and seeding [21, 22].

The most widespread polymer adhesives in agricultural practice are polyvinylpyrrolidone (povidone), polyvinyl alcohol, sodium alginate and carbomethylcellulose [23], however their effectiveness as rhizobia protectors is poorly studied. Of particular urgency is the issue of effectiveness of a polymer and its optimal concentrations, as well as recommendations for its application as bacteria protector for a particular rhizobia strain-variety pair.

In this paper, having analyzed the effects of a number of water soluble polymers, we identified polyvinylpyrrolidone as the most effective rhizobia protector (it extends the allowable interval between seed inoculation and sowing) and for the first time demonstrate that a solid-phase component (activated charcoal) improves the protective properties of polymer composition upon pre-seeding inoculation.

The purpose of this work was the investigation of temporal dynamics of viable rhizobia counts on inoculated soybean seeds as affected by various polymers in different concentration and by the mixes of different components, and also upon optimization of polymer protectors with activated charcoal.

Techniques. In order to obtain *Bradyrhizobium japonicum* preparation, 634b strain (Departmental Collection of Beneficial Agricultural Microorganisms of All-Russian Research Institute of Agricultural Microbiology) was grown in
liquid semisynthetic medium for 1 week at 28 °C on shaker [24].

As polymeric additives to the inoculate, water-soluble polymers were used, i.e. polyvinylpyrrolidone (Sigma-Aldrich, USA), PVA polyvinyl alcohol grades 4-98, 4-88 (Sigma-Aldrich, USA), sodium alginate (Xiamen Huaxuan Gelatin Co., Ltd, China) and carboxymethylcellulose (ZAO Karbokam, Russia). Activated charcoal powder (grade OU-A, OAO Sorbent, Russia) was used as solid-phase component for polymer protectors. Belgorodskaya 7 cv. soybean seeds were inoculated with the preparation in the following way. *B. japonicum* 634b strain (0.25 ml of 20% suspension) was applied to seed portions (25 g) in Petri dishes. The dishes containing inoculated seeds were stored at room temperature in the dark, with periodical collection of seed portions to prepare swabs (for the first time, 1 hour after inoculation, then after 27, 72, 124, 168 and 240 hours).

For quantitation of viable bacteria on a single soybean seed, 8 inoculated seeds from the Petri dish were placed in a test tube containing 8 ml of sterile water and shaken on a vortex for 1 minute. Tenfold dilutions of swabbed sample were prepared followed by plating onto agar-based nutrient medium (0.5 g/l K$_2$HPO$_4$, 0.2 g/l MgSO$_4$·7H$_2$O, 0.1 g/l NaCl, 1.0 g/l yeast extract, 10.0 g/l mannitol, 16 g/l agar-agar; pH 6.8-7.2) in Petri dishes for incubation at 28 °C and counting of the colonies formed. Based on the bacteria titer in the sample, their count was determined per 1 inoculated seed. The experiment was carried out in 4 biological replicates.

Statistical processing was carried out using Microsoft Excel 10. The figures and tables present mean (M) and standard error of the mean (±SEM). Differences were evaluated by Student’s t-test and were considered statistically significant at p < 0.05. Analysis of variance was carried out according to Dospekhov [25]. In variants where the polyvinylpyrrolidone was used as protector, the difference from control group exceeded the least significant difference LSD$_{05}$ values and was statistically significant.

**Results.** Soybean seed surface is an adverse medium for *B. japonicum* (Fig. 1, A). The same figure shows the curve of reduction (RC) of alive rhizobia upon treatment of soybean seeds with bacterial suspension with 100 g/l polyvinylpyrrolidone. Polyvinylpyrrolidone was chosen as a base for protector due to a number of works attributing the polyvinylpyrrolidone such traits as high adhesiveness, water retention, ability to protect bacteria from toxic seed exudates and to enhance overall viability of bacteria on inoculated seeds [22].

![Fig. 1. *Bradyrhizobium japonicum* bacteria strain 634b survivability on inoculated Belgorodskaya 7 soybean seeds in the presence of polyvinylpyrrolidone (A) and in its combination with activated char-

-200 0 500 1000 1500 2000 2500 3000 3500 4000 4500 5000 5500 6000 6500 7000
1 27 72 124 168 240

*Bradyrhizobium japonicum* strain 634b × 10<sup>5</sup> CFU per grain

**A**

0 500 1000 1500 2000 2500 3000 3500 4000
1 27 72 124 168 240

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coal within povidone-charcoal compounds: 1 — control, 2 — polyvinylpyrrolidone (100 g/l), 3 — number of viable rhizobia per 1 seed required for forming effective symbiosis, 4 — polyvinylpyrrolidone (100 g/l) + activated charcoal (0 g/l), 5 — polyvinylpyrrolidone (100 g/l) + activated charcoal (25 g/l), 6 — polyvinylpyrrolidone (100 g/l) + activated charcoal (50 g/l), 7 — polyvinylpyrrolidone (100 g/l) + activated charcoal (75 g/l) (lab test).

The tests revealed that adding polyvinylpyrrolidone materially improves the survivability of soybean rhizobia on inoculated seeds. Thus, in control group (CG), the number of viable bacteria on seeds exceeds the threshold required for effective symbiosis (about 40 000 CFU per seed) in 72 hours. With polyvinylpyrrolidone, CG drops to a stable level of 500 000 CFU per seed in 24 hours and does not suffer any considerable drops for at least 10 days.

Successful practice of modification of polymer solutions was described, particularly, in a paper dedicated to influence of ZnO and MgO additives on the effects of carboxymethylcellulose as a compound promoting survivability of soybean nodule bacteria during storage in liquid culture [26]. In our research, adding to the polyvinylpyrrolidone solution of solid-phase filler (activated charcoal) also enabled us to somewhat increase the effectiveness of composition based on polyvinylpyrrolidone (see Fig. 1, B). Optimal charcoal concentration in the final solution was 50 g/l.

Similarly, the optimal concentration of polyvinylpyrrolidone (75 g/l) in combination with activated charcoal (50 g/l) in a povidone-charcoal composition was identified (Fig. 2).

Fig. 2. Survivability of Bradyrhizobium japonicum 634h strain on inoculated seeds of soybean Belgorskaya 7 variety depending on povidone-charcoal composition: 1 — control, 2 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l), 3 — polyvinylpyrrolidone (50 g/l) + activated charcoal (50 g/l), 4 — polyvinylpyrrolidone (75 g/l) + activated charcoal (50 g/l), 5 — polyvinylpyrrolidone (100 g/l) + activated charcoal (50 g/l) (lab test).

Thus, our experiments showed high effectiveness of polyvinylpyrrolidone as a polymer base for rhizobia protector, though its relatively high cost considerably limits the opportunities for industrial production of polyvinylpyrrolidone-based protectors. Therefore, we have compared a number of analogous inexpensive polymers widespread in agriculture as adhesive that can potentially substitute (in full or in part) expensive polyvinylpyrrolidone in povidone-charcoal mixture.

One of such analogs is polyvinyl alcohol that is described as a seed-encapsulating polymer promoting the resistance of nodule bacteria to stress-factors [27]. Polyvinyl alcohols of both grades were studied according to the methodology similar for studying polyvinylpyrrolidone (Table 1). It turned out that while polyvinyl alcohol improves the survivability of rhizobia on seeds, it is materially inferior to polyvinylpyrrolidone in effectiveness.

We also have tested carboxymethylcellulose and sodium alginate as protectors. These polymers were elected due to the reports of their propitious effect on liquid culture of nodule bacteria during storage [23]. For sodium alginate, a capability of sustaining the viability of growth-promoting bacteria on inoculated glass beads for over 14 years was reported [28]. In our experiments, however, the effect of application of carboxymethylcellulose and sodium alginate as bacteria protectors
was rather insignificant (Table 2).

### 1. Survivability of *Bradyrhizobium japonicum* 634b strain on inoculated seeds of soybean Belgorodskaya 7 variety when polyvinyl alcohol is applied

(10^3 CFU per one seed, N = 4, lab test)

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>Variant</th>
<th>control</th>
<th>50 g/l</th>
<th>75 g/l</th>
<th>100 g/l</th>
<th>200 g/l</th>
<th>LSD_{0.05}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polyvinyl alcohol</td>
<td>255±12.1</td>
<td>300±13.4</td>
<td>470±18.2</td>
<td>580±23.6</td>
<td>845±31.7</td>
<td>35.40</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>25±1.1</td>
<td>25±1.2</td>
<td>30±1.7</td>
<td>55±2.1</td>
<td>105±4.2</td>
<td>5.08</td>
</tr>
<tr>
<td>66</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>52.9</td>
<td>20±1.4</td>
<td>2.45</td>
</tr>
<tr>
<td>93</td>
<td></td>
<td>0</td>
<td>15±0.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Polyvinyl alcohol</td>
<td>85±3.3</td>
<td>180±4.2</td>
<td>255±6.7</td>
<td>305±7.4</td>
<td>425±9.5</td>
<td>8.97</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>76</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

In addition, it should be noted that sodium alginate and particularly carboxymethylcellulose make the solutions much more *viscous* than polyvinylpyrrolidone does in the same concentrations hence these polymers are not only ineffective as rhizobia protectors but also much less feasible practically. Other authors in their papers also demonstrate that sodium alginate and carboxymethylcellulose that successfully support the bacteria survivability in liquid culture during storage were ineffective protectors on seeds [23]. In the same study, polyvinylpyrrolidone was an extremely effective polymer protector of rhizobia on seeds, but inhibited bacteria stored in liquid culture [23]. At the same time it had been reported that in 4% concentration polyvinylpyrrolidone successfully protects *Azotobacter vinelandii* cells in liquid culture during prolonged storage [29], and salutary effect of polyvinylpyrrolidone on the stored nodule bacteria culture was reported [30], which may indicate the species (strain) specificity of effect of the polymer on bacterial cultures.

Given the high cost of polyvinylpyrrolidone, we have studied the possibility of its at least partial substitution with sodium alginate and carboxymethylcellulose in polymer-charcoal composition (Fig. 3).

![Fig. 3. Survivability of *Bradyrhizobium japonicum* 634b strain on inoculated seeds of soybean Belgorodskaya 7 variety depending on partial substitution of polyvinylpyrrolidone for carboxymethylcellulose or sodium alginate in povidone-charcoal composition: 1 — control, 2 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l) + , 3 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l) + carboxymethylcellulose (50 g/l), 4 — polyvinylpyrrolidone (25 g/l) + activated charcoal (50 g/l) + sodium alginate (50 g/l) (lab test).](image)

Adding of sodium alginate to povidone-charcoal composition has reduced the latter’s effect to some extent, and carboxymethylcellulose has almost not affected...
its effectiveness, which shows that polyvinylpyrrolidone within povidone-charcoal composition cannot be substituted with another polymer even partially.

Thus, soybean seed surface is an adverse medium for \textit{Bradyrhizobium japonicum} strain 634b. The count of these bacteria per inoculated seed drops from 620 000 (1 hour after treatment) to 115 000 (27 hours after treatment). Bacteria destruction can be considerably slowed down through use of polymeric additives to inoculants. The most effective protector of rhizobia among the studied polymers is polyvinylpyrrolidone. When applied in concentration of 100 g/l, 500 000 viable rhizobia remained on a single seed for 10 days. Polymeric basis of this protector may be modified with activated charcoal, which enhances the effects of povidone-charcoal composition 1.5-2.0 times (optimal concentration of povidone in the composition is 75 g/l, concentration of activated charcoal is 50 g/l). Potential full or partial replacements of povidone (polyvinyl alcohol, sodium alginate, carboxymethyl cellulose) in the proposed composition are ineffective.

**REFERENCES**

2. Vavilov P.P., Pospyanov G.S. \textit{Bobovye kul'tury i problema rastitel'nego belka} [Legumes and the problem of vegetable protein]. Moscow, 1983 (in Russ.).
7. Kokorina A.L., Kozhemyakov A.P. \textit{Bobovo-ribozial'ni simbiox i primenenie mikrobiologicheskikh preparatov kompleksnogo deistviya — vazhnyi rezerv povysheniy produktivnosti pashni} [Legume-rhizobial symbiosis and complex microbiological preparations are an important reserve to increase arable land productivity]. St. Petersburg, 2010 (in Russ.).
18. Vashan Y., Bashlan L., Prabhu S.R., Hernandez J. Advances in plant growth-promoting bacteri-


