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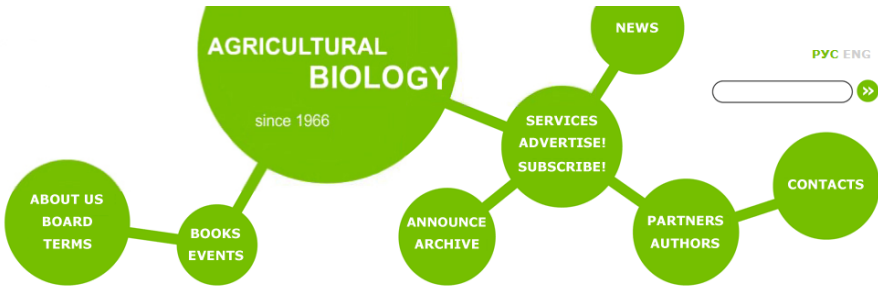
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MOLECULAR BASIS OF THE DWARFISM CHARACTER IN CULTIVATED PLANTS. I. GROWTH DISTORTIONS DUE TO MUTATIONS OF GIBBERELLIN METABOLISM AND SIGNALING

(review)

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Abstract

Development of dwarf cereal varieties with improved mechanical stability of stems preventing their lodging led to significantly increased crop productivity in the 1960-1970s. The creation of novel high-yielding cultivars was one of the main purposes of the «Green revolution» aimed at the reorganization of agriculture in developing countries (G.S. Khush, 2001). At the current time the dwarfism character is of widely use in plant breeding. The dwarf varieties are not only resistant to lodging but also have higher nutrients-absorbing potential and often are more tolerant to diseases than traditional cultivars (K.U. Kurkiev et al., 2006). In connection with that the factors that pre-terminate plant growth have become of increasing scientific interest over recent years. Numerous dwarf cultivars produced during the «Green revolution» possessed mutant genes responsible for metabolism and transmission of gibberellic (GA) signal (M. Ueguchi-Tanaka et al., 2001; T. Sakamoto et al., 2004). GAs are involved in control of many stages of plant development, including seed germination, stem and root elongation (E. Tanimoto, 2012; P. Hedden, V. Sponsel, 2015). However, alternations at different steps of the GA-dependent processes might lead to different results: to plants with reduced height as well as to tall slender plants. Clear understanding the interaction of genetic and molecular mechanisms will facilitate the revealing of key molecular targets the changes in which would result in production of the desired dwarf varieties. The paper considers the ways of gibberellins biosynthesis, deactivation and how pool of active GAs is maintained. Among numerous known GAs, produced by plants, only GA₁, GA₃, GA₄, GA₅ and GA₇ are physiologically active. GA₂₀-oxidases and C3,β-oxidases (or C3,β-hydroxylases) involved in production of the active gibberellins catalyze final reactions of gibberellin biosynthesis. C2,β-oxidases are main enzymes that can quickly inactivate active GAs by adding a hydroxyl group (–OH) to a GA molecule. Modern concept about GA-signaling is reviewed according to the following established steps: GA receptor GID1; DELLA-proteins as the negative regulators in GA signaling; SCF E3-ubiquitin protein ligase and 26S proteasome; transcriptional factors with DNA-binding site; GA-regulated genes. Accumulated up-to-date data consider that in transmission of gibberellic signal a GA molecule initiates DELLA-protein degradation via interaction of GID1-DELLA complex with E3 SCF^{SLY1/GID2} (T.-P. Sun, 2011). Thus, reduced growth in dwarf cultivars can be associated with defects in biosynthesis of the active GAs or with accumulation of repressors of GA signaling, the DELLA-proteins, while GA-constitutive growth in tall slender forms might be caused by disturbance in GA-deactivation system or lack of the DELLA repressive function (H. Claeys et al., 2014). The paper also considers ways of participation of gibberellin in the complex hormone regulation of plant growth which occurs often via control over the repressive function of DELLA-proteins (P. Achard et al., 2003). A special attention is paid to characteristics of the genes that lead to altering plant growth, the dwarfism or gigantism.

Keywords: dwarfism, restrained growth, gibberellin signal transduction, receptor GID1, DELLA protein, proteolysis.

Dwarfing genes are known to be widely used in crop breeding. Dwarf plants are compact and resistant to lodging, which makes them suitable for mechanical harvesting. Additionally, they can possess higher nutrient-use efficiency and be more resistant to diseases. Russian scientists have contributed significantly to the involvement of dwarf forms in breeding rye [1], wheat [2-4], sorghum [5], triticale [6], sunflower [7], fruit plants [8], etc.

In 1920 N.I. Vavilov, in his law of homologous series in variation, paid attention to appearance of dwarf forms and giants in genetically unrelated families (*Gramineae*, *Papilionaceae*, *Urticaceae*, *Solanaceae*, *Rosaceae*, etc.). These hereditary variations naturally repeated in different taxa were considered as a result of convergence, evolution, parallel variation or mimicry [9]. Subsequently, factors determining plant growth, as well as genetic and molecular mechanisms of the phenomenon, have been the subject of numerous investigations. Different dwarf varieties have been shown to carry mutations in identical genes. Among plant dwarf mutations the most thoroughly studied ones are those of gibberellin biosynthesis and signaling pathways. The Green Revolution, led by Norman Ernest Borlaug, an American breeder, plant pathologist and Nobel laureate [10], was exactly due to dwarf forms actively used in 1940-1970s. The Green Revolution demonstrated new opportunities of involving variations of agronomic genes in breeding for higher crop yields [11].

Currently, a lot of data are accumulated on how the genes involved in gibberellin biosynthesis and signal transduction regulate plant growth, and the factors responsible for the altered growth phenotypes were found. The objective of our review was to systematize these data, to give a scheme of gibberellin involvement in dwarfing plants, and to explain the molecular mechanisms underlying dwarf mutants traditionally used in plant breeding.

Gibberellins (GA; the abbreviation comes from the most known gibberellic acid — GA) are tetracyclic diterpenes capable of inducing a strong elongation of stems [12]. Among the currently found numerous GA produced by plants, only GA₁, GA₃, GA₄, GA₅ and GA₇ are physiologically active. The active GA can be rapidly inactivated by adding methyl (—CH₃), hydroxyl (—OH), and other groups to the molecule. GA are involved in many stages of plant development, including seed germination, stem and root growth, meristem formation, flower, fruit and seed development, photomorphogenesis and circadian rhythms. In case of GA biosynthesis disorder and the lack of active GA, development of dwarf plants with dark green leaves is observed, sometimes with abnormalities of flowering, male sterility and a prolonged dormancy of seeds, to induce germination of which it is often necessary to remove the seed coat [13-16].

The plants with altered growth response are divided into GA-sensitive and GA-insensitive mutants (Table).

Growth response in mutant plants deficient in gibberellin biosynthesis, deactivation and signaling

Plant	Mutation	Encoded protein	Loss of protein function (structural damage)	Phenotype	Reference
G A b i o s y n t h e s i s					
Arabidopsis	<i>gal-3</i>	CPS (<i>ent</i> -copalyl diphosphate	Blocking GA synthesis	GA-sensitive, dwarf	[16]
Pea	<i>ls</i>	synthase, EC 5.5.1.13)	at <i>ent</i> -copalyl diphosphate		[17]
Rice	<i>osks</i>	KS (<i>ent</i> -kaurene synthase,	Blocking GA synthesis	GA-sensitive, dwarf	[14]
Maize	<i>d5</i>	EC 4.2.3.19)	at <i>ent</i> -kaurene		[15]
Rice	<i>d35</i>	KO (<i>ent</i> -kaurene oxidase,	Blocking GA synthesis	GA-sensitive, dwarf	[13]
Pea	<i>lh</i>	EC 1.14.13.78)	at <i>ent</i> -kaurene acid		[17]
Arabidopsis	<i>kao1</i>	KAO (<i>ent</i> -kaurenoic acid	Blocking GA synthesis	GA-sensitive, dwarf	[16]
Pea	<i>kao2</i>	oxidase, EC 1.14.13.79) of	at GA ₁₂ aldehyde		[17]
Maize	<i>na</i>	cytR450 88A1 group			[18]
Barley	<i>d3</i>	(CYP88A1) in cytochrome			[19]
	<i>grd5</i>	P450 family (CYP)			

Table continued				
Rice	<i>sd1</i>	GA ₂₀ -oxidase (EC 1.14.11.12)	Blocking oxidation of C ₂₀ -gibberellins to C ₁₉ -gibberellins	GA-sensitive, dwarf [13]
Rice	<i>d18</i>	D18 (Dwarf18) and LE (Length)	Blocking hydroxylation at C3,β position in gibberellin biosynthesis	GA-sensitive, dwarf [13, 20]
Pea	<i>le</i>	(gibberellin 3-β-oxidase, EC 1.14.11.15, or C3,β-oxidase)		[21, 22]
GA deactivation				
Pea	<i>sln</i>	SLN (Slender) (gibberellin 2-β-oxidase, EC 1.14.11.13, or C2,β-oxidase)	Blocking hydroxylation at C2,β position in gibberellin deactivation	Slender form with GA-induced growth [17, 23]
GA perception				
Rice, arabidopsis, rape, soybean	<i>gid1-1</i>	GID1 (Gibberellin Insensitive Dwarf 1; high homology of plant carboxyl esterase with α- and β-hydrolase superfamily)	Replacement of amino acids in the GX SXG motif (lack of GFA-GID1 formation)	GA-insensitive, dwarf [20, 24, 25]
Rice	<i>d1</i>	Gα (α-subunit of heterotrimeric G-Enzyme, GTPase, EC 3.6.5.1)	Function in GA-signaling is not defined	Semi-dwarf (growth response to GA is weakened) [26, 27]

The first group includes mutants (both dwarfs and giants) sensitive to endogenous GA level. In these plants the mutations affect genes encoding enzymes of GA biosynthesis or deactivation. Therefore, dwarf plant growth can be stimulated by treatment with active GA, and giant plants growth can be slowed down by GA biosynthesis inhibitors. In plants of the second group the growth changes may be associated with disorders in gibberellic signal perception and transduction to GA-inducible genes. This group, unlike the first one, is characterized by a reduced response or insensitivity to GA.

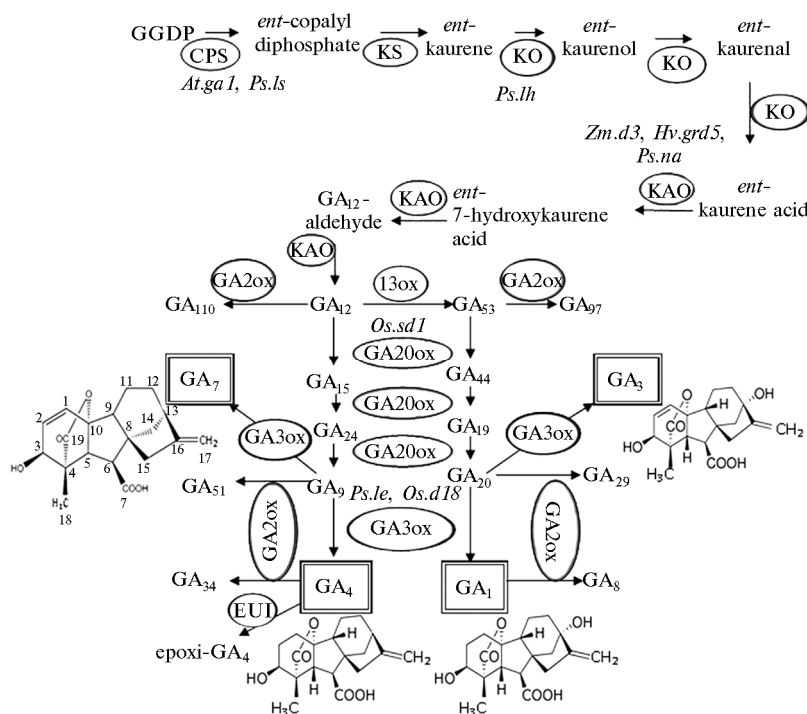


Fig. 1. Scheme of gibberellin biosynthesis [28] (as amended): GGDP — geranylgeraniol diphosphate, GA₁₋₁₁₀ — gibberellins; CPS — *ent*-copalyl diphosphate synthase, KS — *ent*-kaurene synthase, KO — *ent*-kaurene oxidase, KAO — *ent*-kaurenoic acid oxidase; 13ox — monooxygenase hydroxylating gibberellic GA₁₂ aldehyde at C13 position; GA20ox, GA3ox and GA2ox — three groups of 2-oxoglutarate-dependent dioxygenases (GA₂₀-oxidase, C3,β-oxidase and C2,β-oxidase); EUI (Elongated Uppermost Internode) — GA deactivating epoxidase; *At.gal*, *Ps.ls*, *Ps.lh*, *Zm.d3*, *Hv.grd5*, *Ps.na*, *Os.sd1*, *Ps.le*, *Os.d18* — dwarf mutants on relevant genes. Active gibberellins are marked with square frames; enzymes of gibberellin biosynthesis and deactivation are marked with ovals.

Growth disorders due to mutations in the genes of GA biosynthesis enzymes. All gibberellins derive from diterpene geranylgeraniol diphosphate (Fig. 1), which is also the precursor for carotenoids and unsaturated phytol, a diterpene alcohol forming part of chlorophyll. Gibberellin biosynthesis starts with the geranylgeraniol diphosphate cyclization occurring in the proplastid stroma [29, 30]. The final product of the cyclization is hydrophobic *ent*-kaurene. Its formation is catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). Arabidopsis plants with a knockout mutation in the gene encoding CPS (*gal-3*) are characterized by poor germination, dwarf type, underdeveloped root system, late flowering and male sterility [16, 29, 31-33]. Dwarf growth, flowering and seed formation disorders were also observed in pea, corn and rice mutants with knockout in CPS and KS genes, and also in the *ent*-kaurene oxidase (KO) and *ent*-kaurene acid oxidase (KAO) genes of the next phases of GA biosynthesis [13, 15, 17]. Treatment with gibberellin and its precursors restored normal plant phenotype, thus proving the important role of these enzymes in the biosynthesis of gibberellins.

The next stage of GA biosynthesis is a sequential oxidation of *ent*-kaurene to GA₁₂ aldehyde with two monooxygenases from R450 cytochrome family (see Fig. 1), the *ent*-kaurene oxidase (KO) and *ent*-kaurene acid oxidase (KAO) of CYP88A1 subfamily. In *Arabidopsis*, KO is localized on the outer surface of the outer plastid membrane, while KAO is associated with endoplasmic reticulum membranes [16, 19, 29]. Due to the mutations that lead to the loss of KAO activity, dwarf plants are observed in maize *d3* (*dwarf3*), barley *grd5* (*GA-responsive dwarf*) and *na* peas (*nana*) sensitive to GA [17-19]. Further reactions, beginning with GA₁₂ aldehyde oxidation, occur in the cytoplasm and lead to the formation of various forms of gibberellins [28-30].

The production of active gibberellin forms involves soluble dioxygenases, using 2-oxoglutarate as a substrate. One group of these enzymes is GA₂₀-oxidases (GA20ox), and the other group is the C3,β-oxidases (GA3ox, or C3,β-hydroxylases), of which the former oxidize C₂₀-gibberellins to C₁₉-gibberellins, and the latter catalyze hydroxylation of GA molecules at C3,β position at the final stage of the biosynthetic pathway. Rice mutants with deactivated *GA3ox* and *GA20* genes were stunted [13, 34]. Semi-dwarf rice mutant *sd1* (*semi-dwarf1*), used in the Green Revolution, carried the mutation in the *GA20ox* gene [13]. Along with the biosynthetic enzymes, the third group of dioxygenases called C2,β-oxidases (GA2ox) is also important in maintaining active gibberellins pool. These enzymes transform active GA and their precursors to inactive forms by hydroxylating the molecule at C2,β position [29, 35, 36]. In addition to C2,β-oxidase, deactivation of gibberellins in rice plants may involve EUI epoxidase (Elongated Uppermost Internode phenotype) from the P450 monooxygenases family [28, 29], and in *Arabidopsis* methyltransferase (GAMT1 and GAMT2, Gibberellin Methyl Transferases 1, 2) may be involved [37]. Thus, the pool of active GA is sustained by various feedback mechanisms, which regulate expression of GA biosynthesis genes (*GA20ox*, *GA3ox*) and GA catabolism genes (*GA2ox*). Expression of these genes is specific regarding particular time and localization, and depends on lighting conditions, temperature and phytohormones [29]. The expression of genes involved in the final stages of GA biosynthesis is inversely correlated with bioactive GA level. As GA is accumulated, the GA₂₀-oxidase gene expression is reduced, and GA catabolism gene expression is enhanced [38-40]. In the mutant dwarf pea plants (*le*) [21, 22] with a lack of GA due to reduced function of C3,β-oxidases the transcriptional activity of GA biosynthesis gene (*GA20ox*) is high, while it is low for GA deactivation gene (*GA2ox*). In the pea mutants *SLN* (*SLENDER*) with elongated shoots the disorders affect C2,β-

oxidase gene. In these plants due to suppressed GA deactivation the bioactive GAs are accumulated even despite a very small number of GA₂₀-oxidase transcripts [23, 29].

GA reception and transmission of the signal. The currently accumulated data allow to suggest that GA signal reception and transmission events involve gibberellins receptor GID1 (Gibberellin Insensitive Dwarf 1); proteins with conserved DELLA motif, negative regulators of GA-signaling; SCF (SKP-Cullin-F-box) E3 ubiquitin-protein ligase covalently attaching ubiquitin to the target protein, and 26S proteasome; transcription factors (TF) with a DNA-binding domain; gibberellin-regulated genes.

In the early 1990s the existence of a GA receptor at the plasma membrane (PM) was hypothesized. The hypothesis that the binding of gibberellin molecules with PM receptor is a necessary step in GA-signal transduction was experimentally confirmed using aleurone cell protoplasts. For many years aleurone layer of grains has served as a convenient model for the study of GA signaling and responses [30]. During seed germination GA causes α -amylase synthesis in the aleurone cells and secretion of this and other hydrolytic enzymes to endosperm for hydrolysis of stocked substances, which are subsequently used in the heterotrophic nutrition of the developing seedlings. In experiments, GA₄ linked covalently to sepharose granules induced α -amylase gene expression in aleurone protoplasts with no cell walls. The size of the granules prevented GA molecules from penetrating into the cells, therefore, phytohormone reception had to occur on the cell surface [41]. Hypothetical gibberellin receptor on the aleurone cells PM was not found. Nevertheless, a GA signaling scheme involving this hypothetical receptor was proposed. It was designed by analogy with mechanism known for animal cells hormonal signal transduction from transmembrane receptor of GPCR-type (G-Protein-Coupled Receptor) to heterotrimeric G-protein (GTPase, EC 3.6.5.1) [25]. The question whether the GPCR-type receptors in plants exist or not has not yet been resolved [42]. Currently, this role is attributed to several transmembrane proteins [27], while the G-proteins have been discovered, and their participation in the GA-signaling is indeed confirmed experimentally. Thus, the mutation in rice *d1* (*dwarf1*) affects the gene encoding α -subunit of the G-protein. Mutants in which G α -protein activity could not be detected were stunted and characterized by attenuated responses to the GA [26]. However, the mechanism of participation of G α -protein in GA signal transmission has not been conclusively determined [28, 43, 44]. It is known that G α -protein modulated physiological responses not only for GA-signaling but also for other hormones (brassinosteroids, abscisic acid — ABA) and environmental factors (blue light, ozone) [43].

GID1 gene (*Gibberellin Insensitive Dwarf 1*) was identified in rice dwarf mutants not responding to GA treatment [24]. It is believed to be the only GA receptor gene in rice [44]. Such a gene is also found in soybean and rape [45]. The relationship between the dwarf phenotype of plants and reduced *GID1* gene expression in rape is allegedly caused by a mutation in the promoter region [46]. In the *Arabidopsis* genome three genes homologous to *GID1* (*AtGID1a*, *AtGID1b* and *AtGID1s*) are identified. Triple insertion mutant for these genes has a pronounced short form and is unable to reproduce [47].

Soluble GID1 protein is localized in the nucleus and in the cytoplasm [24, 48, 49]. Its C-terminal domain is highly homologous to plant carboxyl esterases of α - and β -hydrolase superfamily. However, GID1 is not esterase-active due to the substitution of key amino acid residues in the catalytic center [49]. In the GID1 there are HGG and GX SXG motifs conservative for carboxylesterase. The important role of GX SXG in GA reception is shown. The substitution of

glycine residue (G) with aspartate residue (D) in this motif leads to the development of GA-insensitive dwarf phenotype in rice *gidl-1* mutants [50]. The possibility of GID1b association with the plasma membrane in *Arabidopsis* leaf tissue has been demonstrated recently [33], but the function of the protein with such localization has not yet been investigated.

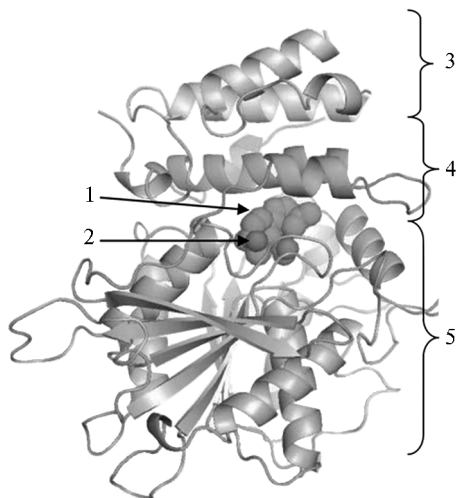


Fig. 2. The structure of HA-GID1-DELLA complex [51]: 1 — gibberellin molecule (GA), 2 — polar groups of GA molecules, 3 — DELLA protein domain containing a conserved Asp-Glu-Leu-Leu-Ala sequence (DELLA), 4 — N-terminal «cap» domain of GID1 protein (Gibberellin Insensitive Dwarf 1), 5 — GID1 protein receptor pocket.

It is known that GID1 is composed of two domains different in size. The main part of the receptor (C-terminal domain) forms a pocket (Fig. 2; 3, A) with the GA-binding site. The second small domain is located in the N-end of the protein [51]. When interacting with GID1, the gibberellin molecule is directed with its nonpolar part into the pocket of the protein, and

the polar groups are bound to the receptor. In this, approximately 10 water molecules are also involved. Interaction with GID1 changes GA protein conformation so that N-terminal domain like a lid shuts the receptor pocket. GID1 binds only the active gibberellins, and its affinity to GA₄ is 20 times higher than to GA₃. GA-GID1 complex is unstable (i.e., it quickly dissociates and then re-associates again) [50]. The affinity of this complex to DELLA-proteins is greatly increased. In turn, the interaction of the complex with DELLA significantly improves the stability of GID1-GA binding. It is shown that in the presence of DELLA protein the rate of dissociation of GA from GID1 decreases [50].

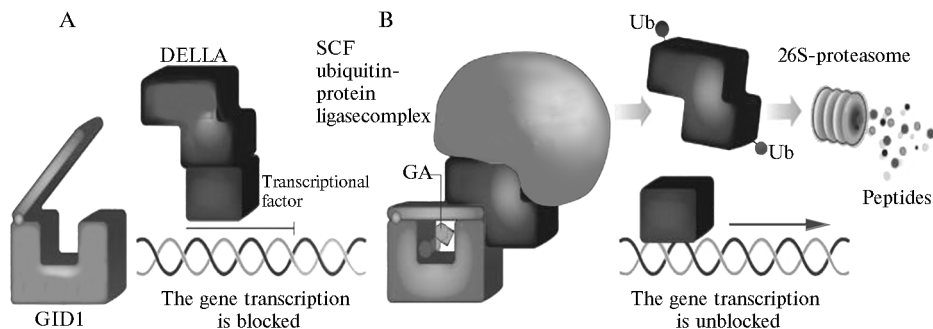


Fig. 3. Model of reception and transduction of gibberellin (GA) signal [52]: A — GID1 receptor (Gibberellin Insensitive Dwarf 1) is not bound with a gibberellin molecule; B — GA signal transduction resulting from interactions of a gibberellin molecule with GID1 receptor; DELLA — protein with a domain with Asp-Glu-Leu-Leu-Ala conserved sequence (DELLA), SCF — E3 ubiquitin protein ligase of the SCF Group (SKP, Cullin and F-box), Ub — ubiquitin tagging DELLA-protein.

When GA-GID1 interacts with DELLA-protein, a triple complex GA-GID1-DELLA is formed. In this complex DELLA-protein with its N-terminal domain containing conserved sequences DELLA and TVHYNP binds with the GID1 lid protein. C-terminal domain of DELLA-protein, known as GRAS [53] interacts with the pocket GID1 protein fixing gibberellin molecule inside the complex [45]. GA-GID1-DELLA complex is recognized by E3 SCF^{SLY1}/GID2

ubiquitin protein ligase [47] (see Fig. 3, B). In the recognition the important role is played by F-box domain-containing ligase subunit, the SLY1 (SLEEPY1) in *Arabidopsis* or GID2 (Gibberellin Insensitive Dwarf 2) in rice. These proteins of high affinity to the GA-GID1-DELLA complex induce interaction with ubiquitin protein ligase, which further leads to ubiquitination and subsequent degradation of DELLA-protein in 26S proteasomes (see Fig. 3) [47, 52, 54, 55]. Interestingly, as a prerequisite for ubiquitination of DELLA-protein serves its relationship with GID1 containing GA inside its receptor pocket. It is shown that DELLA can also bind GID1 without GA, but this does not increase its affinity to the F-box proteins of ubiquitin protein ligase [33, 56].

DELLA proteins suppress GA response because they reduce the expression of GA-dependent genes. Degradation of these proteins blocks their inhibitory effect, thereby triggering the expression of genes regulated by the GA [56-58], the products of which are signals to the GA-induced plants growth and other GA-dependent responses, such as seed germination, flowering, etc. However, DELLA degradation is not always required to suppress its repressive functions. Proteolysis-independent DELLA inactivation is observed in the GA-GID1-DELLA complex and at *GID1* overexpression [40, 49, 59].

Proteins containing F-box. In signal transduction pathway the GA induces degradation of DELLA repressor through interaction of GID1-DELLA with E3 SCF^{SLY1/GID2} ubiquitin protein ligase complex. First the part of the ubiquitin ligase in the GA-signaling was found in *Arabidopsis* mutant *sly1-10* and rice mutant *gid2-1*, having a dwarf phenotype, insensitive to GA treatment. These mutants were also noted for an unusually high content of DELLA proteins [54, 55, 60]. Genes *SLY1* (*Sleepy*) and *GID2* (*Gibberellin Insensitive Dwarf 2*) appeared to be positive regulators of GA signal. They encode proteins containing conserved F-box domain of about 60 amino acid residues, which is required for the interaction with DELLA and its subsequent degradation. Mutations that lead to loss of function of the repressive DELLA proteins partially restored growth in *gid2* and *sly1* plants, thus confirming the assumption that the dwarf type was caused by DELLA accumulation [54, 55].

SLY1 and GID2, containing F-box, form part of the E3 ubiquitin protein ligase complex of SCF-type, the main function of which lies in the electoral ubiquitin tagging proteins for subsequent degradation in the proteasome [60]. The name of SCF group is derived from the proteins that make up this sophisticated protein complex, i.e. SKR1 (S phase Kinase-associated Protein 1), CUL1 (Cullin) and a protein containing F-box domain [61]. The protein with F-box is responsible for target recognition. In *Arabidopsis* genome, 694 genes of proteins with this domain are identified. So proteins comprising F-box seem to form one of the largest protein families in plants [61].

SLY1 or GID2 proteins with their N-terminal part bind SKR1 through F-box, and with C-terminal domain comprising highly conservative GGF and LSL they bind the target DELLA protein [60]. In *gid2* rice dwarf mutants there was a deletion in the region coding F-box domain, and in *Arabidopsis sly1-2* and *sly1-10* mutants SLY proteins was C-terminal end truncated. Because of the damage the proteins lost the ability to associate with DELLA-protein [54, 55].

Two GRAS sites of DELLA-protein are involved in interaction with F-box protein domain, one of which has the conserved VHIID sequence, and the second comprises leucine repeated series (LR) [45, 56]. Thus, as the result of recognition by F-box protein of its target, a triple GID1-DELLA-SLY1 complex is formed. Interestingly, in mutants *sly1-d* due to the replacement of glutamic acid (E138) with lysine (K138) in GRAS domain the SLY acquired the ability to associate with the DELLA protein with its subsequent proteolysis regardless of

the presence of the GA-GID1 complex. These mutants were superior to dwarf plants in height [54].

Interaction between GA and other signaling pathways. Growth and development of the plants and their adaptation to external factors are under the control of plant hormones. Gibberellins are involved in many life processes of plants. However, in the absence of these hormones the development of stems, leaves and roots does not stop, but poor germinating and non-reproductive dwarf plants with altered sensitivity to external conditions are formed, indicating GA signal transduction to be a part of the complex signaling network, transmitting information from other external factors and hormones.

ABA (abscisic acid) and GA are antagonists, and each negatively regulates transcription of the other's biosynthetic genes. In addition, in case of promoters sensitive both to GA and ABA, the gene expression is influenced by these hormones oppositely [38]. It is known that auxin and GA stimulate growth of plant cells and tissues. These hormones have a positive effect on the biosynthesis of each other. Removal of the sprout apical buds, being the main source of auxin, caused a decrease in GA content in pea, poplar and tobacco [62]. Moreover, the interaction of signaling pathways of these hormones was identified so that auxin promoted GA-mediated DELLA degradation, and GA stimulated active polar auxin transport. In the study of poplar stem transcription profiles it was found that about 83 % of the GA-regulated genes were also regulated by auxin. These include genes of transcriptional regulators of auxin (Aux/IAA, auxin/indolyl-3-acetic acid), GA (DELLA) and ethylene signaling (EIN3-like protein, Ethylene Insensitive 3), as well as genes which products regulate the cell wall elongation [62]. Interaction of the signaling pathways of cytokinin, auxin and gibberellin in the shoot apical meristem is well-studied [63]. Low-GA and high-cytokinin levels in apex maintained the proliferating cells undifferentiated. High accumulation of auxin and GA enabled cell differentiation and determined the location of leaf primordia. Interaction between GA, auxin and ethylene is shown on the example of the formation of hypocotylous hook from etiolated seedlings in *Arabidopsis*. Hypocotylous hook protects the apical meristem, helping to move seedling to the soil surface. It is formed due to asymmetric growth of the inner and outer sides of the hypocotyl. Ethylene and auxin control the hypocotyl bending via DELLA degradation. At the same time, ethylene induces retardation of root growth in *Arabidopsis*, reducing endogenous GA level by blocking biosynthesis gene, and increases the stability of DELLA proteins [64].

Thus, the active use of the dwarf forms of agricultural plants in breeding programs in 1940-1970s served as a powerful incentive for the research of genetic and physiological mechanisms of dwarf traits. Data accumulated over 30 years show that most of the known plant mutations associated with dwarf character are caused by disorders in metabolism or GA signaling pathways. However, changes in these processes at various stages may lead to opposite modifications of plant growth resulted in shortened or elongated stem. It is shown that the dwarfism in plants is associated with the lack of GA due to GA biosynthesis disorders or the accumulation of DELLA proteins, the repressors of GA-signaling, while giant plants are caused by an excess of gibberellins due to GA deactivation enzyme damage and(or) to the loss of the repressive function of DELLA proteins. Currently, this GA-induced suppression of repressive DELLA function seems to be the only characterized GA signal transduction pathway and explains well the role of gibberellins in hormonal regulation of plant growth. Nevertheless, probably, there are alternative GA signaling events, independent from DELLA proteolysis or incorporating G-protein, the role of which in the plant growth control has not yet been studied. Also, it remains unclear how the gib-

berellins regulate the growth correlation between roots and stems. It is known that the roots are much more sensitive to GA than stems. The growth of the roots is activated by low GA concentrations insufficient to induce stem growth. Conversely, the GA concentration, being enough to activate stems tension, inhibits roots growth. It is believed that GAs are involved in plant shape control by adjusting stem to root growth rate [65].

In the breeding and genetic studies the response of dwarf forms to exogenous GA is traditionally used as evidence of their differences in the dwarf loci [66]. Understanding molecular mechanisms that determine intraspecific variability of plant height allows forecasting effective crossing and source material selection to create the varieties and interline hybrids with a desired phenotype. Particularly, for optimum adjustment of sunflower, breeding of which was mostly focused on high-yield heterosis hybrids, it is preferable to use dwarf lines as parents. In the VIR (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) genetic collection of sunflower there are lines with dwarf traits determined by nonallelic genes and, therefore, controlled by different physiological and genetic mechanisms [67]. In-depth studies of these forms, along with the elucidation of the physiological and genetic causes of their uniqueness are necessary for effective breeding source material and cross breeding.

Thus, it has been shown that many low-growing varieties involved in the Green Revolution carried mutations in genes responsible for metabolism or transfer of gibberellin signal (GA). GA is involved in seed germination, growth of stems and roots. Changes in different GA-dependent processes can lead both to dwarf and giant plants. Of plant GAs well-known to date, the GA₁, GA₃, GA₄, GA₅ and GA₇ are physiologically active. Their production involves GA₂₀-oxidase and C3,β-oxidase (or C3,β-hydroxylase). C2,β-oxidases are the main enzymes that can rapidly inactivate GA. Experimental data give reason to believe that in GA-signaling the GA induces degradation of DELLA repressor through GID1-DELLA interaction with E3 SCF^{SLY1/GID2} ubiquitin protein ligase complex. Poor growth may be associated with a disorder in GA biosynthesis or accumulation of GA-signaling repressors (i.e., DELLA-proteins), while the high growth may be related to the damage of GA deactivating enzymes or the loss of a repressive function in DELLA proteins. Nowadays, GA-induced suppression of repressive DELLA function is known as the only characterized GA signal transduction pathway. Nevertheless, alternative GA-signaling pathways, which role has not been studied yet, can not be excluded. In-depth physiological and genetic studies of dwarf and tall plant forms are necessary for effective breeding.

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GENETIC ENGINEERING AS A TOOL FOR MODIFICATION OF SEED STORAGE PROTEINS AND IMPROVEMENT OF NUTRITIONAL VALUE OF CEREAL GRAIN

(review)

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«So modern bread wheat is the result of crossing three species barriers, a kind of natural genetic engineering»

(Norman Ernest Borlaug,
Nobel laureate)

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Abstract

In recent years, genetic engineering has become an effective tool for the genetic improvement of cultivated plants including changes in the composition grain storage proteins of cereal crops that are the main source of nutrition for humans. The review describes the approaches used in these studies: the introduction of genetic constructs (i) providing the synthesis of proteins that are absent in recipient cultivars; (ii) inducing RNA-silencing of genes encoding proteins with low nutritional value, (iii) regulating the pool of amino acids in the endosperm. The studies are referred, which reported on the introduction of additional genes of high molecular weight glutenins (*1Dx5*, *1Ax1*, *1Bx17*, *1By18*, *1Dy10* and others) into the genomes of different lines and cultivars of wheat. In these studies, the transgenic lines with increased dough strength and elasticity were obtained. In addition to the practical importance, these studies allow understanding the role of individual genes of high and low molecular weight glutenins in the formation of wheat flour quality traits. The examples of marker-free transgenic wheat lines expressing *1Dy10* and *1Bx14* genes, as well as transfer of high molecular weight glutenin genes into the genomes of other cereals (rye, corn, sorghum) are given. The possibilities of using the RNAi technology to obtain new information about the mechanisms of development of protein bodies, vitreous endosperm formation, and the role of different classes of prolamins and glutenins in the technological properties of flour and dough are discussed. The examples of the creation of transgenic maize with improved nutritional value via RNA-silencing of prolamin genes, transgenic sorghum with improved protein digestibility (obtained by silencing gene of the γ -kafirin, the protein that forms outer layer of the protein bodies, resistant to pepsin digestion), transgenic wheat with suppression of gliadin synthesis, which flour has a low toxicity to humans with celiac disease, forced to comply gluten-free diet, are given. An example of natural RNA-silencing is given. Particularly, in the rice mutant with reduced level of glutelin, a deletion between the two coding sequences, one of which has an inverted orientation has been detected in the *Lgc1* locus. The genetic engineering approaches to increase the lysine content are described, e.g. introduction of genes that enhance its synthesis, such as dihydrodipicolinate synthase (DHPS) insensitive to feedback inhibition and aspartate kinase; suppression of *zlk/sdh* gene regulating its catabolism; introduction of genes that control the synthesis of proteins with high lysine content (histones and other lysine-rich proteins). The prospects of using genetic engineering methods to create varieties with improved nutritional value are associated with the use of marker-free technologies, increasing accuracy of insertion of genetic constructs, using the methods of genome editing by artificially engineered nucleases.

Keywords: transgenic plants, RNAi, prolamins, protein bodies, endosperm, cereal crops.

The studies over the past 20 years have convincingly demonstrated that genetic engineering becomes a highly effective tool of the genetic improvement of cultivated plants. The reality is that genetic engineering has become one of the method for breeding different crops, which allowed to create a significant number of cultivars and lines, resistant to biotic and abiotic stresses, with im-

proved quality of final products, increased photosynthetic and nutrient-use efficiency (<https://www.isaaa.org/gmapprovaldatabase/>).

Among the various methods of genetic engineering, *Agrobacterium*-mediated plant transformation is considered the most effective and least costly approach in obtaining transgenic plants. For a long time, this technique has been poorly used for cereal crops, for which the expensive ballistic transformation served as the only actual way of transgenesis. However, improvement of methods for regenerating cereal plants in tissue culture and the development of various protocols for *Agrobacterium*-mediated transformation [1, 2] greatly facilitated the production of transgenic plants in cereals, significantly expanding the possibilities of using genetic engineering in plant breeding of such an important group of crops.

Among the most promising areas of genetic engineering is the creation of transgenic plants, which are improved nutritionally via the changed composition of grain storage proteins. These investigations are particularly relevant for cereals being the main source of food and feed protein. It is known that up to 50 % protein (or up to 70 % in developing countries) and up to 65 % of calories humans receive from cereals, in which the storage proteins account for up to 80 % of the total protein content in the mature seed [3-5].

The article provides an overview of studies on the production of transgenic cereal crops with modified composition of storage proteins. We investigated the possibilities of using these plants for the study of protein bodies and the formation of endosperm, as well as creating lines with a higher nutritional value.

Fractions of grain storage proteins in cereals. According to the widely used classification by T.B. Osborne, storage proteins are divided into albumin (water-soluble), globulin (salt-soluble), prolamine (spirit-soluble), and glutenin (alkali-soluble) fractions [6].

In bread wheat (*Triticum aestivum* L.), the major cereal crop, storage proteins are represented by glutenins (50 % of the total pool of endosperm proteins) and gliadins (30 %). Glutenins are polymer complexes, which define elasticity of flour, composed of subunits with high and low molecular weight (HMW and LMW, respectively). Bread wheat contains closely linked pairs of genes that encode X- and Y-types of subunits, forming HMW proteins, and are located on the long arm of chromosomes 1A, 1B and 1D. Gliadins consist of α -, γ - and ω -fractions with different electrophoretic mobility [7]. Gliadins are regulated by closely linked gene clusters, called blocks, which are located in the chromosomes 1 and 6 [4, 8].

Prolamins are the main storage proteins in corn *Zea mays* L. and sorghum *Sorghum bicolor* (L.) Moench. Fractions of maize prolamins (zeins) are α - (19 kDa and 22 kDa), β - (15 kDa), γ - (50 kDa, 27 kDa, 16 kDa) and δ - (18 kDa and 10 kDa) zeins, whereas α -zeins in the endosperm of maize kernels account for up to 70 % of the total protein pool [5, 6]. Zeins contain small amounts of valuable amino acids such as lysine, threonine, tryptophan, and therefore having a low nutritional value. In 1960s, the *opaque-2* (*o2*) and *floury-2* (*fl2*) corn mutants were obtained with a reduced content of α -zein, in the endosperm of which a significant amount of non-zein proteins rich in lysine was accumulated, and an increased content of free lysine and tryptophan was also observed [6]. The kernels of these mutants, however, contained no vitreous endosperm, and that increased the fragility of the kernels, their susceptibility to infection with fungal microflora, and, ultimately, hindered the commercial use of such mutants. Later, recombinants with vitreous endosperm and a high content of lysine (QPM, Quality Protein Maize) have been obtained. In these, a compensatory synthesis of non-zein proteins rich in lysine occurred in the kernels,

along with an increase in the γ -zein content [6, 9].

Similarly to corn, sorghum prolamine fractions (kafirins) were designated as α - (25 kDa and 23 kDa), β - (18.7 kDa) and γ -kafirins (28 kDa) [10-12]. Data on molecular weight of γ -kafirin disagreed in different studies, 28 kDa [10] or 20 kDa [13]. According to the last published classification, sorghum kafirins are represented with six proteins, such as α -kafirin 1 (25 kDa), α -kafirin 2 (22 kDa), β -kafirin (19 kDa), γ -kafirin 1 (27 kDa), γ -kafirin 2 (50 kDa), δ -kafirins (18 kDa), with the α -kafirin fraction amounting up to 80 % of the total amount of kafirin proteins [14].

Prolamins synthesized in endosperm cells are deposited in highly specialized organelles, i.e. protein bodies with a well-ordered structure [5]. Protein bodies are formed as the endoplasmic reticulum vesicles. First, γ - and β -prolamins accumulate in these vesicles, and then α - and δ -prolamins begin to accumulate, while γ - and β -prolamins are pushed away to the outer layers. Such an organization of protein bodies is considered as one of the major causes of a lower nutritional value of sorghum grains as γ -kafirin occupying a peripheral position is highly resistant to proteolytic degradation, thereby preventing the digestion of the major storage proteins, i.e. α -kafirins [15, 16].

Storage proteins in rice (*Oryza sativa* L.) are represented by prolamins and glutelins. Prolamins are divided into three subclasses: 10 kDa, 13 kDa (13a-1, 13a-2, 13b-1 and 13b-2) and 16 kDa. Prolamins 13a-1 and 13a-2 belong to proteins rich in cysteine, while 13b-1 and 13b-2 are cysteine-deficient. Genes encoding prolamine 13b-2 exist in 18 copies, genes of the other prolamins have a lower copy number (2 to 4 copies per genome) [17].

Prolamins and glutelins in the endosperm of rice kernels are deposited in the protein bodies. Protein bodies containing prolamine have a spherical shape, 1-2 microns in size and an inner structure with concentric circles of different electron density; protein bodies containing glutelin are irregular in shape, 3-4 microns in size and uniformly coloured [18, 19]. It has been established that prolamine 10 kDa is located in the centre of the protein body, prolamins 13a and 16 kDa are found in the middle layer, while prolamine 13b forms the surface layer and the lining between the inner layer and the middle layer of the protein body [17].

Transgenic plants with additional storage protein genes. Development of genetic transformation has opened the possibility of using genetic engineering techniques to modify the composition of storage proteins in cereal crops. To solve this problem, different approaches are used such as the introgression of genes regulating synthesis of storage proteins, which are absent in the recipient cultivars; the introgression of genetic constructs that induce RNA silencing of genes encoding proteins with a low nutritional value or reduce the degradation of other proteins by proteases; the introgression of genes regulating amino acid synthesis.

To date, the literature contains a considerable number of reports on the introduction of genes of various high molecular weight glutenins (*1Dx5*, *1Ax1*, *1Bx17*, *1By18*, *1Dy10*, etc.) in the genomes of different lines and cultivars of bread wheat. The studies resulted in obtaining transgenic lines with improved quality of flour, increased dough rising potency and elasticity [20-24]. In addition to practical value, these works allow understanding the role of individual genes of high and low molecular weight glutenins in flour quality. Thus, a decline in the SDS-sedimentation index and flour strength was observed in transgenic bread wheat lines with enhanced expression of low molecular weight glutenin genes [24]. Simultaneous expression of three genes of HMW-GS, i.e. *1Ax1*, *1Dx5* and *1Dy10*, increased the flour rising potency, with much more pronounced effect of gene *1Dy10* compared to *1Ax1* and *1Dx5* [25].

There have also been reports on durum wheat transgenic plants with genes *1Ax1* or *1Dx5* encoding high molecular weight glutenins [26]. Among 10 transgenic lines which were obtained by the ballistic transformation of immature embryos from three cultivars and a line of *Triticum turgidum* L. var. *durum*, five ones demonstrated the expression of introduced transgenes. An analysis of the flour mixogram based on the grains from three transgenic lines has revealed the elevation of dough rising potency and stability, which indicates the possibility of using genetic transformation technique to improve the quality of durum wheat grain.

In a similar work done on other cultivars of durum wheat, the transgenic lines with bread wheat genes *1Dx5* and *1Dy10* encoding high-molecular glutenins also were obtained [27]. However, it is notable that transgenes and a marker gene (*bar*) in one of the lines appeared to be located on different chromosomes, so as the segregation resulted in isolation of a markerless transgenic line expressing gene *1Dy10*. The flour from obtained transgenic lines had improved kneading properties.

Durum wheat transgenic plants with genes encoding high molecular weight glutenins LMW-GS [28] have also been obtained, and as a result a transgenic line with enhanced flour strength was developed [29].

Taking into account the possible negative effects of the marker gene *bar* on human health, efforts have been undertaken to obtain markerless transgenic plants of bread wheat with extra storage protein genes [30]. In this study, PCR was used to select transgenic plants with the gene *1Bx14* encoding a high molecular glutenin subunit. Seven transformants (transformation frequency 0.28 %) have been identified among 1219 plants, with three of them demonstrating the transgene expression in the T₁ generation.

Noteworthy experiments were reported in which high molecular glutenin genes were transferred into the genomes of other cereal crops. Thus, it has been reported about a transgenic line of rye (*Secale cereale* L.) with bread wheat glutenin genes *Glu-1DX5* and *Glu-1Dy10*, which boasted 2- to 3-fold increase in the gluten content [31]. Transgenic plants of corn and sorghum with genes *Glu-1DX5* and *1Dy10* introduced in their genomes, respectively, have also been developed, and these genes showed specific expression in the endosperm [32, 33].

Using RNA interference to change the composition of storage proteins. The RNA interference technique is known to be based on destructing mRNA of the target genes via genetic constructs containing inverted repeated sequences of these genes' fragments. The mRNA transcribed from such structures forms a hairpin structure. The resulting double-stranded RNA molecule is exposed to destruction with subsequent formation of single-stranded fragments 21-25 bps long (short interfering RNA), which interact with mRNA of the target gene due to complementarity. As a result, there are new double-stranded RNA molecules that are also subject to destruction [34-36].

In recent years, RNA interference technology has been extensively used to improve the nutritional value of a number of cereals, particularly corn and sorghum, as well as to obtain new information about the mechanisms of protein bodies' formation, the endosperm structure, and the role of various classes of prolamins and glutenins in determining properties of flour and dough.

By means of ballistic transformation using a genetic construct, which contained the γ -gliadin gene fragment sequences in direct and reverse orientations, separated by the intron *Ubi1*, seven transgenic plants with silencing of the γ -gliadin gene were obtained from two lines of the wheat cultivar Bobwhite [37]. Further on, the construction for RNA silencing was moved via crossings into the genomes of other three bread wheat cultivars that led to an increase in the number of high and low molecular weight glutenins and SDS sedimentation value as

a result of silencing [38]. It is noteworthy that such an effect of silencing γ -gliadin gene was not found in the gene pool of the Bobwhite 208 cultivar.

Other experiments with the use of genetic constructs for RNA silencing of the genes of α - and ω -gliadins revealed repression of the synthesis of all gliadin classes (up to 86.8 % compared with that observed in the original non-transgenic plants) [8]. Abnormalities in the development of protein bodies which had an irregular shape have been identified in these lines, whereas repression of the synthesis of γ -gliadin alone did not change the shape of these structures. In lines carrying a genetic construct targeted at silencing genes of α - and ω -gliadins, an increase in the content of globulins was seen. It was also reported the development of a transgenic wheat with silencing of the α -gliadin gene, and this wheat was characterized by a high flour strength index and increased baking volume [39].

Transgenic wheat lines harbouring the genetic construct for silencing ω -5-gliadin gene were obtained [40]. The content of ω -5-gliadin in two lines was decreased by 80 %, while the synthesis of other gluten proteins was not altered. At the same time, two other lines showed both complete suppression of the ω -5-gliadin synthesis and down-regulation of ω -1,2-gliadins. Moreover, one of the lines also demonstrated the decreased synthesis of three proteins from the high molecular glutenins and one low molecular weight glutenin (s-type LMW-GS), and simultaneously increased content of two other low molecular weight glutenins (m-type LMW-GS) and several α -gliadins. This study clearly demonstrates that the same construct for RNA silencing may cause different effects depending on the gene pool.

It should be noted that the flour of transgenic wheat lines with suppressed synthesis of gliadins is less toxic for people suffering from celiac disease and forced to keep a gluten-free diet. It has been showed that the gluten proteins in transgenic wheat lines with silencing of genes of α -, γ - and ω -gliadins may reduce the formation of epitopes (DQ2 and DQ8) [41] specifically associated with the celiac disease and recognized by T-cells. Therefore, transgenic wheat with suppressed synthesis of gliadins may be used in food by people with celiac disease who are not able to consume products based on conventional wheat, rye and barley flour.

In corn, using genetic constructs harbouring inverted repeats of genes of α -zeins (19 kDa and 22 kDa), transgenic lines with suppressed synthesis of these proteins were obtained [42, 43]. It was found that repression of the synthesis of zeins possessing a relatively low nutritional value leads to accumulation of other proteins with a higher nutritional value. Corn plants with gene silencing of α -zeins were characterized by doubled content of essential amino acids tryptophan and lysine in the kernels.

These experiments have found that silencing gene of α -zein with a molecular weight of 22 kDa resulted in the formation of the floury endosperm. Such a modification in the type of endosperm was associated with abnormalities in the formation of the structure of protein bodies, namely the violation of embedding a 19 kDa α -zein into the centre of a protein body, or a modification of its interaction with β - and γ -zeins [42].

Later on, RNA interference of genes of various zein fractions in the QPM maize line with a vitreous endosperm allowed obtaining a variety of mutants with different variations in the protein body structure and types of endosperm [44, 45]. It has been demonstrated that silencing of the γ -zein gene plays an important role in the formation of the floury endosperm, which results in a modification of the protein body structure and a change of their relationship with the starchy granules [45].

A thorough study of various zein subclasses, using the RNA interference technique, has revealed their role in the formation of the vitreous endosperm and development of protein bodies in corn [46]. Thus, it was found that a 27 kDa γ -zein is involved in the initiation of protein body formation. Other proteins belonging to the family of γ -zeins operate at the stage of protein body growth. Co-suppression of two α -zeins (19 kDa and 22 kDa) significantly inhibits the outgrowth of protein bodies, but does not induce their morphological anomalies, which do arise from the reduction of a 22 kDa α -zein alone. The simultaneous suppression of all zein classes leads to a decrease in the number of protein bodies, which at the same time retain their normal size and morphology.

A large number of experiments on the induction of RNA silencing of kafirins in sorghum was carried out by several research groups. The main purpose was to suppress the synthesis of a poorly digestible γ -kafirin and develop sorghum lines with improved nutritional value. RNA silencing was induced by genetic constructs containing inverted repeats of several genes of kafirins ($\delta 2$, $\gamma 1$, $\gamma 2$ or $\alpha 1$, $\delta 2$, $\gamma 1$, $\gamma 2$) separated with a sequence of ADH1 (aldehyde dehydrogenase 1) intron. These constructs were controlled by a promoter of the maize α -zein (19 kDa) gene [14, 47, 48]. In another study [33], for induction of γ -kafirin gene silencing, a genetic construct comprising the complete sequence of the γ -kafirin gene was used, controlled by the γ -kafirin promoter, with the gene sequence of the ribozyme (self-cleaving ribozyme) of tobacco ringspot virus as the terminator. α -Kafirin silencing was induced by a construct consisting of the inverted repeats of the α -kafirin separated with an intron sequence of the *Arabidopsis* gene encoding the D1 protein of spliceosome; the construct was controlled by the α -kafirin promoter [33]. All research groups succeeded in obtaining transgenic lines of sorghum with a floury type of endosperm and improved pepsin digestibility of kafirins. Unfortunately, the floury endosperm is a disadvantage of these lines, since the absence of a vitreous layer increases the fragility of kernels and reduces their resistance to fungal microflora.

By means of transformation using the *Agrobacterium* strain GV3101, carrying a genetic construct pNRKAFSIL, to induce RNA silencing of γ -kafirin gene, we managed to obtain transgenic lines of sorghum with in vitro improved digestibility of grain storage proteins and a modified amino acid composition (i.e., increased relative lysine content) [49]. The construct pNRKAFSIL developed in the «Bioengineering» Centre of RAS (Moscow; authors N.B. Ravin and A.L. Rakitin) was a hairpin insert consisting of the γ -kafirin gene fragments in direct and reverse orientations, with an intron of the maize ubiquitin gene located between them; the construct was controlled by a constitutive 35S promoter of cauliflower mosaic virus (CaMV).

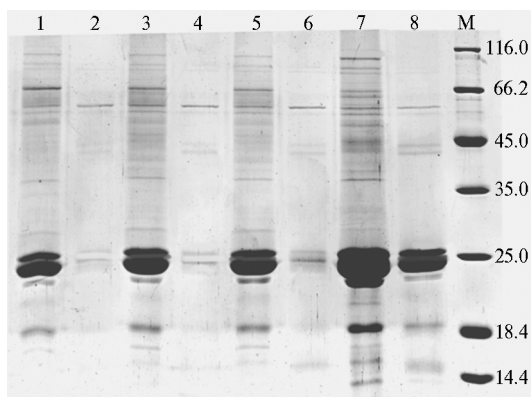


Fig. 1. Electrophoretic protein spectra of kernels from three transgenic plants of sorghum *Sorghum bicolor* (L.) Moench from T₃ generation carrying a genetic construct for the γ -kafirin gene silencing (lines 1 to 6) and a control plant of the original non-transgenic line of Zheltozernoe 10 cultivar (lines 7, 8), pre-treatment (lines 1, 3, 5, 7) and after treatment (lines 2, 4, 6, 8) with pepsin. M (kDa) is a molecular weight marker SM0431 (Thermo Scientific, Lithuania).

In vitro digestibility of proteins showed that in the transgenic

plants the flour is characterized by higher values compared to the original sorghum line (Zheltozernoe 10 cultivar) (Fig. 1). In some transgenic plants, the level of proteins that were not digested by pepsin was 4.7 times lower, and amount of the undigested monomers of kafirins was 17.5-fold lower than that in the original line. However, digestibility (total score of the protein cleavage) has reached 90-92 % compared to that in the control untreated with pepsin, whereas the score in the original non-transgenic line was 60-61 % [49]. Increased digestibility was observed in plants from different generations (T_1 - T_3) thus being inherited.

It is noteworthy that we obtained both plants with a floury type of endosperm and forms with a vitreous or modified endosperm type, which combined high digestibility score of kafirins with the presence of a vitreous layer in the endosperm (Fig. 2). The obtained transgenic plants are of great interest for the breeders as the vitreous layer is required to protect kernels from pathogens and mechanical damage.

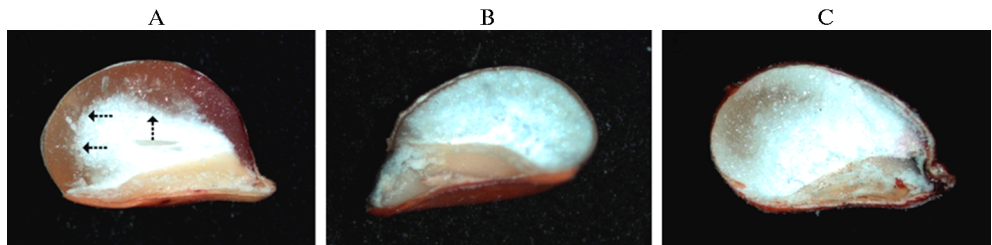


Fig. 2. Texture of endosperm in transgenic plants of sorghum *Sorghum bicolor* (L.) Moench containing a genetic construct for the γ -kafirin gene silencing: A — a kernel of the original non-transgenic line of Zheltozernoe 10 cultivar with a thick layer of vitreous endosperm (boundaries are marked with arrows); B — a kernel of a transgenic plant with the floury endosperm; C — a kernel of a transgenic plant with a modified type of the endosperm, where floury endosperm contains fragments of vitreous endosperm (StereoDiscovery V12, Carl Zeiss, Germany). Zoom by $\times 20.5$.

It should be noted that the structure of the protein bodies, which determines the digestibility and nutritional value of the sorghum grain, has been changed in the transgenic lines with the α -kafirin gene silencing. Protein bodies in the endosperm of conventional sorghum line plants have a regular rounded shape, while the shape of these structures in the transgenic lines or mutants with high digestibility is irregular, with protrusions and grooves on their surfaces [14, 50]. At the same time, the γ -kafirin silencing has not changed the form of protein bodies [33, 47].

The RNA interference technique was used to study the mechanism of the formation and structure of protein bodies in rice. Using a genetic construct for silencing the gene of one of the prolamins, CysR10 (a 10 kDa prolamine rich in cysteine), it was shown that this protein is a constituent part of the prolamine-containing protein bodies. In a line with RNA silencing, protein bodies lost their characteristic spherical configuration [51].

Changes in the shape and size of prolamine-containing protein bodies have been observed in transgenic rice plants with the gene silencing of a 13 kDa prolamine [52]. In these transgenic plants, protein bodies were smaller and deprived of their characteristic lamellar structure. Moreover, it has been observed an elevation in the content of other proteins (a 10 kDa prolamine, glutelins, chaperone) and free lysine that increased the nutritional value of the grain. It has also been showed that gene silencing of a 13 kDa prolamine increases the total lysine content up to 56 % as a result of a compensatory rise in the synthesis of lysine-rich glutelins, globulins and chaperones [53].

Of interest, there has been identified a natural mutation *Lgc1* (*low glute-*

lin content) in rice, which reduces the amount of glutelin in kernels, probably by means of RNA interference. In the *Lgc1* locus, a deletion was detected between two sequences of the glutelin gene, one of which had a reverse orientation. Such a structural organization of the locus during its transcription may result in the formation of a double-stranded RNA molecule, i.e. of the RNA silencing inductor [54]. Thus, the *Lgc1* mutation is a good example of the naturally occurring RNA silencing, and one of the arguments in favour of genetic engineering using the processes taking place in nature and occurring without human intervention.

Changes in the content of essential amino acids. Genetic engineering techniques are quite promising for enrichment of cereal grain with essential amino acids, i.e. lysine, tryptophan, methionine. By using endosperm-specific promoters, expression of the desired genes may be provided exactly in the kernels, rather than in somatic tissues where the increased content of the mentioned amino acids may lead to developmental abnormalities [55]. However, to date, successful results in changing amino acid composition of cereal grains by genetic engineering were obtained only for lysine. To increase the content of this amino acid, various genetic engineering approaches have been used, i.e. the introduction of genes enhancing its synthesis; suppression of genes regulating the catabolism of lysine; inhibition of synthesis of proteins with a low content of lysine, which results in the initiation of lysine-rich protein synthesis in the endosperm; the introduction of genes that regulate the synthesis of proteins rich in lysine.

Lysine biosynthesis is triggered by dihydro dipicolinate synthase enzyme (DHDPS, KF 4.2.1.52). Its special feature is the response to inhibition by the end-product, which allows maintaining the concentration of lysine in the plant cells at a steady level [55]. However, a modified enzyme DHFPS called CordapA, non-responsive to inhibition by the end-product, was isolated from bacteria *Corynebacterium glutamicum* [56]. By means of *Agrobacterium*-mediated transformation, a transgenic maize line M27908 harbouring the CordapA gene was derived, where the content of free lysine increased more than 50-fold [57]. The crossing of the maize lines with RNA silencing of the α -zein (19 kDa) gene with the M27908 line carrying the gene CordapA allowed to obtain F₁ hybrids, in which the total content of lysine in the kernels increased up to 6.9-8.5 % of the total amino acid content (vs 3.3 % to 3.4 % in the control) [57]. Therefore, a combination of two approaches (inhibition of lysine-deficient protein synthesis and the introduction of a gene that enhances the synthesis of this amino acid) allowed developing the hybrid maize with doubled lysine content in the kernel.

The catabolism of lysine is controlled by two enzymes, i.e. by lysine: α -ketoglutarate reductase (LKR, KF 1.4.3.2, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9), which are products of a single gene *lkr/sdh* [5]. Using a genetic construct inducing RNA silencing of the *lkr/sdh* gene, transgenic plants were obtained in maize, which boasted a 20-fold elevation in the lysine content compared to non-transgenic controls [58]. Subsequently, using a shared cassette which contained the CordapA gene enhancing the biosynthesis of lysine, and a genetic construct which induces RNA silencing of the *lkr/sdh* gene and thereby reduces the catabolism of lysine, there have been obtained transgenic corn plants with a 40-fold increase in this amino acid level in kernels [59].

It has been reported the development of transgenic rice plants with genes enhancing the synthesis of lysine, which encode an unresponsive to inhibition by lysine aspartate kinase (EC 2.7.2.4, EC 2.7.2.4) and dihydro dipicolinate synthase (EC 4.2.1.52), and constructs that induce RNA interference of the *lkr/sdh*

gene, which controls the catabolism of lysine [60]. There has been recorded a 60-fold increase in free lysine content in the seeds and a 12-fold increase in the leaves of these plants. Any significant alteration in the plant development and seed germination was not shown.

As mentioned above, another way to enrich the kernels of corn and sorghum with lysine and tryptophan is to inhibit the synthesis of zeins, which contain low amount of these amino acids. The resulting compensatory effect in the kernels provides enhanced synthesis of non-zein proteins, which are richer in lysine and tryptophan [61]. A similar process was observed in transgenic sorghum lines with suppressed synthesis of kafirins and resulting enhanced synthesis of non-kafirin proteins in the kernels [33, 47].

In recent years, an approach based on the introduction of genes that regulate the synthesis of proteins rich in lysine has been used to develop lines of cereals with a high content of lysine. However, to provide the expression of such genes in the endosperm, the genetic construct includes the endosperm-specific promoter and, in some cases, a signal sequence directing the synthesized protein into the protein bodies. Transgenic rice plants were obtained just this way, with enhanced expression of genes encoding the synthesis of high lysine histones RLRH1 and RLRH2 in the seeds. Transgene transcription was ensured by the promoter of the rice glutelin 1 gene. The lysine content in the obtained transgenic plants has increased by 35 % [62]. In another study, the wild potato (*Solanum berthaultii*) gene *sb401* encoding a lysine-rich protein of pollen cytoskeleton was inserted into the corn genome. As a result, the lysine content increased in the progeny of different transgenic plants by 16.1 % to 54.8 % as compared to the original non-transgenic line, and the total protein content increased by 11.6 % to 39.0 %; moreover, increased amount of lysine and protein was consistently inherited in six generations [63]. In another study, in transgenic plants of corn with a cotton gene (*Gossypium hirsutum* L.) *GhLRP* regulating the synthesis of lysine-rich protein the lysine content increased by 65 % compared to a non-transgenic one [64]. It should be noted that the potential allergenic potency of these proteins had been eliminated before the work started, by means of test for homology with known allergenic sequences.

When discussing the prospects of practical application of genetic engineering techniques to create cultivars of cereal crops with improved nutritional value of the grain, it should be noted that the first transgenic corn cultivar LY038 with a higher lysine content was marketed in 2005 [55]. Negative public opinion and concerns about possible harmful effects of genetically modified foods on human health and the environment remain an obstacle to a wider distribution of transgenic cultivars. However, it is obvious that, with improvements of the methods for producing transgenic plants (e.g. using markerless technologies) [65, 66], enhancements in the accuracy of insertion of genetic constructs [67], and application of genome editing methods using artificially engineered nucleases [68], the number of transgenic cultivars of cereals improved on the targeted characteristics (in particular, the nutritional value) will be increasing as these experiments use DNA sequences encoding the beneficial and safe for humans products, with genetic engineering techniques of breeding based on naturally occurring processes.

Thus, the use of genetic engineering methods opens up great opportunities for the modification of the grain storage protein composition in cereals. These exploratory research studies are critical for solving theoretical problems associated with understanding endosperm development and mechanisms of the synthesis and accumulation of essential nutrients, i.e. protein and starch, in ontogeny. In addition, they have a direct practical way, because they allow

creating nutritionally improved cultivars and lines to be used in foods and for forage production.

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GRAIN LEGUMES GENETIC RESOURCES OF MEDITERRANEAN ORIGIN IN VIR COLLECTION: DIVERSITY AND USE IN BREEDING (review)

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Abstract

The introductions from the Mediterranean region to VIR collection of grain legumes genetic resources amounts 12 % of its content. One-fifth of this sample accessions (1272) had been collected by N.I. Vavilov in 1926-1927, in his expedition around this richest floristic region. In the collection there are also large amounts of the accessions collected by P.M. Zhukovskiy, V.F. Dorofeev, K.Z. Boudin and other collectors from VIR. Many other accessions are obtained by the requests or as a result of exchange with breeding institutions of the countries situated around Mediterranean Sea and genebanks worldwide. The Mediterranean, a «hot spot» of the world's biodiversity, is one of the centers of origin of cultivated plants, including some economically important species of grain legumes. This is a region with many endemic species, both cultivated and wild relatives of legumes. In the countries of Mediterranean many species of legumes, including dry beans, cowpeas, vetch, white, yellow and blue lupines, chickpea, vetchling, lentil and grass pea had been domesticated. Common bean and soybean had been introduced and became an integral part of agricultural production in the region. All this makes the germplasm of the region very valuable. The article for the first time analyses the botanical and genetic diversity over 5500 samples of Mediterranean origin, introduced and stored in VIR collection and brief history of the process. In accordance with the N.I. Vavilov's rule of regularity in the geographical distribution of the traits, Mediterranean gene pool is phenotypically differentiated depending on the place of origin. The genotypes cultivated in the countries of western and northern Mediterranean for centuries been subjected to careful breeding, have been planted on fertile soils, in a mild climate and most of them are characterized by strong habit, large fruits and seeds. In the arid conditions of the southern and eastern parts of the Mediterranean basin early-matured plants grow adapted to drought and heat (N.I. Vavilov, 1962). This Vavilov's discovery is the basis of the addressed assignment of the material from VIR collection for the regional breeding programs in accordance with ecological conditions and geographical position of the region. The paper for the first time provides the information about the features of the Mediterranean gene pool stored in the collection of grain legumes. The differentiation of the breeding status of the material is clear depending on the significance of the genus (species) or the crop in agricultural production in Mediterranean region. The collections of peas, soybeans, common beans from this gene pool are represented mostly by commercial varieties. The collections of lupine, cowpea, chickpea, beans, chicklings and vetch are comprised mostly of local varieties. Some genera (*Vicia*, *Lathyrus*, *Lupin*) are represented by numerous species of wild relatives. For all crops the examples of elite germplasm (sources of alleles of genes determining the valuable traits) for breeding identified in the collection of VIR as a result of its assessment are given. The examples of using of Mediterranean gene pool in breeding domestic and foreign varieties are also given.

Keywords: Mediterranean, plant genetic resources, grain legumes, endemic species, elite germplasm, valuable traits, breeding.

The Mediterranean Basin is a historical region, located on the shores of the Mediterranean Sea and uniting countries of Europe, Asia and Africa, as well as numerous islands and archipelagos. Severely rugged terrain and the variety of edaphoclimatic conditions led to a significant variability of the landscape and biological forms on this vast territory (4 million km² or, with the Southwest

Asian highlands, more than 5 million km²) [1]. The region belongs to one of 34 biodiversity «hotspots» [2], thanks to the presence of 13,000 endemic species of vascular plants, or 4.3 % of the global number of endemic species. In this the Mediterranean region is second only to Sundaland (South East Asia) and tropical Andes [3]. Endemic species make up 40 % of the Mediterranean flora [4].

The Mediterranean basin is a region with the highest density of ancient civilizations in the Old World (Egyptians, Greeks, Romans, Phoenicians, Byzantines, Arabs, Franks, Ottomans), which enriched farming methods. The convenient location of the territory on the junction of the continents contributed to the rapid penetration and spread of cultivated plants [5]. It is not surprising that the Mediterranean region, with its «extraordinary concentration of species diversity» [6] and agronomy tradition, was one of the key research targets for N.I. Vavilov, P.M. Zhukovskii and, later, for other collectors from the All-Russian Research Institute of Plant Industry (VIR). The favourable climate, fertile soil and high culture of agriculture have made this region one of the main producers of crop products in the world.

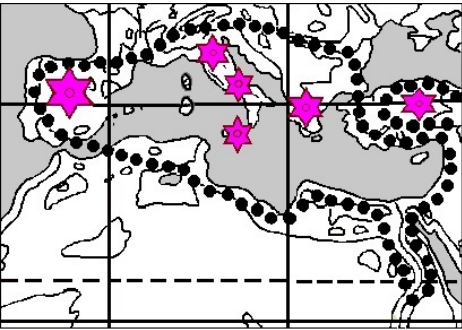
The value of the Mediterranean gene pool is determined by a wide variety of traits, a long history of cultivation of many plants, the active breeding of traditional and introduced forms, which became widespread in the Mediterranean countries, and the presence of wild relatives of various species.

From ancient times, legumes are part of the Mediterranean ecosystem. In this region, the following species had been brought under cultivation and cultured for millennia: *Pisum sativum* L., *Vicia faba* L., *V. sativa* L., *V. monantha* Retz., *V. narbonensis* L., *V. ervilia* (L.) Willd., *Lens culinaris* Medik., *Lathyrus sativus* L., *L. cicera* L. [7]. According to J.R. Harlan [8], one third of the plant species that mankind has brought under cultivation for life sustenance grows here. Currently, legumes are widely used in the Mediterranean region for feed and food purposes. They are an integral part of the Mediterranean diet, contributing to high life expectancy in the countries of the region. Genetic erosion of the Mediterranean phytodiversity (in particular, the Mediterranean gene pool of cultivated leguminous plants and their wild relatives), which takes place here, as well as throughout the world, as a result of climatic, environmental, urban and other factors, makes its conservation an impelling need [9] and the concern of all the world's genebanks. The value of the seed material from the Mediterranean region is evidenced, for example, by the fact that among the Laureates of the «Man and the Biosphere» UNESCO Programme, as part of the International Year of Biodiversity (2010), was Salama El Fatehi (Morocco), the developer of the project «Evaluation of genetic resources of the endangered bean species (*Vicia ervilia*) on the territory of the Mediterranean Intercontinental Biosphere Reserve (Morocco-Andalusia, Spain)». Analysis of modern germplasm collections of *Lathyrus* L., *Pisum* L., *Vicia* L. and *Lens* Mill. revealed a representability of genetic resources from the countries having access to the Mediterranean Sea, as well as from the maximum concentration area, i.e. the Fertile Crescent in Turkey, Syria and Lebanon [10]. The paper clearly demonstrates the wealth of the region's species composition stored ex situ, and suggests the ways of its further conservation in situ.

This article is aimed at reviewing the diversity of cultivated plants and wild relatives of legumes from the Mediterranean region (regardless of the center of their origin) stored in the VIR's collection, and their use for domestic breeding.

History, composition and value of the Mediterranean gene pool of legumes from the VIR's collection. The Mediterranean center of plant diversity and domestication, with its four centers (Pyrenean, Apennine, Balkan and Syrian-Egyptian), is considered one of the largest in the world (Fig.). It is this region along with located in close proximity South-West

Asian center covering three oases (Caucasian, Western Asian and North-Western Indian), which is associated with the origin of the major legumes (peas, lentils, vetch, vetchling, beans, lupine), and a number of forage legumes [11].



The Mediterranean center of origin and centers of diversity of cultivated plants [12].

The mass accessions of legume specimens from the Mediterranean region have been established by the longest expedition of N.I. Vavilov (1926-1927), the route of which covered the territory of all the coastal countries and the largest islands, including Portugal, Abyssinia and Eritrea that have no direct access to the Mediterranean Sea. The number of leguminous specimens collected by N.I. Vavilov and his assistants M. Gaysinskii on Sardinia and R. Gudsoni in Egypt amounted to 1,262 [13]. Later, the collection was replenished due to expeditions of P.M. Zhukovskii to Syria, Turkey, Mesopotamia and other countries and island territories (1925, 1926, 1927, 1954), V.F. Dorofeev to Turkey (1967) and Syria (1974), K.Z. Boudin and V.L. Witkowskii to Algeria (1969), A.V. Pukhalskii and E.V. Mazhorov to Tunisia and Morocco (1970), etc. [14]. Over past two decades the accessions are mainly based on exchange with foreign researchers and genebanks.

Currently, the number of specimens from Mediterranean countries in the VIR's legume collection amounts to 5,563 (Table). Most legumes are known to have come from the South-West Asian center [11]. As floristic studies showed, there are 1,974 species of legumes [15] in the natural and cultural Mediterranean plants, which indicates the significant resource potential for the mobilization into the collection.

Specimens of leguminous crops and their wild relatives stored in the VIR's collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources; as of January 1, 2014)

Genus, species	Quantity, pcs	From the Mediterranean region		
		quantity, pcs	fraction, %	countries represented
<i>Cicer arietinum</i> L.	3,310	779	23.5	15
<i>Glycine max</i> (L.) Merr.	7,267	286	4.0	8
<i>Lathyrus</i> sp.	2,066	367	17.8	11
<i>Lens culinaris</i> Medik.	3,040	606	19.9	13
<i>Lupinus</i> sp.	2,833	300	10.6	15
<i>Phaseolus</i> sp.	7,678	638	8.3	14
<i>Pisum sativum</i> L.	8,057	685	8.5	16
<i>Vicia faba</i> L.	1,964	231	11.8	12
<i>Vicia</i> sp.	5,509	1,499	27.2	17
<i>Vigna</i> sp.:				
total	4,068	92	2.2	11
incl. <i>V. unguiculata</i> (L.) Walp.	1,847	80	4.3	11
Total in the collection	45,792	5,483	12.0	

The natural features of the Mediterranean region, including flora, were found long time ago [16], but only the differential botanical and geographical approach, developed by N.I. Vavilov, revealed intraspecific variation of traits in the forms that grew in different parts of this vast territory, and their specific geographical location. For centuries, plants, handled in the countries of western and northern parts of the Mediterranean basin, have been subjected to careful selection, cultivated on fertile soils under conditions of mild climate, and most of them are characterized by a strong habitus, large fruits and seeds. Early ripening crops adapted to drought and heat are grown in the arid conditions of the south

and eastern parts of the Mediterranean basin [11].

Geographically defined features, or «correctness of the intermutation process, which, upon closer examination, appeared to be a general process» [11], was demonstrated by N.I. Vavilov on a number of crops, including peas, beans, chickpeas and lentils. This discovery by N.I. Vavilov is the basis for addressed assignment of the source material from the VIR's collection for the regional breeding programmes in accordance with ecological and geographical conditions.

Very important gene pool is provided by wild relatives of cultivated plants, preserved in the region and carrying many valuable traits, the introgression of which into the cultigens may contribute to their agronomic improvement. For example, Spanish lupine (*Lupinus hispanicus* Boiss. et Reut.) growing in Spain and Portugal can serve as a donor of cold and drought hardness, as well as disease resistance [17, 18]. Wild and local specimens of Turkish (Anatolian) vetches belonging to *Vicia sativa* possess many economically important traits needed for cultivation in northern conditions of our country [19].

Peas *Pisum* L. Two species, *P. sativum* and *P. fulvum* Sibth. et Sm., are represented in the collection. Among 685 specimens of the Mediterranean origin, commercial and local varieties account for 46.0 % and 38.0 %, respectively, and the wild forms and samples with unknown status make up 1.5 % and 13.0 %, respectively. The primary centers of origin and diversity of peas, according to N.I. Vavilov [11], are mountain regions of the Western and Central Asia and Ethiopia, with the Mediterranean region being a secondary center. Recently, some of these views has been corrected, e.g. the primary center was allocated to the Western Asia (Iran, Afghanistan, Pakistan and Turkmenistan) and the entire Mediterranean region (Greece, Italy, Spain and Morocco), while Ethiopia was given the status of the secondary center [20]. In South-East Asia, due to low human influences, peas preserved many primitive features, while in Europe there was an intensive process of domestication [21] resulted in productive grain and vegetable forms. Here, they began to use recessive traits, which ultimately determined the breeding success. New morphotypes have appeared regulated by combinations or individual recessive alleles, such as leafless (*af*), semi-dwarf with shortened internodes (*le*, *la*, *lm*), with fasciated stem (*fas*, *fā*), etc. These plants are resistant to lodging, suitable for mechanized cultivation and harvesting. The combination of *h* and *v* alleles determines sweet peas. The *nn* genotype results in minimum thickness of the bean pod halves. Good cooking characteristics in *epep* homozygote are due to maximum reduction of bean seed hypodermis [22].

Introgression of these and a number of other recessive alleles to cultivars has determined the phenotype and quality features of modern pea varieties. No wonder that the Mediterranean specimens include primarily commercial varieties. The major part of them (i.e., 268 varieties in the collection) came from France, one of the world leaders in the production of peas. Worldwide known French varieties often served as the initial material for the creation of our local varieties. Thus, the Baccara cultivar (k-8974), which dominated in commercial pea production in France for many years and was involved in pea breeding programmes in many countries, has been used at the All-Russian Research Institute for Legumes and Groat Crops (VNIIZBK, Orel Province); the early-maturing, productive and high-protein cultivar Supraduction Tezier (k-6025) is being involved in pea breeding at the Cheshminskaya Experimental Station. Quite active pea breeding has been undertaken in Spain, Italy, the former Yugoslavia, from where the VIR collection received 66, 39 and 44 samples, respectively. A large set of 125 samples from Turkey consists mainly of local varieties collected in expeditions by P.M. Zhukovskii (1926) and V.F. Dorofeef (1967).

The sources for valuable gene alleles, identified due to multi-year pea

plant evaluations are k-2495 (Turkey), k-6018 (France), k-3108 (Italy), k-7262 (Algeria) (early ripening); k-2975 (Italy), k-3118, k-7573 (Spain), k-2593 (Cyprus), k-4421, (Yugoslavia), k-2415 (Algeria) (large-fruited); k-8577 (Spain) k-8720, k-9263 (France) (leafless); k-5568, k-6017 (France), k-6151 (Morocco) (high seed productivity); k-2629 (Italy), k-6668 (France) (*Fusarium*-resistant); k-6667 (France), k-2249 (Turkey) (*Phomopsis*-resistant); k-9342 (Greece) (resistance to black spot); k-7243, k-6936 (France), k-7644 (Algeria) (polycarpous, three and more beans per node) [23-25].

Broad beans *Vicia faba* L. The primary center of its origin is the South-West Asia, while the Mediterranean region, where the large-seed forms are concentrated, is considered a secondary center. From here, beans penetrated into Europe and formed the locus of third significance, and there, following the impact of other environmental conditions, races with roughly podded fruit and straight and sturdy stems were isolated [26]. Modern research has showed that the domestication of beans and chickpeas could occur in north-western Syria, in close proximity to the Mediterranean coast in the X millennium BC [27].

All the variety of beans belongs to *V. faba* species. Among 231 samples of the Mediterranean origin, the local varieties account for 94 %, and those obtained as a result of scientific breeding make up only 6 %. In Europe, the diversity of local varieties of beans appears to be explained by long history of cultivation, as from ancient until relatively recent times they had mainly been the source of vegetable protein in food and feed. The import of soybeans, which began in the mid-twentieth century, has affected the production of beans and their breeding in Europe. However, in the Mediterranean countries they are still widely used in food and for animal feeding [28]. The bean breeding is developed in Spain and France, but as a food crop, they are more popular in North African countries [29].

The forms widely-spread in the Mediterranean countries are not of great variety, since large-seeded, light-coloured, medium- and late-maturing varieties dominate, with occasional medium-seeded forms. Small-seeded legumes are not grown everywhere, and they are even more uniform. A distinctive feature of Mediterranean beans is their thin pod valves and tendency to lodging. There are both early-maturing and late-maturing forms with a vegetation period of 94 to 132 days. Seed size, light coloration, pod shattering resistance, drought and cold hardiness, rust resistance, and high protein content are valuable features of the Mediterranean forms, however, a weak, subject to lodging stem is a disadvantage of these beans when mechanically harvested.

Long-term investigations of Mediterranean gene pool samples in the VIR experimental stations' network allowed to isolate valuable bean forms such as k-1569, k-1571 (Egypt) (early ripening); k-1579, k-1581 (Sudan), k-1717, k-1832 (Italy), k-1577 (France) (high protein content in seeds); k-1575 (Syria), k-1582, k-1584 (Egypt), k-1416 (Yugoslavia), k-1470, k-1688 (France) (high seed productivity) [30].

Chickpea *Cicer* (Tourn.) L. The chickpea collection is represented by seven annual species, such as a cultivated species *C. arietinum* L. and wild *C. bijugum* K.H. Rech., *C. cuneatum* Hochst. ex Rich., *C. echinospermum* P.H. Davis, *C. judaicum* Boissier, *C. pinnatifidum* Jaubert et Spach, *C. reticulatum* Ladisinsky species, originated from the Mediterranean basin. Of these 779 samples, 90 % are local varieties, 5 % are cultivars resulted from scientific breeding, and 5 % are wild species. The samples came from 15 countries, from the East (Iran, Syria, Turkey, Lebanon, Israel) to the West (Spain, France, Morocco) Mediterranean. According to M.G. Popov [31], the center of origin and distribution of species from the genus *Cicer* was located in the Ancient Mediter-

ranean area, which started from the Atlantic Ocean shores and Morocco and, bordering both shores of the Mediterranean sea, stretched into Asia, including Asia Minor, Syria, Palestine, Mesopotamia, Persia, Transcaucasus, Turkmenistan, Uzbekistan and Afghanistan. E.N. Sinskaya [32] considers that the cultivated chickpea species *C. arietinum* is native to the Eastern Mediterranean (Asia Minor). In the coastal countries of the Mediterranean basin (Spain, Morocco, Algeria, Tunisia, Turkey, Syria, etc.), large-seeded varieties, most valuable for breeding, are distributed. The large-seed character in the significant part of them is combined with drought hardiness and relative resistance to *Fusarium*. Samples of chickpeas from Spain have good taste.

The sources of gene alleles regulating valuable traits of chickpeas from Mediterranean countries are k-277 (France), k-361 (Turkey), k-452 (Algeria), k-626 (Palestine) (early ripening); k-278 (France), k-1886 (Spain) (high attachment of the lower bean); k-277 (France), k-340, k-343, k-608 (Turkey), k-125 (Palestine), k-453 (Algeria), k-798 (Italy) (large-seeded); k-355 (Turkey), k-1943, k-1959, k-1972, k-1991 (Spain), k-2291 (Syria) (high seed productivity); k-278 (France), k-352 (Turkey), k-2648 (Syria) (compact habitus); k-1941, k-1980, k-1981 (Spain) (high protein content in seeds) [33, 34].

Lentils *Lens* Mill. Based on hybridological and molecular genetic data, only two species are currently distinguished in the genus *Lens*, such as *L. culinaris* (cultivated) and *L. nigricans* (Bieb.) Webb et Berth. (wild) [35]. E.I. Barulina, the main monographist of the crop in the Soviet Union [36], divided the cultivated lentils *L. sulinaris* into two subspecies, *macrosperma* (Baumg.) Bar. (large-seeded, with large flowers and seeds of 7-9 mm diameter), and *microsperma* (Baumg.) Bar. (small-seeded, with small or medium-sized flowers and seeds of 3-6 mm diameter). The large-seeded plants are mainly used for breeding.

Among 606 Mediterranean samples, 5 % are the varieties resulted from scientific breeding, the local varieties account for 70 %, and 25 % possess unknown status. The origin of lentils is still debated. E.I. Barulina [37] believed that the cultivated species originated from the area between Afghanistan, India and Turkestan (between the Hindu Kush and the Himalayas), but now this area is considered a secondary center of lentils origin [38], as archaeological and molecular evidence indicate the border areas between Turkey and Syria, and between Syria and Jordan, as the center of domestication and the diversity of species [39, 40]. The accessions from the center of maximum diversity are of particular interest. This is where most of the Mediterranean lentil specimens for the VIR collection were obtained from, namely 113 from Syria and 96 from Turkey, which are on top 10 of the crop producers. A total of 82 specimens came from Israel, and 65 and 61 from Morocco and Spain, respectively (western Mediterranean). Having spread along the shores of the Mediterranean Sea in the Neolithic Age, lentils became popular in many countries of the region.

The sources of valuable gene alleles are k-2722, k-2728 (Syria) (early ripening); k-538 (Turkey), k-1103 (Spain) (tall-growing); k-1084, k-1098 (Italy) (large-seeded); k-1045, k-585 (Turkey) (high protein content); k-1045 (Sicily), k-2727 (Syria) (high seed productivity); k-1829 (Yugoslavia), k-2222 (Italy) (resistance to *Botritis cinerea* Fr.). Using a k-538 specimen as the parent form, Rauza and Svetlaya cultivars have been developed in the VNIIZBK by crossings. These cultivars have high yield (2.84 to 3.05 t/ha), a relatively large seeds with high (28.0 to 29.6 %) protein content, excellent product quality and cooking characteristics. During the observation period (2003-2010), they were not infected with diseases [41]. These cultivars suitable for all climatic zones of lentil cultivation in Russia contribute significantly to its increased production, though crop expansion is still limited due to unstable yields and poor suitability for

mechanized harvesting. Involvement of the Mediterranean lentils with large seeds, high and slightly branching stem in the breeding programmes could help to obtain advanced cultivars.

Vetch *Vicia* L. Many species of the genus are endemics of the Ancient Mediterranean [42]. There are 58 representatives of species of the genus *Vicia* in the VIR collection, with more than a quarter of the specimens and more than half of the species diversity (1,499 samples of 39 species) being accessions from 17 countries of the Mediterranean region. The majority, being mostly wild forms and local varieties, are derived from Turkey, Syria, France, Morocco and Israel (343, 211, 167, 108 and 104 specimens, respectively). From Spain, which is recognized as the leader of vetch production in Europe [43], 209 samples were obtained. There are significant accessions of vetch from Italy, Algeria, Yugoslavia, Greece, Cyprus, Tunisia (91, 56, 55, 42, 44, 45 specimens, respectively), and other countries.

Common vetch (*V. sativa* subsp. *sativa*) is an important forage crop in Russia. The species diversity center is located in the North-Eastern Mediterranean with the greatest concentration in the countries of the Fertile Crescent, i.e. Turkey, Lebanon, Syria, Iran, Iraq and the former Asian republics of the ex-USSR [44]. The collection contains 532 specimens of common vetch, including accessions from Spain, Israel, France, Turkey, Morocco, Syria, Italy, Greece, Algeria (145, 57, 60, 53, 40, 37, 27, 29, 16 specimens, respectively), Albania, Libya, Tunisia, Egypt, as well as about 300 specimens of closely related taxa, mainly *V. sativa* subsp. *nigra* (L.) Ehrh. and *V. sativa* subsp. *cordata* (Wulfen ex Hoppe) Arcang.

Geographical differentiation of the common vetch Mediterranean gene pool in accordance with the origin was clearly demonstrated via AFLP-profiling samples from two genebanks, the VIR and the Institute of Genetics of Plants (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung — IPK, Gatersleben, Germany) [45].

The sources of valuable gene alleles are k-920, k-923, k-33583 (Syria), k-965, k-1058, k-1062 (Turkey), k-102 (Italy) (early ripening); k-1370 (Italy), k-34587 (Yugoslavia) (herbage productivity); k-1019 (Italy), k-35043, k-36035 (Turkey), k-1141, k-35696, k-35915 (Spain) (seed productivity); k-1370, k-35167 (Italy), k-35761 (Spain) (high protein content in the herbage); k-34805 (Greece), k-35262 (France) (high protein content in seeds); k-1152 (Algeria) (drought hardiness) [46].

The samples of Mediterranean origin have been involved in breeding domestic vetch cultivars, e.g. k-33747 (France) for Orlovskaya 4 (VNIIZBK) and LOS-5 (Lgovskaya Experimental Breeding Station) cultivars, k-34456 (France) for Lugovskaya 85 cultivar (V.R. Williams All-Russian Fodder Research Institute, Moscow Breeding Station). The cultivars are listed in the State Register of Breeding Achievements admitted for use (2014) [47]. A Syrian specimen k-33583 was involved in breeding Nemchinovskaya 84 cultivar (the Moscow Research Institute of Agriculture «Nemchinovka»), released in 1989.

The collection of bitter vetch *V. ervilia* (French lentils), the endemic species from the Mediterranean region, includes 334 specimens from the region, with 177 from Turkey, 57 from Spain, 31 from Israel, 27 from Cyprus island, and 15 from Syria. The initial bitter vetch domestication is believed to occur in the eastern Mediterranean region, i.e. Cyprus island, Syria, Palestine, Greece and, in part, Asia Minor, where the greatest diversity is shown. *V. ervilia* is characterized by drought hardiness and early maturity, it can mature even in the far north, with large herbage grown. This plant is mostly from mountainous countries [36].

Some sources of gene alleles in *V. ervilia* are k-161 (Turkey), k-339 (Cy-

prus), k-384 (Spain), k-388 (Italy), k-439 (Greece), k-602 (Morocco) (drought hardiness); k-107, k-141 (Turkey), k-340, k-392 (Cyprus), k-281, k-295 (Israel), k-240, k-535 (Syria) (early maturity); k-253 (Syria), k-18 (France), k-112, k-204, k-213 (Turkey), k-333, k-537 (Greece), k-390 (Spain), k-588 (Algeria) (seed productivity); k-213, k-259 (Turkey), k-356 (Italy), k-537 (Greece), k-588 (Algeria), k-230 (Tunisia) (productivity of herbage) [48].

Vetchling *Lathyrus* L. The Mediterranean region is the center of origin [11, 49] and diversity [50] of many vetchling species. The VIR collection possesses 56 species of *Lathyrus*, and 33 of them are of Mediterranean origin. The specimens are mostly wild species, and such as *L. cicera*, *L. ochrus* (L.) DC, *L. tingitanus* L., are cultivated ones. *L. clymenum* L., *L. articulatus* L., *L. aphaca* L., *L. hirsutus* L. are important only as forage and green manures, while *L. odoratus* L. (sweet pea) and *L. sativus* (grass pea) are mostly known and widespread in the horticulture and agriculture in different countries.

Among 367 specimens of vetchling of Mediterranean origin, 90 % are local varieties, and 10 % are wild forms. Specimens of grass pea first collected in 1926-1927 by N.I. Vavilov and P.M. Zhukovskii dominate in the collection. Currently, there are specimens from 11 Mediterranean countries, mostly from Greece, Italy, Algeria, Syria, Cyprus, France and Turkey. As to the number of species characteristic of these countries, the VIR collection is superior to other 15 world largest germplasm collections, and by the total number of specimens it is second only to the collection of the University of Pays and Adour (Université de Pau et des Pays de l'Adour, France) [51].

Grass pea was cultivated in Egypt and Asia Minor in 9500-7600 BC [52] and on the Balkan Peninsula in the early Neolithic Age at the beginning of the VI century BC [53]. Many local varieties have beneficial properties. Distinctive features of plants from the Mediterranean coast, including the Apennine and Iberian Peninsulas, the islands of Sardinia and Sicily, are high branching, fast growth, drought and cold hardiness, strong demand to heat during ripening, large seeds, white seed colour, productivity, high protein content in seed and hay, resistance to fungal diseases. Specimens from these territories were the source material for all large-seeded varieties obtained from scientific breeding in our country. Many forms and varieties from the Cyprus, Turkey, Egypt, the mountain regions of Algeria and Spain stand out for their early ripening (the vegetative season is 70 days) [54].

The sources of valuable gene alleles are k-775 (Spain), k-1114 (France), k-801, k-865, k-870 (Turkey) (early ripening); k-30, k-742, k-1112 (France), k-791, k-879, k-880 (Italy), k-410 (Cyprus), k-884 (Spain) (high productivity); k-390 (Cyprus), k-420 (Italy), k-1110 (France) (high productivity of seeds and herbage); k-774, k-775, k-778, k-781 (Spain), k-791, k-795 (Italy), k-1112 (France) (large-seeded); k-417 (Algeria), k-706, k-983 (Italy), k-1095, k-1363 (Turkey) (high protein content in seeds); k-773 (Spain) (high protein content in herbage); k-1221 (Yugoslavia), k-703, k-765, k-770, k-879, k-881 (Italy), k-774 (Spain), k-395, k-398, k-409, k-411 (Cyprus), k-30 (France) (*Ascochyta* resistance); k-406 (Cyprus), k-781 (Spain) (resistance to *Erysiphe communis* Grev r. *lathyr* Rabh.); k-836 (Yugoslavia), k-888 (island of Sardinia), resistant to rust pathogen *Uromyces pisi* (Pers.) Schröt [55-57].

Among other species of vetchling from the Mediterranean region, there have also been identified specimens with various valuable features, such as high protein content in seeds, k-200 (*L. tingitanus*) and k-135 (*L. ochrus*) (France); high protein content in herbage, k-387 (Cyprus), k-769 (*L. cicera*) (Italy); resistance to *Ascochyta* and rust, k-200 (*L. tingitanus*) and k-135 (*L. ochrus*)

(France), k-443 (*L. ochrus*) (Turkey); resistance to *Uromyces pisi* (Pers.) Schröt., k-1391 (*L. tingitanus*) (France) [56, 57].

Lupine *Lupinus* L. The number of lupine species from the Mediterranean region as the center of origin is small compared to those from the New World, and among them, the great economic significance is attributed to blue lupine (*L. angustifolius* L.), yellow lupine (*L. luteus* L.), and white lupine (*L. albus* L.). The first two are cultivated from the middle of the XIX century, while white lupine is an ancient plant [49]. The VIR collection includes 50 species of lupine. The Mediterranean region is the origin and formation center of nine of them, such as *L. angustifolius*, *L. luteus*, *L. albus*, *L. cosentinii* Guss., *L. hispanicus*, *L. pilosus* Murr., *L. atlanticus* Glads., *L. digitatus* Forsk. and *L. micranthus* Guss. (841, 800, 494, 25, 18, 15, 9, 6 and 5 samples, respectively). Most of the accessions are scientifically-bred cultivars from Australia, Belarus, Poland and Russia, and approximately 300 of them were obtained directly from Spain, Egypt, Greece, Morocco, Italy, Israel (87, 53, 28, 36, 22, 15 samples, respectively). None of these Mediterranean countries are considered major producers of the crop, but all of them are located in the centers of its diversity. Therefore, most of the specimens (70 %) are represented with local varieties, and the remaining 30 % are wild forms.

Carriers of valuable alleles among *L. albus* are k-3154 (Egypt), k-3293, k-3294 (Israel) (early ripening); k-3109 (Egypt), k-3115 (Greece), k-3118 (Yugoslavia) (early ripening and productivity); k-507 (Egypt), k-682 (Yugoslavia), k-2864, k-2865 (Greece) (resistance to *Fusarium*); k-294, k-295, k-298, k-302, k-306 (Palestine), k-1435 (Greece), k-502 (Egypt), k-2299, k-2298 (Spain), k-313, k-1600, k-1601 (Italy) (high protein content); k-290, k-294, k-295, k-298, k-302 (Palestine), k-1649 (Yugoslavia), k-2297 (Spain) (high oil content) [58]. Among *L. angustifolius* the valuable traits are found in k-3093 (Morocco) (resistance to grey mould); k-91, k-371, k-372, k-373 (Algeria), k-169 (Italy), k-2868 (island of Corsica) (high productivity); k-288 (Palestine) (early ripening); k-3347 (Turkey), k-2666 (island of Crete), k-3345 (Greece) (drought hardiness); k-288 (Palestine), k-169 (Italy) (high oil content). Of *L. luteus*, the k-2072, k-2076, k-2081, k-3343 (Turkey) (high productivity and drought hardiness); k-3341 (Italy) (early ripening, productivity, resistance to virus diseases) were shown to be worthy [46].

The gene pool of the Mediterranean lupine, stored in the VIR collection, is widely used in different countries. With the involvement of Palestinian samples, the Kievskii Mutant cultivar of white lupine was created in the Ukrainian Institute of Agriculture (Kiev Province), which stands out by early ripeness and high productivity, and is grown almost all over the world. In Russia, for the blue lupine breeding, the Apendrilon specimen from Greece is used as a source of high productivity, drought hardiness and resistance to *Fusarium*. Resistance to *Fusarium* from Italian wild forms of yellow lupine was transferred to the Borluta cultivar, developed in Germany [46].

Beans *Phaseolus* L. Bean seeds were brought to Europe (primarily to the Mediterranean region, to Spain and Portugal) from Central and South America (center of origin and species formation) around 1500 [59], and, due to the variety of flower and seed morphology of the introduced plants, the bean seed delivery occurred several times and from different parts of America [60].

The VIR collection comprises five species of the genus. A total of 635 specimens of *P. vulgaris* L. samples (kidney bean) and 3 specimens of *P. lunatus* L. (lima beans) were obtained from the Mediterranean countries. The latter one is a typical subtropical species, which is common and popular in the Mediterranean countries, but almost never used in Russia. According to the breeding status, the

scientifically-bred varieties, local varieties and breeding material amount to 36.4 %, 7.2 % and 8.3 %, respectively, and 48.1 % have no defined status. The accessions were mostly obtained from France (331 specimens), where its scientific selection has been performed from the middle of the XIX century. A significant part of specimens (87, of which 52 are breeding lines) was from the former Yugoslavia, particularly Serbia where bean breeding is being conducted. Large accessions were obtained from Italy, Spain, and Turkey where the crop is popular.

Sources of valuable alleles of *P. vulgaris* are k-12031, k-12063, k-12150, k-13412, k-14673 (France), k-15279 (Greece) (early ripeness); k-11963, k-12037 (France), k-15121 (Italy), k-15347 (Turkey) (productivity); k-11977, k-11993, k-12018, k-12052 (France), k-10312 (Italy), k-12321 (Morocco), k-12952 (Greece), k-15169, k-15171 (Turkey) (protein content more than 27 %); k-12027, k-13403, k-14672, k-14690, k-14694 (France) (low trypsin inhibitor activity) [61]; k-2279 (Italy), k-76, k-14910 (France), k-12682 (Turkey) (weak photoperiodic sensitivity) [62]; k-12034, k-12049, k-12138, k-13967 (France), k-14160 (Spain) (drought hardiness); k-11992, k-13534, k-14664 (France), k-13328 (Tunisia) (cold hardiness); k-11771, k-11959 (France) (resistance to common mosaic virus); k-13063, k-14665 (France) (resistance to a number of disease).

Cowpea *Vigna unguiculata* (L.) Walp. The primary center of origin of this ancient food and fodder plant is allocated by some researchers to East Africa [6, 49, 63], and by others, it is the central and southern parts of the African continent [64], from where cowpea reached the Mediterranean region. The long history of *Vigna* cultivation in the vast territory of the Mediterranean region, along with hybridization and mutations have led to many local varieties, and as a result, modern genetic diversity in the Mediterranean gene pool is broader than that of African and North American [64]. Among the Mediterranean specimens, there are plants with bushy and procumbent form, with small and large seeds (weight of 1,000 seeds amount to 50 to 280 g). The majority of specimens are early ripening (68 to 90 days), with good productivity and high protein content in seeds, and resistance to viral diseases [65, 66]. The varieties are mainly for food purposes. Many specimens are superior to other forms in the herbage, and are used for fodder and as a green manure. In the Mediterranean region, cowpea is grown almost in all countries, but primarily as a garden plant. Production areas are in Croatia and Cyprus [43].

The VIR collection of *V. unguiculata* contains only 80 specimens of Mediterranean origin from 11 countries (Algeria, Egypt, Tunisia, Cyprus, Greece, Turkey, Spain, Italy, Syria, France, Israel). Many specimens are characterized by high parameters, such as k-247 (Turkey), k-481 (Italy), k-1226 (Egypt), k-304, k-1221 (Syria) (seed productivity); k-292 (Algeria) (herbage productivity); k-492, k-495, k-896, k-1226 (Egypt), k-309 (Cyprus) (early ripening); k-309 (Cyprus) (early ripeness and seed productivity); k-247 (Turkey), k-304 (Syria), k-307 (Tunisia), k-309 (Cyprus), k-478, k-481 (Italy), k-518, k-901 (Egypt) (large-seeded); k-292 (Algeria), k-190 (Italy), k-247 (Turkey) (high protein content in the seed) [65, 66].

Soybean *Glycine max* (L.) Merr. Soybean is native to East Asian center of origin, located on the territory of China, Korea and Japan [67]. With the development of contacts with the Southeast Asian countries, a multiple delivery of soybean seeds to Europe occurred, there were repeated attempts to cultivate the plant, and already in 1740, soybeans were grown in the Botanical Gardens in Paris (Jardin des plantes de Paris) [68, 69]. Since 1880, the seed breeding firm Vilmorin-Andrieux (France) has offered soybean seeds in its catalogue for gardeners and farmers [69]. However, low import prices retarded soybeans production in Europe for a long time. At present, soy is cultivated only on

364.9 thous. ha in 28 countries of the European Union, the largest portion of which (153.0 thous. ha) are the areas in Italy. In North Africa, soy is not widely produced [43].

A total of 286 soybean specimens in the VIR collection are of Mediterranean origin. The bulk of the material was donated to the VIR from various research and breeding organizations, and includes varieties resulted from scientific breeding as well as breeding material. Not all the varieties in the VIR collection received from the Mediterranean region (especially in the first half of the XX century) were developed there (for example, a sample obtained by N.I. Vavilov from an Italian seed company Ingegnoli in 1926 was from Japan). Most Mediterranean accessions of those years contained no information about the primary origin. Only since 1949, the large-scale accessions from Mediterranean countries have begun, evidencing the development of breeding and production of soybeans in the region. Specimens were mostly received from France, Yugoslavia and Algeria (137, 85 and 47, respectively), and small sets came from Italy, Israel, Morocco and Syria.

Under the conditions of the Krasnodar Krai and the south of Ukraine the important traits were found in k-5935, k-8216 (Algeria), k-5529 (Israel), k-10089, K-10091 (Italy), k-5895, k-5798 (France), k-9655, k-9931 (Yugoslavia) (high seed productivity); k-5724, k-5749, k-9470 (France), k-5317, k-8268 (Yugoslavia) (early ripening); k-5543, k-8268 (Yugoslavia), k-5865, k-6226 (France) (high protein content in seeds); k-10086 (Italy), k-9233, k-10148 (France), k-9653, k-9936 (Yugoslavia) (high oil content in the seed) [70-72].

Therefore, the recognition of the Mediterranean basin as one of the «hotspots» of global biodiversity, and the territory of the Fertile Crescent as the «hotspot» of species diversity of the major genera of legumes in the Old World (*Lathyrus*, *Pisum*, *Vicia* and *Lens*) indicates unconditional importance of the region to collect and save this plant genetic resources both in situ and ex situ (in germplasm collections). An example of the VIR collection, where the Mediterranean gene pool of legumes, depending on the crop, amounts from 3.1 % to 23.8 %, demonstrates its diversity and value for breeding. The investigation of this gene pool is equally important for botany, ecology, geography, for phylogenetic analysis and understanding the cultivated plant evolution.

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MICROBIAL COMMUNITY OF SOIL: PHYSIOLOGICAL DIVERSITY PATTERNS AND ASSESSMENT

(review)

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Abstract

Study of the taxonomic and functional diversity of soil microorganisms association is of great theoretical significance for understanding the structure of the soil microbial community, the nature of the interaction of individual species of microorganisms belonging to this community, as well as their participation in the processes of soil formation and circulation of substances. This article summarises a brief history of ideas about the functioning of soil microbial complex, providing transformation and mineralization of organic matter in soil formation processes. Soil as habitat for microorganisms is heterogeneous that defines microzonal character of the distribution and activity of microorganisms that live in it. The structure of the association of microorganisms and its physiological profiles vary in time and space (D.G. Zvyagintsev, 1987). This defines methodological difficulties and the significant variability of the results of the evaluation of soil microflora by various authors. This review discusses the methodological approaches in determining the physiological diversity of soil microorganisms association. Traditional methods of elective culture media in over a century allowed to reveal numerous physiological groups of microorganisms and developed an idea about their role in the cycle of matter, processes of soil formation and plant nutrition. However, such work is almost not given anything new in principle, both in environmental studies, as well as in agronomy over the past 20 years. At the end of the 1990s a method of analysis of the carbon source utilization profiles (SUP) of natural microorganisms association by BIOLOG system used previously only in medical and general microbiology was proposed to study the physiological diversity for the test strain (J.L. Garland, A.L. Mills, 1991). This approach was further developed by H. Insam (1997), M.V. Gorlenko and P.A. Kozhevnikov (2005) and others. A number of modifications of this method (Eso-Plates, ECOLOGY and others) characterized by a set of organic substrates, which, as the authors suggest, is most likely present in natural environments were worked out. Instrumentation of ECOLOG (M.V. Gorlenko, P.A. Kozhevnikov, 2005) allows to determine not only the range of organic substrates used by microorganisms, but also to quantify the consumption of each substrate. For the processing and interpretation of a significant amount of information obtained in the course of the analysis of soil samples there are an apparatus of multidimensional mathematical statistics, cluster analysis, rank distribution, and ecological indexes of Shannon and Pielou. SUP method (multisubstrate test) possesses a high performance, good resolution (10^4), a satisfactory reproducibility and is a high-tech and effective tool to assess the physiological diversity. The article deals with the positive and negative aspects of the method. SUP reflects to some extent the potential of aerobic soil microorganisms using low molecular weight organic compounds in the catabolism. However, because of availability of several modifications, as well as some technical problem it is difficult to compare the results obtained by different authors, there is no unified SUP analysis protocol that is required for the comparative environmental studies and the establishment of relevant national and international databases. Thus, analysis of the carbon source utilization profiles (SUP) in BIOLOG system now is under development and testing, and with the accumulation of experimental data and critical analysis, it has good prospects in soil ecology, in research of the relationship between microorganisms and plants, and in assessment of the impact of anthropogenic factors.

Keywords: association, physiological groups, physiological diversity of bacteria, range of consumed substrates, multisubstrate test, system BIOLOG, ECOLOG, EcoPlates.

The founders of soil science V.V. Dokuchaev and P.A. Kostychev were the first to draw attention to the soil as a living system with bacteria as one of the key

factors of its functioning. The soil, as habitat of microorganisms and the product of their activity, is a complex system which includes species various in their physiology that provide the biological circulation of elements, the soil formation and resistance to natural and anthropogenic factors. This determines the theoretical and practical importance of the ecological studies of microbial communities. The key factors of microbial ecology are taxonomic and functional diversity of the microbial community and the nature of interaction of individual microorganism species belonging to this community which provides soil formation and plant mineral nutrition.

This article discusses the models of the soil microbial community functioning and the conditions of microbial activity in the soil. Methodical approaches to the study of soil physiological diversity in microorganism association, and the positive and negative aspects of the methods proposed are considered.

An ecological approach to studying soil microflora, its species and functional diversity is associated with the classical research of S.N. Vinogradsky [1]. Based on own studies and the accumulated experience, he comes to the conclusion that the vital functions of soil microflora are based on the principle of «division of labor, which is expressed <...> in the interoperability of the team members». S.N. Vinogradsky writes: «Microbiological processes in the soil are composed of numerous phases which replace one another, each of the phases being associated with a single pathogen or a small group of pathogens» [1]. Accordingly, he allocated two major consecutive phases of organic matter decomposition in the soil and two large groups (associations) of microbes that differ in their functional roles in these processes. In the first phase, plant and animal residues are decomposed by zymogen microflora which enters the soil with plant and animal residues, while humus is decomposed by soil autochthonous microflora in the second phase [1]. However, he believed that the autochthonous microflora is represented by a specific group of microorganisms using humus as the source of energy nutrition. In his research, S.N. Vinogradsky does not consider the humus formation. But a priori, we can assume that it is derived in the process of plant residue decomposition by zymogen microflora.

Developing S.N. Vinogradsky's concept, N.M. Lazarev [2] considered three large functional groups of microorganisms. The first of them is the actual zymogen microflora which digests protein and monomers that enter the soil with plant residues. The second one is autochthonous microflora A involved in decomposition of a variety of plant biopolymers (with a wide range of the carbon/nitrogen ratio) the transformation of which is followed by the formation of so called α -humates that are rich in nitrogen. As they accumulate in the soil, the activity of the above group fades gradually due to the substrate exhaustion and the accumulation of humates that are the products of these microorganisms' activity and are toxic to them, that is a kind of catabolic repression. Then, a phase of α -humate decomposition by autochthonous microflora B starts. According to N.M. Lazarev, the qualitative composition of organic matter changes in the transformation process, like, accordingly, the structure of the microbial community. In modern understanding, there is a succession of microflora caused by the changes in the sources of nutrition and the physical and chemical parameters of microbial habitat. According to N.M. Lazarev, the surface of minerals, organic matter and microorganisms form a complex which he called a bioorganic mineral complex. Unfortunately, this concept has not been developed further.

Accepting S.N. Vinogradsky's concept [3] in general, E.N. Mishustin introduced two more groups of oligotrophic and chemoautotrophic microorgan-

isms into the system.

S.N. Vinogradsky's ideas were further developed by T.V. Aristovskaya [4] who, basing on the principle of «labor specialization and division» in microbial associations, proposed a concept of elementary soil and biological processes (ESBPs) that include biological transformation of plant residues, humus formation and decomposition, destruction of parent rock minerals, mineralization, hardpan formation and soil salinization, the first five ESBPs of the seven ones listed required for all types of soil formation and reflecting the essence of this phenomenon, in her opinion [4]. Gley, hardpan and bauxite formation, salinization and possibly other transformations involving microorganisms can only characterize the formation of certain specific soil types [4].

G.A. Zavarzin allocates two functional groups as the main differential characteristic of functioning soil microflora, i.e. the microorganisms capable of producing hydrolytic exoenzymes which gives them an advantage in the early stages of biopolymer decomposition and the so-called scattering microflora which utilize the monomers that are the products of biopolymer decomposition [5]. Functionally, these two groups are interconnected in the system acting successively.

Developing G.A. Zavarzin's opinions, V.S. Guzev and P.I. Ivanov [6] proposed a scheme of activity of the zymogen part of the microbial system in the soil where the process of decomposition of biopolymers entering the soil in the form of plant residues is initiated by hydrolytic microorganisms which degrade biopolymers to monomers due to the release of hydrolytic exoenzymes. Being accumulated in the soil, the latter cause the repression of exohydrolase synthesis on the feedback principle, which provides the transition of hydrolytic microorganisms into a resting state. The monomers produced are utilized by another group of copiotrophic organisms, the intensive development of which results in a sharp decrease in monomer content. As a result of the soil monomer pool depletion, copiotrophic organisms go into an inactive state, after which an active phase of oligotrophic organisms starts due to their ability to utilize extremely low amounts of monomers. At the same time, catabolic repression of hydrolase synthesis is removed, and the biopolymer degradation cycle repeats. Thus, the activity of each of the above groups of microorganisms is of pulsating nature. Apparently, the scheme of soil microbial community functioning proposed by these authors can be used for both the zymogen and autochthonous microflora in the same way, since in either case the organic matter consists mostly of biopolymers that are a source of energy and carbon nutrition for the microorganisms.

Thus, steady functioning of soil microbial systems takes place in natural ecosystems with a constant influx of plant residues, including a variety of biopolymers. Both N.M. Lazarev and T.V. Aristovskaya considered the complex chemical composition of plant residues that enter the soil. They noted that transformation of organic matter and minerals by a microbial association includes various sequential and simultaneous biochemical reactions caused by the trophic and other relations in a self-adjusting system. In our opinion, the ESBP concept reflects the essence of microbiological processes of soil formation most fully. T.V. Aristovskaya preferred to consider the taxonomic composition of the microflora involved in organic matter and mineral transformation without going into the details of biochemical substrate processing reactions. N.M. Lazarev and his school were focused on the study of physiological groups of microorganisms, suggesting that the latter may be the indicators of a particular stage in the transformation process.

All the above models suggest that microbial processes in the soil are the result of collective activities of microorganism associations. Performance of indi-

vidual species in the association and their physiological activities are substantially different from those observed in pure culture grown on artificial medium. The formation of these associations and their activities in the soil is greatly influenced by the mineralogical composition, physical and chemical properties and the structure of the soil, as well as by the status and nature of the distribution of organic matter in it.

Soil structure and distribution of microorganisms. Soil, as the microorganism product and habitat, is heterogeneous in its structure and includes particles, micro and macro aggregates which are penetrated by capillaries, have pores and voids of different sizes filled with soil solution, organo-mineral gel and gases composed mainly of nitrogen, oxygen and carbon dioxide [7]. The degree of filling may be different and varies depending on humidity, temperature, specific weather conditions, intensity of biochemical processes in the soil, etc.

Activity of microorganisms is associated with soil aggregates [8-10]. They are distributed in the form of films [11, 12] and microcolonies [13-15] on the surfaces of minerals and organic matter particles which enter the soil with plant residues, in water film on the walls of the pores and capillaries, as single (rarely numerous) cells in the soil solution that fills the pore space of soil aggregates [8]. Typically, formation of colonies and films takes years, sometimes those are formed by a number of microbial species [9, 15].

The interaction of microorganisms with the surfaces of soil aggregates is of a complex nature dependent on the mineralogical composition, the nature of the organic material, the pore space dimensions [9, 10]. Biochemical activity of microorganisms is higher in large pores and is virtually absent in small capillaries commensurate in sizes with the microorganisms [9]. According to D.G. Zvyagintsev [9], bacteria develop better in the films with a thickness over 10 μm . The activity of bacteria adsorbed (immobilized) on the surfaces of soil particles and immersed in organo-mineral gel is generally lower than in the solution [9, 15].

The sizes of the bacterial cells and the fungal mycelium thickness in the soil were noted to be lower compared to the artificial culture of microorganisms grown on nutrient media [16].

Organic matter has various composition and is unevenly distributed at the different stages of plant residue transformation. Accordingly, the topography of microorganism distribution in the soil is characterized by micro zonation and is of mosaic nature both taxonomically and functionally [2, 10, 17, 18]. Therefore, in such a complex heterogeneous environment as the soil, various processes (i.e., decomposition of plant residue organic matter, humus formation and its decomposition, and destruction and formation of minerals) take place simultaneously at different phases of transformation being spatially divided both in the soil profile and horizontally.

Environmentally, the microflora of soil macroaggregates is something like synusia consisting of various microorganism species populations formed as a result of competition for food sources in the specific physical and chemical conditions of soil aggregates. In them, the activity of microorganisms is apparently based on the type of consortium, and the relationship between the populations of individual species are based on cooperation and are manifested in different ways from symbiosis to syntrophy [19], which determines the stability of the system under particular conditions. Changing these conditions leads to competition, restructuring of the association in accordance with the change of physical and chemical soil parameters. We must assume that the taxonomic and functional diversity of microorganisms is subject to significant changes as a result of temporal and spatial variability of physical and chemical properties of soil aggregates.

Among the external factors responsible for these changes, the leading role is played by precipitation, periodic soil drying and the temperature. In waterlogged soil, the pore space of soil aggregates is filled with water, oxygen content is reduced, anaerobic processes develop, and a reorganization of the microbial soil complex takes place [20]. There reduction processes develop that contribute to the formation of ferrous and manganese oxides, hydrogen sulfide, methane, organic acids and toxic compounds [4, 7, 20]. Conversely, soil drying results in aggregate shrinkage, pore space narrowing [7], involving microorganisms in the dehydrated organo-mineral gel matrix, increasing the osmotic pressure of the soil solution concentrated primarily in the capillaries and films [9]. This reduces the biochemical activity of microorganisms and is the cause of their partial death and the restructuring of the microbocenosis as a whole.

The main internal factor of the change in taxonomic and functional diversity of the microflora of soil aggregates is the very activity of microorganisms which causes qualitative changes of organic matter and results in the accumulation of metabolic products leading to the restructuring of the microbial complex [2, 9]. Accordingly, its taxonomic composition and physiological diversity change.

Thus, the soil microflora is a self-adjusting system that is formed at the level of soil aggregates and microzones, including mainly plant residues, humus complexes and mineral surfaces. Taxonomic and functional diversity of soil microflora is highly variable and depend on the freaks of nature, the internal regularities of decomposition processes, the synthesis of organic matter, and the physical and chemical properties of the soil.

Physiological groups of soil microflora. Almost all studies of taxonomic and functional diversity of soil microorganisms are based on the analysis of average soil samples and give us some integrated idea of the qualitative composition of microflora for the sufficiently large soil massif. One should clearly understand that these data reflect the dominant processes and dominant microflora at the date of sampling under certain weather conditions and a certain state of vegetation. The studies of soil samples from the same field areas would provide different results under different conditions which can erase the previously made conclusions.

The study of the functional and taxonomic diversity of soil microflora has been performed since the beginning of the XX century quite intensively. Significant progress has been made after the onset of solid culture media proposed by Robert Koch and especially after the introduction of agar-agar in the practice of research [21]. A great amount of bacteria, actinomycetes, filamentous fungi, yeasts, microscopic algae, and protists have been isolated from the soil; these make up the basis of microorganisms represented in the collections of various scientific centers worldwide. An inestimable role in the study of soil microorganisms is played by elective nutrient media and enrichment cultures, the principles for the use of which have been proposed by S.N. Vinogradsky [1] and M. Beijerinck, [21], respectively. A rapid growth in the studies using molecular genetic techniques has been observed in the two last decades. As a result, the number of microorganism species found in the soil has increased more than 10-fold. A significant part of these relates to the so-called uncultivated forms [22].

The use of elective media made it possible to isolate various physiological groups of microorganisms involved in the small biological cycle of substances which reflects the general regularities of these processes in the soil. Physiological groups are the sets of microorganisms that perform the same function in the chain of substance transformation in the soil [23]. However, it is well known that microorganisms are multifunctional systems. Therefore, under the changing

conditions, they can be rearranged, perform another (sometimes opposite) function or be assigned to another physiological group. For example, at deficiency of mineral nitrogen compounds, the so-called denitrifying bacteria are capable of fixing molecular nitrogen [24], and in the presence of protein and amino acids in the medium, they perform the functions of ammonifying [25].

Hence, it is clear that the taxonomic composition of the microorganisms that represent a particular physiological group is unstable and varies depending on the specific conditions in the soil. Therefore, the physiological groups identified for certain elective media do not necessarily exercise the same processes in the soil.

According to D.G. Zvyagintsev [9], the stability of certain physiological and biochemical functions of microorganism associations in the changing physical and chemical parameters of the soil is due to the presence of several overlapping microorganism species, on the one hand, and to their adaptation to the new conditions, on the other hand.

From the perspective of soil science and practical agriculture, physiological groups of microorganisms capable of being functional indicators of biochemical processes in the soil that are important for its fertility and, accordingly, crop productivity, were of interest. The most popular of them and the ones widely used as the objects of research in soil microbiology are summarized in the Table.

Main physiological groups of soil microorganisms and their roles in soil biochemical processes

Physiological group	Biochemical process
Ammonifying bacteria	Decomposition of organic nitrogen compounds to ammonia
Nitrifying bacteria	Oxidation of ammonium nitrogen to nitrites and nitrates
Denitrifying bacteria	Reduction of nitrates and nitrites to nitrogen dioxide and molecular nitrogen
Nitrogen fixing bacteria	Fixation of molecular nitrogen from the atmosphere
Cellulose decomposing microorganisms	Decomposition of cellulose and hemicellulose
Pectin decomposing microorganisms	Decomposition of pectin
Amylolytic microorganisms	Starch hydrolysis
Humus decomposing microorganism	Depolymerization and mineralization of humic acids
Sulfur bacteria	Oxidation of reduced sulfur compounds to sulfuric acid
Sulfate reducing bacteria	Recovery of sulfates to sulfur and hydrogen sulfide
Iron bacteria	Oxidation of iron and manganese oxides
Phosphate solvent microorganisms	Solubilization of poorly soluble calcium, iron, and aluminum phosphates
Microorganisms capable of decomposing organic phosphorus compounds	Mineralization of phosphorus containing organic compounds

These physiological groups often used in microbiological studies include the microorganisms involved in the processes of nitrogen cycle such as ammonifying, nitrifying, denitrifying, and nitrogen fixing bacteria. The key role in carbon cycle is played by a physiological group of cellulose decomposing microorganisms, since cellulose and hemicellulose make up to 70 % of carbon entering the soil with plant residues.

To account for the number and allocate the corresponding physiological group of microorganisms special elective nutrient media are used; these media are described in the respective manuals, workshops and original papers [26-28].

The main results of nearly a century of studies of physiological groups of microorganisms have identified a significant part of organotrophic and chemo-autotrophic prokaryotes involved in carbon, nitrogen, phosphorus, sulfur, iron, manganese, and other cycles [4, 23]. Various soils have been shown to have their own profiles of the abundance and composition of physiological groups of microorganisms, the boundaries of which are however blurred and can not be used to classify the soil type. The qualitative composition and the abundance of physiological groups of microorganisms of the same soil were found to depend

on the season, vegetation and weather conditions, and vary for the soil profiles. The impact of agricultural activities on the abundance of different physiological groups of microorganisms depends on the soil type, the composition of agrochemicals, applied agricultural technologies, and tillage intensity [29-32].

Previously, the presence of a particular physiological group of microorganisms and their abundance were suggested, with some reservations, to reflect the intensity of the respective processes of substance conversion and the soil fertility [33]. Indeed, this regularity can be seen in general. However, numerous experiments have demonstrated high variability of the results of these observations. The results of analyses are interpreted differently. There are no clear criteria for soil quality assessment based on the analysis of physiological groups of microorganisms. All these led to the lack of reliability of such data as the indicators of soil fertility and land treatment efficiency. Therefore, these parameters have not been used practically in farming [21, 33].

The main disadvantage of the method lies in the fact that the activity of microorganisms, cultured on artificial culture media does not correspond to their behavior in a complex physical and chemical environment of the soil when interacting with other organisms [9]. In other words, considering the abundance of a particular physiological group of microorganisms, we can discuss some of the potential microflora possibilities in the best case, which do not necessarily appear in the soil at the time of sampling.

The risk is that that further studies of this kind result in the accumulation of contradictory and poor (or even falsely) interpreted data with our limited knowledge about the physiology of microbial associations under specific soil conditions (unlike the physiology of individual species and strains of microorganisms cultivated on nutrient media). The way out is to follow the principle proposed by S.N. Vinogradsky, that is to study microflora and microorganism activity in the natural environment, i.e. in the soil. According to S.N. Vinogradsky, «pure cultures acquainted the researchers with general microbial physiology, but a study like this can only lead to analogies and hypotheses to be verified by direct experiments under the conditions as close to natural as possible» [1]. Elective culture media and identification of physiological groups of microorganisms remain an important tool for detecting microorganisms with specific physiological features for biotechnological application.

Carbon source utilization profiles. In 1991, J.L. Garland and A.L. Mills [34] proposed the BIOLOG system which is widely used in medical microbiology and general microbiology for the identification of bacteria, to assess the functional diversity and developed the general approaches to the interpretation of carbon source utilization profile (SUP) using multivariate statistics [35].

The method uses standard 96-well plates to determine SUPs. Low molecular weight carbonaceous substrates and tetrazolium salts are added in each well as the indicators of source utilization. With the growth of microorganisms in the wells filled with a nutrient solution, colorless tetrazolium salts are reduced to formazan which colors the solution burgundy. Substrate utilization rates are assessed by the intensity of medium color in the wells using an optical reader. Application of the BIOLOG system provided extensive data, 80 % of which relate to soil sample SUP analysis [36]. The impact of mechanical processing, crop residues [37], and heavy metals on soil SUPs have been noted [38]. The efficacy of this method for the characterization of potential carbon source utilization by microbial communities in composts [39], industrial wastewater [40] and other natural environments, and in plant rhizosphere [37, 41-43] has been demonstrated.

Modified methods with more rationally selected sources of carbon nutri-

tion corresponding potentially to those of microorganisms from various natural environments were proposed. In an analytical review, J. Preston-Mafham et al. [36] noted a number of modifications which differ mainly in the set of test substrates. H. Insam [44] proposed the plates which contain 31 substrates to study physiological diversity of a microorganism association (SUP) in the soil and the plant rhizosphere. On this basis, BIOLOG, Inc. (USA) launched the EcoPlatesTM plates [45] with a standard set of carbohydrates, amides, amino acids, carboxylic acids, nucleosides and some polymers.

A total of 47 substrates are used in the ECOLOG multisubstrate testing system developed at the Department of Soil Biology of the M.V. Lomonosov Moscow State University [46]. We believe that their composition in the ECOLOG system is mostly reasonable and acceptable to the non-formalized interpretation of the results from an ecological point of view. It contains no exotic chemical compounds. In both cases, the modifications of substrate sets to study the physiological diversity of various natural environments are allowed. It should be noted that the ECOLOG system makes it possible not only to determine carbon source utilization profiles, but also to quantify the utilization individually. Thus, it compares favorably to other BIOLOG modifications. A multidimensional mathematical statistics apparatus, neural network algorithms, the ecological diversity Shannon index, and rank distribution are proposed for processing and interpretation of a considerable amount of information obtained in the analysis of soil samples [46]. The method for multisubstrate testing (MST), or substrate utilization profile (SUP) testing, turned out to be a good tool for a comparative description of the physiological diversity of soil microbial communities and plant rhizosphere, as well as for the assessing the impact of natural and anthropogenic factors on the association of soil microorganisms [46-50].

A number of disadvantages should be noted which, according to some authors [36, 46, 51], limit the scope of SUP based methods and appears to require further development. Thus, the conditions in the wells on artificial liquid culture medium do not meet the conditions of bacterial growth and physiological and biochemical functions in situ [36, 51], that is why the contributions of individual physiological groups in SUPs does not necessarily reflect their relative proportions in the total source utilization profile typical for populations in the soil [52].

Low molecular weight substrates and short-term (2-5 days) incubation of the plates inoculated with soil suspension reflects the growth of R-strategists in an individual well in the given experimental conditions, and not the fact that the activities of these bacteria in the soil is the same under different physical and chemical parameters and in association with other microorganisms. In addition, it is necessary to consider that not a single bacterial species but a number of them may enter the wells with nutrient solution, resulting in a possible manifestation of their synergism or antagonism which would undoubtedly have an impact on the intensity of tetrazolium salt transformation to formazan. One should consider that not all microorganisms are capable of reducing tetrazolium salts [36]. At the same time, K-strategists that provide biopolymer transformation (cellulose, hemicellulose, pectin, lignin, and humic complexes) drop out of sight. There are technical questions for the sample preparation and analysis protocol with regard to timing and methods for soil sampling, methods of preparing soil samples for analysis, nutrient medium composition, buffer solution pH, the temperature and time of plate incubation with the wells inoculated with soil suspension or supernatant, profiles of the substrates used in an experiment, etc., which is to be unified in the case of monitoring studies.

To sum up, we would like to note that the study of taxonomic and func-

tional diversity of soil microbial association is of extremely great theoretical significance for the understanding of the soil microbial community structure, the nature of its constituent species interactions, their involvement in soil formation, soil fertility formation and the circulation of elements. This explains the role of this approach in ecological research and farming practice, especially in the development of effective methods of maintaining and increasing soil fertility.

Analysis of data on the variety of physiological groups of microorganisms in the soil obtained by microbiologists within nearly a hundred years, has shown that the methods of limiting dilution and elective nutrient media used to identify and determine the number of bacteria of different physiological groups are low informative to characterize their physiological diversity in soil. No more than 5-6 of physiological groups of microorganisms were considered in published studies in accordance with the objectives of the studies [30]. The nitrogen cycle (ammonifying, nitrifying, denitrifying, nitrogen-fixing bacteria) and cellulose decomposing bacteria which were considered as the indicators of the relevant processes in the soil were the most common objects of observations. Thus obtained information is used for the analysis of fertility and efficiency of land treatment. However, these methods have not been adequately appreciated in practical farming [21]. Nevertheless, the fact is that the high titers of nitrifying and cellulose decomposing bacteria are typical for active soil organic matter mineralization processes and, consequently, for the favorable regime of plant mineral nutrition. Apparently, the methods for the analysis of physiological groups of microorganisms on elective nutrient media have been exhausted for a comprehensive environmental assessment of the biodiversity of soil microbial associations, however, remaining a good tool to study the role of microorganisms in soil formation and plant nutrition, as well as identification of new physiological groups of microorganisms.

A breakthrough in the research of physiological diversity of soil microorganism associations is related to the methods based on carbon source utilization profiles (SUPs) analysis used with various modifications of BIOLOG [36, 44, 45] and multisubstrate test ECOLOG systems [46] with the appropriate software. J. Preston-Mafham et al. have described the above advantages and disadvantages of these systems critically and in detail [36]. Currently, over 1500 papers have been published which demonstrate the BIOLOG system capabilities to analyze the physiological diversity of the soil, the plant rhizosphere and other natural substrates [46].

The ecological meaning of these data is clear: any (taxonomic or functional) diversity provides a comparative description of the studied ecosystems, so SUP parameters can be used as the indicators of their relevant changes. However, the biological sense is far from being understood, primarily due to the fact that the *in vitro* conditions of bacterial growth and metabolism are different from those *in situ* (in plant rhizosphere or in the soil). As a result, we estimate some physiological potential of soil microorganisms present, but not its implementation in the natural conditions. Therefore, the SUP methods in their present form are, apparently, sufficiently useful and informative as indicators of human impact on the microflora and of physiological diversity in plant rhizosphere microflora. However, according to J. Preston-Mafham et al. [36], they are not applicable for environmental monitoring due to the use of several modifications differing considerably in the set of substrates, and to the lack of a unified analysis and result assessment protocol, which makes them almost disparate. In particular, fresh [53], dried [46], and frozen [54] soil is sampled; according to some protocols, soil suspension is prepared at mechanical shaking [53]; according to other protocols, it is pre-sonicated, centrifuged, and the supernatant is used for

culturing [46]. According to some authors, there are much less species in the liquid fraction compared to the sediment or soil suspension [36]. In frozen soil, the number of bacteria, fungi and actinomycetes reduces considerably, and their ratio changes [55].

Thus, traditional study methods for the functional state of soil microflora revealed the forms involved in the key processes of carbon, nitrogen, phosphorus and other element cycles. Some physiological groups are still used as the indicators of land treatment efficiency and soil fertility, but this area is almost not developing in the latest 20 years. Carbon source utilization profile (SUP) analysis (modified BIOLOG, EcoPlates, and ECOLOG systems) is currently applied to study a more or less limited number of low molecular weight organic compounds and reveals the physiological groups of microorganisms capable of being involved in their catabolism. In other words, SUP analysis reflects to some extent the potential of physiological diversity of organo-heterotrophic aerobic and facultative aerobic soil bacteria that utilize basic carbon chemical compounds. SUPs are applied most efficiently to the study the effects of natural and anthropogenic factors on soil microflora. We believe that multisubstrate testing has good prospects for the study of rhizosphere, especially when combined with the analysis of root exudates, which is a key to understand the mechanism of the formation of rhizosphere bacterial association and its interaction with the plant.

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SSR-genotyping to control the origin

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GENETIC SIMILARITY OF THE AUTOCHTHONOUS GRAPEVINE VARIETIES FROM DON REGION REVEALED BY SSR-ANALYSIS AND MAIN LEAF AMPELOGRAPHIC TRAITS

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Abstract

Native, ancient grape varieties of different cultivation regions are important part of grapevine genetic resources. Many native Don grape varieties represent a significant value for cultivation and use in breeding. The close varieties and more distant groups are distinguished on the main characteristics among the varieties of Don. The main features of the leaves of grape varieties are the key ampelographical characteristics. Currently, the study at the DNA level is considered the most informative method of plant genotyping analysis. Microsatellite markers are widely used for genotyping of grapevine varieties and rootstocks, and successfully applied in the study of the origin of varieties and the analysis of their pedigrees. We evaluated the relationship among the number of Don varieties by microsatellite genotyping. The aim was to study the genetic similarity of native Don varieties based on DNA analysis and compare the results with earlier made conclusions about relationship of varieties, and with data of the analysis of the main features of the leaves. The research was carried out on 16 varieties from the collection of the All-Russian Scientific Research Institute of Viticulture and Winemaking (Novocheboksarsk) and the Russian ampelographic collection (Anapa). Studied Don grapevine varieties were described ampelographically, and the main method we used in the work was the PCR. Six SSR-markers basically recommended for *V. vinifera* fingerprinting were used. DNA was extracted from young leaves of the apical shoots of 4–5 typical bushes. Chardonnay and Cabernet Sauvignon were used as the reference cultivars. Genetic distance matrix was constructed using the coefficients (indices) similarity of M. Nei and W. Li. Based on the data of SSR-genotyping, estimation of the genetic similarity of studied varieties was performed using cluster analysis (UPGMA), and dendrograms were graphically constructed. Data on the morphological characteristics of leaves and SSR-genotyping results were analyzed by means of principal coordinates (PCA). DNA profiles of 16 local Don grapevine varieties were obtained using microsatellite loci VVMD5, VVMD7, VVMD27, VVS2, VrZAG62 and VrZAG79 with an automated genetic analyzer ABI Prism3130 («Applied Biosystems», USA). In the studied Don varieties genotypes, six (for VVS2, VVMD5, VMD7, VrZAG62) and seven (for VVMD27, VrZAG79) alleles per locus were determined. Cluster analysis allowed to divide the varieties into two main groups: one included Sibir'kovyi, Puhlyakovskii belyi, Sivolistnyi, Puhlyakovskii chernyi, Kosorotovskii and Kukanovskii cultivar, being a group of natural seedlings of Puhlyakovskii belyi, the other contained Bezmyannyi Donskoi, Plechistik oboepolyi, Staryi Goryun, Tsimlyanskii belyi, Tsimlyanskii chernyi, Tsimladar, Plechistik, Sypun chernyi, Mahrovatchik and Bessergenevskii № 7 cultivars. Interestingly, the second cluster had three subgroups. One includes varieties Bezmyannyi Donskoi, Plechistik oboepolyi, Tsimlyanskii belyi, Tsimlyanskii chernyi, Tsimladar, Plechistik, Sypun chernyi of the Tsimlyanskii group. The other contained Bessergenevskii № 7 cultivar being presumably a seedling of Puhlyakovskii belyi, and Staryi Goryun of the Tsimlyanskii group. Variety Mahrovatchik (considered to be a seedling of Kokur white variety) was grouped separately. Analysis of the main features of leaves showed no differentiation according to the presumed origin of the studied varieties. As the result of SSR-analysis, most of varieties were distributed in accordance with the earlier made conclusions about their origin. Thus, the study of collections, old varieties, breeding material and introduced samples based on the complex of ampelographic traits

and SSR-markers can be considered as the most informative one.

Keywords: native gene pool, SSR-markers, ampelographic leaf traits, *Vitis vinifera* L., Don grape varieties, the genetic similarity.

Native, ancient grape varieties from different vine cultivation regions, as well as wild forms are the most important part of the world genetic resources of a cultivated plant *Vitis vinifera* L. It is autochthonous varieties, many of which do not even have a local distribution, that may be irretrievably lost. Their genotypes contain rare alleles, and are characterized by unique adaptive properties to specific areas of viticulture. For this reason, investigation of the indigenous gene pool receives special attention in all grape-producing countries [1-6].

The history of viticulture in the Don region extends back to several centuries. Local grape cultivars are diverse and specific. Many of them are of significant value both for cultivation in favourable conditions on the right bank of the Don and for breeding [7]. There is no consensus about the origin of the Don varieties of grapes, but typical local names and common traits indicate their age antiquity. Emergence of groups of varieties, close by the major morphological features, in different wine-growing districts also indicates the regional origin of most of the local Don indigenous varieties.

Based on the similarity of signs (leaves and berry form), it has been established that Kosorotovskii, Sibir'kovyi, Pukhlyakovskii Chernyi, Olkhovskii, Sivolistnyi, and Bessergenevsky № 7 cultivars are natural seedlings of the Pukhlyakovsky Belyi cultivar [7]. According to A.I. Potapenko, the Pukhlyakovsky Belyi cultivar appeared on the Don at the beginning of the XIX century [8]. A.M. Aliev, in contrast, argues that a large group of grape varieties might not emerge and spread over such a short period of time [9]. According to him, the Pukhlyakovsky Belyi cultivar had been known much earlier on the Don, and belongs to the indigenous Don varieties. A group of varieties similar to the Pukhlyakovsky Belyi cultivar is not the only one. There is a much more numerous group of so-called Tsimlyanskii cultivars with similar morphological features.

When determining the origin of grape varieties of local inhabitant selection, methods for their identification are no less important. Studies have shown the effectiveness of using harmonized descriptions of grape varieties developed by the International Organization of Vine and Wine (Office International de la vigne et du vin, OIV, Paris, France) [10]. This system facilitates the evaluation of trait similarity, and thereby assisting in confirming or denying the alleged origin of the variety. The most valuable are the main features of the leaves, the formation of which is almost unresponsive to the artificial selection [7]. Depending on the shoot length and growth conditions, the size and shape of leaves vary within the same variety, but these features remain reliable ampelographic parameters.

Studies at the DNA level are considered the most informative method of plant genotyping analysis. DNA profiles complement the conventional description and agrobiological characteristics of varieties, allowing an accurate determination of the varieties, investigation of their origin, identification of synonyms and impurities in the collections. The works on the investigation of genetic diversity and identification of varieties most commonly use marker systems based on the variability of microsatellite DNA regions.

Microsatellites (simple sequence repeats, SSR) are tandem repeats of simple sequences in the DNA structure. The source of their polymorphism is a site-specific variation of the repeat length due to the difference in the number of its units [11]. Microsatellite sequences are ubiquitous in the genome of higher plants. SSR-markers are characterized by the co-dominant pattern of inheritance, high differentiation capacity and reproducibility of results. Microsatellite

markers are widely used for genotyping of grapevine varieties and rootstocks [12-16], and successfully applied in the study of the origin of varieties and the analysis of their pedigrees [17-22]. Based on the fingerprinting of grapevine varieties carried out in different laboratories, it has been established the basic standard set of SSR-markers for genotyping *Vitis vinifera* [23].

In this paper, for the first time, we evaluated the relationship of autochthonous grapevine varieties in the Don region, mainly from groups of Tsimlyanskii varieties and seedlings of the Pukhlyakovskii Belyi cultivar, based on DNA analysis.

The aim was to study the genetic similarity of the Don varieties based on the analysis of microsatellite locus polymorphism, and compare the obtained results with findings from other authors about the origin of varieties, as well as with the analysis of the main traits of a fully developed leaf.

Technique. The investigations were carried out on a set of the autochthonous Don grapevine varieties, such as Bezymyannii Donskoi, Bessergenevskii № 7, Kosorotovskii, Kukanovskii, Makhrovatchik, Plechistik, Plechistik oboepolyi, Pukhlyakovskii belyi, Pukhlyakovskii chernyi, Sibir'kovyi, Sivolistnyi, Staryi Goryun, Sypun chernyi, Tsimladar, Tsimlyanskii belyi, and Tsimlyanskii chernyi, growing in the collection of the Ya.I. Potapenko All-Russian Research Institute of Viticulture and Winemaking (Novocherkassk) and the Russian Ampelographic Collection (Anapa). Chardonnay and Cabernet Sauvignon were used as the reference cultivars as their allelic composition for the studied SSR-loci was known [23]. All grapevine varieties were described based on the main ampelographic traits of a fully developed leaf, according to the methodology of testing for distinctness, uniformity and stability, proposed for grapes [9].

The main method we used was the polymerase chain reaction (PCR). Separation of PCR products was performed by electrophoresis methods, the 2 % agarose gel electrophoresis when customizing PCR parameters, and capillary electrophoresis, using an automated genetic analyzer ABI Prism 3130 (Applied Biosystems, USA) when performing the SSR-fingerprinting.

The study used SSR-markers, such as VVMD5, VVMD7, VVMD27, VVS2, VrZAG62 and VrZAG79, recommended for molecular genetic genotype certification of *Vitis vinifera* by the European Database and the GrapeGen06 project [23]. DNA was extracted from young leaves of the apical shoots of 4-5 typical bushes of the cultivar by a CTAB (cetyl trimethylammonium bromide) method [24]. The PCR mixture proportion was designed according to a basic protocol, the reaction was carried out using standard parameters, with experimentally found temperatures for annealing primer pairs [25]. Sizes of amplified fragments were determined on an automated genetic analyzer ABI Prism 3130. Results were processed in the Gene Mapper 4.1 software.

The matrix of genetic distances was constructed using similarity coefficients (indices) by M. Nei and W. Li [26]. Cluster analysis was performed by means of the unweighted pair group method with arithmetic mean (UPGMA) using the FreeTreeApplication 0.9.1.50 (ZDATv.o.s.) software. Graphical representation of dendrograms was carried out using the TreeView (Win32) 1.6.6 software.

Data on the morphological characteristics of leaves and SSR-genotyping results were analyzed by PCA method. Calculations were performed in the PAST v. 2.17c software.

Results. The studied sample set was represented mainly with grapevine cultivars belonging to two groups, which have been previously identified ampelographically, i.e. the group of Tsimlyanskii cultivars and the group of natural seedlings of the Pukhlyakovskii Belyi cultivar.

To reduce the cost of analyzes, sets for multiplex analysis were formed

after testing SSR-markers and selecting optimal PCR parameters. SSR-markers were pooled considering the size range of the amplified fragments for each locus and temperatures of annealing primer pairs, using two of the four fluorescent dyes (FAM, R6g, Rox, or Tamra) in one set. Genotyping was performed by the following marker pairs: VVS2 + VVMD7; VVMD27 + VVMD5; VrZAG62 + VrZAG79.

The results of the analysis of microsatellite locus polymorphism demonstrated that each variety had a DNA profile that was different from all other specimens. Considering the studied SSR loci in the set of the Don grapevine varieties, there were six (for VVS2, VVMD5, VMD7, VrZAG62 loci) and seven (for VVMD27, VrZAG79 loci) alleles per locus determined.

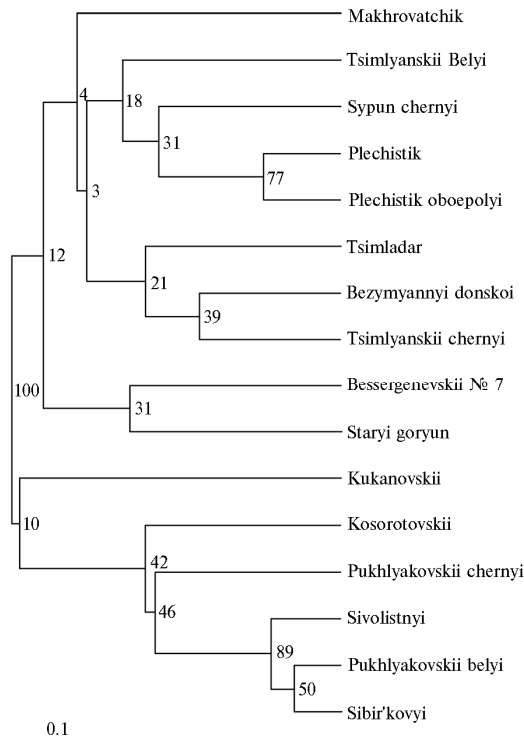


Fig. 1. Dendrogram of genetic similarity between the studied Don varieties of grapes (*Vitis vinifera* L.) based on SSR-analysis (the collection of the Ya.I. Potapenko All-Russian Research Institute of Viticulture and Wine-making, the Russian Ampelographic Collection).

Cluster analysis allowed dividing the cultivars into two main groups based on the fingerprinting by microsatellite loci (Fig. 1). One large group included Sibir'kovyi, Pukhlyakovskii belyi, Sivolistnyi, Pukhlyakovskii chernyi, Kosorotovskii and Kukanovskii cultivars. All of them revealed the genetic similarity, which confirmed the hypothesis of their origin from the Pukhlyakovskii belyi cultivar, with the Sibir'kovyi, Pukhlyakovskii belyi and Sivolistnyi cultivars appeared to be the most similar, based on the results of microsatellite analysis.

The second group contained the cultivars Bezmyannyi Donskoi, Plechistik oboepolyi, Staryi Goryun, Tsimlyanskii belyi, Tsimlyanskii chernyi, Tsimladar, Plechistik, Syapun chernyi, Mahrovatchik, and Bessergenevskii № 7. Interestingly, the second cluster had three subgroups. Genetic similarity can be stated for the cultivars of the so-called subgroup of Tsimlyanskii varieties,

such as Bezmyannyi Donskoi, Plechistik oboepolyi, Tsimlyanskii belyi, Tsimlyanskii chernyi, Tsimladar, Plechistik and Syapun chernyi. The Plechistik and Plechistik oboepolyi cultivars appeared to be genetically the most similar. The Makhrovatchik cultivar showed similarity with the group of Tsimlyanskii varieties. The Bessergenevskii № 7 and Staryi Goryun cultivars appeared to be closer to each other than to other allegedly related cultivars, however, both of them were closer to the Tsimlyanskii group. Considering that based on ampelographic characteristics the Bessergenevskii № 7 cultivar being presumably a seedling of the Pukhlyakovskii Belyi cultivar, it may be assumed that his other parent was a variety from the Tsimlyanskii group or a variety similar to them.

More accurate conclusions can be made with the increased number of SSR-loci to be analyzed. However, even the set of microsatellite markers used in this study yielded results comparable with the findings of other researchers.

The major ampelographic leaf traits in the autochthonous Don grapevine varieties (*Vitis vinifera* L.) (the collection of the Ya.I. Potapenko All-Russian Research Institute of Viticulture and Winemaking, the Russian Ampelographic Collection)

Cultivar	Trait code											
	080	077-1	077-2	078-1	078-2	068	067	065	082	079	084	093
Natural seedlings of the Pukhlyakovskii Belyi cultivar												
Pukhlyakovskii belyi	3	5	3	5	3	3	4	5	2, 3	5	5	3, 2
Kosorotovskii	5	3	3	5	5	3	3	5, 7	2	5, 8	7	4
Sivolistnyi	3	5	5	5	5	3	3, 4	5, 7	2, 3	5, 6	5	2
Pukhlyakovskii chernyi	3	3	3	5	3	3	4	5, 7	2,3	5, 8	3	2
Bessergenevskii № 7	3	3	3	5	5	2, 3	4	7	1	6	5	3
Kukanovskii	5	5	3	5	3	3	4	5	2, 3	3, 5	3, 5	2
Sibir'kovyi	7	5	5	5	5	3	4	5, 7	2	5	5	2, 3
The group of Tsimlyanskii varieties												
Bezmyannyi Donskoi	3	5	3	5	5	3	4	5	2, 3	5, 8	7	2
Plechistik oboepolyi	3	3	3	7	7	3	4	5, 7	2	5, 6	7	2
Staryi Goryun	3	7	3	7	5	3	3	7	2	5	7	2
Tsimlyanskii belyi	3	5	3	7	5	3	4	5, 7	2	5, 8	3, 5	3, 4
Tsimlyanskii chernyi	3	3	3	5	5	3	4	7	2	5	7	4
Tsimladar	7	7	5	5	5	3	4	5	3, 4	8	5, 7	2
Plechistik	7, 9	5	5	5	5	3	4	7	2	5	7	2, 3
Sypun chernyi	7, 9	5	3	5	5	3	4	5, 7	2	5	5	3
Seedling of the Kokur White cultivar												
Makhrovatchik	9	7	5	7	7	3	4	5, 7	2, 4	5, 6	7	2

N o t e. 080 — depth of the upper lateral sinuses, 077-1 — length of an apical serration, 077-2 — length of a lateral serration, 078-1 — the apical serration length to width ratio, 078-2 — the lateral serration length to width ratio, 068 — the number of laminae, 067 — the form of the blade, 065 — the size of the blade, 082 — arrangement of the laminae of upper lateral sinuses, 079 — arrangement of the laminae of the petiolar sinus, 084 — downiness between the primary veins on the underside of the blade, 093 — the petiole length relative to the mid rib length. The figures in the table indicate the expression of a trait [10].

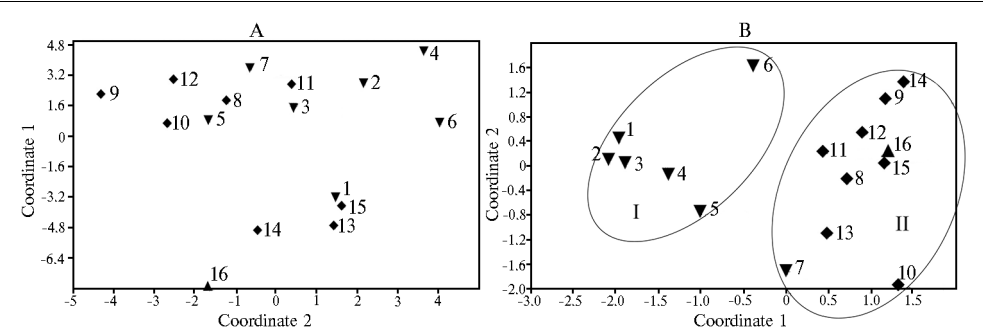


Fig. 2. PCA distribution of the studied Don varieties of grapevine (*Vitis vinifera* L.) based on the evaluation of the leaf blade traits (A) and SSR-analysis (B): 1 — Sibir'kovyi, 2 — Pukhlyakovskii belyi, 3 — Sivolistnyi, 4 — Pukhlyakovskii chernyi, 5 — Kosorotovskii, 6 — Kukanovskii, 7 — Bessergenevskii № 7 (natural seedlings of the Pukhlyakovskii belyi cultivar); 8 — Bezmyannyi Donskoi, 9 — Plechistik oboepolyi, 10 — Staryi Goryun, 11 — Tsimlyanskii belyi, 12 — Tsimlyanskii chernyi, 13 — Tsimladar, 14 — Plechistik, 15 — Sypun chernyi (the group of Tsimlyanskii varieties); 16 — Makhrovatchik (seedling of the Kokur belyi cultivar); I and II — clusters obtained by UPGMA (the collection of the Ya.I. Potapenko All-Russian Research Institute of Viticulture and Winemaking, the Russian Ampelographic Collection).

The major ampelographic traits of a fully developed leaf for each cultivar were described by the index system in accordance with the methodology adopted by the International Organization of Vine and Wine (Table). The analysis of the obtained data by main features of leaves was performed by means of the PCA method. We have found no common factors consistent with the proposed origin of varieties or the results of SSR analysis, i.e. area of distribution of varieties

from a group of Pukhlyakovskii belyi cultivar seedlings significantly overlaps the area of distribution of the Tsimlyanskii group (Fig. 2, A).

Meanwhile, the results of SSR analysis demonstrated that most of varieties were distributed in the space of the principal coordinates in accordance with the earlier conclusions of their origin (see Fig. 2, B). The isolated areas I and II corresponded to clusters obtained by the UPGMA clustering.

Thus, the main features of a fully developed leaf are insufficient to evaluate the relationship of certain genotypes of grapevine. The use of microsatellite markers for these purposes is more efficient. Quite promptly, DNA markers allow drawing conclusions about the genetic similarity of the samples to confirm or rule out the information on the origin. Thus, the study of collections, old varieties, breeding material and introduced samples based on the complex of ampelographic traits and SSR-markers can be considered as the most informative one.

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Landraces and scientifically-bred varieties — factors of adaptation

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COMPARATIVE CHARACTERISTICS OF ROOT SYSTEMS AND ROOT EXUDATION OF SYNTHETIC, LANDRACE AND MODERN WHEAT VARIETIES

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Abstract

Finding ways to increase the wheat adaptation to drought is now considered as a major problem in breeding new varieties of this crop. This study was conducted to evaluate genotypic differences in fundamental root traits which may have effect on wheat adaptation to unfavorable environments, including drought. Three wheat genotypes representing various evolution levels of hexaploid bread wheat (*Triticum aestivum* L.) were used: synthetic wheat developed by crossing variety LEUCURUM 84693 of durum wheat (*Triticum durum* Desf., genome AB, Ukraine) with wild Tausch's goat grass (*Aegilops tauschii* Coss., genome D, Turkey), landrace of bread wheat Albostan (genome ABD, Turkey, province Nevşehir), and a modern cultivar Karahan (genome ABD, Turkey). The varieties differed in root diameter, shoot biomass and shoot/root ratio. The removal of above ground biomass caused increase in the root length, number of tips, number of forks, number of crosses and shoot/root biomass ratio of synthetic wheat and Albostan, but decreased these parameters in Karahan. Averaged values of ten measured growth parameters of the plants with removed above ground biomass relatively to the control plants were +28 % for synthetic wheat, 0 % for landrace Albostan and –37 % for cultivar Karahan. These results showed a higher ability of synthetic wheat but lower ability of Karahan to recover from cutting stress and to revegetate. For the first time a comparative analysis of root exudation (amino acids, sugars and organic acids) by wheat genotypes having different levels of evolution was performed. It was shown that Karahan was characterized by high exudation of tryptophan (0.05 mg/g dry roots per day), histidine (0.12 mg/g dry roots per day) and phenylalanine (0.45 mg/g dry roots per day). Total amount of sugars (mostly fructose, glucose and maltose) exuded by Karahan was 55 mg/g dry roots per day, that was 5 and 3 times higher as compared to synthetic genotype and Albostan. The quantum of organic acids in exudates of all studied genotypes was approximately similar and amounted to about 1.8 mg/g of dry roots per day. The results suggested low ability of Karahan to control the flow rate of carbohydrates from roots to the environment. We propose that high root exudation of tryptophane (precursor in biosynthesis of auxins) and sugars may result in deficit of these compounds and involved in impaired shoot regeneration of Karahan. The results demonstrated differences in the functioning of the root system of primitive hexaploid wheat (synthetic) compared to landrace and modern cultivated variety. The nature of these differences requires more investigation.

Keywords: wheat, root exudation, revegetation, drought stress adaptation.

Wheat (*Triticum* spp.) is one of the main crops providing millions of people with food products. It is estimated (<http://www.wheatinitiative.org>) that by 2050 the production of wheat grains must be increased from the current 650–700 tons to 1 billion tons. However, the possibilities of mobilizing the genetic resources of this crop have been considerably exhausted since the Green Revolution. This, in combination with adverse climate changes, can partially explain

the fact that the varieties used have practically reached their biological yield limit. The recent studies on winter wheat in Europe, Central and Eastern Asia and the USA revealed increased average air temperature in periods critical for plant development [1]. Though warmer winters generally led to a higher yield of this crop, the increased temperature in late spring or in summer reduced it because of drought. Now, the adaptation to drought is considered an important task in wheat breeding which gave rise to the International Winter Wheat Improvement Program (IWWIP, <http://www.iwwip.org/>).

Drought resistance in wheat may be evaluated by the crop yield and biochemical composition, and by the traits contributing to high yield at a lack of moisture, for example by the structure of the root system responsible for the absorption and transportation of water to the shoots [2]. It is demonstrated that the efficiency of moisture absorption from arid soils is generally determined by the contact area of the root system which in its turn is determined by root branching and the length of root hairs [3].

Most vascular plants are capable of improving their mineral and water nutrition by forming associations and symbioses with soil microorganisms at their roots and in the rhizosphere [4-7]. The main carbon and energy sources for such microorganisms are the organic substances (mostly sugars, organic acids and amino acids) excreted by the roots into the rhizosphere [8]. Root exudates also play an important role in supplying plants with nutrient elements the lack of which may be manifested in drought conditions [9, 10].

At drought, the root to shoot weight ratio increases, but the overall dry weight of roots increases very rarely as compared to that in normal conditions. However, with moisture deficiency the density of the root system per unit of leaf area generally grows [11]. The photosynthates that could be used for the development of new roots are used for the growth of the existing ones, resulting in their penetration into deeper soil layers. For wheat, soil drainage restrained root growth in the upper soil layer (30 cm) and made them spread deeper [12]. After normal soil moisture recovers, the plant roots start to grow quickly again in the upper soil layer and cease developing in deeper layers.

The immediate assessment of the root parameters during research and selection in field conditions is rather labor consuming and requires special equipment. Currently, an indirect indicator, the vegetation cover temperature, is often used to determine the capability of roots to supply plants with nutrient substances and water [13]. Wheat genotypes with good water balance and drought-resistance generally have a lower temperature than the plants growing under drought conditions. However, measuring temperature of the vegetation cover, though a simple and quick, still requires special conditions (i.e., a sunny day without wind) that are not always the case. Another indirect method is measuring plant ability to regenerate after the aboveground parts cut. The removal of aboveground parts in wheat and other crops is a widely used for dual-purpose crop practice on the southern valleys of the USA, in South America and Australia, where fields are used in winter as pastures, after which the crops are grown until grain harvest [14]. However, this technique has never been used in research to assess the adaptation of wheat genotypes to drought and the interrelation between shoot regeneration and root exudation.

The diversity of genetic wheat resources is the basis for enhancing the resistance of plants to abiotic stresses, including drought. More and more researchers and breeders look for new drought-resistance genes beyond the existing and well-described genotypes. The potential sources of such genes are traditional local varieties that have been cultivated for centuries in isolated and arid regions. In Turkey, local primitive wheat varieties are still cultivated throughout the

country, especially in mountainous regions, still being the source of genetic diversity and valuable traits. Synthetic wheat obtained by crossing durum wheat (*Triticum durum* Desf.) and wild Tausch's goat grass (*Aegilops tauschii* Coss.) also possesses a number of useful features, including drought-resistance [15].

Our purpose was to evaluate the root and stem systems of three wheat genotypes (i.e., primitive, modern, and synthetic varieties) representing various stages of the hexaploid wheat evolution in normal conditions and under exposure to stress caused by the removal of aboveground parts. For the first time, root exudation of assimilates (organic acids, sugars, and amino acids) was compared in plant genotypes with identified differences in the root system functioning and shoot regeneration.

Technique. The objects of research were the wheat genotypes received under the IWWIP program from the International Maize and Wheat Improvement Center (CIMMYT, Turkey), corresponding to three levels of the crop evolution: a synthetic form produced by crossing durum wheat (*T. durum* Desf.) of the Ukrainian variety LEUCURUM 84693 (AB genome) and wild Tausch's goat grass (*A. tauschii* Coss.) with genome D; the traditional hexaploid wheat variety (*T. aestivum* L.) Albostan (genome ABD) of local selection in the Turkish province Nevşehir; drought-resistant modern wheat variety (*T. aestivum* L.) Karahan (genome ABD) from Turkey bred in the 1990s through standard methods.

Study of growth of shoots and roots and their response to the removal of shoots was conducted based on the Turkish department CIMMYT (Izmir, Turkey). The plants were grown in pots filled with 1 kg of sand (2 plants per pot, and 4 pots per variant) under natural temperature and light conditions (with night temperature of 8-12 °C and daytime temperature of 20-24 °C) in February to March 2014 to the 4th to 5th leaf before shooting. The plants were watered twice a week (200 ml of water per pot). The plants were treated with the nutrient solution (N₂₀P₂₀K₂₀, 250 g/l, Harmony Imports, USA) three times: at the 2nd to 3rd leaf, before tillering and 1 week after tillering. After five to six weeks, an initial assessment was conducted of the parameters studied. The plants from two pots were removed, and the roots were washed and scanned using an Epson Perfection V700 (Epson America, Inc., USA) and a WinRHIZO (Regent Instruments, Inc., Canada). Then, the roots and shoots were dried up in room temperature until constant dry weight. In the two remaining pots, the shoots were cut and the plants were left for 3-4 weeks for revegetation, after which the parameters of shoots and roots were assessed as described above.

The experiments with root exudates were conducted at the All-Russia Research Institute for Agricultural Microbiology using hydroponic culture. To receive root exudates, the seeds were subject to surface sterilization for 6 minutes with 0.1 % HgCl₂, washed with sterile water, and then they germinated for 2 days in Petri dishes in darkness at 27 °C. The sprouted seeds were planted into sterile glass dishes with 100 ml of deionized water and stainless steel meshes (10 seeds per dish, and 3 dishes per genotype). The seedlings were cultivated in the climatic cell ADAPTIS-A1000 (Conviron, Great Britain) for 5 days (at illumination of 200 $\mu\text{lk} \cdot \text{m}^{-2} \cdot \text{c}^{-1}$ and 16-hour photo-period with minimum and maximum temperatures of 18 °C and 22 °C, respectively). After 3 days, 0.1 ml of the solution was taken from each pot and plated on Petri dishes with Bacto Pseudomonas agar (Difco International BV, Netherlands) to control sterility. After 5 days, the plants were taken out of the dishes, dried up, and the dry weight of shoots and roots was determined. The solutions from the three dishes were pooled, vacuum-filtered through nylon filters (0.45 μm ; Coming, Inc., USA) and

vacuum-evaporated to 5 ml using a rotary evaporator BUCHI R-200 (BUCHI Labortechnik AG, Switzerland). Aliquot of 100 µl was taken from each concentrate of root exudates, and the remaining solution was poured through a column with ion-exchange resin DOWEX 50Wx8 (Sigma-Aldrich Co., USA) to obtain a purified fraction of organic acids and sugars, vacuum-evaporated to dryness, and the residue was dissolved in 0.5 ml of deionized water. The chromatographic analysis was conducted using Waters ACQUITY UPLC H-Class (Waters, USA). Sugars were separated using a SUPELCOSIL LC-NH2 column (Supelco Gland, Switzerland). To identify sugars, a refraction index detector Waters 2414 (Waters, USA) was used. Amino acids, except L-tryptophan, were analyzed using Waters AccQ-Tag (Waters, USA) with a fluorescence detector by the manufacturer's standard method. L-tryptophan in root exudates was determined by separation using a column Waters UPLC RP-18 Shield (Waters, USA) with a fluorescence detector ACQUITY UPLC (Waters, USA). Organic acids were separated using ACQUITY CSH C18 (Waters, USA) and determined with an UV-detector Photodiode Array ACQUITY UPLC (Waters, USA) at $\lambda = 210$ nm. The freshly prepared mixtures of sugars, organic acids, non-proteinogenic acids (1-amino-cyclopropane-1-carbonic acid, β -alanine, α -aminobutyric acid, β -aminobutyric acid, γ -aminobutyric acid, N-butyryl-DL-homoserine lacton, L-canavanine, L-citrulline, dopamine, DL-homoserine, D-glucosamine, L-mimosine, L-ornithine, serotonin) (analytical grade, Sigma-Aldrich Co., USA), L-tryptophan (analytical grade, Fluka Chemie GmbH, Switzerland) and Amino Acid Hydrolysate Standard H (Thermo Fisher Scientific, Inc., CIIIA) served as standards for identifying components of root exudates. Three replicates were conducted.

For data processing (i.e., determining standard errors, Student's *t*-test LMD, correlation analysis), STATISTICA v. 7.0 (StatSoft Inc., USA) and the dispersion analysis program DIANA were used [16].

Results. In the synthetic wheat obtained due to crossing durum wheat (*T. durum* Desf.) of the LEUCURUM 84693 variety (genome AB) and goat grass

Root and shoot growth in the wheat genotypes representing various levels of the crop evolution (pot test with and without shoot removal)

Parameter (per plant)	Control			Plants with removed shoots					
				total			vs. control, %		
	1	2	3	1	2	3	1	2	3
Root length, cm	823 ^a	923 ^a	1373 ^a	1274 ^a	1404 ^a	1052 ^a	+55	+52	-23
Root area, cm ²	144 ^a	206 ^a	204 ^a	160 ^a	153 ^a	103 ^a	+11	-26	-50
Root volume, cm ³	2.1 ^{ab}	3.7 ^{ab}	2.4 ^{ab}	1.6 ^{ab}	1.3 ^{ab}	0.8 ^a	-22	-64	-67
Average root diameter, mm	0.55 ^c	0.71 ^d	0.48 ^{bc}	0.40 ^{ab}	0.35 ^{ab}	0.31 ^a	-27	-51	-34
Number of root tips	1596 ^a	2022 ^a	2311 ^{ab}	2365 ^{ab}	3398 ^b	2534 ^{ab}	+48	+68	+10
Root fork number	6650 ^a	9723 ^a	11069 ^a	9529 ^a	10268 ^a	6299 ^a	+43	+6	-43
Number of root overlaps	926 ^a	1213 ^a	2121 ^a	1963 ^a	2318 ^a	1618 ^a	+112	+91	-24
Dry root weight, g	0.29 ^{ab}	0.53 ^b	0.31 ^{ab}	0.19 ^a	0.17 ^a	0.12 ^a	-35	-69	-63
Dry shoot weight, g	0.56 ^a	1.30 ^b	1.19 ^b	0.64 ^a	0.54 ^a	0.43 ^a	+15	-59	-64
Shoot to root weight ratio	2.0 ^a	2.5 ^{ab}	4.0 ^b	3.5 ^{ab}	3.2 ^{ab}	3.7 ^b	+78	+32	-8

Note. 1 — synthetic form created by crossing durum wheat (*Triticum durum* Desf.) of the Ukrainian variety LEUCURUM 84693 (genome AB) and wild Tausch's goat grass (*Aegilops tauschii* Coss.) (genome D); 2 — traditional variety of hexaploid wheat (*T. aestivum* L.) Albostan (genome ABD) of local Turkish selection; 3 — modern Turkish drought-resistant wheat variety (*T. aestivum* L.) Karahan (genome ABD). In an experiment two replicates were performed per each variant. Different Latin characters are used to indicate significant differences between the variants for each parameter (LMD test, $P < 0.05$).

(genome D), the resulting hexaploid genotype (genome ABD) reconstructs semi-wild wheat that existed 4,000-6,000 years ago when it was cultivated in the Middle East in the area of the so-called Fertile Crescent. The genotype is resistant to abiotic and biotic stresses due to the genes received with genome D. Albostan (genome ABD) is a local ancient traditional primitive variety of hexaploid wheat (*T. aestivum* L.) well adapted to drought that was cultivated in Turkey hundreds

years ago without any improvements through modern breeding methods. Karahan (*T. aestivum*) (genome ABD) obtained by standard breeding is drought-resistant and used in Turkey in development of drought-resistant varieties.

The vegetation experiment showed that Albostan had a bigger root diameter as compared to the synthetic genotype and Karahan (by 29 % and 48 %, respectively), while the shoot weight and the shoot to root ratio in Albostan and Karahan plants were higher than those of the synthetic genotype (by 130 % and 113 %, and by 25 % and 100 %, respectively) (Table).

Shoot removal resulted in a 27-50 % decrease in the root diameter of all genotypes, and in a 68 % increase in the number of new roots in Albostan plants. The weight of shoots in Albostan and Karahan plants reduced 2.4-fold and 2.8-fold, respectively, and the weight of roots in Albostan plants reduced 3.1-fold (see the Table). With shoot removal, some characteristics of the root system (e.g., root length, root number, number of forks, shoot to root ratio) changed depending on the wheat genotype, namely increased in the synthetic form and the Albostan variety, but decreased in Karahan plants (see the Table). These data can be considered as an evidence that even in modern scientifically-bred varieties with effective root system its recovery after stressing caused by shoot removal is still problematic. In the control variant, the shoot weight in the modern cultivar Karahan was 4.0 times higher than the root weight (see the Table), while in synthetic wheat plants and in the traditional local variety Albostan it was only 2.0 times and 2.5 times higher, respectively. After shoot removal, the root system of the synthetic wheat form and the Albostan variety demonstrated high efficiency in re-vegetation and contributed to the growth of new shoot weight to the values that are 3.2-3.5 times higher than the root weight. Comparing average growth values of the control plants exposed to stress confirmed that the effect of the shoot removal depends substantially on wheat genotype (Fig. 1). For synthetic wheat, seven parameters out of those ten studied (see Table) demonstrated a positive reaction to the shoot removal, the Albostan variety under stress showed higher values of five parameters, while in the modern variety Karahan only one parameter changed positively.

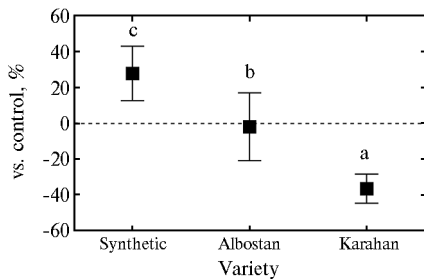


Fig. 1. Average values of growth parameters in wheat plants with removed shoots versus the control ones (%).

The forms representing various levels of the crop evolution have been studied: synthetic, traditional landrace Albostan, and modern Turkish cultivar Karahan. The data and variety descriptions are provided in the Table. The vertical sections indicate the standard error of mean. Different Latin characters indicate significant differences between the variants (LMD test, $P < 0.05$).

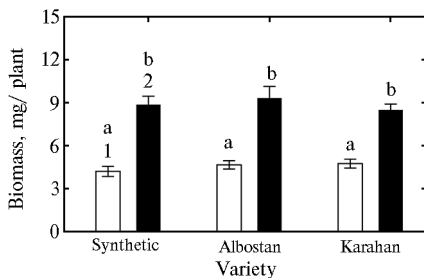


Fig. 2. Dry weight of roots (1) and shoots (2) in hydroponic wheat plants.

The forms representing various levels of the crop evolution have been studied: synthetic, traditional landrace Albostan, and modern Turkish cultivar Karahan. Average data of three experiments are provided in one replication for each variant. The vertical sections indicate the standard error of mean. Different Latin characters indicate significant differences between the variants (LMD test, $P < 0.05$).

Hydroponic plants of the studied wheat genotypes practically do not differ in the root and shoot weight (Fig. 2), which suggests that the plant biomass has no effect on the root exudation. The analysis of sugars in root exudates showed that the plant roots in all the three genotypes exuded mostly fructose,

glucose, and maltose (Fig. 3). The sugar exudation was minimum in synthetic wheat plants, and the modern cultivar Karahan plants excreted more glucose and maltose than the other two genotypes. The root exudates of the three genotypes also contained an insignificant amount of arabinose, ribose, and xylose (see Fig. 3).

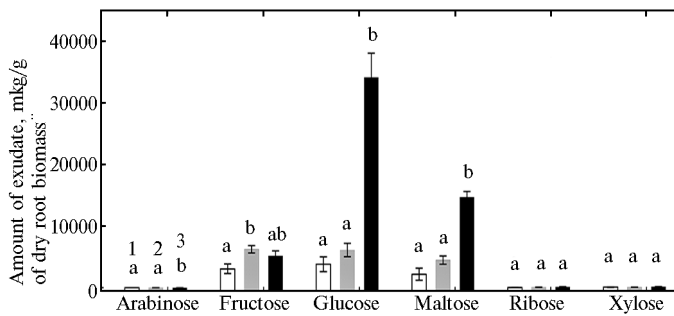


Fig. 3. Root exudation of sugars in hydroponic wheat plants: 1, 2, 3 — the forms representing various levels of the crop evolution (synthetic wheat, landrace Albostan, and modern Turkish variety Karahan). Average data of three experiments are provided in a replication for each variant. The vertical sections indicate the standard error of mean. Different Latin characters indicate significant differences

between the variants (LMD test, $P < 0.05$).

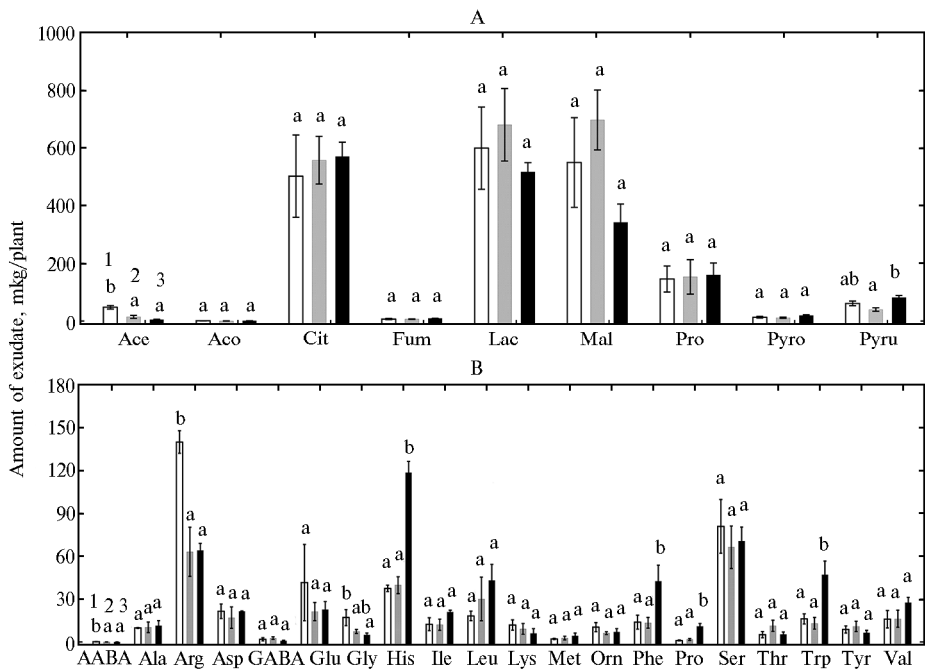


Fig. 4. Root exudation of organic acids (A) and amino acids (B) in hydroponic wheat: 1, 2, 3 — the forms representing various levels of the crop evolution (synthetic wheat, traditional landrace Albostan and modern Turkish cultivar Karahan); Ace, Aco, Cit, Fum, Lac, Mal, Pro, Pyro, and Pyru — acetic, aconitic, citric, fumaric, lactic, malic, propionic, pyroglutamic, and pyruvic acids, respectively; AABA and GABA — α -aminobutyric and γ -aminobutyric acids, and generally accepted abbreviations are indicated for proteinogenic amino acids. Average data of three experiments are provided in a replication for each variant. The vertical sections indicate the standard error of mean. Different Latin characters indicate significant differences between the variants for each acid or amino acid (LMD test, $P < 0.05$).

The content of organic acids was similar in all the genotypes (Fig. 4). The main components were citric, lactic, malic and propionic acids. The genotype differences included increased root exudation of acetic acid in synthetic wheat plants and pyroracemic acid in Karahn as compared to Albostan plants.

The root exudates of the wheat genotypes studied contained 17 proteinogenic amino acids mostly presented by arginine, asparaginic and glutamic acids, histidine, leucine, phenylalanine, serine, tryptophan and valine (see Fig. 4), and

only three of the 13 analyzed non-proteogenic amino acids, the α -aminobutyric, γ -aminobutyric acids and L-ornithine, were found. Synthetic wheat differed from other forms by increased exudation of α -aminobutyric acid, arginine and glycine, while the modern cultivar Karahan was characterized by intensive exudation of histidine, phenylalanine and tryptophane.

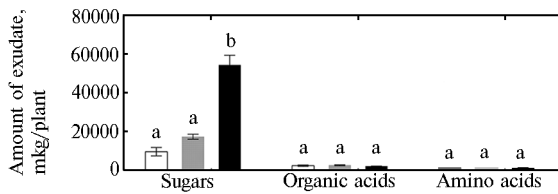


Fig. 5. Total amount of sugars, organic acids and amino acids in root exudates of hydroponic wheat: 1, 2, 3 — the forms representing various levels of the crop evolution (synthetic wheat, landrace Albostan, and modern Turkish variety Karahan). Average data of three experiments are provided in a replication for each variant. The vertical sections indicate

the standard error of mean. Different Latin characters indicate significant differences between the variants (LMD test, $P < 0.05$).

The total amount of sugars secreted by the roots of the Karahan variety plants was 5- and 3-fold higher, respectively, compared to synthetic wheat and Albostan plants, however, we identified no genotype differences by the total exudation of organic acids and amino acids (Fig. 5). The comparison of the total amounts in three analyzed fractions of root exudates showed sugars to be the main component in all the genotypes.

Thus, the basic initial hypothesis in the pot experiment was the assumption that shoot removal is a stress resulting in the mobilization of plant regeneration. Its rate depends on the ability of the root system to supply the plant with water and nutrients. Consequently, more active functioning of the root system will probably lead to accelerated and effective plant regeneration. Shoot removal demonstrated higher stress resistance in synthetic wheat as compared to Albostan and Karahan plants. Accordingly, the regeneration efficiency determined by the biomass of new shoots and the plant growth parameters under stress as compared to control turned out to be better in synthetic wheat. At the same time, in the control the modern cultivar Karahan produced 2-times more aboveground biomass per root weight unit. It can be assumed that long-term wheat breeding indirectly contributed to higher efficiency of plant root system. Our results showed that Karahan was unable to increase the shoot to root ratio in response to stress. Moreover, the average ratio in stressed and control Karahan plants was substantially lower than that of synthetic wheat and Albostan (see Fig. 1). The root systems of the synthetic genotype and Albostan plants demonstrated higher stability in response to stress caused by shoot removal. It is probable that these genotypes are more drought-resistant than Karahan. Currently, field experiments are being conducted to verify this assumption. Previously, the research comparing the root systems of old and modern wheat varieties showed that in modern forms the root weight and size are somewhat less at blooming [17]. If the shoots of winter wheat are eaten by animals, it does not have a substantial effect on the development of the root system in mature plants [18]. However, comparing these data with our results is problematic, as we analyzed the parameters of the root system before shooting. It is possible that the effective response of the synthetic wheat root system to shoot removal is unique and may be attributed to the mechanisms of adaptation to abiotic stresses.

Substantial variety differences in the root exudation of sugars, organic acids and amino acids were described for various plants, including tomato [19], pea [20] and potato [21] which indicates a high variability of genotypes by these traits. A characteristic feature of Karahan plants is a very intensive exudation of sugars (fructose, glucose, and maltose) that accounted for the main amount of

carbohydrates secreted into the rhizosphere. In Karahan plants, as was already mentioned, it is 5- and 3-times higher, respectively, compared to synthetic wheat and Albostan plants (see Fig. 5). Earlier, we showed that modern varieties of hexaploid wheat (*T. aestivum* L.) exude sugars in the rhizosphere more intensively than diploid genotypes *T. boeoticum* Boiss. and *T. monococcum* L. [22]. Based on these data, it can be assumed that the modern cultivar Karahan has a lower capacity of controlling sugar exudation through the root into the soil. It would also be appropriate to assume that after shoot removal the high intensity of sugar exudation in Karahan plants can result in reduced transportation of carbon sources from the root to the aboveground part and, thus, slowing down the re-vegetation. Therefore, it is of interest to compare the root exudation in the studied varieties with removed shoots, and the amount and composition of xylem sap coming to the regenerated shoot. In particular, we demonstrated stronger exudation of xylem sap in the cadmium-resistant pea mutant SGECD¹ [23] and intensive transportation of nutrients from the root to the shoot [24] resulting in better growth of plants when exposed to cadmium stress [23]. Thus, the increased transportation of nutrition and energy resources from roots to shoots can at least partially explain the high re-vegetation potential of synthetic wheat plants.

In our research, we have for the first time compared the root exudation of organic substances (sugars, organic and amino acids) in wheat genotypes corresponding to the main levels of wheat evolution. The most significant result was the identification of substantial differences in the intensity of sugar exudation. The character of organic acids exudation in the studied varieties was similar, but there were certain genotype peculiarities in the exudation of amino acids. Thus, the roots of synthetic wheat plants exuded more arginine, while the modern cultivar Karahan demonstrated increased exudation of histidine, phenylalanine, and tryptophane. Tryptophane is known as a precursor in the biosynthesis of auxins [25], the phytohormones playing an important role in many physiological processes of plants, including root formation and growth, cell division, xylem tissue formation, shoot regeneration and sugar transportation to the stem meristem [26, 27]. The need for tryptophane and auxins increases under stress caused by the loss of shoot tops (in our experiment this stress was caused by shoot removal), and these compounds are required to repress apical domination and induce a new shoot growth [27]. Intensive exudation of tryptophane by Karahan plants can reduce its content in tissues resulting in reduced biosynthesis of auxins and their transportation to the regenerating shoots. Moreover, the deficiency of auxins can influence negatively the transportation of sugars from the roots to the regenerating shoots. To verify this hypothesis more detailed research is required, including the determination of auxin level in tissues and xylem sap.

So the obtained results show that there are difference in the root systems of the three studied wheat genotypes (primitive, modern, and synthetic varieties), which represent various stages of hexaploid wheat evolution, with regard to the re-vegetation of shoots and the root exudation. The nature of the identified differences is subject to further investigation.

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ADAPTIVENESS OF PRODUCTIVITY AND PHOTOSYNTHESIS IN BUCKWHEAT (*Fagopyrum esculentum* Moench) LANDRACES AND VARIETIES PRODUCED AT DIFFERENT PERIODS

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Abstract

Crop breeding if aimed mainly at the highest productivity results in a significant loss of defense system activity thus causing a decreased plant resistance to adverse environment factors. Reasonably, more attention is now being paid to evolution base in breeding. With this, we studied the norm of reaction to environment changes in buckwheat (*Fagopyrum esculentum* Moench) cultivars and landraces as reflecting plant adaptation potential for photosynthesis and yield production to be further involved in breeding. A total of 11 buckwheat cultivars of which Kalininskaya, Bogatyr' and Shatilovskaya 5 have been derived in 1930-1970, and Chatyr-Tay, Batyr, Devyatka, Dizain, Demetra, Dikul' and Bashkirskeya krasnostebel'naya are the modern cultivars, together with landraces k-406 and k-1709 (VIR collection, St. Petersburg) were investigated. For the first time it was shown that in the course of buckwheat breeding no improvements in photosynthesis and production sustainability, as well as in homeostasis of grain formation have been achieved. Modern buckwheat cultivars possess high photosynthesis and productivity under favorable weather conditions, whereas at stresses do not have any significant advantage over their predecessors. In dry 2010 the seed production in modern cultivars was not reliably different from that in landraces and old cultivars, while in 2011-2013 at more favorable water supply and temperature it was on average 67.5 % higher, mostly due to the response of photosynthetic system to growing conditions. When drought occurred during the seed filling phase the photosynthetic activity in leaves decreased on average by 32.1 %, dry mass of the aboveground parts and seeds was lower by 46.7 % and 67.5 %, respectively, compared to those under favorable conditions in 2011-2013. With increasing water deficit the situation becomes worse. At soil moisture of 30 % of full capacity the activity of photosynthesis in buckwheat plant leaves was on average 4.4 times less, and seed production was 41.8 % less compared to the optimal moistening. At that, the losses were significantly higher in modern cultivars, e.g. in the k-1709 plants a 66.1 % decrease was found compared to 78.8 % in Dikul' and Dozhdik plants. Thus the obtained data suggests a low adaptive potential of modern buckwheat varieties. So we propose to improve the seed formation homeostasis in buckwheat plants. In this regard, the selection of autogamous form and the creation of self-pollinating varieties can be used as more effective approaches. The hybridization with *F. homotropicum* can significantly improve the viability of self-pollinated inbred lines of buckwheat, which can be successfully used in breeding programs to create autogamous varieties. Moreover, the adaptiveness of the of yield formation processes which are not sustainable enough to guarantee the high and sustainable crop production must be improved. An increased activity and effectiveness of photosynthesis and initial growth seem to be of interest. It is shown that the leaf photosynthetic rate in buckwheat varies plants varies widely from 4.65 to 17.8 $\mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, which allows to select forms both by hybridizing and using selection within a population.

Keywords: crop, buckwheat, selection, adaptation of variety, dry mass, photosynthesis, transpiration.

Over the past 50 years, the productivity of winter wheat, barley, buckwheat, soybeans, peas, broad beans and other crops has grown twice and more [1]. Undoubtedly, this is determined by the breeding process, since cultivar contributions in crop yields make over 50 % in many countries [2-5]. At the same time, the resistance to biotic and abiotic factors has been deteriorated considera-

bly [6-9]. According to the researchers, the latter is due to the fact that agriculture in general and breeding in particular aimed primarily at achieving maximum productivity weaken protective plant systems substantially and reduce their resistance to adverse conditions, consequently [10-13]. The effects of artificial and natural selection differs more and more distinctly, because the cultivar productivity and high yields (production efficiency) have been basic in crop breeding for a long time while the ability to survive (adaptability) is the determining feature of evolving species.

Therefore, the change of crop breeding priorities, in particular an objective to develop adaptive varieties based on evolutionary principles, is becoming increasingly urgent in recent years [10, 12, 14]. In Russia adaptive cultivars are needed due to contrasting local conditions exacerbated by global climate change and the increasing weather unpredictability [15-17].

This problem is relevant for buckwheat, the productivity of which remains low in Russia averaging 0.75 t/ha primarily due to lack of modern cultivar sustainable under extreme weather [18]. According to G.E. Martynenko et al. [19], average buckwheat cultivar productivity correlates negatively with their ecological plasticity ($r = -0.737$). Therefore, the further increase in the crop yield is planned to be achieved through the adaptive reconstruction of its genome [12] which requires a comprehensive research.

In connection with the problem of preserving and enhancing adaptive crop capacity, we for the first time studied the seed production process and the photosynthesis response under the extreme environment changes in pot and field experiments with buckwheat cultivars derived in various breeding periods.

The purpose of this study was to determine the adaptive potential of photosynthesis and seed yield in buckwheat plants with regard to breeding.

Technique. The research was performed in 2010-2013 (Orel region) in the following 13 buckwheat (*Fagopyrum esculentum* Moench) cultivars divided provisionally into three groups depending on the peculiarities and time of breeding: local cultivars (k-406, k-1709, VIR collection, Saint Petersburg) derived by breeders in the 1930-1970s (Kalininskaya, Bogatyr', and Shatilovskaya 5) and modern cultivars (Chatyr-Tay, Batyr, Devyatka, Dizain, Dozhdik, Demetra, Dikul', and Bashkirskaya krasnostebel'naya).

Plants were grown in breeding crop rotation (All-Russia Research Institute of Legumes and Groat Crops). At the experimental plot the medium loamy gray forest soil predominated. For sowing in rows with a seeding rate of 3 million seeds/ha the SKS-6-10 seeding machine (Russia) was used. The plot area was 10 m² with a randomized seeding in 4 replicates. Sowing and harvesting were performed according to the regional guidelines. Pot experiments to study cultivar drought resistance were carried out in 6 replicates for each cultivar under controlled greenhouse conditions using special 10 dm³ vegetation pots with soil moisturized to 30 % and 45 % of total moisture capacity (MC) and plants grown at the soil moisture of 70 % MC as a control.

The amount of dry matter accumulated by plant leaves, stems, side branches, inflorescences and seeds was registered in different growth phases by sampling 10 plants of each cultivar from plots (3 replicates) followed by drying the samples at 105 °C in a SM 50/250-1000 ShS drying chamber (SM Klimat, Russia). Harvest index was calculated as a percent of the seed weight to the total plant dry weight.

The rates of photosynthesis (RP) and transpiration (RT) were estimated in intact plants in real time using a Li-COR-6400 portable gas analyzer (Li-COR Bioscience, USA) according to the attached manual.

The experimental data statistical processing was performed using Micro-

soft Excel.

Results. Weather during the study has been contrasting. The growing season of 2010 was characterized by high daytime temperatures and a pronounced shortage of rainfall, whereas in 2011 and 2013 it was more favorable for buckwheat as a heat-loving and moisture-loving crop.

1. Dry weight and efficacy of seed formation in 13 buckwheat (*Fagopyrum esculentum* Moench) cultivars studied for the years of experiments (breeding crop rotation, Orel region)

Parameter	2010	2011	2012	2013	On the average
Dry weight of aboveground parts at harvesting, g/plant:					
average for cultivars	3.22	5.18	6.89	6.04	5.33
range	2.51-4.18	3.91-6.21	6.47-6.88	4.65-7.10	4.57-5.90
Harvest index, %:					
average for cultivars	16.2	27.1	25.4	26.5	23.8
range	10.4-24.7	18.7-36.0	20.9-32.4	14.1-46.5	19.1-30.7

Note. Please refer to Methods section for cultivar description (local forms, modern cultivars and cultivars derived in 1970s)

Studies demonstrated that the dry matter accumulation in buckwheat plants averaged 6.04 g under moderate moisture and optimum air temperature (in 2011-2013), reaching 7.10 g when the weather was favorable (Table 1). But under the extreme conditions (the year of 2010) with high temperatures and moisture deficiency, total plant productivity declined not less than 1.9-fold. With this, the efficacy of assimilate use for seed formation dropped especially sharply. Thus, in 2010 the harvest index was 16.2 % being 1.6 times less than in the years of 2011-2013 which were more favorable in humidification and temperature (see Table 1).

As a result, plant seed productivity under the arid conditions in 2010 was very low averaging 0.52 g/plant, or 33 % of this value for 2011-2013 (Fig. 1). With this, seed weight decreased more significantly than the weight of vegetative organs. Under the pronounced lack of moisture and high temperatures, buckwheat seed productivity was 3.0 times lower, and the dry weight of vegetative organs was 1.9 times lower compared to the more favorable years. Seed productivity in buckwheat cultivars ranged from 0.33 to 0.87 g in 2010, from 0.73 to 2.43 g in 2011, from 1.32 to 2.20 g in 2012, and from 0.77 to 2.58 g in 2013.

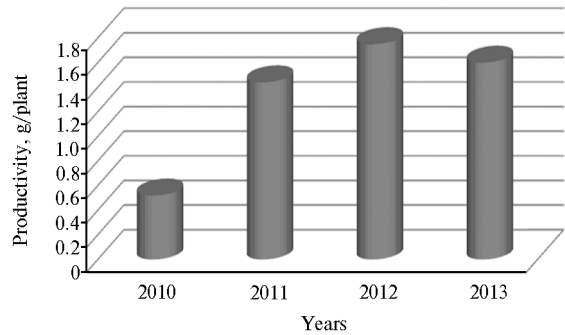


Fig. 1. Buckwheat (*Fagopyrum esculentum* Moench) seed productivity for the study years (averages for 13 cultivars of various breeding periods; breeding crop rotation, Orel region). Please refer to Methods section for cultivar description (local forms, modern cultivars and cultivars derived in 1930-1970s).

Differences between the aboveground organs for the dry matter accumulation may be due to the entomophilous pol-

lination and to the capability of maintaining high vegetative growth intensity almost throughout the growing season, including reproductive development. The last feature is typical for this crop that adversely affects seed formation [12, 18].

According to our data, in modern buckwheat cultivars, the number of seeds per plant varied almost 2 times greater than the weight of 1000 seeds, with 23.6 to 52.4 g for the first parameter, and 27.2 to 28.5 g in the second one, the later being within the experimental error. Due to such a high dependence of

seed formation on weather, the number of seeds formed per buckwheat plant in the dry year of 2010 was 51.5 % lower on average compared to 2011 and 2013, while their sizes were almost unchanged. The weight of 1000 seeds in 13 buckwheat cultivars studied averaged 28.5 ± 1.2 g in 2010, 27.2 ± 1.4 g in 2011, and 28.4 ± 1.3 g in 2013. In other words, seed yield is largely limited by the processes of seed formation. The low rate of fertile pollen under extreme weather, a characteristic feature of buckwheat as a cross-pollinated entomophilous crop, may be one of the reasons [20].

It should be noted that the stability of buckwheat seed formation is hardly increased via breeding [21], and the crop is widespread (from the Southern China subtropical areas to the northern border of agriculture lands), at the low physiological adaptations, mainly due to the population polymorphism in the growing period duration [22]. As a result, the species fertility is provided by long-term mass flowering at the very low seed formation efficacy, particularly only 10 % of flowers form seeds [18, 23].

In this regard, active work to improve seed formation productivity is held through the selection of the specified trait in cultivar populations [24, 25]. It is believed that the breeding of such type may be mostly efficient in hybrid populations involving buckwheat cultivars from the mountainous regions of India and Southeast Asia for which sharp fluctuations in weather conditions are typical [26]. Creation of self-pollinating buckwheat forms is another approach [27, 28]. It is assumed that creation of autogamous varieties would primarily reduce the buckwheat dependence on pollination by bees. Interest in the development of autogamous buckwheat increased sharply after the discovery of the wild self-pollinating *F. homotropicum* Ohnishi in the mountains of Southern China which is closely related to common buckwheat *F. esculentum* Moench [29]. As a result of successful crossbreeding, fertile interspecific hybrids have been reported in a number of laboratories, and their genetics is now being studied in detail [30-33]. A particular interest is due to the adaptive ability to regulate the time of seed formation in the wild type lacking in crop buckwheat that has been discovered by us [34]. The attempts to create self-pollinated common buckwheat cultivars based on this source material have not been successful so far mainly due to the difficulties of overcoming the inherent buckwheat inbreeding depression and poor adaptability of interspecific hybrids to temperate climate conditions [35]. However, this approach is very promising.

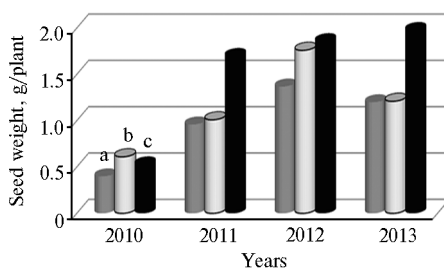


Fig. 2. Seed production in different buckwheat (*Fagopyrum esculentum* Moench) cultivars for the study years: a — local cultivars (k-406, k-1709, VIR collection, St. Petersburg); b — Kalininskaya, Bogatyr', and Shatilovskaya 5 cultivars (bred in 1930-1970s); c — modern cultivars Chatyr-Tay, Batyr, Devyatka, Dizain, Demetra, Dozhdik, Dikul', and Bashkirskaia krasnostebel'naya (breeding crop rotation, Orel region).

Apparently, more attention should be paid to the stable seed formation at the adverse weather factors to improve its efficiency in buckwheat, as this parameter tends to decrease. As a result, the dry weight variability of aerial plant parts (seeds especially) grows. During our study, the range of genotypic variability for seed weight per plant was 0.41-1.37 g in local populations, 0.61-1.76 g in old cultivars, and 0.53-1.85 g in modern cultivars. In the arid year of 2010, modern cultivars had almost no differences in seed production compared to the local and old ones, whereas this value was on average 67.5 % greater in the years of 2011-2013 with relatively favorable temperature

and water regime (Fig. 2).

In other words, modern buckwheat cultivars, like many other crops, have a pronounced advantage over their predecessors mainly in the favorable, but not in the extreme conditions. The weak development of the plant root system can also be considered a possible cause. According to results obtained by A. Lakhanov et al. [36], the proportion of roots formed in the total plant weight in buckwheat cultivars is significantly lower compared to the ancestral forms (*F. homotropicum* and *F. esculentum* ssp. *ancestrale*).

The low sustainability of reproduction in modern buckwheat cultivars may be caused by the high dependence of photosynthesis in plant leaves on the external factors which results in a dramatic reduction in yield under unfavorable conditions [37]. Photosynthetic crop productivity is known to depend significantly on the environmental factors such as temperature, light, moisture, and on the species and cultivar adaptive specificity [38-43]. This is clearly evidenced by our results. Thus, in the arid year of 2010 with a dry and hot weather almost throughout the whole growing season, the rate of photosynthesis in the leaves at seed filling was reduced by an average of 32.1 %, the dry weight of the aerial organs decreased by 46.7 %, and the seed weight was 67.5 % lower compared to the years of 2011-2013 (Fig. 3). The limits of RP genotypic variation (in $\text{mmol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) were 4.65 to 10.80 in 2010; 9.81 to 14.38 in 2011; 14.74 to 17.8 in 2012, and 7.92 to 12.9 in 2013.

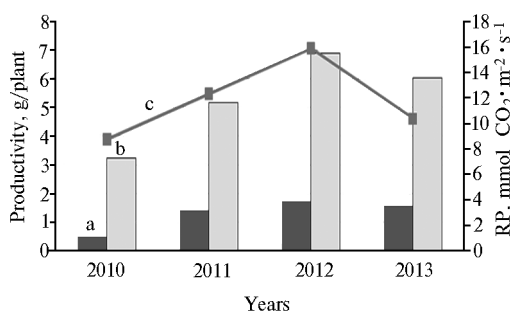


Fig. 3. Productivity and the rate of photosynthesis (RP) in leaves of 13 buckwheat (*Fagopyrum esculentum* Moench) cultivars: a, b — seed productivity and total aerial biomass, respectively; c — RP curve (blooming +10 days; breeding crop rotation, Orel region).

In acute drought, the situation is further getting worse. A model pot experiment demonstrated that at soil moisture of 30 % MC of the total moisture capacity the rate of photosynthesis in buckwheat leaves decreased 4.4 times on average, and seed yield was 41.8 % lower as compared to those obtained at optimal moistening. At the same time, a significant reduction in both parameters was primarily observed in modern varieties. While in k-1709 local cultivar the rate of photosynthesis decreased by 66.1 %, in Dikul' and Dozhdik cultivars the reduction averaged 78.8 % (Table 2).

2. Photosynthesis (RP) and transpiration (RT) rates in buckwheat (*Fagopyrum esculentum* Moench) leaves of different cultivars depending on soil moisture (pot experiment, 2013, blooming +30 days)

Cultivar	RP, $\text{mmol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$		RT, $\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$		Leaf temperature, °C	
	control	experiment	control	experiment	control	experiment
k-1709 (local form)	6.22	2.11	2.80	0.96	20.2	21.3
Bogatyr' (cultivar of the group derived in 1930-1970s)	6.51	1.03	2.64	0.55	24.2	25.1
Dikul' (modern cultivar)	6.70	1.73	3.26	0.66	25.0	25.5
Dozhdik (modern cultivar)	8.23	1.37	2.81	0.65	22.2	23.7
Average	6.92	1.56	2.88	0.71	22.9	23.9
HCP ₀₅	1.69	1.47	1.12	0.52	0.23	0.22

Note. Control — 70 % of the total moisture capacity (MC), experiment — 30 % MC.

In our opinion, these differences may be due to an increased capacity of local cultivars for transpiration which provides appropriate leaf tempera-

ture and an increased uptake of water from the soil under the water deficit. A relatively high positive correlation between the rates of photosynthesis and transpiration ($r = 0.68$; $P_0 < 0,05$) was observed in all buckwheat samples during the study.

In our experiments, the activity of gas exchange in buckwheat leaves varied significantly within cultivar populations as well. Thus, the analysis of 159 Di-kul' cultivar plants demonstrated the RP range in the leaves of 0.2 to 14.8 mmol $\text{CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Thus, the rate of photosynthesis was from 12.0 to 14.5 mmol $\text{CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ in 15 %, from 9.0 to 12.0 in 26%, from 3.0 to 9.0 in 41 %, and from 0.2 to 3.0 mmol $\text{CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ in 18 % of the examined plants. The findings provide a conclusion of a targeted buckwheat breeding for the rate of photosynthesis not only by hybridization, but also by a massive selection in a particular cultivar population, which will level the plants in their ability to provide assimilates for themselves thus increasing the total population productivity.

Thus, the analysis of the crop adaptability problem shows that resistance to adverse environmental factors in modern cultivars has decreased significantly as a result of breeding aimed primarily at providing the maximum productivity [10-13]. Creation of self-pollinating cultivars based on autogamous forms may be the solution. Difficulties associated with the emerging inbreeding depression that accompanies any attempt to obtain cross pollination based autogamous material are almost inevitable but can be overcome as shown by our experiments. Hybridization with *F. homotropicum* can significantly improve the viability of self-pollinated inbred crop buckwheat lines [34, 35]. Another approach is to increase ecological plasticity and stability of the reproduction to ensure high and stable yields. In this, breeding for increased activity and efficacy of photosynthesis remains a reserve [44, 45].

So we are the first to demonstrate no significant increase in the stability and adaptability of photosynthesis, seed production and seed formation as a result of buckwheat breeding. In our experiments, the rate of photosynthesis and dry matter accumulation, as well as the efficient use of assimilates for seed formation under the dry conditions were on average 38.4 % lower compared to those in the years that were favorable for moisture conditions and temperature. Modern cultivars demonstrated high rates of photosynthesis and formed high productivity under the favorable conditions only, but under stress had no advantages compared to cultivars derived in earlier breeding periods and to local forms. The leaf photosynthetic rate in buckwheat plants was found to be a widely varying genotypic trait (from 4.65 to 17.80 mmol $\text{CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), which makes it possible to select the forms for this parameter both by hybridizing and using mass selection within an individual cultivar population.

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STUDY OF ISOZYME POLYMORPHISM IN SPRING BARLEY (*Hordeum vulgare* L.) CONTRASTING IN TOLERANCE TO LEAD

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Abstract

Lead is one of the most hazardous heavy metals (HM), for which the mechanisms of plant resistance is not completely clear. One of the mechanisms for implementing toxic effect of lead is the generation of reactive oxygen species (ROS), which normally perform important regulatory function, but cause multiple violations of vital activity of cells at increased concentrations. ROS level adjustment is carried out by antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (PER). The effectiveness of most enzymes depends upon the existence of multiple isoforms, ensuring the most optimal operation under changing environment conditions. As a working hypothesis, it has been suggested that the level of plant tolerance to the HM can be determined by differences in composition of isoforms of antioxidant enzymes differently contributing to neutralization of the ROS excess. To test this hypothesis the study of isoenzymatic spectra of superoxide dismutase and peroxidase enzyme systems and key enzyme of protein metabolism, the glutamate dehydrogenase (GDH), was carried out using embryos of germinating seeds of the spring barley (*Hordeum vulgare* L.) cultivars. A total of 12 cultivars of spring barley contrasting in resistance to Pb^{2+} (6 tolerant cultivars and 6 sensitive cultivars) were studied. To analyze the composition of isoenzyme the germinating embryos of viable seeds were used with root length of 1 mm after 1 day germination. Homogenized embryos of seeds were subjected to electrophoresis using 7.5 % polyacrylamide gel (PAGE) units with an alkaline buffer system (pH 8.9). The analysis showed the presence of following isoenzymes from enzyme systems of superoxide dismutase, peroxidase and glutamate dehydrogenase: an obligate SODII, PERI and GDHI as well as rare SODI, SODIII + SODIV (always occurring together), SODV, PERII and GDHII. The frequencies of rare alleles were calculated and the highest frequencies were found for alleles SODI and SODIII + SODIV being the same in sensitive and resistant cultivars. A reliability of differences in the frequencies of rare allelic forms in the sensitive and resistant cultivars was assessed using ϕ -criterion of the angular Fisher transformation. The calculated value of Fisher's ϕ -criterion was proved to exceed the critical level. Thus the studied rare enzyme isoforms could be regarded as biochemical markers of sensitivity or tolerance to lead in barley plants. As our research ascertained, SODV and PERII are more common in lead sensitive cultivars, while GDHII is mostly found in tolerant ones. Frequency of SODIII and SODIV also reliably differed in the resistant and tolerant cultivars. The paper discusses the molecular genetic mechanisms of plant HM-resistance. It is first experimentally shown that spring barley plant polymorphism on resistance to lead is associated with biochemical polymorphism and correlates with a specific complex of superoxide dismutase, peroxidase and glutamate dehydrogenase isoenzymes. The data obtained can be used in selection programs aimed to producing lead-resistant barley cultivars.

Keywords: lead, barley, intraspecific polymorphism, contrast variants, isoenzymes.

The problem faced by modern agriculture under a decrease of usable territories and, consequently, the need to enhance production efficiency, is environment contamination with anthropogenic pollutants, in particular heavy metals (HM). Heavy metals are known to have an adverse effect on the growth and development of plants and animals [1-3]. Therefore, it is crucial to create crops with a high resistance to the toxic effect of HMs making it possible to have a sufficient amount of high-quality products.

Earlier [4], we determined the lead concentrations having a real toxic ef-

fect on barley seeds and investigated the intraspecific polymorphism by the resistance to lead in spring barley distinguishing 12 varieties with a contrast response (six resistant and six sensitive ones). A question arises on the relation between the polymorphism by morphological indicators as described in our previous research and the intraspecific variability on deeper levels of biological organization. For example, the genetic nature of the intraspecific polymorphism in radiation resistant hexaploid wheat plants has long been demonstrated [5]. It is reasonable to assume that contrast reactions of barley varieties to lead are related to their genetic peculiarities.

The development of methods for marking biological properties in plant and the productivity traits remains the main practical application of the biochemical and molecular genetics as a theoretical basis of modern breeding. Initially, the morphological traits (shape, color, pubescence, etc.) were mostly used for genetic marking as they are more easy to detect and more available to the researcher [6, 7]. However, the instability of these traits, their dependence on the cultivation conditions, the polygenic nature and a high probability of subjective interpretation led to the fact that a key role in genetic marking belonged first to protein but then to molecular-genetic markers [8].

The main difficulty in marking biological traits in plants is due to the fact that the majority of such traits is related to many life functions, metabolic and morphological processes which are under complex genetic control, vary phenotypically in a wide range, and therefore, are not available for a classical genetic analysis. Complex properties may often be subdivided into simpler ones, thus showing a hierarchic structure. In this, some of the complex elements are monomorphic (or, in other words, are common for all species), others are polymorphic and specific for varieties and biotypes [9]. Such subdivision makes it possible to reveal the genetic nature of a complex trait, and to identify the availability and localization of locuses and simple genetic systems in the genome that correspond to a particular trait [5].

Many enzymes are known to have several isoforms of specific structure and conformations. These differences often led to different activity [10, 11]. Biotypes and cultivars may have only some of the isoforms with various portion and prevalence of each one. It contributes to intraspecific variation formed on the biochemical level. The isozyme polymorphism is generally neutral [12] and related to the role of enzyme in cell functions. There are a number of reports about association of isoforms dissimilar in the activity with environment stress resistance, which can be used to differentiate plants on their response to adverse environmental factors. Thus, an increased expression of the superoxide dismutase (SOD) genes involved in the LpFe-SOD and LpCu/Zn-SOD isoforms' control and higher activity of these isomers in the plant roots were found in two rye grass (*Lolium perenne* L.) varieties after plant exposure to aluminum [13]. However, the expression differed substantially between the varieties, and thus, according to the authors, influenced stress resistance. The work [14] dedicated to the oxidative stress in the root cells of field beans (*Vicia faba* L.) grown in lead-contaminated soil also reports that various SOD isoforms differ in capability to detoxicate reactive oxygen species produced in such conditions at higher rates. However, SOD isoforms can respond to environment pollutants in different ways. It is reported [15] that copper increased activity of all SOD isoforms in *Arabidopsis thaliana* leaves, while cadmium induced an increase in the activity of only Fe-SOD and Mn-SOD, while the Cu-SOD activity reduced compared to control.

We have analyzed the isozyme patterns of barley plants contrasting in the response to lead contamination, with special attention to glutamate dehydro-

genase, superoxide dismutase and peroxydase as related to plant resistance under environmental stress, and shown the tolerance identified at a morphological level to be pre-determined by biochemical peculiarities of the varieties. In this, we have identified the biochemical traits of barley plants associated with resistance or sensitivity to lead.

The purpose of our research was to identify the enzyme isoforms more frequently found in the varieties resistant or sensitive to lead.

Technique. A total of 12 varieties of two-rowed spring barley (*Hordeum vulgare* L.) contrasting in resistance to Pb²⁺ were studied. To analyze the isoenzyme composition, the embryos of viable seeds with root length of 1 mm after 1 day germination were sampled. For germination the roll method was used [16]. A total of 15 samples (embryo extracts) of each variety were analyzed for each of the enzymes studied. The experiment was repeated with 1-year ($n = 180$ per enzyme of each variety) and 3-year ($n = 540$ per enzyme of each variety) harvest samples, with a total number of 1,620.

The isoforms of glutamate dehydrogenase (GDH, K.F. 1.4.1.2), superoxide dismutase (SOD, K.F. 1.15.1.1), and peroxydase (PER, K.F. 1.11.1.7) were analyzed with Tris glycine electrode running buffer solution (pH 8.3) [17, 18] in 7.5 % polyacrylamide gel plates (pH 8.9) [19]. The germinating seeds were homogenized in glass mortar with 50 μ l of extraction solution of 1 % Triton X-100 and 0.2 % β -mercaptoethanol at 1:1 (v/v) and equal volume of 50 % saccharose. The separation was performed using a vertical electrophoresis P9DS apparatus (Owl, USA) at ~350 V and ~50 mA for 3 hours with 1 ml of 0.1 % bromphenol blue as an internal reference dye. Then the gel was cut into three parts and prepared for histochemical assay as described [20]. Photos of the stained gels were analyzed to evaluate the frequency of each enzyme activity.

The obtained data were compared for the groups of resistant and sensitive varieties using criterion ϕ of Fisher's angle-transformation [21] as recommended [5].

Results. Based on morphological parameters, 12 varieties contrasting in lead resistance (i.e. 6 resistant and 6 sensitive ones) (Table 1) were selected out of 100 barley varieties of various geographic origin harvested in 2009 [4]. Additionally, adequacy of the grouping was confirmed on seed samples harvested in 2008 and 2010.

1. The varieties of two-rowed spring barley (*Hordeum vulgare* L.) of various origin contrasting in resistance to lead used in the study

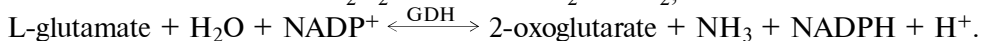
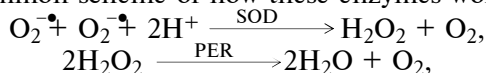
Name and origin	Variety	VIR catalog number
Resistant		
Vyatsky (Kirov Region)	<i>nutans</i>	k-30848
Teo (Great Britain)	<i>nutans</i>	k-29871
Zarya (Kirov Region)	<i>nutans</i>	k-4731
Donum (Czechia)	<i>nutans</i>	k-30863
Symphony (Kharkov Region)	<i>medicum</i>	k-30996
Pongo (Sweden)	<i>nutans</i>	k-30946
Sensitive		
Medicum 336 (Samara Region)	<i>medicum</i>	k-30962
Myt (Ukraine)	<i>medicum</i>	k-30993
Jelen (Yugoslavia)	<i>nutans</i>	k-30955
NSGL 1 (Yugoslavia)	<i>nudum</i>	k-30956
Zavetny (Rostov Region)	<i>medicum</i>	k-30959
Rubezh (Belarus)	<i>nutans</i>	k-29446

Note. VIR — Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg.

Obtained electrophoregrams showed the distribution of the isoforms as illustrated in the scheme (Fig.). The found alleles are numbered depending on the band distance to the reference dye position.

mitochondria, and peroxisomes [27]. Normally, ROS production is controlled by the antioxidant systems [28]. Under stress, however, there is a risk of cell damage due to excess ROS production [29]. An increased ROSs level can lead to crucial cell failures because of DNA modification, and oxidization of proteins and lipids [30, 31]. At the same time, ROSs can cause an increased expression of genes encoding SOD and PER responsible for their neutralization, thus also influencing cell homeostasis.

This is a common scheme of how these enzymes work:



SOD is one of the main enzymes responsible for stress protection under ROS generation [32]. Primarily, SOD must transform superoxide anion $\text{O}_2^{\bullet-}$ into hydrogen peroxide with subsequent production of water and molecular oxygen. Adverse environments lead to unequal development of oxidative stress in cell compartments which results in expression of SOD genes encoding isoforms to protect cell structures [33]. The presence of SOD isozymes in plants and their genetic control was first demonstrated in corn [34, 35]. In our study, a higher frequency of the rare SOD and PER isoforms was found in the lead-resistant barley varieties compared to sensitive ones. It may be assumed that the tolerance is related to a higher genetic diversity of antioxidant systems enabling the plants to endure stress better. Higher polymorphism is known to increase the capability of withstanding technogenic stresses [36]. At biochemical level this polymorphism appears as optional SOD isomers. The higher their frequency and diversity, the higher the level of polymorphism is. As to our data, the rare isoforms contribute to a higher plant tolerance.

The appearance and increased frequency of optional SOD isozymes in HM-resistant varieties compared to sensitive ones may be due to the ROS-inducing environments (e.g. arid or saline soils, metal deposits, etc.) at breeding. The available publications [37, 38] suggest that the responses of the plant antioxidant systems to HMs and natural factors are similar. Here, the technogenic load should also be taken into account. Three of six resistant varieties are from the industrial countries (Great Britain, Czech Republic, and Sweden) with an increased level of heavy metals in soils in many regions. It can be considered as an evidence of the above hypothesis. Indeed, the varieties grown at high natural level of arsenic differ in their tolerance [39]. In such conditions, adaptively more efficient isoforms could be fixed by selection with conservation of their alleles in the genome. At the same time, the breeding for economically important traits could also contribute to the revealed biochemical peculiarities. Thus, in the seeds rich in fats and exposed to atmospheric oxygen, an increased levels of organic peroxides and, as a result, free radicals can be produced. These could provoke development of additional antioxidants required to maintain homeostasis. Such peculiarities of the enzyme system can be fixed in gene pool [40] which was shown while studying the transcription of the gene encoding the Mn-SOD isoform. As it has turned out, mercury causes increased expression of this gene and, as a result, the isozyme activity rises, which is quite in line with our hypothesis. Another evidence for better withstanding to stresses in polymorphic samples can be found in the report of V. Rancelis et al. [36]. The authors showed that after treating bean plants with cobalt the occurrence of rare SOD variants grew. In this regard, we can also mention the increased frequency of optional antioxidant enzyme isoforms (SOD, PER, etc.) in chronically irradiated pine populations (*Pinus sylvestris* L.) after Chernobyl accident [41]. This example also indicates the uniformity of an organism's response to various stressors.

H₂O₂ produced in cells due to SOD is toxic [42], and its concentration must be kept low. Hydrogen peroxide, similar to other ROSs, can also be induced by metal ions and pathogens. For example, it is reported [43, 44] that in sensitive plants the H₂O₂ level is substantially higher than in resistant ones. At the same time, at low concentrations H₂O₂ activates a number of protective mechanisms [45], including cell wall regeneration [43], binding and neutralization of pathogens and harmful ions [46], a response to hypersensitivity, and synthesis of proteins and phytoalexins [47]. That is why it may be assumed that in resistant varieties these compounds are produced in relatively small amounts and stimulate the protective functions enabling better capability to withstand stress caused by HMs and making the varieties more adapted to adverse environments. As for the sensitive varieties, they have an excess of H₂O₂, which on the contrary leads to weaker protection and damages. The above mechanisms of peroxide action in cells can explain how the plants of the investigated varieties respond to the presence of Pb²⁺.

However, the obtained data should be discussed not only with regard to the effects of low-molecular compounds. As it was already mentioned, the anti-oxidant enzyme system protects plants from excessive ROS concentrations caused by environmental stress. The excess of peroxides is controlled by PER. The high frequency of PERII that we observed in sensitive varieties is probably related to higher sensitivity of plants to HM ions. Peroxidase is a highly labile enzyme that can respond to most homeostatic abnormalities. Thus, it was ascertained [48] that one of the peroxidase isozymes determines high resistance to virus infections in various red clover lines. This isozyme activates peroxidase cleavage, so the authors offered using this form as a general resistance marker in view of similarity in plant response to various stressors. Such data show that peroxidase markers make it possible to provide more comprehensive characterization of plant protection to various environments which can be taken into account in breeding.

The biological role of GDH is to catalyze the reductive amination of 2-oxoglutarate to glutamate and the reverse oxidative deamination [49] with involvement of reductive and oxidative NAD forms as coenzymes. Mostly due to this fact, the nitrogen compounds are taken up by plants and metabolized with synthesis of nitrogen-containing organic compounds and their destruction resulted in releasing nitrogen as ammonium salts. Thus, this enzyme is a link between the two fundamental processes characteristic of an autotrophic organism, i.e. nitrogen and carbon assimilation, and plays an important role in controlling plant development. GDH has a number of isoforms, in particular plants have light and heavy forms [50, 51] catalyzing amination and deamination, respectively. The differences in the level of these forms regulate the organism's balance between catabolism and anabolism. According to the Chatelier's principle, a catalyst speeds up chemical reaction bidirectionally, that is the enzyme enhances amination and deamination with equal probability, and kinetic disbalance [52, 53] may depend on environmental conditions and stress exposure. Accumulation of various compounds (e.g. sugars, amino acids, ammonium ions) is related to the GDH function in cells, which may indirectly contribute to the development of resistance to adverse environments such as drought or salination, to biomass accumulation and higher tolerance to toxicants, for example, herbicides and perhaps also HMs [54, 55]. The substantial disbalance towards the formation of 2-oxoglutarate was reached due to plant genetic modifications.

In view of the significant electrophoretic mobility of GDHII as compared to GDHI, it can be assumed that the first of them corresponds to the lightest of the described isoforms, mainly catalyzing the amination. Conse-

quently, in such plants the anabolism is more active compared to those in which this isomer is absent. With regard to the data we obtained, this tendency is of a special interest, as excessive glutamate is mostly produced so a direct reaction of deamination must prevail. Since there were cases when an isozyme catalyzing primarily the glutamate synthesis was revealed, the identified isoform had to have an increased activity. As we have showed the GDHII to be more often found in the resistant varieties, it could be assumed that their tolerance is related to the higher activity of the synthesis processes contributing to intensive accumulation of biomass. Similar data were obtained by R. Ameziane et al. [56]. As a result, the plants with GDHII allele developed quicker and were more adapted to adverse environments. The results on genetic control of *Pinus sylvestris* L. tolerance to air pollution [57] was in line with our findings of the GDH two isoforms, of which the lighter one (GDHII) was found in resistant plants.

At the same time, there is evidence [58] that the catabolic-type GDH is activated not only by ammonium ions, but also by single- and double-charged metal cations, and the affinity with such cations may be higher than with NH_4^+ . It is reasonable to assume that in sensitive varieties the relatively high concentration of the Pb^{2+} ions may cause a shift in the enzyme synthesis balance towards such GDH form, and it can be inherited. That is why such isozyme in sensitive varieties is capable of expressing even without HM exposure. From this, it becomes clear why a light GDH form responsible for anabolism, and, consequently, less sensitive to cations is found in sensitive barley varieties more rarely.

So, the data we obtained enable us to conclude that the lead resistance polymorphism of spring barley is related to biochemical polymorphism and correlates with a certain complex of superoxide dismutase, peroxydase and glutamate dehydrogenase isozymes. Evidently, there are specific alleles encoding the synthesis of isoforms of these enzymes and in some way determining the tolerance or the sensitivity. We have identified the isozymes of superoxide dismutase and peroxydase more frequent in lead-sensitive varieties, and ascertained the increased occurrence of the glutamate dehydrogenase isozyme in resistant ones. The differences between the groups, contrasting in resistance, with regard to isozyme frequency are confirmed statistically. Thus, it is offered to consider such isoforms of superoxide dismutase, peroxidase and glutamate dehydrogenase as biochemical markers to differentiate resistant and sensitive two-rowed spring barley varieties. The obtained data can be used in breeding lead-resistant barley.

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RESISTANCE OF SWEET PEPPER GENOTYPES TO ABIOTIC STRESSES IN GROWING CONDITIONS OF LOW-CAPACITY HYDROPONICS

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Abstract

The modern trend of vegetable crop breeding is a development of new cultivars and hybrids resistant to abiotic and biotic stresses and suitable for fully mechanized agriculture. It can provide the maximal commercial output and high profitability of vegetable growing. Pre-breeding is based on knowledge of impact of various abiotic factors on the yield and productivity of certain genotype in specific growing conditions. Traditionally the pepper plants are cultivated in greenhouses on low-volume soil or artificial medium with drop irrigation. Main advantage of low-volume technologies is a labor saving and improved phytosanitary conditions. In the present paper, the response of different sweet pepper (*Capsicum annuum* L.) genotypes to various abiotic factors has been studied aiming at development of initial breeding material for new varieties and hybrids under the hydroponic system conditions. The investigation has been conducted in 2008-2014 in the All-Russian Research Institute for vegetable breeding and seed production (Moscow Province). The breeding and collection samples of sweet pepper as well as F₁ hybrid Raisa (standard) were used in the present study. Plants were grown in a greenhouse on sphagnum peat mixed with calcified substance as a potting substrate and mineral fertilizers. The concentration of nutrient solution for irrigation of seedlings conforms to the recommended standard. The growth conditions were partially controlled. The parameters of outside environment were recorded during growing period using an automated climate control system KISTOCK KH-100 (France). The soil temperature was also controlled. Yield sample characteristics and assessment were done by UPOV system (Union Internationale pour la protection des obtentions végétales, France). The most adverse factors were recorded in the beginning (February-May) and the end (September-October) of growing season regardless of year of investigation, that resulted in decreasing marketability of the yield. The unmarketable part of yield was represented by fruits with excessive growth, deformation, and affected by blossom-end rot. Long-term screening of pepper genotypes on responsiveness to unfavorable factors of environment allowed to divide the cultivars into the following groups: susceptible (cv. Agapovskii), low-susceptible (cv. Elisa), and tolerant (cv. Zheltiyi buket). At hydroponics, productivity of the susceptible cultivar Agapovskiy was decreased up to 40 % as compared with the tolerant cultivar Zheltiyi buket. It was shown that adaptability of sweet pepper varieties to the conditions of low-capacity hydroponics is defined by the norm of reaction to changes of environmental factors such as daily temperature, difference of night and day temperatures, humidity, and its combinations. Changes of the light intensity are not so crucial for cultivars productivity; it may have negative impact on varieties with low adaptability when combined with other environment parameters. The correlation between the rate of non-standard part of yield, the damage of fruits caused by blossom-end rot, and the microclimate parameters differed among the varieties. Development of non-standard fruits in the susceptible and the low-susceptible cultivars is due to low humidity (at r value ranged from -0.65 to -0.72), while in the tolerant cultivars it depends on a big difference between the day and night temperatures (at $r > +0.70$). Damage of fruits caused by blossom-end rot is more genotype dependent. In all genotype, it is promoted by huge temperature changes, being stronger related to this factor in the susceptible genotypes at $r = 0.65-0.75$. For development of new pepper varieties and hybrids suitable for low-capacity hydroponics, the initial breeding material with high norm of reaction to temperature and high resistance to low humidity must be selected.

Keywords: sweet pepper (*Capsicum annuum* L.), adaptivity, temperature, humidity, environmental factors, yield, productivity, blossom end rot, norm of reaction, breeding.

Sweet pepper (*Capsicum annuum* L.) is cultivated in greenhouses using soil and soil-containing or artificial substrates with drip irrigation. The small scale technology of growing vegetables improves the fertilizer utilization coefficient, increases the yield and prevents environmental pollution. The main advantages of this technology are savings of labour and substrates costs, improved phytosanitary conditions, and enhanced production standards [1, 2]. However, the cultivation of pepper varieties, intended for other technologies, under conditions of low-capacity hydroponics often leads to an increase in a non-marketable part of the yield, especially under adverse conditions of early spring and autumn to winter periods [3].

Sweet pepper culture is responsive to even minor changes in environmental factors [4, 5]. Thus, with a dramatic decrease in night temperatures, fruit formation does not occur quickly enough, pistil and anthers grow along with the fruit resulting in excessive growth or irregularly shaped fruits. When the temperature difference increases by more than 10 °C, decreases in the yield may be as high as 1 kg/m² [6, 7]. Too high temperature (35 °C or above) results in an overall depression of the plants, causes elongation of the pistil column, abscission of flowers and ovaries, especially at the lack of air and soil humidity [8-10]. The optimum of soil temperatures for sweet pepper ranges within 20-25 °C. Its reduction to 18 °C stimulates abundant flowering, but the fruits appear to be deformed, flattened, and non-marketable. Lower temperatures (15 °C) will cause vegetative growth retardation and transition to the reproductive phase of development, reinforce the abscission of flowers and young fruit, and increase the risk of root rots [11, 12].

Low lighting conditions significantly extends all the stages of pepper organogenesis, delaying the transition to the reproductive stage, and causes abscission of buds and ovaries. Abscission of flowers and fruits is associated with a reduction in the photosynthesis activity and reduced formation of photoassimilates, as well as with alterations in their distribution within the plant [5, 11, 13].

Solanaceae plants are also demanding to substrate and air humidity. At the stage of the reproductive organ formation, the substrate moisture should be maintained within the range of 60-70 % PPV before fruiting and 80 % during the period of fruiting. Insufficient air humidity slows the growth of leaf mass, causes abscission of flowers and young ovaries. Relative humidity of at least 70-80 % is considered suitable for sweet pepper plants. Very high humidity or soggy soil reduce the growth [14-16].

Drip irrigation in the small scale technology of plant growing involves the use of high salt concentrations, with subsequent gradual salinization of the substrate, increased osmotic pressure, and an effect of «physiological drought», which causes blossom-end rot (BER) on fruits, especially at high temperature and high fruit burden on the plant [16-19]. This physiological disease is associated with changes in metabolism as a result of violations in moisture regime and transpiration, when the plant being in the ripening phase is suffering from a lack of moisture, especially when combined with periods of enhanced transpiration, or when water loss through the leaves is more higher than its absorption by roots. Calcium deficiency also increases the manifestation of BER symptoms, although the lack of this element in the soil does not always become the direct cause of the BER [20-22].

The cultivars and hybrids being created should include the desired combination of commercially important traits with high resistance to biotic and abiotic stresses. The plant resistance is a genetically regulated trait, which is characterized by the reaction norm of genotype to the exposure to adverse factors. Under optimal conditions, it is latent, being realized only when the plant is

exposed to an extreme factor.

Years of our research have resulted in the first ever identification of the combination of the most significant factors affecting the obtainment of marketable sweet pepper yield when using a small scale technology. Furthermore, we obtained lines with a high reaction norm that can be used to develop advanced, highly adapted sweet pepper forms.

The aim of our work was to study the response of different genotypes of sweet pepper plants to a variation of main abiotic factors and creation of source material to produce competitive cultivars and hybrids to be used under the small scale hydroponics.

Technique. The investigation has been conducted in 2008-2014 in All-Russian Research Institute for Vegetable Breeding and Seed Production (VNISSOK) using a modern greenhouse complex by Richel Group (France). Cultivars, breeding and collection samples of sweet pepper from the gene pool of the VNISSOK were used, and the reference was the F₁ hybrid Raisa originated by the breeding company Enza Zaden (The Netherlands). Sweet pepper plants under trial were grown on the «Professional» (Russia) substrate, which included sphagnum peat mixed with lime substances and mineral fertilizers. Samples were planted by 20-30 plants without repetitions, and a reference was placed between every 10 samples.

The concentration of the nutrient solution for irrigation of cassettes with seedlings, seedling pots and mats conformed to the recommended standards for solanaceous crops [23-25]. Supply of the nutrient solution into the mats was carried out through droppers. One day before the planting, the substrate was moistened with a nutrient solution with electrical conductivity (EC) 2.3-2.5 mS. During the season, EC of the solution under high lighting conditions was 3.5-4.0 mS, on cloudy days it was increased to 4.0-4.5 mS. Plants were grown with tying on a trellis to form two stems.

For seedlings (January to February), optimal temperature and light conditions were maintained considering the phase of plant development [12, 24, 25]. For young and adult plants the growing conditions were partially controlled (due to the absence of autonomous heating, air-conditioning and additional lighting) with the main parameters recorded continuously during the entire growing period by an automated climate control system KISTOCK KH-100 (France) and the substrate temperature measured by laboratory thermometers.

Fruit yield sample characteristics were assessed by the UPOV system (Union Internationale pour la protection des obtentions végétales, France) based on the complete yield structure analysis, i.e. marketable (standard) and non-marketable (non-standard and sick fruits) parts. In addition, phenological phases of development of the plant reproductive organs were recorded over time, with simultaneous recording of the main environmental parameters.

Mathematical and statistical processing of the results was carried out based on appropriate methods [26] using the Microsoft Excel 2003 application package.

Results. Many years of observations have revealed changes in the main parameters when growing sweet pepper plants according to the small scale technology with no strict control of the microclimate in the greenhouses.

Mean annual values of day and night temperature during the entire growing period did not usually go beyond a favourable range for the crop. The day air temperature was 21-28 °C, and night air temperature was 16-18 °C, with the exception of two months (February and September), when night temperature averaged to 1-3 °C below the optimum (Fig. 1, A).

However, average daytime air temperatures within each month of the grow-

ing period (see Fig. 1, B) often did not meet the optimum values (+21 ... + 23 °C). This can be explained by significant fluctuations in temperature and, accordingly, by a wide variation of differences between daytime and night-time temperature, from 1 to 17 °C (see Fig. 1, A, B).

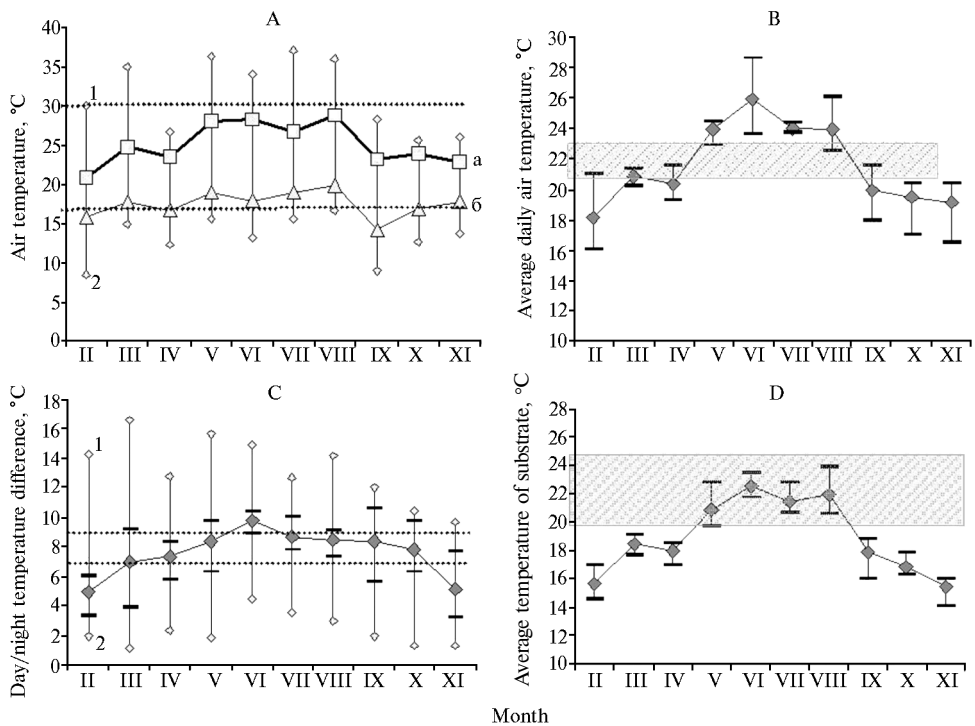


Fig. 1. Air and substrate temperature recorded monthly during the growth of sweet pepper (*Capsicum annuum* L.) plants in greenhouse under small scale technology: A — day (a) and night (b) air temperature, B — average daily air temperature, C — temperature difference (day/night), D — average temperature of substrate; II-XI — February to November, respectively (Moscow region, 2008-2012). The diagrams show average annual values and a range of mean deviations by year; the diagrams A and C represent the upper (1) and lower (2) limits of daily fluctuations. Dotted lines and shaded areas designate areas of optimal settings for the culture of sweet pepper [24, 25].

According to annually recorded data, a more favourable average daily temperature appeared to be in March and April, while in February and autumn months it was most often below the optimum, and in the summer months it was above the optimum (on average of 1-5 °C). The average temperature of the substrate in summer months was favourable for the development of sweet pepper plants, i.e. +20 ... + 24 °C, while in spring and autumn it dropped markedly below the optimum (see Fig. 1, D). Lowering the temperature in the root zone (< 16 °C), especially in spring, restrained the vegetative growth and increased the abscission of flowers and ovaries. However, too high substrate temperature (> 25 °C), which have been registered, for example, in August 2007 and June 2008, also resulted in poor fruit set and emergence of BER.

Another distinctive feature of small scale technology of cultivation was reduced humidity in the greenhouse (Fig. 2, A). Its average annual values were significantly lower than the optimal values for almost entire vegetative period (< 60 %), and only in certain years they were within the required limits (62-70 %), mainly in the summer months, with the maximum fluctuations in August (38 to 76 %).

Illuminance was changed in accordance with the characteristics of the Moscow region (see Fig. 2, B). The highest light intensity was recorded in June

and July, with a significant variation of the average values seen in some years (24,000 to 45,000 lx).

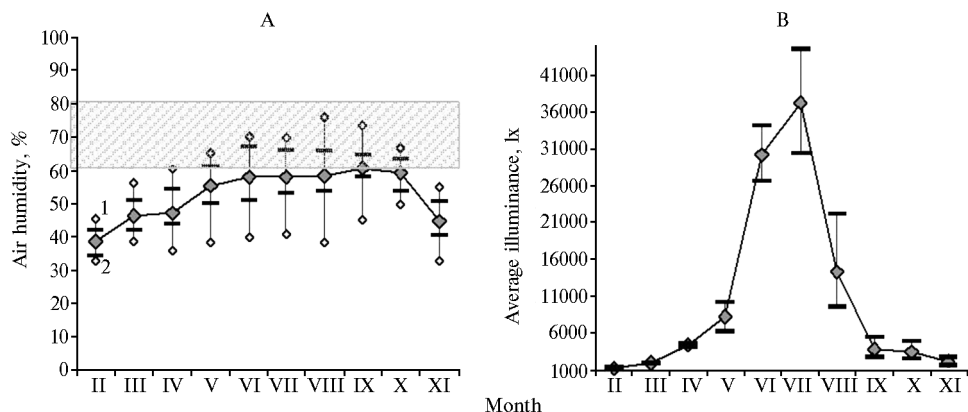


Fig. 2. Air humidity (A) and illuminance (B) recorded monthly during the growth of sweet pepper (*Cap-sicum annuum* L.) plants in greenhouse under small scale technology: II-XI — February to November, respectively (Moscow region, 2008-2012). The diagrams show the average annual values and a range of mean deviations by year; the diagram A represents the upper (1) and lower (2) limits of daily fluctuations. The shaded zone designates an area of optimal settings for the crop [24, 25].

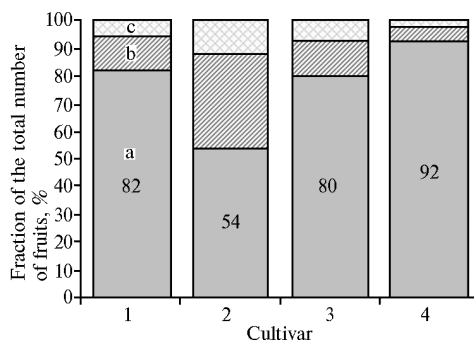


Fig. 3. The yield structure in sweet pepper (*Cap-sicum annuum* L.) cultivars from different groups of susceptibility to growing in greenhouse under small scale technology: 1 — F₁ Raisa (reference); 2 — Agapovskii (susceptible); 3 — Elisa (low susceptible); 4 — Zheltiyi buket (resistant); a — a marketable yield part; b — a non-standard part; c — fruits with blossom-end rot (Moscow region, 2008-2012).

Thus, the most unfavourable combination of exogenous factors occurred at the beginning (February to May) and in the end (September to November) of the vegetative period, regardless of the year of research, that led to a decrease in the marketability of the yield. At this, a non-marketable part was represented by non-standard fruits (with excessive growth, deformities, etc.) and those affected by diseases, mainly the blossom-end rot.

When creating breeding material, it is important to know the relationship between the non-standard yield part and that affected by blossom-end rot, and the microclimate parameters for different samples. Changes in the yield structure allowed to identify groups of genotypes based on their responsiveness

to unfavourable environmental factors [7], i.e. susceptible, low susceptible and tolerant (resistant), the most typical representatives of which were the Agapovskii, Elisa and Zheltiyi Buket cultivars, respectively (Fig. 3). Marketability (productivity) of the susceptible cultivar Agapovskii was decreased on average up to 40 % as compared to the tolerant cultivar Zheltiyi Buket. These specimens have clearly showed that they have different requirements to environmental conditions, especially to temperature and humidity profile (Fig. 4).

A non-standard portion of the yield in the Agapovskii cultivar, consisted mainly of fruits with extensive growth, was largely determined by low air humidity, especially at lowered average overnight temperatures and low illuminance. A non-standard fruits in the Elisa cultivar also emerged mainly at the lowered air humidity and lightening (see Fig. 4). The emergence of such fruits in the cultivar Zheltiyi Buket and the hybrid F₁ Raisa was mainly determined by the tempera-

ture profile, especially the difference between day and night temperature. At the same time, the effect of the average air temperature was more pronounced in the cultivar Zheltiyi Buket (see Fig. 4).

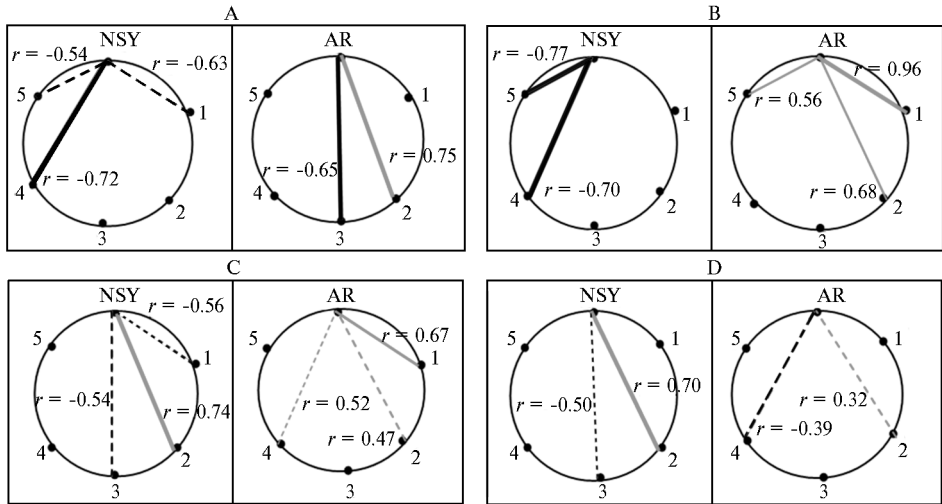


Fig. 4. Correlations between environmental factors and the portion of non-standard fruits (NSY) and those affected by the blossom-end rot (BER) in the sweet pepper (*Capsicum annuum* L.) cultivars Agapovskii (susceptible, A), Elisa (low susceptible, B), Zheltiyi Buket (resistant, C), and F₁ Raisa (reference, D), when grown in greenhouse under small scale technology: 1 — average daily temperature, °C; 2 — day/night air temperature difference, °C; 3 — the substrate temperature, °C; 4 — relative air humidity, %; 5 — illuminance, lx (average values per month of vegetation) (Moscow region, 2008–2012). Positive correlation is showed in grey, negative correlation in black; the degree of contingency is represented by line thickness.

Other patterns were observed in the blossom-end rot. In all specimens, the appearance of BER was provoked by dramatic changes in temperature, and the impact of this factor was more pronounced in the susceptible samples. Air humidity exerted a lower effect on the BER development, and this relationship was cultivar-specific. In addition, increased BER in fruits was caused by a low temperature of the substrate in the Agapovskii cultivar, or by an increase in average air temperature in the Zheltiyi Buket cultivar, or by an increased air temperature at high illuminance in the Elisa cultivar (see Fig. 4).

A small part of close correlation between the emergence of the non-marketable yield part and the environmental parameters appeared to be attributable to the complex character of the response of sweet pepper cultivars to changes in the factors investigated.

It has been established that the response of the samples to changes in exogenous factors can be assessed by comparing the marketability by the specific harvesting date and the average values of the studied environmental parameters during the interphase period of blooming to fruit biological ripeness. This period averaged to 60 days, but it must be specified individually for each sample. Plotting the influence curves of the non-standard or BER damaged fruit output against the investigated external parameters revealed the causal factors for the decline of marketability, and allowed to determine the reaction norm of the genotype to each of them. This is clearly exemplified by changes in the yield structure (the proportion of marketable, non-standard and BER damaged fruits) depending on humidity and average air temperatures. The revealed relationships were nonlinear and polynomial cubic and quartic in the curve shape (Fig. 5).

The obtained results indicate significant differences in the reaction norm of sweet pepper cultivars from different groups of responsiveness to the main en-

vironmental parameters. We managed to determine the optimal range, providing high marketability of the fruit under low-capacity hydroponics.

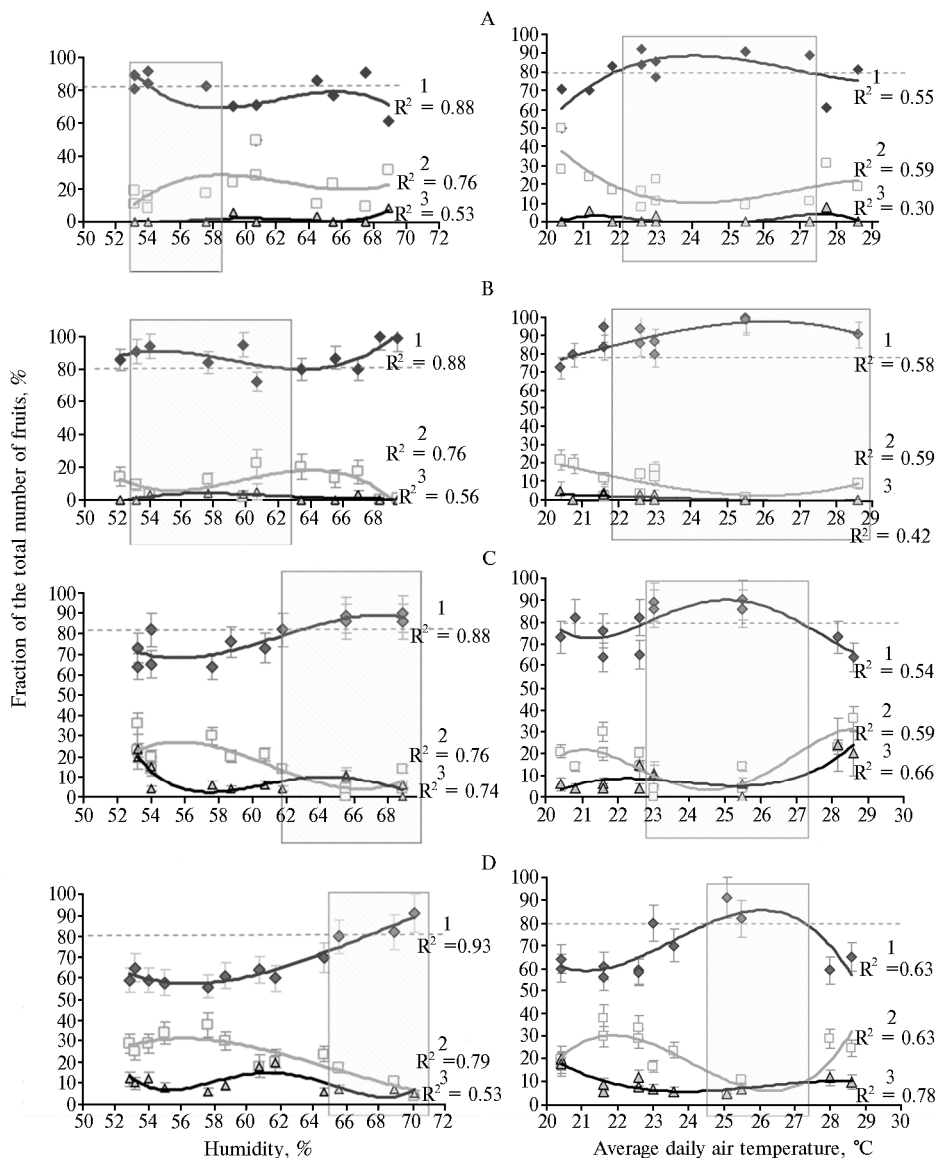


Fig. 5. The yield structure in the sweet pepper (*Capsicum annuum* L.) cultivars *F₁ Raisa* (reference, A), *Zheltiyi Buket* (tolerant, B), *Elisa* (low susceptible, C), *Agapovskii* (susceptible, D) depending on the humidity (left) and average daily air temperature (right) in the greenhouse when growing under small scale technology: 1 — fruit marketability; 2 — a non-standard yield portion; 3 — the yield affected by blossom-end rot (Moscow region, 2008-2014). Hatching covers the range of optimal values of the exogenous parameters for the cultivars.

For the *F₁* hybrid *Raisa* (reference) grown under cover in the Nonchernozem zone, the optimal conditions were air temperature up to 22.5-27.5 °C and 54-58 % humidity. A decrease or increase in temperature led to an increase in the non-standard portion of the yield or, when combined with high humidity, to the appearance of the fruits affected by blossom-end rot. Another key factor was the difference between day and night temperatures, optimal values of which ranged from 5.7 to 9.5 °C. A significant temperature difference (over 10 °C) influenced the fruit yield most adversely.

In general, based on the evaluation of responsiveness to exogenous fac-

tors, the chosen reference cultivar appeared to be between susceptible and low susceptible groups of specimens. Thus, the Zheltiyi Buket cultivar (susceptible group) was reported to have a higher reaction norm to the changes for the most of the studied parameters. The humidity optimum was within 52 % to 70 %, and the temperature optimum averaged from 21 °C to 29 °C with a day/night difference of 5 °C to 10.5 °C. This explains the high yield and marketability of the Zheltiyi Buket cultivar fruits throughout the entire vegetation season.

Representative specimens of low and high susceptible groups (Agapovskii and Elisa cultivars) required a more strict optimal range to ensure the highest marketability, such as air humidity at least 62 % for the Agapovskii cultivar and 65 % for the Elisa cultivar; the optimum of average daily temperatures was 24.5–27.5 °C and 23–27 °C, respectively with day/night difference lying within 9 °C to 10.5 °C. Low adaptiveness of these cultivars to the small scale technology seems to be related to their selection for growing in plastic film greenhouses, which are characterized by a higher humidity and drastic temperature fluctuations.

Many years of our research have resulted in the development of the sweet pepper F₁ heterotic hybrid Mila with a high reaction norm, which appeared to be capable of fruiting at different edaphic factors. The hybrid is characterized by high crop yield of 25 kg/m² on average, marketability over 95 %, and fruit quality.

Therefore, the average daily temperature, the difference between day and night temperatures, relative air humidity and their combination are essential for ensuring the standard sweet pepper production output when plant growing in greenhouse under the small scale technology. Illuminance has a less significant impact on the product merchantability, and may act as an additional adverse factor, together with other environmental parameters, affecting cultivars with low adaptive capacity. Therefore, when developing sweet pepper cultivars and hybrids to be used under the small scale technology, the breeding material with a high norm of reaction to the temperature and high resistance to low humidity must be initially selected. An important criterion for the adaptiveness of individual genotypes and selection samples on the pre-breeding stage is the index of marketability. Based on long term evaluation and breeding (2008–2014), we have produced sweet pepper lines with a high reaction norm, which was involved in hybridization to create promising heterotic hybrids and new highly adapted forms with a combination of features essential for growing under conditions of low-capacity hydroponics.

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***Fusarium* AND *Alternaria* FUNGI IN GRAIN OF OATS GROWN IN THE NORTH-WESTERN RUSSIA REGARDING CULTIVAR SPECIFICITY**

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Abstract

Oat (*Avena sativa* L.) is widely cultivated in a moist, cool climate and it is important crop particularly in Northern Europe. The most general usage of oat is for livestock feed. However, oat consumption as human food has recently increased, perhaps due to its reported health benefits. The abundance and species composition of the microbiota are the important factors in determining the quality of the grain. *Fusarium* fungi produce toxic metabolic products called mycotoxins. Mycotoxin contamination of food commodities can affect both human and animal health. Species of the genus *Alternaria* have rather less significance as the pathogens of cereals and sources of grain contamination. The aim of this study was to evaluate the natural infestation by *Fusarium* and *Alternaria* fungi of oat grain harvested in 2014 in the northwestern Russia. Asymptomatic seeds of 56 oat samples harvested in five provinces of the northwestern Russia (7 samples from Arkhangelsk and Pskov provinces, 20 samples from Vologda Province, 12 samples from Leningrad province and 10 samples from Novgorod Province) were assayed for the presence of filamentous fungi. Grain samples (except for a few unnamed) belonged to 14 varieties (Adamo, Argamak, Borets, Borrus, Krechet, Lev, LOS 3, Skakun, Scorpion, Teremok, Fukhs, Chernigovskiy, Chernigovskiy 83 and Yakov). Seeds were surface sterilized and placed on Petri dishes with Potato-dextrose agar (PDA). The resulting fungal colonies from each kernel were isolated and identified based on cultural and morphological features. Fungal contamination and the percentage of species belong to *Fusarium* or *Alternaria* fungi (%) were calculated in each sample. Mycological analysis revealed presence of different species of fungi belonging to the genera *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, and others. The main representatives of mycobiota were *Fusarium* and *Alternaria* fungi. Infection of *Fusarium* fungi was detected in 88.9 % analyzed oat samples (average grain infestation in different regions ranged from 6.1 to 18.7 %, the maximum value was 64.0 %). *Alternaria* fungi were found in 91.0 % grain samples (average infestation ranged 1.5 to 48.0 %, the maximum value was 85.0 %). Among *Fusarium* fungi the *F. poae*, *F. sporotrichioides* and *F. langsethiae* which belong to trichothecene-producing species were detected with the highest frequency. A number of species, such as *F. anguoides*, *F. avenaceum*, *F. graminearum*, *F. incarnatum*, *F. subglutinans*, and *F. tricinctum*, were low-frequent. For the first time *F. langsethiae* was found on the territory of Arkhangelsk Province which is the most northern border of this fungus areal in Russia. The high positive correlation between portion of grains damaged by *F. langsethiae* and accumulation of T-2/HT-2 toxins was found. Fungi of *Alternaria* genus were mainly presented by toxin-producing species *A. tenuissima* and *A. arborescens* (66-86 % of the total number of *Alternaria* isolates). Cultivars Lev, Adamo, Yakov, Krechet were the most infected by *Fusarium* (an average infestation rate was more than 20 %). The significantly higher grain infection by *Alternaria* fungi was detected in cultivar Lev in comparison with another analyzed oat cultivars. The obtained data are in line with earlier reported results of toxicological analysis (A.A. Burkin et al., 2015) in which the presence of mycotoxins in the grain from all provinces of the northwestern Russia was revealed, particularly T-2/HT-2 toxins were found in 60.7 % of samples, deoxynivalenol was detected in 62.5 % and alternariol was presented in 29.0 % of the tested oat samples.

Keywords: oats, cultivars, infection, mycotoxins, *Fusarium* and *Alternaria* fungi.

Currently, the research of oat grain infestation with toxin-producing fungi species is particularly relevant, since the demand for this crop as a source

of raw material for food products, including baby and dietetic food has increased considerably [1]. Therefore, more attention is paid to the safety of oat-containing products.

The abundance and species composition of the microbiota are the important factors in determining grain quality. *Fusarium* fungi produce toxic secondary metabolites that can adversely affect the health of cereal product consumers. Currently, the maximum permissible concentrations (MPC) of deoxynivalenol (DON) trichothecene fusarium toxins of 700-1000 µg/kg, T-2/HT-2 toxins (T-2/HT-2) of 100 µg/kg, and zearalenone (ZEN) of 200-1000 µg/kg [2] are set for cereals and a variety of processed products in Russia.

Annual mycotoxicological analysis showed a high level of *Fusarium* fungi infestation and grain contamination with fusarium toxins. It averaged 17.0 % in the northwest of Russia in 2008. T-2/HT-2 and DON mycotoxins were found in 46 and 47 % of samples, respectively, and their maximum concentration was 182 and 2505 µg/kg [3]. Studies performed in Norway indicate that oats are significantly more susceptible to *Fusarium* infection than wheat. In addition, trichothecene mycotoxin producers, the *F. poae* (Peck) Wollenw. and *F. langsethiae* Torp et Nirenberg fungi, were also more frequent in oats than in wheat and barley [4]. Within 10 years of observations, Finland, Norway and Sweden researchers have shown that the permissible content of DON (1750 µg/kg) was exceeded in 3-28 % of the samples of grain used for food, depending on the region [5]. However, they failed to identify the meteorological factors and agricultural practices that affect the increase in mycotoxin content in cereals. In 2010-2011, analysis of oat samples from three regions of Sweden demonstrated that the majority of samples (90-100 %) were infected with *F. poae*, *F. langsethiae* and *F. avenaceum* (Fr.) Sacc. A positive correlation of not less than $r = 0.52$ at a 99 % significance level between the presence of *Fusarium* species and the amount of fusarium toxins in grain [6] was identified. The study of 98 grain crop samples of oat grown in 2009-2011 in Poland indicate a high prevalence of T-2/HT-2, nivalenol (NIV), DON and ZEN in the grains and derived products. According to the authors, the greatest risk is caused by the maximum content of NIV of 655 µg/kg [7].

Grain infestation with *Alternaria* and contamination with mycotoxin produced by the fungi of this group has also been reported [8-14]. *Alternaria* species are rather less significant as cereal pathogens and the source of grain contamination compared to *Fusarium* [15]. Toxicity of secondary metabolites formed by some species of this genus is investigated actively [16, 17]. Alternariol (AOL) is one of the most common and hazardous *Alternaria* spp. secondary metabolites [18, 19].

The first report on fusarium toxins found in oat grains from several regions of Russia was published in 2009 [20]. High incidence of *Fusarium* and *Alternaria* on cereals, widespread cultivation of oats in the regions with temperate climate and its extensive use in the production of feed and food suggest the need for the quality control of raw grain material and for the improvement of mycotoxicosis prevention measures.

The purpose of this study was to evaluate oat grain infestation by *Fusarium* and *Alternaria* fungi considering the varietal characteristics, and to search for the correlations between the intensity of grain infestation by dominant fungi species and mycotoxin accumulation.

Techniques. In 2014, 56 oat seed samples harvested in five provinces of the northwestern Russia were studied for the presence of fungi infestation of which 7 samples were from the Arkhangelsk regions, 7 samples were from Pskov regions, 20 samples were from the Vologda region, 12 samples were from the Leningrad region, and 10 samples were from the Novgorod region.

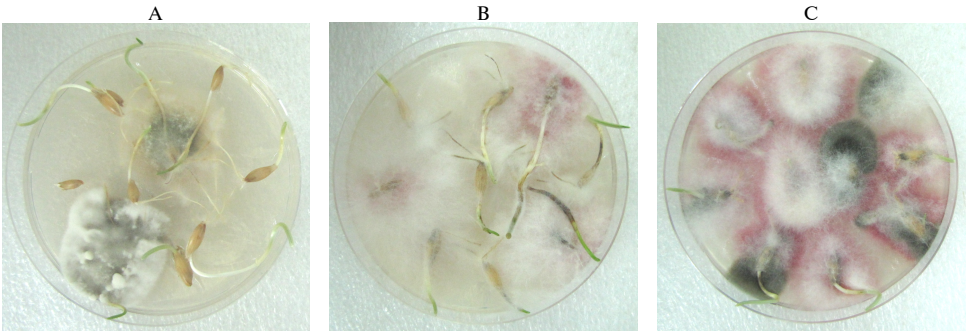
Except for a few not attributed to a certain variety and unnamed, the grain samples belonged to 14 varieties: Adamo ($n = 1$), Argamak ($n = 2$), Borets ($n = 2$), Borrus ($n = 15$), Krechet ($n = 1$), Lev ($n = 9$), LOS 3 ($n = 2$), Skakun ($n = 4$), Scorpion ($n = 1$), Teremok ($n = 1$), Fukhs ($n = 4$), Chernigovskiy ($n = 2$), Chernigovskiy 83 ($n = 1$), and Yakov ($n = 4$).

To study sample infestation, at least 100 grains of a pooled sample were surface sterilized with 70 % ethanol and placed on potato-dextrose agar (PDA); the fungi colonies grown from grains were registered in 10-14 days [21]. Fungi species was attributed using the keys [22-24]. Grain sample infestation with fungi (%) was calculated as the portion of grains infested with certain fungal species or genus to the total number of grains analyzed. Proportions of particular species (%) in the *Fusarium* or *Alternaria* pathogen complexes were determined in each sample as the ratio of grains infected with particular fungi species to the number of grains infected by all species of the genus.

Statistical processing, including calculation of correlation coefficients was performed using Microsoft Excel 2010 and Statistica v. 6 software.

Results. Among the studied samples of oats grown in the Northwest region, the largest group was represented by variety Borrus (Germany) with the proportion of 32 %. This variety has been regionalized in Russia since 1982 and is still in demand from the agricultural manufacturers due to its high yield and tolerance to diseases. Lev (Nemchinovka Moscow Agricultural Research Institute) with 18 % was the next most common variety. Skakun, Fukhs, and Yakov varieties were more rare, and their portions not exceeded 8 %.

Mycological analysis of oat infestation showed the presence of different fungal species of the genera *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, etc. *Fusarium* and *Alternaria* species were most common among the micromycetes identified (Fig.).



***Fusarium*, *Alternaria*, *Bipolaris*, *Epicoccum* infection in oat grains** (surface sterilized, 10 days culture on potato-dextrose agar): A — Borrus variety (Leningrad region), B — Skakun variety (Arkhangelsk region), C — Lev variety (Pskov region) (2014).

***Fusarium* and *Alternaria* fungi infection in oats grains in the North-West district regions**

Region	<i>Fusarium</i>			<i>Alternaria</i>		
	portion of infested samples, %	grain infestation, %		portion of infested samples, %	grain infestation, %	
		average ($M\pm m$)	min-max		average ($M\pm m$)	min-max
Arkhangelsk region	71.4	9.3±10.8	2-27	71.4	1.5±1.4	1-3
Vologda region	100	18.7±14.8	2-64	100	48.0±13.0	24-70
Leningrad region	83.3	6.1±8.5	1-26	91.6	23.7±20.2	1-78
Novgorod region	90.0	17.5±17.6	5-40	80.0	24.1±23.3	1-57
Pskov region	100	8.7±10.3	1-29	100	42.8±34.6	14-85
Total for the region	88.9	12.1±14.5	1-64	91.1	32.0±24.0	1-85

Fusarium infection was found in 88.9 % samples analyzed. Average grain

infestation in the North-West district regions ranged from 6.1 to 18.7 %, the maximum value was 64.0 %. *Alternaria* fungi were found in 91.0 % grain samples studied. Average infestation ranged 1.5 to 48.0 % in the regions, the maximum value was 85.0 % (Table).

Analysis of *Fusarium* species composition demonstrated predominance of *F. poae* fungi on oat grains from all the studied areas. Its portion in the fusarium complex was 73.9 % in the Arkhangelsk region, 80.5 % in the Vologda region, 47.8 % in the Leningrad region, 88.0 % in the Novgorod region, and 59.0 % in the Pskov region. The relatively weak *F. poae* pathogen is usually localized in the flower film and does not penetrate deep inside the grain. However, a high level of this fungus infestation reduces both forage and seed grain quality. Usually, *F. poae* is frequent in oats [25]. It is capable of producing the NIV trichothecene metabolite with highly toxic properties, but its MPC in the grain has not been standardized. *F. sporotrichioides* Sherb. and *F. langsethiae* species were the next ones in occurrence. *F. langsethiae* was detected for the first time in the territory of the Arkhangelsk region. All isolates of this species were obtained from the cereals grown in the Velsk region, bordering with the Vologda region, where *F. langsethiae* is a typical representative of fusaric fungi in the grain [26]. Currently, it covers the entire area of the European part of Russia, which requires careful monitoring of T-2/HT-2 in this area harvest.

Besides these species, *F. anguioides* Sherb., *F. avenaceum*, *F. graminearum* Schwabe, *F. incarnatum* (Desm.) Sacc., *F. subglutinans* (Wollenw. et Reinking) P.E. Nelson, Toussoun et Marasas, *F. tricinctum* (Corda) Sacc. were found at a low frequency in the North-West.

Compared to *Fusarium*, *Alternaria* species were more common. Toxin producing *A. tenuissima* (Nees et T. Nees: Fr.) Wiltshire was the predominant species, infectious *A. arborescens* E.G. Simmons. was less common. The species of the *A. infectoria* complex were low frequent. This species composition and the degree of contamination have also been detected in the cereals in the European part of Russia in previous years [27-29]. Toward the south of the Arkhangelsk region, average *Alternaria* infestation varied depending on the region, but reached high values everywhere (57-85 %). In most cases, the proportion of *A. tenuissima* and *A. arborescens* was 66-86 % of the total count of *Alternaria* isolates.

Lev, Adamo, Yakov, and Krechet were the most *Fusarium*-infected varieties with an average infestation rate of more than 20 %, therefore they were attributed to the group of highly susceptible oat varieties cultivated in the north-west of Russia. A significantly higher rate of *Alternaria* infestation was observed in variety Lev compared to other varieties analyzed ($p \leq 0.05$).

Identification of different amounts of mycotoxins in oat grains in the samples from all areas has been reported earlier [10]. Thus, T-2/HT-2 toxins produced by *Fusarium* fungi were found in 60.7 % samples. MPC exceeding was recorded for these mycotoxins in five grain samples from the Vologda (Vologda, Gryazovets, Totem districts), Leningrad (Lomonosov district), and Novgorod regions (Shimsk district). According to our data, *F. langsethiae* was most responsible for grain T-2/HT-2 contamination. Correlation between *F. langsethiae* grain infestation and the content of these mycotoxins was positive with r amounted 0.7 ($p \leq 0.05$). No significant relationship between the presence of *F. sporotrichioides* — a widespread T-2/HT-2 producer — and the amount of mycotoxins in the grain has been found. The DON mycotoxin was detected in 62.5 % of the analyzed oat samples [10], and the MPC excess was recorded in two samples only, i.e. in Borrus (Khvoinsk district, the Novgorod region) and Skakun varieties (Ust'yansk district, the Arkhangelsk region) at 1159 и 1990 $\mu\text{g/kg}$, respectively. *F. graminearum* and *F. culmorum* were the main DON producers.

F. graminearum fungus which has been previously considered as a typical pathogen of cereals cultivated in warm and humid climate was recently first identified by us in the north-west Russia [30]. The problem of the emergence of dangerous pathogens and their adaptation to new areas is of particular importance due to global warming [31]. This once again confirms the need to conduct a thorough seed phyto expertise to prevent the introduction of pathogenic organisms uncharacteristic for the seeds into new regions.

A total of 29 % of these oat samples were found to contain AOL [10]. A considerable amount of this metabolite was identified in the grains from the Novgorod (Khvoyninsk district) and Pskov (Pechera district) regions — 1159 and 1545 µg/kg, respectively. No significant relationship between AOL contamination and *Alternaria* grain infestation has been found neither for individual species, nor for the species taken together.

Fungal growth and mycotoxin production proceed at different intensities and largely depend on many factors (i.e. fungus species and strain, host plant genotype, infection timing and environmental conditions). Furthermore, coexistence of various microorganisms, including toxin producing ones, on the same nutrient-rich substrate involves various types of interaction. In addition to the direct competition for nutrients and living space, the interaction of organisms may result in the changes in metabolic activity and affect mycotoxin production [32, 33]. Evaluation of the mutual effects of mycobiotal components on the quality of the grain is the priority direction of modern research. Close monitoring of the species composition of pathogens in crops makes it possible to track the emergence of new dangerous fungal toxin producing species and prevent a decrease in the quality of feed and food.

Thus, we have estimated *Fusarium* and *Alternaria* infestation of 56 oat grain samples (*Avena sativa* L.) in 14 varieties (Adamo, Argamak, Borets, Bor-rus, Krechet, Lev, LOS 3, Skakun, Scorpion, Teremok, Fukhs, Chernigovskiy, Chernigovskiy 83 and Yakov, as well as uncertain and unnamed ones) from five regions (Arkhangelsk, Pskov, Vologda, Leningrad, and Novgorod regions). We calculated sample fungal infestation and the portions of particular species (%) in the *Fusarium* or *Alternaria* pathogen complex for every sample. Mycological analysis revealed the presence of different fungal species belonging to the genera *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, etc. *Fusarium* and *Alternaria* fungi appeared to be most common. *Fusarium* infection was detected in 88.9 % analyzed oat samples (with average grain infestation ranged from 6.1 to 18.7 %, and the maximum value of 64.0 %), and *Alternaria* fungi were found in 91.0 % grain samples (with 1.5 to 48.0 % infection, and the maximum value of 85.0 %). Three *Fusarium* species, the *F. poae*, *F. sporotrichioides* and *F. langsethiae*, which are the producers of trichothecene mycotoxins were the most high frequent *Fusarium* fungi species. *F. anguioides*, *F. avenaceum*, *F. graminearum*, *F. incarnatum*, *F. subglutinans*, and *F. tricinctum* were identified as low frequent in the North-West region. *F. langsethiae* was first found in the territory of the Arkhangelsk region, the most northern border of this fungus habitat in Russia. A high positive correlation between *F. langsethiae* detection and accumulation of T-2/HT-2 toxins in grains was found. *Alternaria* fungi were mainly presented by toxin producing species *A. tenuissima* and *A. ar-borescens* (66-86 % of the total number of *Alternaria* isolates). Lev, Adamo, Yakov, and Krechet varieties were the most infected by *Fusarium* (average infestation rate of more than 20 %). A significantly higher *Alternaria* infection in variety Lev compared to other oat varieties was observed. Evaluation of the mutual effects of mycobiotal components on the quality of the grain should be considered the priority of modern research. To track the emer-

gence of new dangerous fungal toxin producing species and prevent a decrease in the quality of feed and food, close monitoring of the species composition of pathogens in crops is required.

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IDENTIFICATION OF ROOT ROT PATHOGENS ISOLATED ON SPRING GRAIN CROPS IN REPUBLIC OF MORDOVIA

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Abstract

The root rot caused by fungi of *Fusarium* and *Bipolaris* genera has damaged both winter and spring cereals. The specific range of the disease agents is characteristic to certain ecological and geographical areas. Root Rot symptoms on wheat (*Triticum* L.), rye (*Secale cereale* L.), oats (*Avena* L.) and barley (*Hordeum* L.) plants are similar. The soil is the basic source of the infection. Also mass infection has been transferred by seeds in the years of abundant precipitations. Pathogenic properties of the root rot agents is due to their ability to develop hydrolytic enzymes and toxins, such as helminthosporol, helminthosporal, victoxin in *B. sorokiniana* strains, isomarticin, zearalenone, diacetoxyscirpenol, nivalenol in *Fusarium* spp., etc. The *Fusarium* and *Bipolaris* species are facultative parasites. In the paper the study of fungal species causing root rot and leaf spot of cereals in the Republic Mordovia territory, and also pathogenic properties of widespread and infrequent *Fusarium* spp. has been represented. Damaged plants of spring wheat, barley and oats (a total of 38 cultivars) in heading stage were sampled. The infected leaves and roots were cut into fragments, aseptically sterilized and placed in Petri dishes on 2 % potato-glucose agar. During growth, the mycelia slices were transferred on a new nutrient medium. As a result, the pure cultures were isolated and the morphology of their colonies were studied. For the evaluation of morphology uniformity of strains, we isolated not less than 20 single spore cultures of fungi using serial cultivations of spore suspensions. Pathogenic and toxic properties of *Fusarium* spp. and *Bipolaris* sp. strains were studied in bio-test on seeds of susceptible wheat cultivar Mironovskaya 808. Finally, there were a total of 457 isolates of pathogenic and saprophytic fungi. No clear specialization of fungi species to cereals was shown. The several species of genera *Fusarium* (*F. heterosporum*, *F. sporotrichioides*, *F. oxysporum*) and *Bipolaris* (*B. sorokiniana*) prevailed on roots of wheat, barley and oat plants. Isolates of *F. redolens*, *F. verticillioides*, *F. tricinum* were few. There were many accompanying saprophytic isolates allocated together with pathogenic fungi, such as *Alternaria alternata*, *Mortierella elongata* var. *elongata*, *Papulaspora appendicularis*, *Clonostachys rosea* f. *catenulata*, *Acremonium strictum*, *Trichoderma hamatum*, etc. As a result of monosporous selections, 24 strains of *Fusarium* spp. and *Bipolaris* sp. with stable morphology have been isolated. The wide range of plant-hosts was found out for *F. heterosporum*, *F. sporotrichioides*, *F. oxysporum* and *Bipolaris sorokiniana*. The widespread species of fungi had various pathogenic and toxic properties, indicating their strong intraspecific variability. Rare fungal species possessed toxicity to test-plant seedlings, but were not pathogenic. This fact possibly explains their low frequency in the root rot mycobiota. *B. sorokiniana* strains were highly pathogenic and toxic to tests plants.

Keywords: root rots, *Fusarium*, *Bipolaris*, isolates, morphology of colonies, culture, pathogenicity, toxicity.

The root rot caused by *Fusarium* and *Bipolaris* fungi is known to be prevalent in various areas of cereal cultivation [1, 2]. They infect both winter and spring cereals throughout the growing period [3], and the direct loss of grain is estimated at 20-50 % and more [4, 5]. The species composition of root rot pathogens is associated with specific ecological and geographic regions and is mixed, as a rule. Thus, fusarium helminthosporal root rot with predominance of *Fusarium* species is found in the areas of winter wheat cultivation, that is in the Central Black Earth and the North Caucasus regions, while *Bipolaris sorokiniana* is found mainly in spring crops in the non-Chernozem zone (North-West, Central, and Volga-

Vyatka regions) [6, 7].

Root rots in wheat, rye, oats, and barley are manifested with similar symptoms [8, 9]. This type of disease is characterized by uneven local spreading. The soil is the basic source of infection. In the years with abundant precipitation, infection transfer via seeds is intensified. Root rot epiphytoticities are usually preceded by a long period of infection accumulation in the soil. Pathogenic properties of the root rot agents are due to their capability of developing hydrolytic enzymes and toxins, e.g., *B. sorokiniana* produces helminthosporol, helminthosporol, victoxin, and cytokinin; *Fusarium* produces isomarticin, zearalenone, diacetoxyscirpenol, nivalenol, etc. [10]. Fungal metabolites have toxic effects on the sprouts and shoots in the period of active mycelium growth [11, 12].

B. sorokiniana (*Cochliobolus sativum* Drechs.) mycelium and conidia are the main forms of fungi reproduction, dissemination and dormancy when wintering [13]. In the spring, plant root and stem tissues are infested via the penetration of endogenous mycelium and germinating conidia. Damp weather promotes the development of conidial sporulation which looks as velvety plaque on the roots and plant residues. Conidia spread aerogenically or with raindrops to other plants, including the emerging ears. *B. sorokiniana* infects wheat and barley plants throughout the growing season, and the spores are formed in autumn and spring on stubble. Fungi persist as conidia and chlamydospores in the soil. They may produce pseudothecia with ascus and ascospores on the overwintered affected plant remnants [14].

Fusarium species (*F. culmorum*, *F. graminearum*, *F. heterosporum*, *F. avenaceum*, *F. oxysporum*, *F. solani*, etc.) are the fusarium root rot pathogens [15]. With seed germination in rolls, a thin, fluffy, fast-growing snow-white or bright-crimson mycelium develops [7, 16]. Formation of microconidia and macroconidia is characteristic of the fungi in this genus. Microconidia are unicellular, rarely with 1-2 septa, oval and egg-shaped; macroconidia have 3 to 9 septa, are of various shapes, sizes and curvatures. Most fungi species form colorless or brown unicellular chlamydospores or sclerotia [17]. Pathogenic *Fusarium* species may develop saprophytically on crop roots, but become parasitic with plant weakening so that they destroy the root system, and inhabit the tillering node and stem bases. Conidia are spread by air flow.

Root rot causal agents have a wide specialization, are capable of infecting not only cultivated and wild cereals, but also other families [4, 13, 18]. By the nature of their interactions with plants, *Fusarium* and *Bipolaris* species are facultative parasites. Wheat, rye, barley, and oat root rots cause very harmful diseases in cereals despite its hidden symptoms. In some cases, root rot can lead to almost total loss of plantings.

In recent years, cereal root rots have widely spread and cause considerable damage [5, 6]. Failure to comply with crop rotation, monocultures of one or another cereal species, and low agricultural machinery create unfavorable conditions for plant growth and contribute to the accumulation of pathogenic fungi in the soil [13, 19]. Root rot infections lead to the rotting of roots and root collar, resulting in the inhibition of growth, yellowing and drying of leaves, delayed ear formation, grain undersizing, and destruction of productive stems. A detailed study of *Fusarium* and *Bipolaris* species complex makes it possible to identify the environmental aspects of plasticity, confinement to specific climatic conditions, and trophic specialization in fungi.

The purpose of this work was to identify the fungal species that cause cereal root rot and leaf spot in the regions of the Republic of Mordovia, and to study the pathogenic properties of common and rare *Fusarium* species.

Technique. Spring wheat (*Triticum* L.), barley (*Hordeum* L.), and oats

(*Avena* L.) samples (a total of 38 variety samples) were collected in 2011 in Chamzinka, Temnikovo, Dubyonky, Staroe Shaigovo, Atyur'ev and Elniky districts of the Republic of Mordovia (Middle Volga region of the Russian Federation) from industrial crops. The plants in the heading stage had the symptoms of leaf spots and root rot infestation.

Fungal pathogens were isolated as described [20]. Plant samples with the symptoms of infestation were thoroughly washed with water and dried on filter paper. The areas of infected tissues (leaves and roots) were cut into 3-7 mm fragments, sterilized in 50 % alcohol for 1 minute and aseptically placed in Petri dishes on 2 % potato-glucose agar, 4-5 cuts per dish. Petri dishes were placed in a thermostat at 22-24 °C. The growth of fungi was daily recorded. With the growth, mycelium pieces were passed to a new growth medium in the centers of Petri dishes. As a result of 2-3 successive passages, pure cultures (isolates) were obtained and examined for the presence of spores for species identification using an Olympus CX41 microscope (Olympus Corporation, Japan) at $\times 850$. Species were identified using the V.I. Bilay [21], B.A. Khasanov [18], F.M. Dugan [22], W. Gerlach and H. Nirenberg [23], W.C. Snyder and H.N. Hansen [24], E.G. Simmons [25] keys. To evaluate the uniformity of morphological and cultural traits, at least 20 monoconidial fungal cultures were repassaged. Monoconidial cultures were obtained by subculturing serial spore suspension dilutions.

Pathogenic and phytotoxic properties of the strains were studied using seed bioassays [11]. The sprouts of wheat variety Mironovskaya 808 susceptible to all root rot pathogens were used as the host plant. To detect pathogenic properties, the tested plant seeds were germinated in conidia suspension (10^6); to detect phytotoxicity, the seeds were treated with the medium filtrate. Pathogen strains were differentiated into four groups according to their pathogenicity and toxicity, i.e. nonpathogenic and nontoxic (0-30 % plant growth inhibition), low pathogenic and low toxic (31-50 % inhibition), moderately pathogenic and moderately toxic (51-70 % inhibition), and toxic and pathogenic (inhibition above 70 %).

Fungal isolate pathogenicity and toxicity were estimated by inhibition of germination, and by slowing down the sprout development and especially root development so far as the root length is the most informative indicator of diseases of this etiology. The root length in the seeds germinating in water (control) was taken as 100 %.

All experiments were performed in 3 replicates. Statistical processing was conducted using Microsoft Excel software package.

Results. In mycological studies, a total of 457 pathogenic and saprophytic fungi isolates of various species were obtained as pure cultures from infected roots and leaves of spring wheat, barley, and oats. No clear specialization of fungi species to cereals has been shown (Table 1).

Fusarium (*F. heterosporum*, *F. sporotrichioides*, *F. oxysporum*) and *Bipolaris* (*B. sorokiniana*) isolates were predominant among the hemibiotrophic fungi. *F. redolens*, *F. verticillioides*, and *F. tricinctum* species were low frequent. Along with pathogenic species, a large number of isolates belonging to concomitant microflora were isolated as pure cultures. These were saprophytic fungal species *Alternaria alternata* (Fr.) Keissl., *Mortierella elongata* var. *elongata* Linnem., *Papulaspora appendicularis* H.H. Hotson, *Clonostachys rosea* f. *catenulata* (J.C. Gilman et E.V. Abbott) Schroers, *Acremonium strictum* W. Gams, *Trichoderma hamatum* (Bonord.) Bainier, etc.

The clones of pathogenic fungi species isolated from cereal leaves and roots had heterogeneous colony topography. To determine the morphological and

1. Incidence of some fungi species isolated from cereals in the Republic of Mordovia (2011)

Pathogen	Wheat	Barley	Oats
<i>Fusarium oxysporum</i>	+++	+++	++
<i>F. heterosporum</i>	+++	+++	++
<i>F. sporotrichioides</i>	+++	+	+
<i>F. redolens</i>	-	+	-
<i>F. verticillioides</i>	-	-	+
<i>F. tricinctum</i>	-	-	+
<i>Bipolaris sorokiniana</i>	+++	+++	+
<i>Alternaria</i> sp.	+++	+++	+++
<i>Arthrinium</i> sp.	++	+	++
<i>Epicoccum</i> sp.	++	++	++
<i>Trichoderma</i> sp.	+++	+++	+++
<i>Acremonium strictum</i>	+++	+++	++

Not e. «+» — 1 to 5 isolates of the same species; «++» — 6 to 10 isolates of the same species; «+++» — 11 or more isolates of the same species. Dashes mean that the species isolates have not been identified.

2. *Fusarium* and *Bipolaris* strains isolated from infested cereal samples in the Republic of Mordovia (2011)

Isolate	Species	Origin	Symptoms
Spring wheat			
RMDsug-1k/3	<i>F. heterosporum</i>	Dubyonky district	Root rot
RMDsug-1k/11	<i>F. heterosporum</i>	Dubyonky district	Root rot
RMD-4k/4	<i>B. sorokiniana</i>	Dubyonky district	Root rot
RMA-6l/5	<i>F. sporotrichioides</i>	Atyur'ev district	Leaf spot
RMA-6l/2	<i>B. sorokiniana</i>	Atyur'ev district	Leaf spot
Spring barley			
RMCh-2k/2	<i>F. heterosporum</i>	Chamzinka district	Root rot
RMCh-2k/3	<i>F. heterosporum</i>	Chamzinka district	Root rot
RMT-3k/2	<i>F. heterosporum</i>	Temnikovo district	Root rot
RMD-3k/1	<i>F. heterosporum</i>	Dubyonky district	Root rot
RMCh-2k/4	<i>F. oxysporum</i>	Chamzinka district	Root rot
RME-5k/1	<i>F. oxysporum</i>	Elniky district	Root rot
RME-5k/3	<i>F. oxysporum</i>	Elniky district	Root rot
RMA-7k/1	<i>F. oxysporum</i>	Atyur'ev district	Root rot
RMT-4k/1	<i>F. redolens</i>	Temnikovo district	Root rot
RMK-2k/1	<i>B. sorokiniana</i>	Krasnoslobodsk district	Root rot
RMD-3l/1	<i>F. heterosporum</i>	Dubyonky district	Leaf spot
RMTem-3l/2	<i>F. heterosporum</i>	Temnikovo district	Leaf spot
RMA-7l/1	<i>F. oxysporum</i>	Atyur'ev district	Leaf spot
RMK-2l/2	<i>B. sorokiniana</i>	Krasnoslobodsk district	Leaf spot
RMT-4l/1	<i>B. sorokiniana</i>	Torbeevo district	Leaf spot
RME-5l/2	<i>B. sorokiniana</i>	Elniky district	Leaf spot
RME-5l/4	<i>B. sorokiniana</i>	Elniky district	Leaf spot
Spring oats			
RMS-1k/1	<i>F. verticillioides</i>	Staroe Shaigovo district	Root rot
RMS-1k/7	<i>F. tricinctum</i>	Staroe Shaigovo district	Root rot

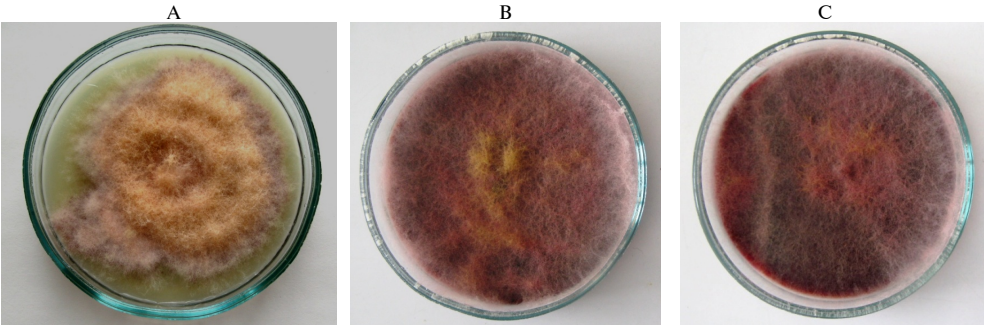


Fig. 1. *Fusarium sporotrichioides* isolates from wheat (*Triticum aestivum* L.) plant roots cultured on 2 % potato-glucose agar: A — fungus clone unstable for morphological and cultural features, B — mono-spore culture of RMA-6l/2, C — mono-spore culture of RMA-6l/5.

cultural characteristics in the populations of various fungal species, to select the strains stable for these traits and to study their pathogenic and toxic properties, isolated clones were subjected to conidial selection. As a result of 2-3 mono-spore

selections, 24 *Fusarium* and *Bipolaris* fungal strains characterized by stable morphological and cultural features were obtained (Table 2).

Typically, morphological and cultural features of pure culture fungal colonies (clones) isolated from the samples of infested plants differed from those subjected to mono-spore selection. Thus, *F. sporotrichioides* clones formed the colonies with inhomogeneous mycelium and uneven edges; sectors with different types of mycelium emerged frequently. Mono-spore culture colonies (strains RMA-6l/2 and RMA-6l/5) had homogeneous mycelium texture, and mycelium was pink, raised by the middle (Fig. 1).

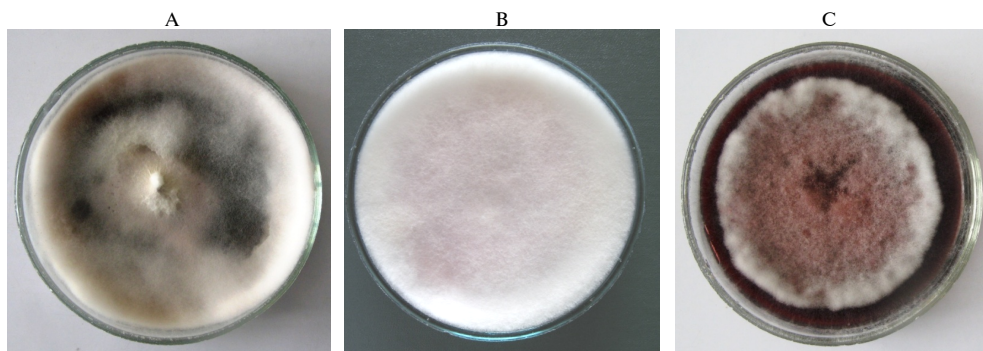


Fig. 2. *Fusarium oxysporum* isolates from wheat (*Triticum aestivum* L.) plant roots cultured on 2 % potato-glucose agar: A — fungus clone unstable for morphological and cultural features, B — mono-spore culture of RME-5k/3, C — mono-spore culture of RMA-7k/1.

This was characteristic of *F. oxysporum* as well. Fungal isolates from infested roots and leaves formed colonies with mycelium uneven in topography, often with membranous and zones of lysis. After 2-3 mono-spore selections, fungal strains usually formed the colonies of homogeneous consistency and color, e.g. white arachnoid mycelium with purple (or olive) inclusions, or stocky burgundy mycelium (Fig. 2). The presence of colonies with different morphological and cultural properties was characteristic of some *F. heterosporium* strains even after repeated conidial selections. Thus, most mono-spore colonies of strains RMCh-2k/2 and RMTem-3l/2 had abundant mycelium of a cream shade, but white homogeneous mycelium was observed in 10-20 % colonies (Fig. 3).

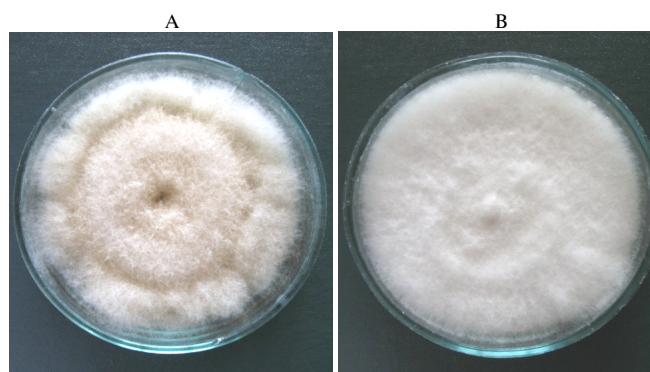


Fig. 3. Mono-spore strain *Fusarium heterosporium* colonies grown on 2 % potato-glucose agar: A — RMDsug-1k/11 isolated from wheat roots, B — RMD-3k/1 isolated from barley roots.

Morphology of *Bipolaris sorokiniana* colonies was typical for the species. Fungal isolates from the roots of cereals usually formed inhomogeneous abundant gray colonies, often containing black sectors. Fungal strains obtained after a series of mono-spore selection had black, smooth, velvety mycelium consisting of conidia plaque. The colony reverse side was

black in both cases (Fig. 4).

The diversity of fungal colonies for morphological and cultural properties within the species depended largely on the genetic peculiarities of multicellular conidia pathogens. It should be noted that morphological traits of mono-conidial

culture colonies of the same species were not associated with fungal specialization. Thus, strain *F. heterosporum* colonies isolated from wheat (PMDsug-1k/3, PMDsug-1k/11) and barley (RMCh-2k/2, RMCh-2k/3, RMT-3k/2) roots had similar morphological and cultural features on 2 % potato-glucose agar. Within a species, fungal strains were characterized by high mono-conidial isolate colony heterogeneity for morphological and cultural features (80-100 %).

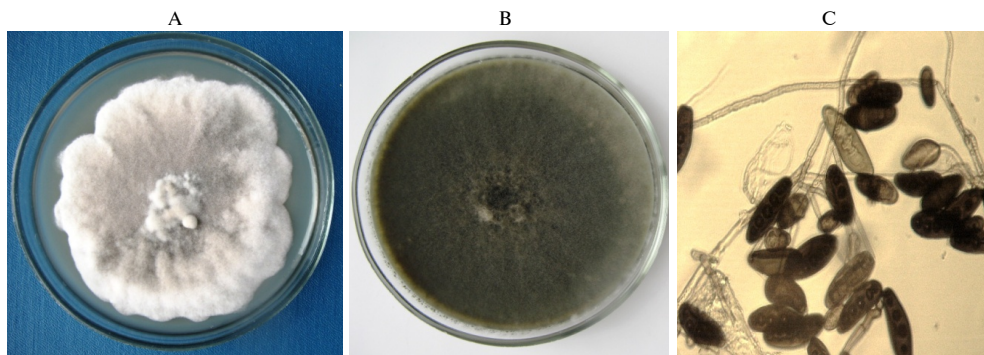


Fig. 4. *Bipolaris sorokiniana* isolates from barley (*Hordeum vulgare* L.) plant roots cultured on 2 % potato-glucose agar: A — fungal clone unstable for morphological and cultural features, B — mono-spore culture; C — strain RME-5l/2 conidia ($\times 850$, Olympus CX41 microscope, Japan).

3. Characterization of *Fusarium* spp. and *Bipolaris* sp. strains isolated from various crops for their pathogenicity and phytotoxicity on the sprouts of the susceptible test variety Mironovskaya 808

Isolate	C	Pathogenicity				Toxicity			
		GA	length, mm ($\bar{X}\pm x$)		PD	GA	length, mm ($\bar{X}\pm x$)		PD
			germs	roots			germs	roots	
<i>F. heterosporum</i>									
RMDsug-1k/3 Wheat		100.0	105.3±3.3	84.4±3.4	NP*	96.7	31.4±2.5	24.1±2.0	T
RMDsug-1k/11 Wheat		46.2	26.8±12.1	26.6±10.0	P	42.9	27.9±13.8	8.7±3.6	T
RMCh-2k/2 Barley		109.4	95.0±3.8	77.7±3.5	NP	90.0	38.7±4.7	21.3±2.3	T
RMCh-2k/3 Barley		103.5	100.7±3.0	64.6±2.5	LP	95.0	9.8±1.0	6.5±0.7	T
RMT-3k/2 Barley		103.4	104.8±4.2	77.9±3.8	NP	93.3	16.7±2.0	12.8±1.6	T
RMD-3k/1 Barley		30.8	15.8±10.3	17.8±8.8	P	—	—	—	—
RMD-3l/1 Barley		103.4	113.7±3.9	95.6±3.3	NP	86.7	28.8±2.7	12.7±1.2	T
RMTem-3l/2 Barley		61.5	40.6±14.2	33.7±10.3	MP	114.3	99.6±16.3	44.8±6.8	MT
<i>F. oxysporum</i>									
RMCh-2k/4 Barley		66.7	63.1±4.3	54.7±11.4	LP	93.8	31.3±5.6	7.0±1.4	T
RME-5k/1 Barley		100.0	109.3±6.4	114.2±5.2	NP	81.3	21.3±3.5	15.7±3.8	T
RMA-7k/1 Barley		50.0	54.1±5.9	49.5±2.7	MP	93.8	11.6±2.4	4.9±0.3	T
RMA-7l/1 Barley		22.2	12.5±6.8	8.5±3.9	P	12.5	3.0±2.2	1.2±0.8	T
<i>F. sporotrichioides</i>									
RMA-6l/5 Wheat		15.4	3.1±2.2	1.2±0.8	P	42.9	17.7±7.9	4.3±2.1	T
<i>F. redolens</i>									
RMT-4k/1 Barley		69.2	85.9±3.0	88.5±3.4	NP	100.0	79.1±6.4	49.6±9.5	MT
<i>F. verticilloides</i>									
RMS-1k/1 Oats		100.0	106.8±4.5	91.0±2.9	NP	103.4	29.6±2.1	24.2±3.0	T
<i>F. tricinctum</i>									
RMS-1k/7 Oats		69.6	64.3±9.7	73.2±9.8	NP	30.0	1.3±0.6	3.2±1.3	T
<i>B. sorokiniana</i>									
RMD-4k/4 Wheat		46.2	4.6±2.5	13.3±2.1	P	62.1	20.1±2.5	13.8±2.2	T
RMA-6l/2 Wheat		34.6	10.7±1.9	17.8±2.7	P	71.4	6.7±3.8	8.5±0.9	T
RMK-2k/1 Barley		42.3	10.0±1.9	18.9±2.6	P	75.0	13.2±2.3	12.2±2.2	T
RMK-2l/2 Barley		61.5	18.4±1.5	11.1±2.4	P	7.1	1.0±0.5	0.8±0.3	T
RMT-4l/1 Barley		57.7	36.4±2.8	25.5±5.3	P	60.0	22.2±4.1	10.1±1.7	T
RME-5l/2 Barley		20.7	5.3±3.9	1.3±3.5	P	64.3	15.7±2.9	14.4±2.7	T
RME-5l/4 Barley		34.5	12.1±3.1	8.1±2.7	P	27.6	7.6±3.6	5.1±3.1	T

Note. C — crop, GA — germination ability, PD — pathogenicity; NP — nonpathogenic, LP — low pathogenic, MP — moderate pathogenic, MT — moderate toxic, P — pathogenic, T — toxic. Dashes mean that tests were not performed under the option.

Fusarium and *Bipolaris* strains are known to have intraspecific variability, which largely determines pathogenesis in this group of fungi. We have studied pathogenic and phytotoxic properties of common (*B. sorokiniana*, *F. hetero-*

sporum, *F. oxysporum*, *F. sporotrichiodes*) and rare (*F. redolens*, *F. verticilloides*, *F. tricinctum*) fungal species (Table 3).

The wide range of plant hosts — wheat, barley, oats — was typical for the first ones. Almost all hemibiotrophic species strains were toxic to the test seedlings. The nature of pathogenic properties in common *Fusarium* species strains was controversial indicating their strong intraspecific variability for the studied features. The strains of rare fungal species were toxic but nonpathogenic to the test seedlings, which may explain their low frequency in the root rot mycobiota. Of all fungal species studied, *B. sorokiniana* strains only were highly pathogenic and toxic to test plants.

Thus, mycological studies of root parts in wheat, barley, and oats harvested from industrial crops in the Republic of Mordovia made it possible to determine the species composition of the major pathogens of root rot which belong to *Bipolaris sorokiniana*, *Fusarium heterosporum*, *F. sporotrichioides*, *F. oxysporum*, *F. redolens*, *F. verticilloides*, *F. tricinctum*, and *B. sorokiniana* species. *F. redolens*, *F. verticilloides*, and *F. tricinctum* species were rare. The clones of pathogenic fungal species isolated from the roots or leaves of cereals, and the strains of the same species resulting from mono-spore selection, differed in their colony topography. The first ones had uneven edges with the sectors of different consistencies mycelium, while even growth and homogeneous mycelium were typical for the second ones. As a result of successive mono-conidial selection, 24 *Fusarium* and *Bipolaris* fungal strains characterized by stable morphological and cultural properties were isolated. The features of the same species strains isolated from various cereals did not differ on 2 % potato agar. Host-related specialization (wheat, barley, oats) of the *B. sorokiniana*, *F. heterosporum*, *F. sporotrichioides* and *F. oxysporum* strains has been identified. Perhaps this is due to their high prevalence in hemibiotrophic populations which persist in the soil, on the roots of weeds and in many crop residues. The frequency of isolates of helminthosporal root rot and brown leaf spot caused by *B. sorokiniana* in all the plant crop samples studied was the highest. This prevalence of *B. sorokiniana* isolates is due to the high toxicity and pathogenicity of this fungus for various cereals.

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Bacillus thuringiensis STRAINS FROM NATURAL SOURCES IN THE LENINGRAD REGION: ISOLATION AND IDENTIFICATION

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Abstract

Recently crystal-forming bacilli of *thuringiensis* group are considered the main microbial producers of insecticides. For these bacilli the high adaptability is characteristic leading to wide distribution of these anaerobic spore-forming bacteria in nature. The same *Bacillus thuringiensis* subspecies and variants were isolated on different continents regardless the presence and prevalence or absence of the host insects of this entomopathogen. In different countries and regions the researchers are searching for new *B. thuringiensis* isolates. In the paper the data are represented on *B. thuringiensis* isolation from natural substrates in the territory of Leningrad province. A total of 24 samples of soil, litter, water, silt, sick and died insects have been collected. The samples were cultivated on fish agar. Among more than 3,000 colonies, 62 ones with specific morphology were found. By microscopy with black aniline dye a total of 12 isolates of 62 isolates tested were found out to form both spores and differently shaped crystals of the endotoxin. The microorganisms were selected with regard to entomocidal and larvicidal activity and identified using H. De Barjac, A.A. Bonnefoi (1968) and O. Lysenko (1985) schemes. The investigation made it possible to classify isolates as *B. thuringiensis* of H₁ (var. *thuringiensis*, isolates №№ 12, 20, 40, 41), H_{3a3b} (var. *kurstaki*, isolates №№ 15, 29, 49) and H₁₄ (var. *israelensis*, isolates №№ 14, 25, 33, 38, 44) serovars. With regard to biological properties (production of acetyl methyl carbonate, lecithinase, pigment, β -exotoxin; pellicle in broth culture; sucrose, mannose, cellobiose, salicin fermentation; starch degradation; proteolytic activity) these isolates are close to standard strains. Isolates are characterized by high productivity, entomocidal and larvicidal activity and can be used as producers of biologicals against insects and larvae. In the isolates of BtH₁, BtH_{3a3b} and BtH₁₄ serovars the titers varied as 2.42×10^9 - 2.78×10^9 ; 1.85×10^9 - 2.15×10^9 and 2.65×10^9 - 3.28×10^9 CFU/ml, respectively. The activity against *Leptinotarsa decemlineata* Say larvae in isolates №№ 12, 41 of the BtH₁ serovar was the same as in standard strain BtH₁ with LD₅₀ at 0.19 %. Entomocidal activity of the isolates №№ 15, 29 and 49 of the BtH_{3a3b} serovar expressed as LD₅₀ for *Ephesia kuehniella* of the 2nd instar was 0.88; 0.82 and 0.92 %, respectively, while in the standard strain BtH_{3a3b} the LD₅₀ was 0.86 %. In the isolates №№ 33, 44 of the BtH₁₄ serovar the titer was the same as in the standard strain, and the activity was even higher compared to the standard. In the isolates №№ 33, 44 the LD₅₀ for the 4th instar *Aedes aegypti* larvae was 0.17×10^{-3} and 0.16×10^{-3} %, respectively, when in standard strain BtH₁₄ it was 0.18×10^{-3} %. Thus, a total of 12 of the isolates which have been identified as *B. thuringiensis* are close to the type isolates on their biological characteristics and promising as producers of biologics with insecticidal action.

Keywords: *Bacillus thuringiensis*, isolation, identification.

High adaptive capabilities of aerobic spore-forming bacteria *Bacillus thuringiensis* in various extreme conditions are the reason for their wide natural occurrence. The bacilli are reported to be isolated from mountain resources [1]. According to the data of a number of authors [2-6], spore-forming bacteria are widely distributed in soil. The bacillus strains are described that can grow at temperature lower than 45-50 °C, while spores withstand heating up to 102 °C [7].

Earlier, it was considered that the crystal-forming bacteria can be mainly found due to screening sick or dead insects from natural populations. However,

currently it is ascertained that these microorganisms are everywhere, i.e. in soil, water, plants, live and dead insects, forest cover, places of insect habitation [8-13]. Entomopathogens have their habitats related to the migration of host insects. The *B. thuringiensis* bacteria are widely spread in the Crimea where they affected wide range of insects due to favorable local conditions with warm and dry climate. In Asia *B. sotto* and *B. dendrolimus* are isolated, while *B. thuringiensis*, *B. entomocidus* and *B. finitimus* are typical of the USA. In Europe, *B. alesti* strains are typical of the areas where mulberry grows [14, 15]. In recent years, however, the same variants of *B. thuringiensis* were isolated on continents differing by their natural conditions, regardless of the host insect population presence or density [16-19]. Annually, *B. thuringiensis* group is replenished with variants (serotypes) differing not only taxonomically, but also by the range of entomocide effects [20-29]. To date, the scientists from various countries have found and identified over 70 isolates of *B. thuringiensis*. The benefits of these bacteria include their safety for people, homoiothermal animals, useful insects and environment [30, 31].

The purpose of this research was to isolate and identify the bacteria belonging to the *thuringiensis* group, and to select strains prospective as producers of biological preparations with entomocide effect on harmful insects.

Technique. A total of 24 samples were collected from various substrates (soil, forest cover, plant parts, sick and dead insects, water, silt) in St. Petersburg, its suburbs and the Leningrad region.

To isolate microorganisms from insects, a drop of hemolymph or a suspension of tissues taken from sick insects was mixed with a physiological solution and plated on fish agar (FA) in Petri dishes sterily by an exhaustive smear technique. The same way, inoculation was performed from other substrates (soil, foliage, etc.). After incubation at 28-30 °C for 7 days the cultures capable of forming crystal endotoxin were identified by microscopy of smears using black aniline dye [32].

The isolates were preliminarily screened for entomocidal and larvicidal activity, and the selected variants were identified using the schemes for *B. thuringiensis* (Bt) offered by H. De Barjac, A.A. Bonnefoi [33] and O. Lysenko [34].

To study the biochemical properties of isolates, instead of liquid differential diagnostic media the indicator paper disks were used (Microgen, Russia) which contain certain substrate amounts in combination with the respective indicator stabilized with film-forming polyvinyl alcohol. To determine the ability to utilize hydrocarbons, daily agar culture (in the amount of one microbiological loop) grown at 29 ± 1 °C was suspended in 0.3 ml of sterile 0.85 % NaCl solution ($\text{pH } 7.3 \pm 0.1$) and then a disk with a hydrocarbon was placed into the test tube. The disks in the sterile 0.85 % NaCl solution were used as a control. The results were recorded in 5-18 hours. Similarly, paper indicator disks were used to assess the indole production, urease activity, and the production of hydrogen sulphide and acetyl methyl carbinol (AMC).

The bacterial yield was estimated on yeast-polysaccharide media in deep culture using Erlenmeyer flasks for 72 hour incubation at 28 °C on a shaker with aeration (220 rev/min). The cell titer was determined by common technique of serial dilutions with FA.

The biological activity of isolates was determined based on the entomopathogen titer causing lethal effect in 50 % of the tested insects which freely ate the inoculated fodder. Several dilutions of liquid culture were prepared that caused death of 10 to 96 % of the insects. Each variant was tested in three replications, and in the control the fodder was not inoculated.

To assess the biosynthesis of thermostable exotoxin, the liquid culture of

the isolate was centrifuged for 15 minutes at 8,000 rev/min. The supernatant was autoclaved at 105 °C for 20 minutes. Dry milk water suspension (2.5 %, 11 ml), 7 g of wheat bran and 2 ml of supernatant (exotoxin), or 2 ml of sterile water (in the control), were placed into glass vessels of 200 ml. Each vessel contained 20 g of substrate (fodder) and 2 ml of supernatant (0.1 ml/g, or 100 µl/g). The supernatant was used undiluted and diluted at 1:2, 1:4, 1:8, 1:16, and 1:32 corresponding to 50.0, 25.0, 12.5, 6.25, and 3.125 µl of exotoxin per gram. The *Musca domestica*, 3-day-old larvae, 25 insects per vessel, were placed on the substrate. The vessels were kept at 28 °C, and in 5 days the puparia were picked out. The flies that flew out were counted, and the percent of flies that died (X), with an adjustment for those in the control, was calculated by the W.S. Abbot's formula [35]:

$$X = \frac{K - B}{K} \times 100 \%,$$

where K and B are the number of flies that flew out in the control and tested samples. LD₅₀ expressed as the amount of exotoxin in microliters per 1 g of fodder was calculated by the Kerber formula [36].

The entomocidal activity was assessed on 2-day-old larvae of the Colorado potato beetle *Leptinotarsa decemlineata* Say. A water suspension of the bacterial culture liquid (CL) was diluted at 1:10, 1:50, and 1:250 corresponding to the content of 10 %, 2 %, and 0.4 %. A potato branch with five leaves was treated on the two sides with the bacterial culture in the appropriate dilution (vs. water in the control), and put into a vial with water, and then the vials were placed at the angle of 45 into the crystallizer with filter paper at the bottom. Using a brush, 25 larvae were placed on each branch. The vessels were left at room temperature (22-25 °C) for 3 days, after which the fodder was replaced with fresh fodder (untreated). The dead larvae were counted on day 7. The death rate was calculated by the W.S. Abbot's formula for each dilution as adjusted for the death rate in the control [35]. LD₅₀ was calculated as to the Kerber formula [36].

When determining the sensitivity of meal moth caterpillars *Ephestia kuehniella* to the isolates, liquid bacterial culture was tested. In this, 2 ml of the dilution (1.0, 0.5, and 0.25 %) was poured into glass vessels of 200 ml with 5 g of wheat flour and then 2-day-old caterpillars, 25 insects per vessel, were placed there and kept at 26 °C with the death rate recorded on day 10. LD₅₀ was calculated using the Kerber formula [36].

The larvicidal activity of isolates was assessed in accordance to the World Health Organization recommendation [37] on the 4-day-old *Aedes aegypti* larvae of the insectary population. The CL suspension was prepared by 200-, 400-, 800-, and 1,600-fold dilutions with tap water, which corresponds to the conditional CL content of 0.5×10^{-3} ; 0.25×10^{-3} ; 0.125×10^{-3} ; 0.0625×10^{-3} %, or 5.0; 2.5; 1.25; 0.625 µl of CL per liter. An aliquot of 50 ml of the dilution was poured into Petri dishes and then 25 mosquito larvae were placed into each. The dishes were kept in thermostat for 24 hours at 28-30 °C after which the larva death rate was recorded. The death rate for each concentration, with an adjustment for that in the control, was calculated by the formula:

$$X = \frac{M_o - M_k}{100 - M_k} \times 100 \%,$$

where M_o and M_k are the arithmetic mean of dead species in the tested and control variants, respectively. Based on the obtained data, LD₅₀ was calculated in per cent of the larva death rate using the Kerber formula [20]:

$$\lg \text{LD}_{50} = \lg C_M - \sigma (\sum X_2 - 0.5),$$

where C_M is the maximum tested content; σ is the logarithm of ratio of each previous dilution to the next one (dilution factor logarithm); $\sum X_2$ are the ratios between the dead insects and the total number of those for the dilution.

The obtained data were processed using the dispersion analysis method [38] with a confidence interval of 95 %.

Results. On FA, 62 colonies were selected out of 3,000 ones based on characteristic morphological features of *B. thuringiensis* (color, type and consistency). The microscopy revealed endotoxin crystals of various shapes, along with spores, in 12 isolates of 62 those tested.

The obtained isolates were the bacilli with peritrichous flagellation. These are gram-positive facultative anaerobes. They formed vegetative cells (single or forming short chains of 2-4 cells) of $2.5 \times 0.9 \mu\text{m}$ in size, and grew well on solid media such as meat peptone agar (MPA) and potato agar (PA). The optimum growth temperature was 28-30 °C. After 48 hours, flat gray-white colonies of round or irregular shape, fine-grained or rough, of viscous consistency, were formed on agar without any changes in the color of the nutrient medium. The cells contained oval spores of $1.1-1.3 \times 0.8-0.9 \mu\text{m}$ (length \times width) and crystalline endotoxin (crystal) located subterminally. In isolates №№ 12, 20, 40, and 41, the crystal of $1.1-1.3 \times 0.9-1.2 \mu\text{m}$ (length \times width) was a regular rhombus in shape with blunt ends and distinct edges, isolates №№ 15, 29, and 49 had crystals of $0.9-1.4 \times 0.9-1.3 \mu\text{m}$ (length \times width) with a prolate regular rhombus shape, and isolates №№ 14, 25, 33, 38, and 44 had crystals of irregular shape sized $0.2-1.1 \times 0.1-0.9 \mu\text{m}$ (length \times width).

On physiological, biochemical and serological properties the isolated bacilli were classified as *B. thuringiensis* and grouped into three serovars (BtH₁, BtH₁₄ and BtH_{3a3b}) (Table 1).

1. The main physiological and biochemical properties of the isolates *Bacillus thuringiensis* (Bt) from natural substrates in the Leningrad region

Isolate №	1	2	3	4	5	6	7	8	9	10	11
12	+	+	-	+	+	+	+	+	+	+	+
20	+	+	-	+	+	+	+	+	+	+	+
40	+	+	-	+	+	+	+	+	+	+	+
41	+	+	-	+	+	+	+	+	+	+	+
BtH ₁ (standard)	+	+	-	+	+	+	+	+	+	+	+
15	+	+	-	-	-	-	-	+	+	+	+
29	+	+	-	-	-	-	-	+	+	+	+
49	+	+	-	-	-	-	-	+	+	+	+
BtH _{3a3b} (standard)	+	+	-	-	-	-	-	+	+	+	+
14	+	+	-	-	+	-	-	-	-	+	+
25	+	+	-	-	+	-	-	-	-	+	+
33	+	+	-	-	+	-	-	-	-	+	+
38	+	+	-	-	+	-	-	-	-	+	+
44	+	+	-	-	+	-	-	-	-	+	+
BtH ₁₄ (standard)	+	+	-	-	+	-	-	-	-	+	+

Note. 1, 2, 3, 4 — production of acetyl methyl carbinol, lecithinase, pigment, and β -exotoxin, respectively; 5 — film on beef-extract broth (BEB); 6, 7, 8, 9 — utilization of saccharose, mannose, cellobiose, and salicin, respectively; 10 — starch decomposition; 11 — beef-extract gelatin (BEG) proteolysis; BtH₁, BtH_{3a3b}, BtH₁₄ — serovars; "+" and "-" — manifestation or no manifestation of trait. The data were processed by dispersion analysis with a confidence interval of 95 %.

To the *B. thuringiensis* var. *thuringiensis* BtH₁ serovar the authors referred isolates №№ 12, 20, 40, and 41 that utilize peptone, meat and fish broth, and protein-rich yeast as nitrogen sources, decompose glucose, mannose, levulose, saccharose, maltose, cellobiose, glycerine, and salicin with acid produced, and do not utilize galactose, arabinose, xylose, rhamnose, lactose, raffinose, manitol, dulcitol, sorbitol, inulin, and inositol. These isolates decomposed gelatine, peptonized milk, hydrolyzed starch, utilized citrates, and produced acetyl methyl carbinol, with no pigments or urease formed. They did not utilize indole or hydrogen

sulphide, and reduced nitrates to nitrites. They were positive in flagellar antigen test with standard antiserum at 1:6400 as *B. thuringiensis* var. *thuringiensis* BtH₁.

Isolates №№ 15, 29, and 49 were classified as the *Bacillus thuringiensis* var. *kurstaki* BtH_{3a3b} serovar. They utilized peptone, meat and fish broth, and protein-rich yeast as nitrogen sources, decomposed glucose, levulose, maltose, cellobiose, glycerine, and salicin with acid production; they did not utilize galactose, arabinose, xylose, mannose, saccharose, rhamnose, lactose, raffinose, mannitol, dulcitol, sorbitol, inulin, and inositol; and they decomposed gelatine, peptonized milk, hydrolyzed starch, and utilized nitrates. They synthesized acetyl methyl carbinol, with no pigments or urease synthesized. They did not utilize indole or hydrogen sulphide, and reduced nitrates to nitrites. They were positive in flagellar antigen test with standard antiserum at 1:6400 as *B. thuringiensis* var. *kurstaki*.

The *Bacillus thuringiensis* var. *israelensis* BtH₁₄ serovar included isolates №№ 14, 25, 33, 38, and 44. They utilized peptone, meat and fish broth, and protein-rich yeast as nitrogen sources, fermented glucose, maltose, levulose, trehalose, glycerine; they did not ferment saccharose, xylose, lactose, arabinose, galactose, rhamnose, raffinose, mannose, dulcitol, sorbitol, mannitol, inulin, or salicin. They did not assimilate cellulose, or decompose esculin, or release hydrogen sulphide. They produced ammonia, acetyl methyl carbinol, and lecithinase. They reduced nitrates, decomposed gelatine, peptonized milk, and discolored lacmus. Based on the test with standard antiserum diluted at 1:6400, they were classified as *B. thuringiensis* var. *israelensis* (BtH₁₄). They produced crystal endotoxins of an irregular shape, and did not produce exotoxin.

The obtained data (Table 2) evidence a high workability of the BtH₁ isolates. By their biological characteristics, the isolates № 12 and № 41 were not inferior to standard BtH₁.

The results of assessing the productivity and larvicidal activity for the mosquito larvae of the BtH₁₄ isolates (Table 3) also indicate a high workability of the isolates. The isolates № 33 and № 44 that are not inferior to standard by the spore titers of and LD₅₀ for mosquito larvae are of special significance.

2. Biological characteristics of *Bacillus thuringiensis* var. *thuringiensis* BtH₁ isolated from natural substrates in the Leningrad region ($\bar{X} \pm x$)

Isolate №	Cell titer, $\times 10^9/\text{ml}$	Amount of exotoxin (LD ₅₀ for L ₂ of <i>Musca domestica</i>), $\mu\text{l/g}$ of fodder	Entomocidal activity (LD ₅₀ for L ₂ of <i>Leptinotarsa decemlineata</i> Say), %
12	2.78±0.11	3.4±0.2	0.19±0.04
20	2.58±0.13	4.0±0.2	0.26±0.04
40	2.42±0.14	4.3±0.2	0.30±0.04
41	2.61±0.10	3.8±0.2	0.19±0.04
BtH ₁ (standard)	2.68±0.11	3.7±0.2	0.19±0.04

Note. The data were processed by dispersion analysis with a confidence interval of 95 %.

3. Biological characteristics of *Bacillus thuringiensis* var. *israelensis* BtH₁₄ isolated from natural substrates in the Leningrad region ($\bar{X} \pm x$)

Isolate №	Spore titer, $\times 10^9/\text{ml}$	LD ₅₀ for L ₄ of <i>Aedes aegypti</i> , $\times 10^{-3}$ %
14	2.65±0.13	0.25±0.03
25	2.15±0.14	0.23±0.03
33	3.12±0.12	0.17±0.03
38	2.81±0.14	0.24±0.03
44	3.28±0.13	0.16±0.03
BtH ₁₄ (standard)	3.38±0.14	0.18±0.03

Note. The data were processed by dispersion analysis with a confidence interval of 95 %.

The yield in the BtH_{3a3b} isolates (№№ 15, 29, 49) on the yeast-polysaccharide medium varied from 1.85±0.15 to 2.15±0.14 billion spores per ml. The entomocidal activity for isolates №№ 15, 29, and 49 was 0.88±0.04;

0.82±0.04 and 0.92±0.04 %, respectively, vs. the LD₅₀ in the reference strain of 0.86±0.04 %.

Thus, our research confirm the established opinion that the entomopathogenic crystal-forming *Bacilli thuringiensis* (Bt) are found everywhere — in soil, water, forest cover, dead insects, and insect habitats. The identification and biotests showed that the isolates referring to the BtH₁, BtH₁₄, and BtH_{3a3b} serovars by their biological properties and practical significance are close to standard strains. Obviously, analytical selection and proper nutrient media and regimes of incubation can help enhance the practically valuable properties of isolated Bt which can be successfully used as producers of biologicals to control the number of harmful insects.

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