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THE USE OF CARBOHYDRATE METABOLISM GENES FOR POTATO (*Solanum tuberosum* L.) IMPROVEMENT

(review)

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Abstract

Potato (*Solanum tuberosum* L.) is one of the most important crop species in the world. Its nutritional and industrial qualities depend on starch content in tubers. Starch consists of linear (amylose) and branched (amylopectin) glucose polymers. Three main goals of modern potato breeding programs include increment of tuber starch yield, development of potato cultivars with improved amylose or amylopectin content and prevention of cold-induced sweetening. Nowadays some molecular and biotechnological approaches to vary plant characteristics have been developed. Among them the most popular are marker-assisted selection, transgenic technologies, genome editing. But, regardless of the chosen approach, the fundamental stage of successful work is the proper choice of the target gene, which in turn requires detailed understanding of the metabolic pathways for the synthesis and degradation of carbohydrates in plant tissues. Starch metabolism includes rather big number of reactions and requires synergetic work of a great number of enzymes. Moreover, it should be mentioned that in starch formation and degradation participate not only carbohydrates modifying proteins, but some regulatory proteins that are also involved in such pathways. Taking into account the previously published review (V.K. Khlestkin et al., 2017), in which attention is paid to genes that determine the specific physical, chemical and technological starch properties, in the present review the emphasis is made on the current understanding of the starch biosynthesis and degradation processes and the key genes of carbohydrate metabolism enzymes in potato tubers. In the present review, among proteins involved in plant carbohydrate metabolism we have chosen those that play the key roles in potato tubers starch formation and retention. The key proteins are sucrose synthases, starch-phosphorilases, granule-bound starch synthase, α- and β-amylases, acid vacuolar invertase, as well as invertase and amylase inhibitors. The main candidate genes that may influence potato agronomical traits are described. The future work requires analysis of allelic polymorphism of the candidate genes in a wide range of potato species, cultivars and lines, looking for associations with desired agronomic traits. It will allow us to use these genes for marker-assisted selection and as target genes for gene editing.

Keywords: potato, starch, amylose, amylopectin, cold-induced sweetening, starch metabolism

Potato (*Solanum tuberosum*) is the most important world food, fodder, and technical crop. Potato is under cultivation throughout the entire territory of the Russian Federation, in different climatic zones located on a huge space from the southern borders to the polar circle, being one of the main food products.

Starch is the basis of the nutritive value of potato tubers. According to its structure, food starch can be divided into glycemic and resistant one, which is determined by the quantitative ratio of the two polymers, amylose and amylopectin. Amylose represents a direct chain of glucose molecules that is digested longer. Amylopectin has some branches of small glucose chains and is digested faster. Thus, the energy and dietary characteristics of potato depend on the qual-

itative composition of starch grains. Potato as a technical crop is valuable for starch content, which is used in the production of glue, glucose, bioethanol, bioplastics and other products and materials [1-3]. In this regard, one of the important directions of potato breeding is the increase in the specific weight of tuber starch and the creation of cultivars with an increased content of amylose or amylopectin. It is also important to remember that the economic effectiveness of potato cultivation depends not only on the production volume and tubers starchiness but also on the duration of their storage, where the weak link again is starch. Tubers contain an average of 12-18% of starch and 0.5-1.5% of sugars under normal conditions. Storage temperatures below +3 °C cause a protective response of tubers to overcooling, which is accompanied by intense starch deterioration and the accumulation of reducing sugars (glucose and fructose). This is the so-called cold-induced sweetening process, which worsens the commercial qualities of potato tubers.

Therefore, three main tasks are considered relevant for today: the increase in the starch proportion in potato tubers (starch content), modeling the qualitative composition of tubers starch (the ratio of amylose and amylopectin), and prevention of the cold-induced sweetening process and the decrease in the amount of reducing sugars. To solve them, it is necessary to determine the ways of carbohydrate metabolism in tubers first, to identify the key enzymes that regulate these ways, and to identify alleles of genes coding them associated with economically valuable characteristics of tubers. It will give an opportunity to carry out a targeted selection based on the modeling of tubers carbohydrate metabolism to produce potato with the desired properties.

The carbohydrate composition of potato tubers is a compound-complex feature, which is controlled by a set of genetic and external factors [4]. A few decades ago, the sequence of biosynthesis reactions and starch decay in a plant cell, which seemed to be well studied, was determined at the physiological level [5]. However, the modern analysis of genomic and transcriptomic data showed that the schemes of carbohydrate metabolism of plant cells are much more complicated: there are alternative metabolic pathways, and the same reaction can be catalyzed by different enzymes. A large number of proteins, regulating the activity of the main enzymes of carbohydrate metabolism, and, for example, carrier proteins, which determine the spatial localization of key reactions, were revealed.

Thus, the understanding of carbohydrate metabolism mechanisms will make it possible to carry out targeted selection, choose useful alleles of key genes and obtain new cultivars with the desired properties. Therefore, the search for genes affecting the content of sugars and starch in potato tubers arouses great interest in many researchers nowadays [2-4, 6].

Carbohydrate metabolism in potato tubers. Potato starch consists of two polymers: branched amylopectin and linear amylose, the structural unit of which is α-glucose. Starch synthesis occurs in plastids (mainly in chloroplasts and amyloplasts), where both polymers form insoluble granules. Starch can vary in grain structure, the degree of molecules polymerization, and physicochemical properties [1, 6-7].

The metabolism of starch occurs in leaves (in chloroplasts) as well as in tubers (in amyloplasts). Most reactions proceed predominantly equally, but some organ-specific differences also exist. For example, a consistent change in the processes of starch synthesis and decay takes place in leaves within 24 hours. Potato tubers, in turn, synthesize starch throughout their development, accumulating it as an energy-intensive substrate. In leaves, ATP, necessary for starch synthesis, is formed during photosynthesis, and in the amyloplasts of tubers,

ATP is imported from photosynthetic organs. The substrate for the starch synthesis in leaves chloroplasts is ADP-glucose, formed as a result of the Calvin-Benson cycle, while in developing potato tubers sucrose becomes such a substrate, coming from photosynthetically active leaves [3, 8]. Biochemical differences in the ways of starch biosynthesis in potato leaves and tubers imply the existence of distinctive features in the genetic basis of the discussed metabolic processes in these organs.

Despite deceptively simple biochemical reactions, there are still many unresolved issues about carbohydrate metabolism. The presence of many enzymes, the opportunity to carry out reactions by alternative pathways, the sequence of intermediate reactions that is still not determined and their subcellular localization complicate the understanding of the process. For example, it is not clear in which organelles the intermediate stages of starch metabolism happen, which proteins carry out and control the intracellular transport of sugars from the cytosol to amyloplasts. Therefore, several alternative hypotheses exist instead of a single starch metabolism pattern [3, 8-9]. It is also important to understand that different periods of plants life are characterized by different metabolic pathways. In particular, starch biosynthesis in the leaves, when stolons are initiated, during the development of stolons, in the ripened tubers, and in the collected tubers during storage vary significantly.

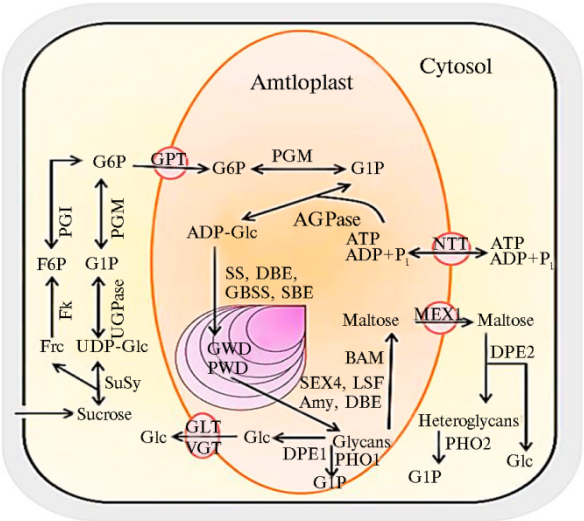
This review will be devoted to the issues of starch biosynthesis in growing potato tubers, in which intense starch accumulation happens.

Sucrose, which is delivered to the cells through the symplast or apoplast, is formed during photosynthesis in the leaves. In the case of the apoplast pathway, sucrose enters the tuber directly through the intercellular space, where it is hydrolyzed to glucose and fructose by apoplast invertases. These generated monosaccharides penetrate into the cells of tubers using hexose transporters. Sucrose enters the cells of the tubers by the symplast way simultaneously, by using sucrose-transporter proteins. By entering the cytosol of tuber cells, sucrose is hydrolyzed by sucrose synthase to UDP-glucose and fructose.

Thus, UDP-glucose accumulates in the cytoplasm of tuber cells. The issue of further UDP-glucose transformations and localization of biochemical reactions remains a controversial one. According to some reports, UDP-glucose is converted into glucose-1-phosphate, which is then converted into ADP-glucose, in its turn, entering the amyloplast and involved in the reactions of polysaccharides biosynthesis there [8]. The alternative model implies that in the cytosol of tuber cells UDP-glucose is first converted into glucose-1-phosphate and then into glucose-6-phosphate; in this form, it is transported using the triose-6-phosphate translocator in the amyloplast. Glucose-6-phosphate is converted into ADP-glucose inside of amyloplasts, from which, under the influence of starch-synthesizing enzymes (starch synthase, starch-branching enzymes, etc.) starch is formed [8, 10]. Both alternative ways imply that ADP-glucose is the direct substrate for the synthesis of amylose and amylopectin in amyloplasts [9]. The residue of ADP-glucose joins the increasing chain with starch synthase (SS, EC 2.4.1.21). While the polysaccharide chain is growing, starch-branching enzymes (BE, EC 2.4.1.18) introduce branching, and amylopectin is synthesized this way [9]. The synthesis of the linear molecule of amylose, in its turn, is carried out by the enzyme of granule-bound starch synthase (GBS, EC 2.4.1.242).

Starch granules with a semi-crystalline structure are formed at the final stage. Although the exact mechanisms of the process are still unclear, it is considered that the final stage of starch granule formation depends on amylopectin only [9]. The very process of starch grains formation is specific for different types of plants and different organs: if there are many small granules in the leaf chloro-

plast, then there are only a few granules in the tuber amyloplast, and they are very large [9].



Potential starch metabolism way in potato tubers [3]. Enzymes: SuSy – sucrose synthase, Fk – fructokinase, UGPase – UDP-glucose-pyrophosphorylase, PGI – phosphoglucoisomerase, PGM – phosphoglucomutase, AGPase – ADF-glucose-pyrophosphorylase, SS – starch synthases, LSF – SEX4-alike enzyme, Amy – amylases, DBE – debranching enzyme; DPE – disproportionating enzyme; PHO – starch phosphorylase, BAM – β-amylases. Translocator proteins: GPT – glucose phosphate transporter, MEX – maltose transporter, NTT – nucleotide translocator; GLT – glucose transporter; VGT – vacuolar glucose transporter. Substances: Frc – fructose, Glc – glucose, UDF-Glc – UDP-glucose, F6P – fructose-6-phosphate, G1P – glucose-1-phosphate, G6P – glucose-6-phosphate, ADP-Glc – ADF-glucose, ATP – ATP, P_i – inorganic phosphorus.

Thus, the starch biosynthesis, starting from the monosaccharide substrates formation and to the starch grains formation, includes many reactions and requires coordinated work of many different enzymes. In addition, it has already been noted that the carbohydrate metabolism involves not only enzymes that modify mono-, di-, and polysaccharides but also regulatory proteins that affect these reactions indirectly, the work of which must also be taken into account.

The primary stage of starch synthesis in tubers depends on the work of saccharolytic enzymes directly, as they contribute to the hexoses accumulation, which is included in starch synthesis further on. However, the final starch accumulation is determined not only by the speed of its synthesis but also by the intensity of its decay, since these processes have been carried out simultaneously and continuously. A sufficient number of enzymes destroying starch, which are specific to the glycoside bond and influence various substrates (amylose, amylopectin, dextran), were described. These enzymes are of different genetic origins and belong to different families [7, 11-14].

Enzymes destroying starch can be divided into two categories, i.e. hydrolytic (α- and β-amylase) and phosphorolytic (α-glycan-phosphorylase). Their comparative activity may vary depending on the stage of development or environmental conditions. Which of enzymes groups has bigger importance is a rather controversial issue. According to some reports, the main contribution to the starch decay is made by the hydrolytic way, although the phosphorolytic one is less energy-consuming [15]. However, starch phosphorylation may not be a sufficient factor by itself [16]. Perhaps, this process makes the starch grains surface more hydrophilic and, thus, more accessible to hydrolytic enzymes, creating selective protein-carbohydrate and protein-protein interactions additionally [14, 16-18].

Starch grains turn into branched or linear forms of polyglycans during the process of decay. Further on, the branched forms are converted into linear glycans as a result of the work of enzymes that remove branching, for example, isoamylase (EC 3.2.1.68) or dextrinase (dextrin 6-α-glucanohydrolase; EC 3.2.1.142) that are specific to the α-1.6-glycosidic bond. At the final stage, linear glycans can be destroyed by β-amylase (EX 3.2.1.2) or starch synthase (EC 2.4.1.21) to neutral sugars [12].

As a result of consistent starch decay, metabolites (triosephosphate, maltose, glucose) are formed in the amyloplasts; then, they are transported to the cytosol with the help of specific transporters [19]. They are involved in the glycans metabolic way there, exposed to the cytosolic phosphorylase – transglucosidase (DPE2, disproportionating enzyme, EC 2.4.1.25), turning into the hexoses phosphates eventually, which, in their turn, are required for the sucrose biosynthesis.

Key genes of carbohydrate metabolism in potato tubers. Many starch metabolism genes are united into genetic families [9]. Different members of one family may play different roles in photosynthesizing and storage organs [3]. The activity of starch metabolism enzymes is regulated both at the transcriptional level (e.g., by circadian rhythms or the presence of sugars) [3] and at the posttranslational level, which includes protein-protein interactions and protein phosphorylation [8].

A major study to identify all genes associated with starch metabolism in the potato genome was carried out in 2017 [3]. As a result, 77 genomic loci coding enzymes of starch metabolism were identified. For comparison, the genome of mustard weed (*Arabidopsis thaliana*) has 46 known genes of starch metabolism, 44 of which have homologs in the potato genome [3]. In addition, new isoforms of many enzymes have been found in the potato genome [3].

The potato genes encoding starch metabolism enzymes that are currently known are given in the table [3, 20-22].

It was shown that among 77 described genomic loci associated with starch metabolism in the potato plant, some genes are expressed in the leaves only, others in all starch synthesizing organs, and the third, the most interesting ones (in the materials of this review) in tubers. In all appearance, the latter group may include genes associated with economically valuable features [3]. The bioinformatic analysis of transcription data [3] revealed several genes, the expression of which is specific for potato tubers. The highest level of tuber-specific expression was observed in genes of the glucose-6-phosphate translocator *GPT2.1*, sucrose synthase *SuSy4*, phosphoglycan phosphatase *SEX4*, starch synthase *SS5* and starch-branching enzyme *SBE3*.

It is known that the most intensive starch synthesis occurs in the process of tubers formation [23]. Therefore, not only genes with high expression in tubers deserve special attention but also those genes, the expression of which grows while the tubers are initiated and developed [3] because they can be regulators of the tubers formation process. Such genes include sucrose synthase *SuSy4,* starch-branching enzyme *SBE3,* glucose-6-phosphate translocator *GPT2.1* and dextrinase *LDE* genes [3]. It is interesting that the level of transcription for the phosphoglycan phosphatase *SEX4* gene, characterized by high expression in the very tubers, is inversely related to the intensity of the tuber growth and the starch synthesis [3].

On the basis of modern concepts of carbohydrate metabolism in tubers, one may offer several candidate genes to solving the above-formulated main objectives of modern potato breeding (the increase in the starch content, the increase in the amylose or amylopectin content, inhibition of cold-induced sweetening). Let us analyze them in details.

Genes determining starch content. Among the genes, the expression of which correlates with the tubers growth, the most interesting one is the sucrose synthase *SuSy4* gene. The largest amount of information was collected for *SuSy4*, indicating its key influence on the starch content in potato tubers [3, 24-27].

Proteins of the sucrose synthase family (EC 2.4.1.13) catalyze the reaction of reversible hydrolysis of sucrose in the presence of UDP to UDP-glucose

and fructose and are found in all higher plants [28]. In the plant cell, SuSy4 is present in the soluble form in the cytosol [24]. Sucrose synthase is the main enzyme resolving sucrose in the endosperm of cereals and potato tubers; it provides a substrate for starch synthesis in the storage organs. Super expression of the sucrose synthase gene *SuSy4* in potato plants leads to the increase in the starch content in tubers and the increase in yield [30].

Genes encoding carbohydrate metabolism enzymes of potato (*Solanum tuberosum* L.) (cit. ex. 3 with supplements)

Protein/protein family	Genes	Expression specificity
ADP-glucose pyrophosphorylase large subunit	*AGPL1, AGPL2, AGPL3*	*AGPL1* in leaves
ADP-glucose pyrophosphorylase small subunit	*AGPS1.1, AGPS1.2, AGPS2*	
Alpha-amylase	*AMY1.1, AMY1.2, AMY23, AMY3, AMY3-like*	*AMY1.1* in leaves
Alpha-glucan phosphorylase	*PHO1a, PHO1b, PHO2a, PHO2b*	*PHO1b* in leaves, *PHO1a* in tubers
ATP-ADP antiporter	*NTT1, NTT2*	
Beta-amylase	*BAM1, BAM2, BAM3.1, BAM3.2, BAM4, BAM6.1, BAM6.2, BAM6.3, BAM7, BAM9*	*BAM3.1* in leaves
Branching enzyme	*SBE1.1, SBE1.2, SBE2, SBE3*	*SBE3* in tubers, expression accelerates with the growth of tubers
Disproportionating enzyme	*DPE1, DPE2*	
Glucan water dikinase	*GWD*	
Glucose transporter	*GLT1*	
Glucose-6-phosphate translocator	*GPT1.1, GPT1.2, GPT2.1, GPT2.2*	*GPT2.1* in tubers, expression accelerates with the growth of tubers
Granule bound starch synthase	*GBSS1*	Expression in tubers is higher than in leaves
Inorganic pyrophosphatase	*PPase, PPase-like*	
Isoamylase	*ISA1.1, ISA 1.2, ISA2, ISA3*	
Limit dextrinase	*LDE*	Organ specificity is not shown, but *LDE* expression accelerates with the growth of tubers
Maltose excess	*MEX1*	
Phosphoglucan phosphatase	*LSF1, LSF2, SEX4, SEX4-like*	*SEX4* in tubers, expression decreases with the growth of tubers
Phosphoglucan water dikinase	*PWD*	
Phosphoglucoisomerase	*PGI, PGI-like1, PGI-like2*	
Phosphoglucomutase	*PGM1, PGM2.1, PGM2.2, pPGM*	
Starch Synthase	*SS1, SS2, SS3, SS4, SS5, SS6*	*SS5* in tubers
Sucrose Synthase	*SuSy1, SuSy2, SuSy3, SuSy4, SuSy6, SuSy7*	*SuSy4* in tubers, expression accelerates with the growth of tubers
Triose-phosphate/phosphate translocator	*TPT, TPT-like*	
UDP-glucose pyrophosphorylase	*UGPase1, UGPase2*	
Vacuolar Glucose Transporter	*VGT3-like*	
Vacuolar invertase	*Pain-1*	
Invertase inhibitor	*INH1, INH2*	
Amylase inhibitor	*SbAI*	

N o t e. Gaps mean the absence of data..

Another important gene, for which the association with high starch content in tubers is shown, is α-glycan phosphorylase. α-glycan phosphorylases (starch phosphorylase, EC 2.4.1.1), the members of the glycosyltransferase family 35 (GT35), play a significant role in the carbohydrate metabolism of plants, animals, and prokaryotes [31-32]. Analogs of α-glycan phosphorylases in plants are also known as starch phosphorylases. This enzyme carries out phosphorolytic starch degradation and catalyzes the reaction of reversible transfer of the glycosyl residue at the end of α-1.4-D-glycan chain in the presence of phosphate to form glucose-1-phosphate. All plants have two different forms of starch phosphorylases – plastidic and cytosolic ones. In its turn, the potato plastidic starch phosphorylase PHO1 is encoded by two homologous genes, which are characterized by the tissue-specific expression: *PHO1b* is expressed in leaves mainly, *PHO1a* – in tubers [3, 33-34]. Even though starch phosphorylases can carry out the reactions of both starch destruction and

starch synthesis, it is shown in vitro that the plastidic form plays a more significant role in the process of starch destruction [35-36]. However, in vivo the evidence of this fact is absent. Also, some information about the ability of starch phosphorylase to synthesize oligosaccharide primer, which is then completed by starch-synthase, was obtained in vitro [15, 37-38].

Genes determining the qualitative composition of starch. As it was said before, the field of the starch use is extensive, and in relation to the specific tasks, it is necessary to obtain starch with different physical and chemical properties, which are determined by the quantitative ratio of amylose and amylopectin directly. Starch with a high content of amylopectin (glycemic starch) has an increased value of nutritional energy and is used for the production of infant and dietetic nutrition. In the industry, such starch is also preferable (economically beneficial) as a raw material for the production of glucose-fructose syrups and bioethanol. High-amylose (resistant) starch is more resistant to the influence of α-amylases, whereby it is used in the production of bioplastics. By having a low glycemic index, such starch is also valuable in dietetics [39]. Starch qualitative composition depends on the work of two enzyme groups – starch synthases (including granule-bonded starch synthase) and starch-branching enzymes.

The genes of six starch synthases isoforms (*SS1*, *SS2*, *SS3*, *SS4*, *SS5*, *SS6*) and the homologous gene of granule-bonded starch synthase *GBSS1* [3] were found in the potato genome. Starch synthases SS synthesize amylopectin polysaccharides and can be found either in the dissolved form or joined to a starch granule. Genetic and biochemical information proves that every isoform of starch synthase SS (EC 2.4.1.21) plays its unique role in the process of amylopectin synthesis. It is considered that SS1, SS2, and SS3 isoforms work one by one directly, synthesizing short, middle, and long chain correspondingly. It is also known that 80% of starch synthase activity in potato tubers is from SS3 [9]. *SS5* isoform starch synthase gene is characterized by the tuber-specific expression, although there is confirmation in vivo of the SS5 direct impact on the starch accumulation and yield of potato. At the same time, the homologous gene *SS5* of corn presumably controls starch accumulation at the stage of grain ripening [3, 40]. However, it is believed that the activity of starch synthase (with the exception of SS5) in tubers does not exceed the same in leaves greatly and the agronomic significance of genes encoding these enzymes is not as important as that of the homologous gene *GBSS1* [3].

Granule-bound starch synthase GBSS1 (EC 2.4.1.242) controls amylose biosynthesis in the forming of starch granules. Many investigations indicate the important economic value of this enzyme [41-44]. GBSS1 join the starch granule directly. *GBSS1*expression in tubers is a little bit higher than in leaves. *GBSSI* was revealed and characterized in many potato cultivars [36, 45-46]. Inactivation of this gene allows obtaining potato, the tubers of which contain amylopectin mainly [47-50].

Starch branching enzyme SBE (EC 2.4.1.18) influences the accumulation of the particular form of starch polysaccharides. SBE catalyzes the formation of points for α-1.6-branches in the polysaccharide chain with different frequency and length of the branched chain. Starch branching enzyme activity was revealed in potato first. The polysaccharide structures formed by the starch branching enzyme are then modified by enzymes that remove branching (DBEs, debranching enzymes, EC. 3.2.1.68), and thus insoluble granules are formed. The activity of the starch-branching enzyme affects the degree of branching of amylopectin directly [52-53].

Many plant species have differences in the expression of particular classes of the starch-branching enzyme [39]. Mutant plants with SBE activity defi-

ciency have the indicative phenotype due to inhibition of starch synthesis and accumulation of large amounts of sucrose and other soluble sugars [39]. For example, pea (*Pisum sativum* L.) has wrinkled fruits, and starch content is reduced by 50% [54]; for corn, the mutation of *amylose extender* (*ae-*) is known, which is accompanied by the decrease in starch synthesis by 20% [55]. At the same time, the starch of such plants consists of amylose mainly, and amylopectin found in them is small branched. High amylose starch in potato was obtained only by inhibiting the activity of several isoforms of the starch-branching enzyme at the same time [56].

Genes determining resistance to cold-induced sweetening. In case of storage at temperatures below +10 °C, reducing sugars accumulate in potato tubers, which, when interacting with α-amino acids, lead to the accumulation of acrylamide and deterioration of taste [57-59]. Therefore, the prevention of potato cold-induced sweetening is extremely important for the food industry [60-62]. Cold-induced sweetening occurs due to the hydrolysis process of polyglycan chains by amylases and the destruction of sucrose by invertases.

As it was said before, starch degradation can be carried out either hydrolytically or phosphorolytically. The hydrolytic way is catalyzed by α-amylases (AMY, alpha-amylase, EC 3.2.1.1) and β-amylases (BAM, beta-amylase, EC 3.2.1.2). Both families include proteins with many isoforms. Nowadays, at least five genes of α-amylases and at least ten genes of β-amylases were identified in the potato genome [3]. α-amylases hydrolyze α-1.4-glycan bonds to form various linear and branched maltooligosaccharides. Two genes of α-amylases – *StAmy1* and *StAmy23* work in potato tubers. In case of low-temperature storage, only amylase StAmy23 is active [63]. β-amylases realize hydrolysis of the non-reducing end of glycan chains associated with α-1.4-glycoside bonds, with the formation of β-maltose [64]. It is shown that the activity of β-amylases of potato increases in the first week of storage at +4 °C significantly [65]. The expression of β-amylases is also closely correlated with the accumulation of reducing sugars in potato tubers stored at positive temperatures of 3-5 °C [66], thus, confirming the importance of β-amylases in the process of cold-induced sweetening. It is considered that among the known genes of β-amylases, *StBAM1* and *StBAM9* have the highest level of transcription in tubers [63].

The sucrose hydrolysis by invertases with the formation of glucose and fructose [4] also leads to the formation of reducing sugars during the storage of potato tubers. Nowadays, it is clearly shown that the main role in cold-induced sweetening of potato is played by acidic vacuolar invertase (Pain-1) (beta-fructofuranosidase, EC 3.2.1.26), catalyzing the irreversible hydrolysis of sucrose. Inactivation of the *Pain-1* gene reduces the accumulation of reducing sugars in tubers at low temperatures [22, 67-70]. This gene was identified in potato, its structure and expression were studied, and single-nucleotide substitutions (SNPs) were found to determine the activity of the enzyme [71-74].

Potato cultivars resistant to cold-induced sweetening have a low transcription of the vacuolar invertase gene, but some lines show high expression of this gene at low enzyme activity [69]. It was found that, in addition to the regulation of the vacuolar invertase work at the transcriptional level, post-translational modification of protein occurs with the participation of inhibitors [75]. Therefore, it is necessary to consider a group of enzymes that indirectly affect the cold-induced sweetening process separately, although they have no affinity for a glycosidic bond and do not interact with sugars and polyglycans. This group should include inhibitors of invertase and amylase.

The sequences of invertase inhibitors' genes were determined for different plant species [76]. Two inhibitors, the St-Inh (*INH1*) and StInvInh2

(*INH2*), were found in the cultivated potato species, affecting the invertase activity and, consequently, the cold-induced sweetening of tubers, which was confirmed by the effect of their overexpression in potato tubers [77]. The potato haploid genome contains one copy of the *INH1* and *INH2* genes localized on the 12th chromosome in tandem orientation and subjected to alternative splicing, and the gene products inhibit apoplast (INH1) and vacuolar (INH2) invertase [76]. It was shown in vitro that *INH2* [78] has the greater inhibitory effect, which is confirmed by a significantly higher level of *INH2* expression in potato genotypes resistant to cold-induced sweetening than in the sensitive ones. In addition, the association of some splice variants of the *NAT2* gene and the variability of its promoter region with the degree of exposure of potato tubers to cold-induced sweetening was reported [76, 79].

Another example of post-translational regulation of genes involved in the process of cold-induced sweetening is associated with the work of the amylase inhibitor. The activity of amylase for potato inhibits by the SbAI gene, which was first cloned from the *Solanum berthaultii* species [21]. The growth of SbAI activity leads to the suppression of amylases and, consequently, to the decrease in the accumulation of reducing sugars in tubers [21]. The presence of protein-protein interactions between SbAI and StAmy23, StBAM1, and StBAM9 [21] proteins was shown with the help of a dihybrid system. Therefore, amylase inhibitor is considered a key regulator of cold-induced sweetening processes of potato tubers caused by the amylase activity.

So, the molecular and biotechnological approaches (marker-mediated selection, derivation of transgenic plants, genomic editing, etc.) already allow changing the desired characteristics of plants. However, regardless of the used approach, the fundamental step that determines the successful result of the work is the right choice of the target gene. In this review, the key enzymes that directly and indirectly can carry out the most important stages of the starch synthesis and decomposition in tubers are identified in a large number of proteins involved in the carbohydrate metabolism in potato tubers. The range of encoding these enzymes candidate genes, allelic variants of which can be associated with economically valuable traits of potato, was determined. Further work requires analysis of allelic variants of these candidate genes in a wide range of cultivars, lines, and samples of wild potato species and identification of associations with the required agronomic traits. It will allow using them as target genes for the development of molecular markers and editing sites for the selection of cultivars with specified characteristics.

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EVOLUTIONARY-GENETIC BASES FOR SYMBIOTIC ENGINEERING IN PLANTS — A MINI REVIEW

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Abstract

Microbe-plant symbioses have a great role in development and evolution of plants providing their mineral (nitrogenous, phosphorous) nutrition, resistance to pathogens and phytophagans and the developmental regulation under stress conditions (R.J. Rodriguez et al., 2009). Construction of the highly efficient symbioses should be based on the knowledge on pathways and mechanisms of partners' coevolution occurring in the natural ecosystems and agrocenoses. Using the model of N_2-fixing legume-rhizobia symbiosis we show that three major stages of its evolution should be simulated using the methods of symbiotic engineering. It should be aimed at: (i) optimization of partners' exchange by C- and N-compounds; (ii) suppression of partners' competition for nutrients and energy obtained from the environment; (iii) activation of partners' altruistic interactions based on the decrease of microsymbiont survival, for example, development of non-reproducible bacteroids by rhizobia. The first approach may be achieved by an increased assimilation by bacteria of the plant-delivered dicarboxylic acids required for the bacteroid nutrition. It is based on the generation of rhizobia recombinants containing the amplified copies of *nif* and *dct* genes encoding for the synthesis and energy supply of nitrogenase. However, this approach is limited by disbalancing the biochemical and developmental processes: a significant (by 70-80 %) increase in N_2-fixing activity is accompanied by a limited increase of plant biomass (by 15-20 %). This limitation can be overcome via construction of bacterial strains optimizing the plant development using the biologically active substances (phytohormones, vitamins, lumichrome) ensuring a complete involvement of N_2 fixation products in the yield formation. The second approach may be implemented by improving the ability of commercial rhizobia genotypes to compete for inoculation of host plants with the aboriginal strains which possess a high virulence combined with a low N_2-fixing activity. Realization of this approach is based on inactivation of genes regulating negatively the early stages of symbiosis development and on the amplification of genes regulating this development positively. The third approach may be realized via manipulations with the rhizobia *eff* genes identified using Tn5 mutants selected directly in fast-growing alfalfa rhizobia (*Sinorhizobium meliloti*) for an increased symbiotic efficiency, i.e. the impact of bacteria on the plant yield. This increase is achieved by knockout of the functions required for autonomous (ex planta) bacteria survival in soil but interfering with the symbiotic cooperation. These functions include synthesis of storage compounds (poly-β-hydroxybutyrate, glycogen), assimilation of «non-symbiotic» (not involved in the nutrition bacteroid) carbon sources (sugars) and formation of the cell surface components inducing the host defense responses (lipo- and exopolysaccharides). Prospects for the further increasing the input of «biological» nitrogen in crop nutrition are associated with establishing the nodular symbiosis in the non-legume (e.g., cereal) plants. The relevant approaches include establishment of the plant ability to form N_2-fixing nodules based on modifications of homologs of legume *Sym* genes (G. Oldroyd et al., 2014), introduction of *nif* genes into mitochondria or plastids which originated from N_2-fixing bacteria during symbiogenes of eukaryotic cell (G. Lypez-Torrejyn et al., 2016), and the construction of novel N_2-fixing cellular organelles (ammonioplasts) providing the optimal conditions for the nitrogenase synthesis and operation.

Keywords: microbial-plant interactions, biological N_2 fixation, nodule bacteria, genetic construction, symbiotic engineering, cellular organelles, symbiotrophic plant nutrition, sustainable crop production

Microbe-plant symbioses (MPS) play a key role in plant nutrition (N_2

fixation, assimilation of soil nutrients), protection of plants from pathogens and phytophages (synthesis of antibiotics and toxins), and well as in the regulation of development and adaptation to stresses (synthesis of phytohormones and vitamins that affect growth processes) [1]. The ecological significance of MPS is determined by the fact that terrestrial plants are a form of life, symbiogenic in its origin: they have colonized the land in close cooperation with microbial communities, consisting of mycorrhizal fungi — glomeromycetes, associated with photo- and heterotrophic bacteria [2]. The creation of environmentally sustainable agrocenoses, the high productivity of which is achieved with the minimal use of chemical fertilizers and protective equipment, requires a significant increase in the symbiotic activity of plants [3, 4].

Despite the high genetic coverage of studies on MPS, microbial preparations for the inoculation of crops are still made almost exclusively on wild-type strains, isolated from natural sources (plants and soil) using analytical breeding [5, 6]. Although genetic control of the symbiotic efficiency (SE), the ability of microorganisms to increase plant productivity, has been studied in great detail [7], genetically engineered and biotechnological approaches have not yet been widely applied to improve this characteristic. The reasons for this lie in the complexity of SE control, which depends on the multifactorial interaction of the genotypes of several partners, which are under the influence of varying external conditions, and also in the absence of genetically grounded programs for managing symbiotrophic plant development. These programs should be based on the mechanisms of their natural co-evolution with microorganisms, which can be studied at the phenotypic [8], genomic [9] and transcriptomic [10] levels.

The optimal model for the development of the methodology for designing the MPS is N_2-fixing legume-rhizobia symbiosis, the development of which from the side of bacteria is determined by two groups of genes. These are *nod* genes that control the synthesis of lipo-chitin oligosaccharide Nod-factors (NF), which activate the nodule development program, and the *nif/fix* genes that determine the synthesis and functioning of the nitrogenase complex in planta [1]. The study of legume-rhizobia symbiosis has shown that in natural conditions the evolution of MPS is aimed at increasing the effectiveness of the cooperative (mutualistic) interaction of partners, determined on the basis of the indicators of their biological productivity, i.e. the number of populations, the rate of reproduction and biomass. In this case, three stages of the symbiosis evolution can be distinguished, on which the SE increases.

Pleiotropic symbiosis is its least specialized form, characterized by a mobile equilibrium of cooperative and antagonistic effects. It depends not only on the manifestation by microorganisms of characteristics, favorable for the host (for example, N_2-fixing activity) but also on the interaction of symbionts with the protective systems of plants, that control the homeostasis of their internal environment. Pleiotropic symbioses are based on the negative feedbacks of the partners, ensuring the stable coexistence of plants and microorganisms, as well as their balanced polymorphism on the grounds of symbiosis [11].

Mutual exploitation of partners is a more specialized and effective form of the symbiosis, based on the equivalent metabolism of plants and microorganisms, including the formation of counterflows of carbon and nitrogen [12, 13]. Thanks to the formation of superorganismal metabolic pathways and energy, positive feedbacks are established between partners: the more N_2-fixation products the plant receives, the higher the activity of photosynthesis is and it supplies more C-compounds to its microsymbionts. An important role in increasing SE is played by the weakening antagonism of the partners, for example, the loss of

rhizobiotoxin synthesis by slow-growing rhizobia [14], and the modification of signal receptor complexes, including the formation by rhizobia of NF and surface polysaccharides, as well as effector proteins, which are transferred to plant cells through the Type III secretion systems [15].

Interspecific altruism is a deeply specialized form of symbiosis, based on the loss of viability by intracellular microsymbionts, modified to perform functions, which are beneficial to the host, for example, rhizobia bacteroids, developing abnormally high nitrogenase activity, which is accompanied by a "refusal" from reproduction. At the same time, the general adaptation of microsymbiont populations increases due to group (interdeme, kin) selection in favor of altruistic clones with elevated SE [16]. In the process of evolution, the symbiotic integrity increases, based on stable regulatory links between the microbial and plant cells of the nodule, as well as between the nodules and the plant organs, in which N_2 and CO_2 are fixed.

The article presents an evolutionarily valid scheme for the design of a highly effective microbe-plant symbiosis. It includes the activation of the target metabolic function of symbionts in combination with an increased ability to compete for the infection of hosts; giving symbionts new growth stimulating functions, which provide a switch of hosts to symbiotrophic development; a consistent increase in the morphometric and biochemical parameters of plants by enhancing the "altruistic" properties of their symbionts.

1. Increase in the nodulation competitiveness (NC) **of rhizobia with changes in genes, controlling the early stages of symbiosis** [17]

Species *Rhizobium* and *Sinorhizobium*	Host plants	Genes (their products)	Increasing NC (%)
Amplification of genes that positively regulate NC			
R. leguminosarum bv. *trifolii*	*Trifolium pratense*	*rosR* (exopolysaccharide synthesis activator)	41 → 69
S. meliloti	*Medicago sativa*	*cmp-107* (hydrophobic protein with unknown function)	40 → 51
S. meliloti	*M. sativa*	*putA* (proline dehydrogenase)	71 → 87
Inactivation of genes that negatively regulate NC			
S. medicae	*M. truncatula*, *M. sativa*	*nolR* (repressor of *nod* genes)	25 → 71
S. meliloti	*M. sativa*	*SMb21195* (ABC-oligopeptide transporter)	57 → 85
S. meliloti	*M. truncatula*, *M. sativa*	*truB* (NADP-dependent dehydrogenase)	50 → 68
R. leguminosarum bv. *viciae*	*Pisum sativum*	*praR* (repressor of biofilm formation)	10 → 90

Note. The increase in NC was determined by joint inoculation of plants with genetically modified and parental strains (1:1).

Competitive processes. The mutually beneficial cooperation of microorganisms and plants in the systems of mutualistic symbiosis is accompanied by intense competition, occurring both between the interacting partners and in the populations of each of them. The most severe competition is observed between different genotypes of symbionts when the hosts are infected. In rhizobia, the ability to compete for the nodule formation (nodulation competitiveness, NC) is determined by an extensive system of *cmp* genes, including positive and negative regulators of early symbiotic functions, i.e. recognition of hosts and signal interaction with them, colonization of the rhizosphere and rhizoplane, as well as root infection [17]. Their study permitted to offer a variety of genetic approaches to increasing the NC, including amplification of positive regulators of this trait and inactivation of its negative regulators (Table 1), as well as combining factors of high SE and NC in one microbial genotype.

In the late stages of nodule development, associated with the transition of plants to symbiotrophic nutrition with nitrogen, the competition between bacteria and plant cells of the nodule for photosynthetic products increases, which come from aboveground organs and are used by rhizobia to provide energy for

nitrogen fixation and reproduction. In this competition, there may be the factors of antagonism of microsymbionts with hosts, including rhizobiotoxins, for example, 2-amino-4-(2-amino-3-hydro-propoxy)-trans-3-enoic acid, formed by evolutionarily primitive and slow-growing symbionts *Bradyrhizobium elkanii*, as well as effector proteins transmitted by bacteria to plant cells through the Type III secretion system (T3SS).

These factors, characteristic of primitive forms of the symbiosis, as it increases its effectiveness, evolve towards mitigating pathogenic effects. Thus, in the evolution of slow-growing rhizobia, a loss of rhizobiotoxin synthesis occurred, which was accompanied by an increase in the N_2-fixing activity of the nodules [14]. Other patterns are characteristic for the evolution of T3SS, which, in the process of diversification of rhizobia, also became more complicated in rapidly growing rhizobia (*Rhizobium*, *Sinorhizobium*) and acquired functions of host specificity regulators, which are additional in relation to NF [15]. Moreover, in evolutionally advanced legume-rhizobia symbiosis, antagonism factors are used to enhance the effectiveness of partner cooperation. For example, cysteine-rich NCR proteins of legumes of the galegoid complex, similar to the factors of plant defense against pathogens (defensins), serve as inducers of the differentiation of endosymbiotic *Rhizobium* and *Sinorhizobium* cells into bacteria, which are unable to reproduce and which possess extremely high nitrogenase activity [18].

Symbiotrophic development of plants. Attempts to construct strains of rhizobia with elevated SE have shown that the enhancement of only one objective function (N_2 fixation) has a limited effect: recombinants of alfalfa rhizobia (*Sinorhizobium meliloti*) with an increased (from 1 to 2-5) number of copies of genes controlling the synthesis of nitrogenase (*nif*) and the supply of bacteroids by dicarboxylic acids (*dct*), provide 70-80% of the increment in plant nitrogen accumulation, but their mass increases only by 15-20% [19]. This disproportion is probably connected to the fact that plant and microbial cells compete for carbohydrates, which are required for supplying energy to the nitrogenase reaction and assimilating its products. With the increased competition of partners for carbon, it is possible to slow the outflow of nitrogen compounds to the above-ground organs. However, such competition can be limited by creating strains of rhizobia that stimulate the development of shoot meristems due to the synthesis of phytohormones or vitamins [20]. In this case, the ratio between the length and mass of shoots and roots increases (the habitus of plants changes), which leads to the most complete use of fixed nitrogen by plants, as well as to the overcoming of the metabolic imbalance of the symbiotic system, which is determined by the energy overexposure due to the enhancement of N_2-fixing activity.

In the evolution of legume-rhizobia symbiosis, the increase in SE was achieved in two ways [1]: a sharp increase in the N_2-fixing activity of bacteria converted into non-viable bacteroids, which is characteristic of microsymbionts of the legumes of the galegoid complex (including *Galegae*, *Trifolieae* and *Vicieae* tribes), and the transfer of plants to the deterministic structure of the nodules, which are typical of the tribes *Loteae* and *Phaseoleae* (in this case, the bacteroids retain the ability to reproduce and, after the death of the nodules, become part of the soil populations). The implementation of the second ("economical") method provided a significant reduction in the energy intensity of the symbiosis, and in the *Phaseoleae* tribe – diversification of the assimilation pathways of fixed and soil nitrogen. This diversification is related to the fact that in nodules of beans and soybeans, fixed nitrogen is included in the composition of special transport forms – ureides (allantoin and allantoic acid), which are transferred to the aboveground organs, allowing plants to combine symbiotrophic and auto-

trophic nitrogen nutrition. At the same time, while implementing the first ("costly") method, symbiotrophic nutrition is limited, since in the nodules of alfalfa, pea, and clover, the transport forms of nitrogen are the same amides, formed in the assimilation of nitrogen fertilizers.

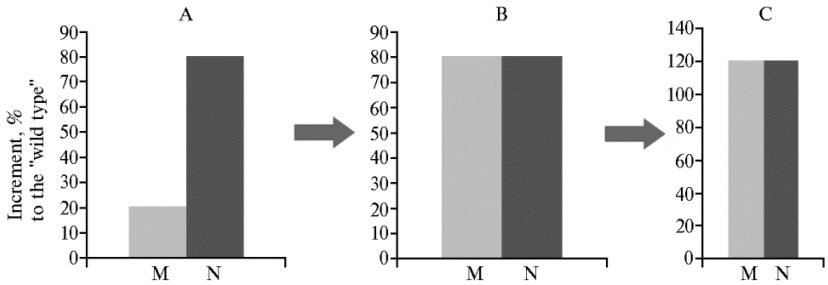
In this connection, one of the directions for constructing symbiotrophic plants can be the combination of a deterministic nodule structure with the ability to activate rhizobia differentiation into non-viable bacteroids [21]. The possibility of such a combination was shown in experiments on the transfer from a one-year barrel medick (*Medicago truncatula*) to birdsfoot trefoil (*Lotus japonicus*) of the *dfn1-1* gene, which controls the development of bacteroides. As a result of the introduction of the wild allele of this gene, *Lotus japonicus*, having retained the deterministic structure of the nodules, acquired the ability, which is characteristic for *Medicago truncatula*, to form monobacterial symbiosomes with an increased degree of differentiation of bacteroides [18].

Interspecific altruism. Loss of free-living functions by bacteria is the most important factor in their transition to mutualistic interaction with plants. In the early stages of the nodule symbiosis evolution in slow-growing rhizobia (*Bradyrhizobium*), phototrophy was lost (it was functionally replaced by the nodule formation system, which provided bacteria with access to plant photosynthesis), as well as diazotrophy (in connection with the specialization of *nif* genes for functioning in planta) [22]. This trend developed in the late stages of the symbiotic evolution, when the rapidly growing rhizobia (*Rhizobium*, *Sinorhizobium*) had the ability to transform themselves into nonviable bacteroids with extremely high N_2-fixing activity. NCR proteins of plants that stimulate this transformation [23] have played a major role in the transition of partners to altruistic relations, which, as the integrity of the symbiosis increases, are transformed from an intraspecific adaptive mechanism limited by a symbiont population into host-controlled "interspecific altruism" [16, 24].

In the framework of genetic engineering programs, this trend can be strengthened by inactivating *eff* genes of rhizobia, which determines the increase in SE due to the loss of functions that are necessary for the autonomous survival of bacteria in the soil, but interfere with the development of effective symbiosis. These include the synthesis of reserve nutrients by bacteria (competes with the catabolism of photosynthetic products supplying the nitrogenase complex with energy), the assimilation of "non-symbiotic" (not participating in the nutrition of bacteroids) sources of carbon (for example, sugars), and the formation of surface components of a microbial cell (exo- and lipopolysaccharides), which serve as the elicitor of plant protective reactions that limit the reproduction of endosymbionts [25]. In the *S. meliloti* – *M. sativa* system, the loss of these functions by bacteria is accompanied by a balanced increase in biomass and nitrogen accumulation in plants, which indicates the optimization of the ratio of their biochemical and morphometric parameters. The relevance of this branch of symbiotic engineering is determined by the fact that when bacteria are developed in the soil, they often lose the signs of mutualism, but they remain virulent and pass to parasitizing on plants [26]. Obviously, the adaptive potential of the supraspecific system can be fully realized only using the methods of symbiotic engineering and agrobiotechnology, which will prevent the loss of signs of mutualism by bacteria, often occurring under stressful conditions.

Perspectives of symbiotic engineering. The examined approaches to the design of high-performance MPS can be combined into a universal genetic and engineering algorithm with the following components: strengthening of the target biochemical functions of symbiosis; their coordination with growth processes, providing symbiotrophic plant development; a decrease in

the survival rate of microsymbionts in the external environment, which ensures an increase in the effectiveness of their interaction with hosts (Fig.).



The main stages of designing highly effective microbe-plant symbioses (on the example of N_2-fixing legume-rhizobia symbiosis): A — an increase in nitrogenase activity of bacteria, B — optimization of the habitus of host plants, C — inactivation of bacterial genes − negative regulators of symbiosis; M — aboveground biomass of plants, N — accumulation of nitrogen in the aboveground biomass.

A study of the legume-rhizobia symbiosis showed that the switching of legumes from autotrophic feeding with nitrogen (assimilation of fertilizers and nitrogen compounds of the soil) to symbiotrophic nutrition (assimilation of N_2-fixation products) is associated with a change in the overall plant development plan. When the rhizobia are inoculated, their habitus changes in favor of the aboveground part. This may be due to the activation of the development of shoot meristems by microsymbionts or to the inhibition of root meristems, which increases the efficiency of using N_2 fixation products for forming vegetative mass and seeds [20].

The proposed algorithm of symbiotic engineering can be used to increase the effectiveness of already existing forms of MPS; however, fundamentally different approaches are required to design new symbioses. At present, the creation of nitrogen-fixing systems in non-leguminous (cereal) crops is widely discussed, which was defined over 40 years ago as a priority task of genetic engineering of plants [27]. However, it turned out that the simplest way of solving this problem associated with the introduction of the nitrogen-fixation genes into nuclear plant genomes is not optimal: the expression of bacterial *nif* genes and the formation of active nitrogenase in planta are hindered, since the work of this enzyme for incomprehensible reasons is incompatible with the metabolism of eukaryotic cell [27, 28].

2. Approaches for the design of nitrogen-fixing plants [27, 39]

Approach	Advantages	Experimental rationale	Restrictions
Introducing *nif* genes in nuclear chromosomes of plants	Stable inheritance of *nif* genes	Insufficient (N_2-fixing eukaryotes are unknown)	Absence of expression of *nif* genes (synthesis of a functionally active nitrogenase) in the cytosol of a eukaryotic cell
Creating N_2-fixing organelles based on mitochondria or plastids	Isolation of nitrogenase from plant cell cytosol	The genetic relationship of organelles and free-living N_2-fixatives	Limited volume and low stability of the genomes of organelles
Creation of nonleguminous (cereal) plants capable of forming N_2-fixing nodules	Opportunity of using the homologies of *Sym* genes, which are widespread in higher plants	Activation of *Sym* gene homologs by bacterial signals (Nod factors). Formation with the action of 2,4-dichlorophenoxyacetic acid (2,4-D) nodule-like structures populated by rhizospheric N_2-fixatives (*Azospirillum*)	Incomplete expression of *Sym* genes in nonleguminous plants when inoculated with rhizobia, low stability of symbiotic structures formed in this case

It is more realistic to use the approaches of the symbiogenesis, including the introduction of *nif* genes into plant cell organelles, the mitochondria or plas-

tids, many free-living analogs of which (α-proteobacteria and cyanobacteria) are capable of nitrogen fixation (Table 2). It was shown in model experiments that functionally active proteins, the nitrogenases, can be synthesized in yeast mitochondria, but these proteins remain inactive in their cytosol even if the yeast is cultivated under anaerobic conditions, which are favorable for the work of nitrogenase (Table 3).

3. Formation of a functionally active small subunit of nitrogenase (Fe-protein NifH) in recombinant yeast [40]

Localization of the NifH synthesis in yeast cells	Co-synthesized proteins	Reduction activity	
		C_2H_2	N_2
Mitochondria	NifM	1600±27	826±60
Mitochondria	Absent	0	0
Cytosol (+ O_2)	NifM	0	0
Cytosol (– O_2)	NifM	102±2	0
Control proteins from *Azotobacter vinelandii*		1652±23	849±25

N o t e. Reduction activity was measured in vitro, based on 1 mg of MoFe protein NifDK (large subunit of nitrogenase): for C_2H_2 — by the formation of ethylene, nM/min, for N_2 — by the formation of ammonium, nM/min.

It is important to note that the symbiosomes with bacteroids, formed in the nodules of legumes and representing the structural and functional analogs of mitochondria and plastids, with which symbiosomes enter into close metabolic relations, can be considered as prototypes of new cellular organelles — ammonioplasts [29]. The initial stages of their appearance are illustrated by genetically reduced cyanobacteria *Nostoc azollae*, strictly obligate symbionts of fern *Azolla filiculoides*, transmitted vertically during its spore multiplication [30]. In the case of legume-rhizobia symbiosis, one of the approaches to involve such organelles in the cell cycle of plants can be their regeneration from cell cultures, containing symbiosomes with bacteroides.

Another promising direction of N_2-fixing symbiosis engineering is the formation of genetic programs for the nodule development in nonleguminous plants. For this, the homologues of the *Sym* genes of legumes can be used, many of which (LysM- and LRR-containing receptor genes) are present in all higher plants and, under certain conditions, are activated by Nod factors of rhizobia [31]. In cereal crops (wheat and maize), by processing with the auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D), it was possible to induce the development of nodule-like structures that turned out to be convenient niches for hosting rhizospheric nitrogen fixators, for example, *Azospirillum*. Settling in these "pseudo-nodules", *Azospirillum* bacteria develop a much higher nitrogenase activity than on the surface of the roots, and effectively transfer the N_2 fixation products to the plants [32].

Further development of symbiotic engineering can be connected with the use of endophytic bacteria [33], primarily those that are inherited by plants through seeds [34]. This ability is also possessed by the endophytic fungi of the *Neotyphodium* family, capable of inhibiting pests of cereal crops by producing toxic alkaloids [35]. For symbiotic engineering, the β-proteobacteria of the genus *Burkholderia*, which include phytopathogenic and symbiotic bacteria, including N_2-fixing forms, are of interest. It is shown that biocontrol of parasitic strains *B. glumae* can be carried out by the symbiotic strain *Burkholderia* sp. with the *iiaA* gene introduced into it, which disrupts the expression of virulence genes of the pathogen, determined by the "quorum sensing" mechanism [36]. A significant phytostimulant potential is possessed by leaf endophytes, an evolutionarily young group of symbiotic bacteria capable of stimulating photosynthesis and inhibiting the development of leaf pathogens [37]. Limitations imposed on the evolution of the listed types of MPS by the conditions of natural ecosystems can be overcome with the help of methods of gene and cell engineering,

symbiogenetics and biotechnology [38].

Thus, at the present time, extensive data on genetic control, molecular organization and evolution mechanisms of microbe-plant symbioses have been accumulated, which makes it possible to form genetically grounded programs for their design for adaptive farming and plant growing. This will permit to replace environmentally hazardous agrochemicals (fertilizers and plant protection products) with completely safe and much cheaper microbial preparations. Creating economically valuable microbe-plant symbioses includes two tasks: i) genetic improvement of symbioses, formed in the process of natural co-evolution of microbes and plants, and ii) the construction of fundamentally new symbioses. Successful development of symbiotic engineering requires extensive cooperation of specialists of different profiles and remains one of the most urgent tasks of modern agrobiology.

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LIMITED PROTEOLYSIS AS A MEANS TO REDUCE THE ALLERGENICITY OF SEED STORAGE GLOBULINS

(review)

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Abstract

According to SDAP (structural database of allergenic proteins, http://fermi.utmb.edu/), storage 11S and 7S globulins from seeds of peanut, soybean and some other plants are allergens. A β-barrel conjoined with a group of α-helices represents the structural basis of domains of the two-domain 11S and 7S seed storage globulins. During evolution, extended disordered inserts of enhanced susceptibility to proteolytic attack appeared in the amino acid sequences of storage globulins outside the β-barrel-α-helix structural module. Regularities of storage globulin limited proteolysis during seed germination and in vitro are determined by these inserts. In this review, available information on successive reactions of limited proteolysis specific to 11S and 7S globulins from peanut, soybean and some other plants is collected. It was demonstrated that limited proteolysis of 11S globulin from peanut (A. Cherdivară et al., 2017) calls forth destruction of a C-terminal region of α-chains, including the region forming the group of α-helices. Three of the four antigen determinants (IgE epitopes) identified in the peanut subunit Ara h3 (P. Rabjohn et al., 1999) belong to this region. Thus, the limited proteolysis leads to a significant decrease in the allergenicity level of the subunit Ara h3. The presence of IgE epitopes in homologous regions of conserved sequences of α-helices from most other subunits of the peanut 11S globulin, non-identical to Ara h3, is very probable. Thus, the limited proteolysis of not only the Ara h3 subunit, but also the whole hetero-hexamer molecule of peanut 11S globulin can be accompanied by a significant decrease in the level of its allergenicity. Prospects for reducing allergenicity of soybean 11S globulin by limited proteolysis are not so unambiguous. On the one hand, the limited proteolysis of the subunit Gly m G1 leads to the destruction of the C-terminal region of the α-chains (A. Shutov et al., 2012), where both identified IgE epitopes are present (T.A. Beardslee et al., 2000). On the other hand, only one of the IgE epitopes identified in the Gly m G2 subunit (R.M. Helm et al., 2000) can be removed using limited proteolysis of this protein (A. Shutov et al., 1993). The detachment of the α-chain α-helices during limited proteolysis of several other 11S globulins was observed as well. A high degree of conservation of this region in the primary structures of 11S globulins allows suggesting the presence of IgE epitopes, similar to those identified in peanut and soybean 11S globulins, in many other storage proteins of the 11S globulin family. Prospects for the reduction of allergenicity of seed 7S globulins by limited proteolysis are advantageous as well. Limited proteolysis of peanut 7S globulin Ara h1 starts with complete destruction of a disordered N-terminal extension (A. Cherdivară et al., 2016), which contains one third of IgE epitopes identified in the amino acid sequence of this protein (D.S. Shin et al., 1998). Further limited proteolysis calls fourth destruction of another disordered region inside the N-terminal domain that contains an additional IgE epitope identified in the Ara h1 sequence. Summary information considered in the review on the structure of seed storage globulins, as well as on the IgE epitopes identified in their amino acid sequences, evidences the availability of limited proteolysis as a means of considerable reduction of the level of allergenicity, not only of peanut and soybean 11S and 7S globulins, but also of those from other plants whose seeds are used as food either directly or as additives to various food products.

Keywords: seed storage globulins, proteolysis, allergenicity, IgE epitopes, *Arachis hypogaea* L., peanut, *Glycine max* L., soybean

The main part of food plant proteins is seed storage proteins. The overwhelming number of seed storage proteins are grouped in two conserved fami-

lies, the 7S (vicilins) and 11S (legumins) globulins [1]. Their amino acid sequences are inherited from the vicilin- and legumin-like proteins of spore plants [2]. In turn, the latter derived from bacterial oxalate decarboxylase [3]. All these proteins belong to the extensive cupin superfamily, combining dozens of functionally diverse families of proteins, in the structure of which there is a β-barrel from antiparallel β-strands [4].

The tertiary structure of subunits of oligomeric molecules of oxalate decarboxylases and storage globulins is supplemented by a group of α-helices [2]. In the subunits of these proteins, there are two domains, each of which is formed by a structural module of the β-barrel-α-helix. The two-domain structure of oxalate decarboxylases was formed at an early evolutionary stage as a result of duplication of this module which is present in their single-domain bacterial precursor [3].

In the course of evolution, extended hydrophilic variable insertions, taken to the surface of their oligomeric structures, appeared in the amino acid sequences of seed storage globulins, conserved ones on a whole [2, 3]. These insertions determine the sensitivity of native molecules of storage globulins to fast and limited proteolysis, from which their degradation begins in germinating seeds and in vitro [2]. The subsequent massive proteolysis of storage globulins occurs by a sequential mechanism [5], leading to the complete destruction of their molecules [3].

In seeds of peanut [6-8], soybean [9-11] and many other cultivated plants used in the dietary intake, e.g. nuts [12-14], almonds [15] and mustard [16], storage 7S and 11S globulins are allergens. This information, as well as some additional data, is included in the SDAP database (Structural Database of Allergenic Proteins) (http://fermi.utmb.edu/) [17]. In some of the storage globulins, 7S globulins of peanut [18] and lentils [19], 11S globulins of peanut [20], soybean [21, 22] and buckwheat [23], antigenic determinants (IgE epitopes) responsible for IgE binding are identified. Many of the IgE epitopes belong to the regions of storage globulins sequences with elevated sensitivity to proteolytic attack, which shows the principal possibility of reducing the allergenicity of seed storage globulins by means of their limited proteolysis.

In this review, to confirm this hypothesis, the experimental data obtained in the study of limited proteolysis of storage globulins of peanut seeds (*Arachis hypogaea* L.), soybean (*Glycine max* L.) and some other plants, have been analyzed.

Limited proteolysis of 11S globulins and IgE epitopes, identified in their sequences. The structure of the α-chains of the subunit Ara h3 (pdb|3c3v) of peanut 11S globulin [24], typical for 11S globulins of soybean seeds [25, 26] and a number of other plants [27, 28], is formed by a β-barrel of antiparallel β-strands BCDEFGHI, connected to the group of α-helices h1, h2, h3, and complemented by β-strands Z, A′-A, E′-F′ and J-J′, as well as by α-helices h0 and h1′. Three hydrophilic disordered regions in the α-chains of 11S globulins are potentially sensitive to limited proteolysis [2]: the loop between the β-strands E and F (E′-F′), the loop between the β-barrel and the α-helices and the C-terminal segment (Fig. 1, Table 1, respectively, the regions a, b and c).

For 11S globulins, the following sequence of limited proteolysis reactions is characteristic [2]. The process begins with the cleavage of the hydrophilic C-terminal region, sensitive to proteolysis (see Fig. 1, Table 1, region c). The further action of proteinases leads to splitting of the loop between the β-barrel and the α-helices (see Fig. 1, Table 1, region b). Depending on the individual features of the structure of 11S globulin and the specificity of proteinase, the loop

between the β-strands E and F can either remain intact or split (see Fig. 1, Table 1, region a).

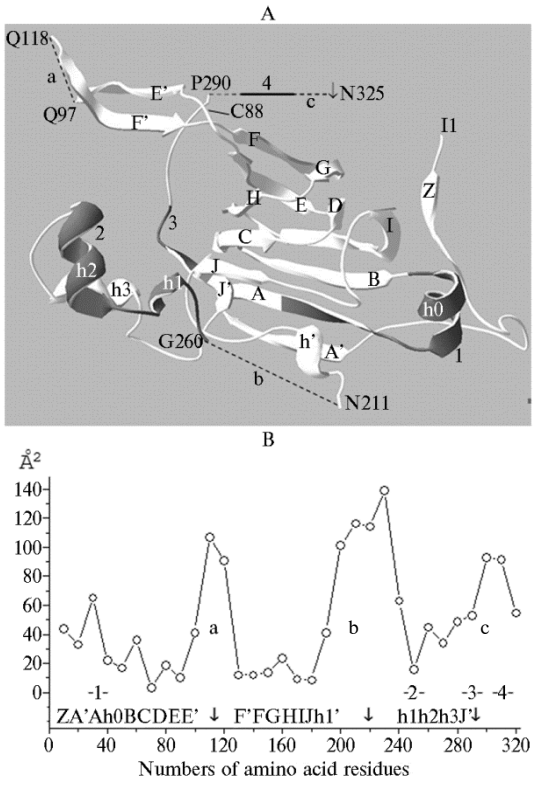

Рис. 1. Третичная структура α-цепи 11S Fig. 1. Tertiary structure of the α-chain of peanut 11S globulin (*Arachis hypogaea*) Ara h3 (pdb|3c3v).

A — the ribbon diagram. Non-ordered regions (a, b, c) in the α-chains of 11S globulins are potentially sensitive to limited proteolysis [2]. The arrow marks the peptide bond N325-G326which is splitted upon maturation of the Ara h3 molecule. The Cys88 residue participates in the formation of a disulfide bond between the α- and β-chains. The dark sections of the diagram correspond to sequences of IgE epitopes 1-4.

B — available to the solvent area of the amino acid residues ASA (accessibility surface area) in the sequence of the α-chain. The reported ASA values [31], expressed in Å^2, correspond to the average values calculated for groups of 10 residues in the amino acid sequence of the α-chain. The graph corresponds to the model quaternary structure of the homohexamer Ara h3 constructed using the pdb|3c3v [31] monomer template. Arrows indicate the location of the cleavage points of the α-chain with limited hydrolysis by trypsin [32]. Figures show the position of IgE epitopes 1-4 in the amino acid sequence Ara h3.

The position of the points of splitting of the α-chains of 11S globulins is determined by the results of N-terminal sequencing of fragments [29, 30] or by the combination of indirect data (specificity of the proteinase, sequence of fragments formation and their molecular weights). The potential sensitivity to the limited proteolysis of disordered regions (see Fig. 1, a, b, c) in the amino acid sequences of 11S globulins is indicated by their relatively high availability to the ASA solvent, shown in the example of Ara h3 (see Fig. 1, B).

The cleavable C-terminal region, encompassing the region of the α-helices h1h2h3 and the β-strand J' (see Table 1), is destroyed to short peptides, which is characteristic not only of Ara h3 [31] but also of other 11S globulins [2]. It should be noted that the N-terminal region of the α-chain

1. Sites of α-chain cleavage (↓) with limited proteolysis of storage 11S globulins of soybean *Glycine max* Gly m G1-G5, peanuts *Arachis hypogaea* Ara h3 and abl14270 (ARAhy), sunflower *Helianthus annuus* aaa33374 (HELan), oat *Avena sativa* aaa32720 (AVEsa), cedar *Pinus sibirica* caa77569 (PINsi) and pumpkin *Cucurbita maxima* pdb|2e9q (CUCma)

Secondary structure A′A-BCD--E--FGHIJ----h1 h2h3J′---- a b c	Subunit	Proteinase
↓ ↓	Gly m G1-G5	Papain [30]
** **↓ * ↓*	Gly m G2	Trypsin [34]
** ↓ **↓ * ↓*	Gly m G2	Trypsin [32]
↓ ↓	Gly m G5	Papain [35]
* ↓ ↓ * ↓**	Ara h3	Papain [31]
↓ ↓ ↓	ARAhy	Trypsin [32]
↓ ↓ ↓	HELan	Papain [36]
↓ ↓ ↓	AVEsa	Papain [37]
↓ ↓	PINsi	Papain [38]
↓ ↓	CUCma	Papain [39]

N o t e. The region h1h2h3J' that is destructed in the limited proteolysis is underlined. The asterisks indicate the position of IgE epitopes identified in the subunits of soybean 11S globulins Gly m G1 [21]and Gly m G2 [22] and peanut Ara h3 [20].

of Ara h3, like all the examined 11S globulins [2], is insensitive to proteolytic attack. Three of the four IgE epitopes identified in the subunit of peanut 11S globulin Ara h3 [20] (see Fig. 1, Table 1) belong to the C-terminal region of the α-chain that degrades by means of hydrolysis with papain [31]. That is, limited proteolysis of the subunit Ara h3 can lead to a significant reduction in its allergenicity.

The hetero-hexamer molecule of peanut 11S globulin contains nine subunits, highly conserved in the C-terminal region of the α-chains where three of the four IgE epitopes Ara h3 are present (see Figs. 1, 2, Table 1). The potential ability to binding of IgE to each of the sequences of other subunits of peanut 11S globulin, homologous to the second and third IgE epitopes Ara h3, can be judged from the results of the definition of PD indexes (property-based peptide similarity index for two sequences) [40]. The method is based on a comparison of the physical and chemical properties of each of the amino acids of the IgE epitope sequence, identified in the protein allergen, with each of the amino acids, which are close in the primary structure of the region of the examined protein's sequence. As the number of differences between the segments of these sequences increases, the value of PD indexes increases from 0 (the sequences are identical) to the limiting value of 10 above which the presence of the corresponding IgE epitope is unlikely [40].

The presence of the second and third IgE epitopes Ara h3 (see Fig. 1, B) in the homologous regions of the sequences of six of the eight subunits of peanut 11S globulin, not identical to Ara h3 [31], is considered very likely (the corresponding PD indexes do not exceed 2.9). Finally, the sequences of the fourth IgE epitope Ara h3 (see Fig. 1, B) and the corresponding putative IgE epitopes in the other two subunits of peanut 11S globulin (aag01363 and abf93402) are identical. That is, the limited proteolysis of not only the subunit Ara h3, but also the whole hetero-hexamer molecule of peanut 11S globulin can be accompanied by a significant decrease in its allergenicity.

Fig. 2. Amino acid sequences of the C-terminal region of the α-chains of 11S globulin of peanut *Arachis hypogaea* (A1 — pdb|3c3v, A2 — abl14270) and soybean *Glycine max* (G1 — pdb|1fxz; G2 — baa00154). The figures on the left correspond to the numeration of amino acid residues in the complete sequences of the subunits. The lower lines show identical amino acid residues (*) and their conserved (:) and semi-conserved (.) substitutions. The arrows correspond to peptide bonds, which are cleavable by trypsin (↓) [29, 32, 34] and papain (↑) [30, 32]. The sequences of IgE epitopes are indicated in bold, and the amino acid residues Arg and Lys of increased accessibility to the solvent (ASA >100 Å) in the model oligomeric structures A1 and G1 are underlined.-

Prospects for reducing the allergenicity of soybean 11S globulin by means of limited proteolysis are not so unambiguous. On the one hand, the limited proteolysis of the subunit Gly m G1 leads to the removal of the region of α-helix h1h2h3-β-strand J', where both identified IgE epitopes are present (see Table 1, Fig. 2). The presence of two corresponding IgE epitopes in homologous sequences of the subunits Gly m G3, Gly m G4, and Gly m G5 is not excluded

(PD indexes from 0 to 5.9). On the other hand, only one of the IgE epitopes identified in soybean 11S globulin Gly m G2 (see Table 1) can be removed by means of its limited proteolysis by papain (see Fig. 2). In this case, the presence of the corresponding IgE epitope in the homologous regions of the sequences of other subunits of soybean 11S globulin is unlikely (the value of PD indexes is close to 10).

Limited proteolysis of 7S globulins and IgE epitopes identified in the sequence of peanut 7S globulin Ara h1. The N- and C-terminal domains of the subunits of 7S globulins are structurally equivalent to the α- and β-chains of 11S globulins, but differ from the latter in several features [41], i.e. the interdomain linker region in 7S globulins is not split, and their structures lack an interdomain disulfide bond. In the 7S globulins of the known tertiary structure from the seeds of jack bean [42], bean [43], soybean [44], cowpea [45, 46] and pine [47], there is a number of disordered regions, which are potentially sensitive to limited proteolysis [2] (Table 2, Fig. 3).

2. Sites of cleavage (↓) of the N-terminal domain with limited proteolysis of seed storage 7S globulins of *Arachis hypogaea* Ara h1, soybean *Glycine max* Gly m α, Gly m α′, Gly m β, and bean *Phaseolus vulgaris* (PHAvu)

Secondary structure ----------Z-A′ABCDEFGHIJ-h1-h2-h3--J′h4- a b c	Subunit	Proteinase
******↓↓ * * * ↓* ↓	Ara h1	Papain [50]
↓ ↓	Gly m α	In vivo [51]
↓ ↓		C2 [52]
↓ ↓	Gly m α′	Trypsin [32]
↓ ↓		C2 [52]
↓ ↓	Gly m β	In vivo [51]
↓ ↓		CPPh [53]
↓ ↓		Trypsin [54]
↓ ↓	PHAvu	In vivo [55]
↓ ↓		CPPh [53, 56, 57]
↓ ↓		Trypsin [58]
↓ ↓		C2 [52]
↓ ↓		LLP [56, 57]

N o t e. The asterisks indicate the position of IgE epitopes identified in the Ara h1 subunit [18]. C2 and CPPh are endogenous papain-like proteinases from germinating seeds of soybean and bean, respectively.

The variable N-terminal extension of the N-terminal domain (see Table 2, region a), characteristic of 7S globulins of the convicilin type, shows the greatest sensitivity to proteolysis [2]. The region between the α-helix h3 and the β-strand J′ (see Table 2, region b), which is not non-ordered in all 7S globulins, is relatively short; there is no information on its sensitivity to proteolysis. The exception is the peanut 7S globulin Ara h1, in which this region is elongated [48, 49] and cleaved by hydrolysis with papain (see Table 2, see Fig. 3, B). The unregulated region of the interdomain linker is sensitive to proteolysis in all the investigated 7S globulins [2] (see Table 2, region c). Finally, the elongated loop between the β-strands E and F in the C-terminal domain of 7S globulins is known to be sensitive to the limited proteolysis [2].

In the subunit of Ara h1 peanut 7S globulin, exhibiting the greatest allergenicity among the storage seed globulins [59], the presence of 21 IgE epitopes [18] is established. IgE epitopes 1-3 belong to the N-terminal sequence deleted post-translationally [60]. IgE epitopes 4-9 (see Fig. 3, A) localized in the sensitive region of the N-terminal extension (see Fig. 3, B, region a), which is replete with residues corresponding to the substrate specificity of papain [61], are removed at the initial attack by this enzyme [50]. The subsequent action of papain leads to the destruction of the sensitive region between the α-helix h3 and the β-strand J′ (see Table 2, region b), specifically elongated in the Ara h1 sequence, where the IgE epitope 13 is localized (see Fig. 3, A).

It should be noted that there is a relatively high availability for the solvent in the α-helix region h1-h3, which is 3.5 times higher than the ASA for the rest of the N-terminal domain Ara h1. Therefore, it is tempting to try to find

conditions for limited proteolysis that ensure the destruction of this potentially sensitive region of the α-helix, where IgE epitopes 11 and 12 are present (see Fig. 3, A).

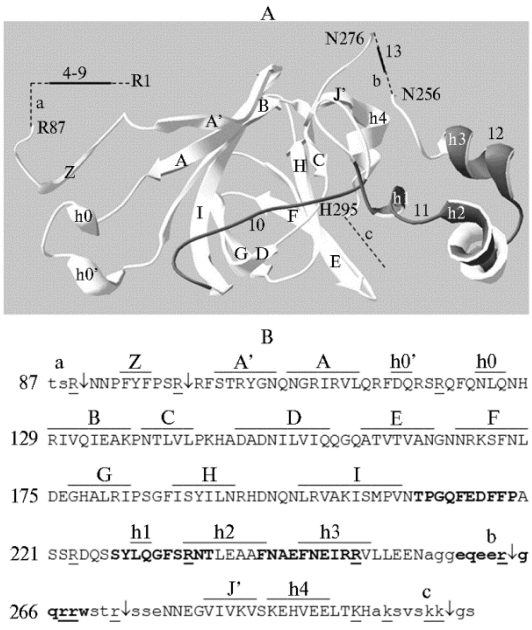

Fig. 3. Structure of the N-terminal domain of peanut 7S globulin Ara h1 (pdb|3smh).

A — the ribbon diagram of a tertiary structure. The disordered regions a, b, and c are shown by dashed lines. The dark sections of the diagram correspond to sequences of IgE epitopes 4-13 in the mature molecule Ara h1 [18].

B — the primary structure. Non-ordered regions: a — N-terminal elongation, b — between the α-helix h3 and β-strand J′, c — C-terminal region. The arrows correspond to peptide bonds cleavable by papain [50]. The amino acid residues with increased availability for the solvent (ASA > 100 Å), corresponding to the sub-stratified specificity of papain [61], are underlined. These residues are present in the crystalline structure of the pdb|3smh oligomer, as well as its model (pdb|3s7e as a template) [50] in regions b and c. The sequences of IgE epitopes 10-13 are in bold.

The subunits of peanut 7S globulin in the composition of its heterotrimeric molecule are extremely conserved. It is very likely that IgE epitopes, identified in Ara h1, are also present in other subunits of this protein: the corresponding PD indexes do not exceed 2.5. Limited proteolysis by papain leads to a significant reduction in the allergenicity of the whole hetero-oligomeric molecule of peanut 7S globulin, in connection with the removal of more than a third of the IgE epitopes.

Thus, the reviewed herein data on limited proteolysis of 11S and 7S globulins of peanut, soybean and some other plants suggest that this method is promising for substantially reducing the allergenicity of storage seed globulins.

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THE PROBLEM OF SAFE SUNFLOWER (*Helianthus annuus* L.) USE FOR FOOD AND FODDER PURPOSES

(review)

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Abstract

Risks associated with the contamination of agricultural products with mycotoxins have been and still remain under the close attention of the world's biological science. In recent decades, special concern was related to the state of the grain harvest, intended for food and feed purposes. In most grain-producing countries, significant progress has been made in the identification of toxin-forming micromycetes and assessing the risk caused by the spread of mycotoxins (T.Yu. Gagkaeva et al., 2004; G.P. Kononenko et al., 2008, 2009; P.M. Scott et al., 2012). For the second most important group of agricultural plants, the oilseeds represented mainly by sunflower, soybean, peanut, rapeseed and cotton, there is a significant lag in such studies, which only has to be overcome. *Helianthus annuus* L. is cultivated everywhere, practically in all regions of the world, suitable for agriculture, and the area of its commercial cultivation is also extremely wide. The group of world leaders in the production of sunflower seeds includes the Russian Federation, Ukraine, Argentina, India and China, but this crop is also cultivated on a significant scale in other countries. The purpose of this review was to systematize and compare the world data on the composition of mycobiota and the nature of contamination by mycotoxins of sunflower (*Helianthus annuus* L.) seeds and the products of their processing. In surveys conducted in the Middle East, Africa, South and South-East Asia, the mycobiota of the seeds revealed the dominance of fungi of the genus *Aspergillu*s with the typical species *A. flavus* and the frequent detection of *A. niger*, and for less common fungi *Penicillium*, *Alternaria* and *Fusarium* common regularities were not traced. In India, Pakistan, Tanzania, Malaysia, Iran and Egypt, the contamination of seeds by aflatoxins was assessed as very high, and the seed processing products for oil retained the same type of contamination, but with an increased detection rate and a more intensive accumulation of mycotoxins (S. Dawar et al., 1991; S.K. Abdullah et al., 2010; H.R. Beheshti et al., 2013; J.A. Mmongoyo et al., 2017). In a number of works, experimental confirmation was obtained that, when storing seeds, especially in conditions of high humidity and temperature, the accumulation of aflatoxins sharply increases (H.H. Casper et al., 1982; P. Jeswal et al., 2013). In the countries of South America (Argentina, Brazil), fungi of the genera *Alternaria*, *Fusarium* and alterniotoxins predominated in sunflower seeds (C.R. Pozzi et al., 2005). In European countries, the fungi of the genera *Alternaria* and *Fusarium* are also classified as the main components of the mycobiota of sunflower seeds, but the data on the species composition of these micromycetes and the contamination of the seeds with mycotoxins are very limited. Long-term studies performed in the Russian Federation show widespread distribution of fungi of the genus *Alternaria* on the vegetative plants and sunflower seeds, most often of small-spore unspecialized species *A. tenuissima*, *A. alternata* and '*A. infectoria'* complex (M.V. Ivebor et al., 2012). *Fusarium* infection in the European area of cultivation is shown annually, while the species diversity is very significant (A.A. Vypritskaya, 2015). Nevertheless, until now, mycotoxicological evaluation of the yield of sunflower seeds in the main areas of commercial cultivation of the crop in our country has not been carried out. During monitoring surveys of sunflower oil cakes and meals, multiple combined contamination by mycotoxins was established with dominance of alternariol and ochratoxin A and a significant contribution of T-2 toxin, as well as citrinin, emodin, mycophenolic acid and cyclopiazonic acid (E.V. Zotova et al., 2017). The nature of the contamination of this feed raw material in the Russian Federation is fundamentally different from that described in the countries of the Middle East, Africa, South and South-East Asia, primarily due to the absence of aflatoxin B_1 and the significant occurrence of ochratoxin A, often in conjunction with citrinin. The generalization and comparative analy-

sis of the broad database of scientific data, undertaken in this paper, allow us to identify ways of eliminating shortcomings in restricting the standardization of mycotoxins and to outline the most relevant areas for future research.

Keywords: *Helianthus annuus* L., sunflower, seeds, sunflower meal, oilcake, micromycetes, fungal diseases, mycotoxins

Risks associated with the contamination of agricultural products with mycotoxins have been and remain at the center of close attention of the world's agricultural science. In recent decades, the state of the grain harvest produced for food and feed purposes has been of special concern. In most grain-producing countries, significant progress has already been made in identifying the main toxin-forming micromycetes and in assessing the prevalence of mycotoxins in grain products [1-3]. The main result of mycological and mycotoxicological examinations performed in the areas where fusariosis of grain and corn is registered is the understanding that this global problem is associated with a region, and its success requires special approaches, taking into account the places of growth [4-6].

For the second most important group of agricultural plants, the oilseeds, mainly represented by sunflower, soybean, peanut, rapeseed and cotton, there is a significant lag in these studies, which only has to be overcome, and the existing database needs to be generalized and analyzed.

The purpose of this review is to systematize and compare the world data on the composition of the mycobiota and the nature of contamination of sunflower (*Helianthus annuus* L.) seeds with mycotoxins while harvesting and storing the harvest, as well as in the processed products used for animal feed.

Sunflower is widespread virtually in all regions of the world, suitable for agriculture. The area of its industrial cultivation is also extremely wide. Seeds of this plant are a valuable food product and raw material for the confectionery and fat-and-oil processing industries, and waste from the production of sunflower oil (oil cake and meal) are in demand as feed raw materials. Green mass of tall varieties, rich in protein, is considered suitable for preservation (silage and haylage). Livestock eagerly eats heads of plants harvested during flowering; the remains of mature plants after harvesting (stems, leaves and heads) are also used for animal feed [7].

Concerns about the negative effects of sunflower consumption are mainly related to the long-established fact that plants have predisposition to diseases caused by imperfect mycelial, pycnidial and sclerotial fungi, as well as basidial, lower and marsupial fungi [7]. The main mycotoxins, having a sanitary importance, for most phytopathogens are not included in the number of physiologically active metabolites. However, the causative agents of Alternaria blight (*Alternaria* spp.), seed spotting syndrome (*Alternaria alternata*, *Cladosporium* sp.), tracheomycotic wilting and pink rot of heads (*Fusarium* spp.) are of interest from a toxicological point of view, and for *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp. and *Cladosporium* sp. fungi, which cause widespread seed molding, the ability to toxin production is known [8, 9]. Many of them are found in the composition of mycopopulations, accompanying the pathogenesis processes.

The group of world leaders in the production of sunflower seeds includes the Russian Federation, Ukraine, Argentina, India and China, but this crop is also cultivated on a significant scale in other countries. Soil-climatic, ecological and agro-technical conditions are known to have a decisive significance for the formation of plant mycobiota [10]. In connection with this, the risks of accumulation of mycotoxins in agricultural products in areas should be assessed by the main taxa of fungi, identifying potentially toxicogenic species and confirming the possibilities for the realization of their genetically determined abilities.

Describing the diversity of the mycobiotic composition of sunflower seeds before harvesting, during the post-harvest period and during storage, researchers in the countries of the Middle East, Africa, South and South-East Asia emphasized the explicit dominance of some genera and species (Table 1). *Aspergillus* fungi were constantly detected, their typical representative was *A. flavus*, *A. niger* was also often found, *A. terreus* and *A. fumigatus* were less common. By the species composition of less common fungi belonging to the genera *Penicillium*, *Alternaria* and *Fusarium*, the general patterns were not traced. The results of the examination of the seed harvest in India [11, 12, 18], Pakistan [13, 14] and the data of local national projects, carried out in different years in Iran [19], Nigeria [20], Sudan [21], the Republic of South Africa [17], Colombia [22], Iraq [15], Malaysia [16] and Tanzania [23], showed that the fungi of the genus *Aspergillus* are characterized by the highest occurrence and intensity of infection in comparison with representatives of other genera.

1. Main representatives of mycobiota of sunflower seeds (*Helianthus annuus* L.) in the countries of the Middle East, Africa, South and South-East Asia

Region	Taxon (genus)	Species	Reference
India (State of Tamil Nadu)	*Aspergillus*	*A. flavus*	[11]
India (State of Bihar)	*Aspergillus*	*A. flavus*	[12]
	Penicillium	*P. citrinum*, *P. verrucosum*	
	Fusarium	*F. moniliforme*	
Pakistan	*Aspergillus*	*A. flavus*, *A. niger*	[13]
	Alternaria	*A. alternata*, *A. tenuissima*	
	Fusarium	*F. moniliforme*, *F. solani*	
	Aspergillus	*A. flavus*, *A. niger*, *A. terreus*	[14]
	Alternaria	*A. alternata*	
	Fusarium	*F. moniliforme*, *F. pallidoroseum*	
Iraq	*Aspergillus*	*A. flavus*, *A. niger*, *A. fumigatus*, *A. terreus*	[15]
	Penicillium	*P. expansum*, *P. brevicompactum*	
	Alternaria	*A. alternata*	
	Fusarium	*F. oxysporum*, *F. solani*	
Malaysia	*Aspergillus*	*A. flavus*, *A. niger*	[16]
Republic of South Africa (Province of KwaZulu-Natal)	*Aspergillus*	*A. flavus*	[17]

The species *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*, identified in sunflower seeds in these areas, are known as toxin-forming ones [24]. The strains of *Aspergillus flavus*, isolated from seeds in India, produced aflatoxin B_1 [25]. For isolates of the same species in Pakistan, the ability to biosynthesize aflatoxins was experimentally confirmed: 29 strains out of 41 produced aflatoxin B_1, and 8 also aflatoxin B_2 [26]. To simulate conditions that are close to the natural ones, the toxin formation of fungi was studied on sunflower seeds. However, whether the use of the same substrate for the correct approach to assessing fungi involvement in the contamination of biological entities by toxic metabolites is fundamental, is still to be determined. For two strains of *A. flavus*, it was found that the process of accumulation of aflatoxins on seeds was decelerated, compared to grain substrates, and the largest amounts of aflatoxins were formed only after 12 and 18 days [27].

In India, Pakistan, Tanzania, Malaysia and Iran, the contamination of seeds with aflatoxins was assessed as very high (Table 2). In India, along with aflatoxins, ochratoxin A, citrinin and zearalenone [12] were detected in seeds, as well as cyclopiazonic acid, which is a toxin, characteristic of *Aspergillus* fungi [29]. The by-products of seed processing for oil in these countries retained the same type of contamination that was inherent in seeds, but the detection rate and the accumulation of mycotoxins increased. In India, in the study of oil

cakes, aflatoxins were detected in all examined samples in amounts exceeding 30 μg/kg [30], cyclopiazonic acid in 10 samples (300-29000 μg/kg) [28], ochratoxin A in 48 and 76% of samples in the summer and winter periods in amounts of 31.84 and 76.52 μg/kg, respectively [31]. In Tanzania, according to the data of 2014-2015, 80.4% of the cake samples (n = 92) from local oil producers contained aflatoxins in amounts ranging from 1.4 to 598.4 μg/kg [23], which exceeded the detection rate in the seed crop with the same contamination (Table 2). For the oil cake sample from the eastern part of the Republic of South Africa, contamination with aflatoxins (84 μg/kg) was established, severely contaminated by the species *A. flavus* and *A. tamarii* [17]. Recently, aflatoxins B_1 (37.8 μg/kg), G_1 (57.8 μg/kg) and B_2 (7.0 μg/kg) [32] have been found in the consignment of meal imported to Malaysia.

2. Mycotoxins found in sunflower seeds in the countries of South America, the Middle East, Africa, South and South-East Asia

Region of cultivation, n	Mycotoxin	Detection rate, amount, μg/kg	Reference
India (State of Bihar), seeds before and after harvesting (n = 240)	Aflatoxin B_1	30 % (43-1070)	[12]
	Aflatoxin G_1	20 % (17-247)	
	Ochratoxin A	17 % (49-248)	
	Citrinin	15 % (23-433)	
	Zearalenone	3 % (111-125)	
Pakistan, seeds from different provinces (n = 24)	Aflatoxin B_1	54 % (≤ 437)	[26]
	Aflatoxins B_1 + B_2	21 % (≤ 14)	
Tanzania, seeds from different places, harvest 2014, 2015 (n = 90)	Aflatoxins	59 % (1,4-662,7)	[23]
South Africa (KwaZulu-Natal province) (n = 1)	Aflatoxins	5,6	[17]
Malaysia	Aflatoxin B_1	82,1 % (0,54-5,33)	[16]
Iran (Khorasan region), various stores (n = 50)	Aflatoxin B_1	13 % (≤ 168)	[28]
	Aflatoxin B_2	8 % (≤12,8)	

N o t e. n — the number of samples examined.

In Egypt, regional differences in the composition of seeds mycobiota were found. In 36 samples of the 1985 harvest selected in the country's markets, 63 species and 3 varieties of fungi, belonging to 18 genera, were identified [33]. Among them, according to the detection rate, representatives of the genera *Aspergillus* (100%) and *Penicillium* (88.9%) dominated, followed by fungi belonging to the genus *Fusarium* (36.1%). Contamination of seeds with *Aspergillus* spp. was the most intensive, and it was considerably less intensive for *Penicillium* and *Fusarium*. Fungi of the genus *Aspergillus* were represented by the species *A. niger*, *A. flavus*, *A. fumigatus*, *A. terreus*, *A. flavus* var. *columnaris*, *A. nidulans*, *A. sydowi*, *A. tamari*; among *Penicillium*, *P. chrysogenum* and *P. corylophilum* were found, among *Fusarium* — *F. oxysporum*, *F. moniliforme*, *F equiseti*, *F. semitectum* (first two more often), the species *Alternaria* spp. were extremely rare. In another study, in 16 seed samples collected at the experimental stations of the universities of Alexandria, Nobariya and Minya there were *Alternaria alternata*, *F. proliferatum*, *F. semitectum* and *F. semitectum* var. *majus* [34]. In 10 samples from Asyut Governorate, *A. niger* (16.5%), *A. flavus* (13.8%) and *Alternaria alternata* (15.3%), prevailed, and *P. digitatum* and *F. oxysporum*, *F. moniliforme*, *F. semitectum* [35] were less frequent.

These results completely correspond to the nature of seed contamination with mycotoxins. In the 1985 harvest [33], 26 samples (72%) were positive, with aflatoxins found in more than half of them (15 of 26). The group of aflatoxins in 10 positive samples was represented by all four metabolites, in 5 samples by only two ones (aflatoxins B_1 and B_2). Fusariotoxins, i.e. diacetoxyscirpenol (5/26), T-2 toxin (4/26), zearalenone (2/26), as well as sterigmatocystin (3/26) and ochratoxin A (1/26) were less common, but, unfortunately, quantitative measurements were not carried out. Later, it was reported that zearalenone and alternariol were

found in seeds in the same region [36].

In the United States, information on the detection of aflatoxin B_1 in sunflower seeds was first obtained by scientists from the University of North Dakota: while analyzing 11 samples of the 1978 crop, aflatoxin B_1 was found in 9 samples in amounts ranging from 10 to 225 μg/kg [37, 38]. This fact was the reason for more thorough research, during which the important role of storage conditions for the accumulation of aflatoxin B_1 was demonstrated. For example, in 1979-1981, in consignments from different manufacturers providing half of the oil production in this state, the contamination with aflatoxin B_1 did not exceed the allowable level (20 μg/kg); however, in the samples from storage facilities where molding and caking were observed, its content reached 100-1100 μg/kg [37, 38]. All this indicates that the key factor in the growth of contamination is post-harvest storage. When examining food and forage, imported into the United States during 1982-1986, aflatoxins in the amount of 179 μg/kg were found in one sample of sunflower seeds [39]. Subsequently, it was stated that in a sample from a consignment of moldy sunflower seeds that caused intoxication of pigs, cyclopiazonic acid was detected in an extremely high concentration, the 10000 μg/kg [40]. Apparently, under certain conditions, a sharp intensification of the growth of individual highly competitive toxigenic species from the number of mold-inducing seeds is possible.

Increased accumulation of mycotoxins during seed storage, especially in conditions of high humidity and temperature, is confirmed in other countries. According to a researcher from India, the content of aflatoxins in freshly harvested and stored sunflower seeds was 1000 and 2200 μg/kg, respectively [30]. When storing a batch of sunflower seeds in a specialized metal hangar at 25-32 °C, after 7 months the amount of aflatoxin B_1 increased more than 2-fold (from 205 μg/kg to 520 μg/kg) [41]. The increase in seed contamination during post-harvest storage is also indicated by the recent data of Indian authors obtained in the state of Bihar [12]. Not only the detection rate of four mycotoxins increased, but also the degree of contamination: aflatoxin B_1 (16 of 120, 43-355 μg/kg), aflatoxin G_1 (11/120, 17-85 μg/kg), ochratoxin A (14/120, 1-3 μg/kg) and citrinin (11/120, 23-65 g/kg) were identified before harvesting, aflatoxin B_1 (56/120, 463-1070 μg/kg), aflatoxin G_1 (36/120, 129-338 g/kg), ochratoxin A (27/120, 121-415 ⌈g/kg), citrinin (26/120, 65-433 μg/kg) and zearalenone (7/120, 111-125 μg/kg) were found after harvesting.

Judging by the published data, in the countries of South America the mycotoxicological situation with sunflower differs radically from the described one for other regions. Here, seed mycobiota is represented mainly by the fungi of the genera *Alternaria* and *Fusarium* [42]. In Brazil, in seeds harvested in one of the states, the contamination with *Fusarium verticillioides* was 70%, *Alternaria alternata* — 46%, *Cladosporium* spp. — 18%, and the rest (*A. flavus*, *Penicillium* spp., *Scopulariopsis* spp.) — from 2 to 10% [42]. In Argentina, seeds were often contained by alternariotoxins, the alternariol and its methyl ether (in 76% of 50 samples) [43]. In Brazil (Sao Paulo, Nova Odessa), contamination with these toxins was 18% and 10%, respectively, with a content of 24.9-170.9 and 14.1-108.6 μg/kg [41]. In cakes, among the most frequent contaminants there were alternariol (35-792 μg/kg) and its methyl ether (9-630 μg/kg); tenuazonic acid was also found [44].

In European countries, fungi of the genera *Alternaria* and *Fusarium* are often mentioned among the main components of mycobiota of sunflower seeds, but there is little data on the species composition. In Serbia (Province of Vojvodina), fungi belonging to 8 genera and 13 species were isolated from seeds, kernels and husk: *Alternaria alternata*, *Arthrinium phaeospermum*, *Aspergillus can-*

didus, *A. flavus*, *A. niger*, *A. ochraceus*, *A. versicolor*, *A. wentii*, *Cladosporium cladosporioides*, *Eurotium herbariorum*, *Penicillium aurantiogriseum*, *Rhizopus stolonifer* and *Trichoderma harzianum* [45]. In Finland, 20 species of fungi were distinguished in affected plant organs, among which there were 4 species of *Fusarium*, i.e. *F. avenaceum* (Fr.) Sacc., *F. equiseti* (Corda) Sacc., *F. oxysporum* Schlecht. and *F. sambucinum* Fuckel [46]. Assessment of the role of toxin-forming fungi *Fusarium* and *Alternaria* is difficult not due to the complexity of the composition of the represented complex of species, but because of the problems with species identification. The contribution of these fungi to the contamination of seeds, green mass and products of processing for food has been little studied so far. The ability to form toxins is known only for a few species of *Fusarium*, found in seeds and contaminated terrestrial organs of these plants. Many species of the genus *Alternaria* Nees are also classified as potentially toxicogenic ones. However, the authors did not find any information on the production of mycotoxins by isolates from sunflower seeds belonging to specific species of *Fusarium*, *Penicillium*, *Cladosporium*, *Scopulariopsis* and *Aspergillus* (*A. niger*, *A. fumigatus*, *A. terreus*, *A. candidus*, *A. ochraceus*, *A. versicolor*, *A. wentii*, *A. nidulans*, *A. sydowii* and *A. tamari*).

Reports on the occurrence of fusario- and alternariotoxins in seeds and products of their processing are still very few. Thus, in southern Italy, in one seed sample, infected with *A. alternata,* alternariol (360 μg/kg) and its methyl ether (130 μg/kg) were detected [47, 48]. In Hungary, contamination by T-2 toxins with a frequency of 13.6% and high accumulation (237-500 μg/kg, in average 230 μg/kg) was established for 22 samples of oil cake. Deoxynivalenol was detected less frequently and in smaller quantities (4.5%, 150 μg/kg), and zearalenone was absent [49].

The results of the annual food monitoring since 1983 in the US and EU countries, show that sunflower seeds supplied to the food markets of these countries are generally poorly contaminated with mycotoxins and do not pose a threat to the population. Nevertheless, in Italy, when the imported seeds were inspected, the content of aflatoxin B_1 in two samples was 50 and 90 μg/kg [50]. Recently, aflatoxins in an amount of less than 20 μg/kg were found in three samples of mixtures prepared with the addition of sunflower seeds, and seeds contained ochratoxin A (20 μg/kg) [51]. In 11 samples of seeds tested in January-February 2013 in a trade network of Uppsala (Sweden) and having the confirmation of Chinese production or without any indication of their origin, aflatoxins and ochratoxin A were not detected, but in the mycobiota everywhere there were fungi of the genus *Penicillium* represented by the toxicogenic species *P. expansum*, *P. chrysogenum*, *P. verrucosum*, *P. crustosum*, *P. albocoremium*, *P. brevicompactum*, *P. citrinum*, *P. rugulosum* and *P. polonicum* [52]. A study in the Netherlands in 2013-2014 showed that the contamination of food products with alternariotoxins (seeds, oil and paste prepared by means of its hydrogenation) is stable and the seeds are more often contaminated by tenuazonic acid (8/10, 240 μg/kg), rather than alternariol (1/10, 5.4 μg/kg) and its methyl ether (1/10, 1.1 μg/kg) [53, 54], but the modified forms of these toxins (sulfates and sulfoglycosides) were not detected.

The first reports regarding the detection of mycotoxins in the products of processing seeds for oil in the European market appeared when little was known about the problem of seed contamination [50]. In Germany, aflatoxin B_1 (17 μg/kg) was found in one of the four samples of oil cake imported in 1972-1973. In Hungary, 9.6% of the 73 consignments, imported in 1975, contained aflatoxins. In all 22 samples of oil cake imported into the UK from Argentina, India and the EU countries, contamination with alternariol was found

(180 μg/kg), its methyl ether (100 μg/kg) and tenuazonic acid (1900 μg/kg) [56]. In 2011, the European Commission based on research data, including those obtained from Russian scientists, raised the issue of contamination of food and forage with the toxins of *Alternaria* fungi and concluded that the risks of contamination of seeds, oil cakes and meal from sunflower seeds are especially high [57].

In the Russian Federation, sunflower accounts for up to 70% of the sown areas of oilseeds, which are concentrated mainly in the Southern, Central and Volga Federal Districts. The study of causative agents of diseases and fungi associated with this plant has a rich history, significant successes in the present and future development prospects. Long-term studies have shown a wide distribution of fungi of the genus *Alternaria* in vegetative plants and sunflower seeds, and in recent years it has been possible to specify their species composition. According to the phytoexamination data, in most of the 62 consignments of seeds from the Krasnodar Territory, Voronezh and Volgograd regions harvested in 2010-2011, there were often small-spore unspecialized species: *A. tenuissima* (52%), the complex of *A. infectoria* (25%) and *A. alternata* (14%) [58]. The results of a 25-year monitoring in the Krasnodar Territory showed significant damage caused by *Alternaria* to roots, stems, leaves, heads and seeds [59]. In the Tambov region, in all the surveyed areas in 1992-2015, the fungi of the genus *Alternaria* were found in 4.3-100% of vegetating plants (most often on heads and seeds) given the insignificant intensity of contamination [60]. A detailed description of the species composition of alternariosis pathogens in different regions of the country is given in the work of F.B. Gannibal [61].

In recent decades, more attention has been paid to fusariosis of sunflower in the European range of cultivation of this crop [62]. The prevalence and species composition of pathogens in the southern regions of Russia was studied [63-65]. In the Tambov region, the dominance of *F. oxysporum* (24.1%) and *F. verticillioides* (20.4%) was shown on plants, *F. oxysporum* (21.8%) and *F. oxysporum* var. *orthoceras* (20.0%) on seeds [66]. Fusariosis manifested itself annually to varying degrees (from single cases of diseases to spreading to 13.3% of plants and more), while the species diversity was quite significant, up to 20 species [60].

Domestic researchers attribute such fungi as *Aspergillus* spp., *Aureobasidium pullulans*, *Cladosporium* spp., *Epicoccum* spp., *Monilia sitophila*, *Mucor* spp., *Penicillium* spp., *Stachybotrys* spp., *Oedocephalum* spp. and *Trichothecium* spp. to accompanying ones, since they are found, as a rule, together with other causative agents and with a frequency of less than 1% [58-60].

Inspection of forage products from sunflower seeds for contamination with mycotoxins commenced in our country in 2002 [67]. For 42 samples of oil cake and meal, the contamination with mycotoxins proved to be quite high (54%) and in half of the samples was provided mainly by ochratoxin A. T-2 toxin was found in 16% (7.5-39.5 μg/kg), zearalenone only in one sample (77.5 μg/kg), aflatoxin B_1 and sterigmatocystin were not detected. Subsequently, significant contamination of oil cakes and meals with ochratoxin A was confirmed, 45.5% and 58.8% respectively, with a content of more than 50 μg/kg in 6.6% of the samples from the positive ones and 4-48 μg/kg in the rest [68].

In 2003-2006, during the analysis of production consignments of oil cakes and meals in 28.4% of 116 samples, another nephrotoxin was detected, the citrinin (from 14 to 300 μg/kg), often together with ochratoxin A (30 out of 33 positive samples) [69]. Among the contaminants, the toxin of the anthraquinone series emodin (in 4 samples out of 7) was found for the first time in an amount of 20-30 μg/kg [70], and in two samples of oil cake out of 58 there was cyclopiazonic acid (50 and 63 μg/kg) [71]. According to generalized data, in 2004-2009

the frequency of occurrence of ochratoxin A and citrinin was 50% (58/116) (as a rule, jointly present). Combined contamination was accompanied by a general quantitative pattern in their ratio: the citrinin content was higher than the one of ochratoxin A, although coincidences or similar values were also observed [72]. Taking into account that citrinin is known as bioactivator of ochratoxin A [73], the importance of this fact for the assessment of the risk of animals exposure to these toxins should be recognized. The obtained results permitted to state the hypothesis of probable sources of contamination which are the species *Aspergillus* and/or *Penicillium*, capable of producing these toxins separately or jointly.

3. Frequency of occurrence (%) and content of mycotoxins (minimum-average-maximal, μg/kg) **in sunflower cakes and meals in Russia [74]**

Mycotoxin	Meals, oil cakes, n = 334 (1997-2016)	By types of raw materials (2009-2016)	
		meals, n = 57	cakes, n = 45
T-2 toxin	17 (4-16-93)	23 (4-9-16)	36 (5-13-25)
Diacetoxyscirpenol	–	–	–
Deoxynivalenol	4 (40-92-375)	2 (375)	–
Zearalenone	0,6 (66-72-78)	2 (66)	–
Fumonisins	–	–	–
Ergoalkaloids	3 (5-17-40)	2 (11)	7 (5-19-40)
Alternariol	77 (19-262-1990)	77 (19-315-1990)	78 (20-205-955)
Roridine A	–	–	–
Aflatoxin B_1	0,3 (3)	–	–
Sterigmatocystin	6 (4-7-12)	9 (4-7-12)	20 (4-6-11)
Cyclopiazonic acid	21 (50-80-142)	18 (50-79-125)	44 (50-82-140)
Emodin	26 (20-217-5000)	32 (20-93-280)	31 (20-440-5000)
Ochratoxin A	59 (4-19-200)	82 (4-15-93)	69 (4-15-62)
Citrinin	33 (19-85-1020)	46 (20-93-1020)	20 (50-88-125)
Mycophenolic acid	25 (20-93-379)	35 (25-95-380)	16 (20-93-335)
PR-toxin	–	–	–

N o t e. n — the number of examined samples. A dash indicates that the samples containing mycotoxin are not found.

In 2008-2010, the first cases of detecting alternariol, mycophenolic acid, T-2 toxin, deoxynivalenol, sterigmatocystin and cyclopiazonic acid in cakes and meals were recorded.

Based on the results obtained for the entire monitoring period from 1997 to 2016 (Table 3), a large combined contaminant with the most frequent detection of alternariol and ochratoxin A and a slightly smaller spread of citrinin, emodin, cyclopiazonic acid, T-2 toxin and mycophenolic acid is characteristic for oil cakes and meals. The remaining mycotoxins (deoxynivalenol, zearalenone, ergoalkaloids and sterigmatocystin) were found in single cases or were not found at all (diacetoxyscirpenol, fumonisins, roridine A and PR-toxin). Both types of raw materials (see Table 3) had a similar type of contamination: the same set of major contaminants and similar accumulation rates, but for the oil cakes there was a slightly higher incidence of ergoalkaloids, sterigmatocystin and cyclopiazonic acid. In one sample, an abnormally high accumulation of emodin (up to 5000 μg/kg) was observed [74].

The long-term examination of oil cakes and meal on a large sample (334 samples) permitted to establish that the type of their contamination in Russia is fundamentally different from the one, described many times in other countries, primarily due to the absence of aflatoxin B_1 and a high rate of occurrence of ochratoxin A, often jointly with citrinin. The presence of ochratoxin A in meals was previously found in Hungary (18.2% of 22 samples, 100-260 μg/kg at an average of 160 μg/kg) [49] and in Yugoslavia [75].

The study of production consignments of raw materials (seeds intended for oil production) in our country has just begun, but the first assessments indicate a lesser degree of contamination compared to the one, established for processing products. In 2017, the first results of the analysis of mycotoxins in sun-

flower seeds proposed for sale to the population were obtained. Contamination with mycotoxins was weakly expressed. Out of 27 seed samples selected in Moscow farmers' markets in 2015-2016, only 6 were positive: two samples had alternariol (42 and 48 μg/kg) and emodin (58 and 208 μg/kg), in single cases T-2 toxin (158 μg/kg) and mycophenolic acid (250 μg/kg) [74]. In connection with this, attempts to discuss the role of fungi of the genus *Aspergillus*, capable of producing aflatoxins, which are actively undertaken in the domestic literature in response to the growing demands for the ecological safety of sunflower products in our country [76], do not have any grounds.

Undoubtedly, in the future, for drawing reasonable conclusions about the mycotoxicological status of this raw material, intended for consumption by the population and processing, it is necessary to continue monitoring sanitary-relevant indicators on a whole scale. However, even now, based on the results of assessing the contamination of seeds and oil cake/meal, it can be concluded that the post-harvest period, storage and processing are key stages for the possible increase in contamination. The need for long-term seed maintenance prior to implementation (the ripening phase, raw material stocks), as well as long processing and storage periods, creates favorable conditions for the dynamic development of mycobiota with a change in composition, in which growth activation and enhancement of metabolic activity of competitive species are possible. In this regard, it is extremely important to identify and control those factors, which are significant for ensuring the mycotoxicological safety of seed stocks, technological intermediates at processing plants and in ready-to-use products.

One should also take into account such a feature of post-harvest seed treatment as rapid self-warming of the wet threshed heap, which can (in case of large content of impurities) significantly affect their quality. Due to the uneven nature of seeds maturation, the moisture of consignments is not uniform. In those cases, when activation of mycobiota occurs with the advantage of competitive toxigenic species, a real threat of focal accumulation of mycotoxins is considered probable.

In our country, to ensure the safe use of sunflower seeds for food and fodder purposes, it is necessary to revise the approaches to the regulation of mycotoxins content and introduce reasonable control criteria. Obviously, the current indicators do not correspond to the real situation. In seeds, supplied for food purposes, the content of aflatoxin B_1 (5 μg/kg, not more) [77-79] is limited, and for feeding purposes there are limits of aflatoxin B_1 (20 μg/kg), ochratoxin A (50 μg/kg), T-2 toxin (100 μg/kg), deoxynivalenol (1000 μg/kg) and zearalenone (1,000 μg/kg) [79]. In sunflower meal, mycotoxins are not normalized [80], and T-2 toxin (not more than 100 μg/kg), deoxynivalenol (not more than 1000 μg/kg) and zearalenone (not more than 1,000 g/kg) [81] are regulated for cake.

Modern science has accumulated a significant amount of information on the extent of occurrence of micromycetes and mycotoxins in other important agricultural plants (soybean and peanut), which also requires generalization and critical analysis. Efforts are made to implement a similar approach for rapeseed, the individual segment of which is constantly growing in the modern market for agricultural products [82]. With regard to less commonly used oilseed and textile crops (castor plant, sesame, safflower, mustard, false flax, colewort, lallemantia, cotton plant, flax and kenaph), such studies have not yet been carried out.

The actual data on mycotoxins in vegetative sunflower plants is not sufficient to discuss the problem. However, the first experimental data indicate that at the initial phase of growth, before the heads are formed, multiple contamination occurs, and during the ripening period, mycotoxins are distributed unevenly

across leaves, stems, heads and seeds [74]. As compared to green parts of mature plants (leaves and heads), the contamination of achenes proved to be very moderate, which may be a general phenomenon associated with the action of protective mechanisms of generative organs from bio-damage in plants, which is of scientific interest and deserves a more detailed consideration. Until now, the processes associated with the emergence of metabolism products of microscopic fungi in sunflower plants from the beginning to the end of vegetation, as well as their localization in the plant, have not yet been studied. However, in recent years, the work has commenced to identify low-molecular compounds in glandular sunflower trichomes [83], which are supposed to be involved in protecting the organism from pathogens.

Thus, the comparison of scientific facts on the contamination of sunflower seeds by toxigenic fungi and mycotoxins, undertaken in the present paper, gives convincing confirmation of the urgency of the problem under discussion and its extreme complexity. Differences in the composition of mycobiota and components of the toxins complex are quite contrasting in the areas of cultivation of this crop. With prolonged seed storage before processing, the probability of a rapid and unpredictable exacerbation of the situation is very high due to the emergence of competitive advantages among highly active producers. To guarantee the safe use of sunflower for food and fodder purposes, it is necessary to continue research for grounded approach to mycotoxicological control of seed raw material and products of its processing. The consideration of known information and accumulation of new data on the peculiarities of seed contamination with toxin-forming fungi and mycotoxins in the main areas of commercial sunflower cultivation in the future will permit to recommend more effective preventive measures to reduce or prevent the threats of mycogenic intoxications of people and animals.

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CLONNING OF *DREB1* GENE IN WHEAT WILD RELATIVES AND DEVELOPMENT OF A DNA MARKER FOR ITS MONITORING IN WHEAT BACKGROUND

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Abstract

The *DREB* gene encodes the transcription factor DREB involved in the response of the plant to drought, salinity and heat. The DREB transcription factors induce the expression of multiple genes linked by signal transmission, abscisic acid-dependent and independent, in response to abiotic stress. Many wild species have evolved under extreme environmental conditions (drought, salinity), so they can serve as sources of new genetic variants of *DREB* in breeding wheat for stress resistance. The study of *DREB* orthologous genes in wild relatives of wheat will permit to expand the set of genes in its breeding improvement using wide hybridization, and the design and application of molecular markers will facilitate transfer of these genes into a genome of bread wheat. Among the diversity of genes encoding DREB proteins, *DREB1* is of the greatest interest due to its involvement in control of plant resistance to various abiotic factors, such as drought, salinity, low temperatures. We were the first to study the *DREB1* orthologs in members of the genera *Thinopyrum*, *Dasypyrum* and *Pseudoroegneria*. Using primers designed on the basis of *DREB1* conserved regions we amplified fragments of the *DREB1* orthologs in *Thinopyrum intermedium*, *Th. ponticum*, *Th. bessarabicum*, *Dasypyrum villosum*, *Pseudoroegneria spicata*, and *P. stipifolia*. The obtained PCR products were cloned and sequenced. As a result, 30 unique sequences were shown to be highly homologous (92-98 %) to the *TaDREB* genes of bread wheat. Between the sequences, we identified multiple single-nucleotide polymorphisms (SNPs) and several large insertions/deletions. The resulting DNA sequences were translated in silico into hypothetical amino acid sequences. All nucleotide sequences found by us are capable of encoding a complete protein that has a DNA-binding domain specific for DREB AP2. Comparison of the amino acid sequences of the AP2 DNA-binding domain in the studied samples showed the presence of polymorphisms for individual amino acids. In all hypothetical amino acid sequences, except for one the sequence described, amino acids conserved for the DREB AP2-domain of cereals were found. We developed the CAPS marker P18_FokI, which in most cases can differentiate the *DREB1* orthologs between wheat and wild relatives due to the presence of polymorphisms in the restriction sites, the fragment amplified from the genome of bread wheat has a size of about 570 bp. A *DREB1* ortholog was localized in the homeological group 3 of *Th. elongatum* (3Je) using P18_FokI and a series of addition bread wheat lines with *Th. elongatum* chromosomes. Analysis of 10 wheat-wheatgrass hybrids revealed the presence of both *TaDREB* bread wheat and the *DREB1* ortholog in all analyzed accessions. In this case, the wheat-type fragment was absent in bread wheat with a substituted chromosome 6J (6D), which also serves as a proof of localization of the *DREB1*

ortholog on the chromosome of homeological group 3. Thus, the CAPS marker P18_FokI developed by us helps to effectively transfer the *DREB1* gene from the wild cereals to the genome of bread wheat, so that we can study the effect of the alien *DREB1* gene on the resistance of bread wheat to drought, salinity, low temperatures, and, farther, to create valuable breeding forms using MAS.

Keywords: bread wheat, *Triticum*, *Thinopyrum*, *Pseudoroegneria*, *Dasypyrum*, resistance genes, orthologous genes, drought, salinity, DREB1, DREB, TADREB1, polymerase chain reaction, molecular markers, sequencing

Significant losses in the agrarian sector are due to such abiotic factors as soil salinity and moisture deficit. The tolerance of plants to these stresses is complex, that is, it is the result of the interaction of many genes and biochemical factors. A key role in plant resistance is played by various transcription factors [1-2]. Among them, the DREB group (dehydration-responsive element binding) can be distinguished from the AP2/ERF family proteins. The transcription factors of DREB, in response to the abiotic stress, induce the expression of multiple genes associated with the abscisic acid-dependent (ABA-dependent) and ABA-independent signal transmission [3-5]. The transcription factor of DREB specifically binds to the regulatory sequence Dre (5′-TACCGACAT-3′), which was first detected in the promoter of the *rd29A* gene [6]. The DREB protein contains an alkaline N-terminal amino acid region acting as a nuclear localization signal. To the right of the N-terminal region, there is the AP2 DNA-binding domain, which consists of three-chain β-folds and one α-helix almost parallel to β-folds [7]. The seven amino acid residues in the AP2 domain [8] are key for binding to the DNA GCC box. The conserved region with a high content of serine and threonine (ST-rich region), which can serve as a phosphorylation site [9], adjoins the AP2/ERF DNA-binding domain. DREB has an acidic C-terminal region, which is believed to possess transcriptional activation activity [10, 11].

Among the diversity of genes, encoding DREB proteins, *DREB1* is of the greatest interest due to involvement in plant resistance to various abiotic factors, such as drought, salinity and low temperatures [12-14]. *DREB1* genes are sequenced in many plant species, including bread wheat [15-17]. B. Wei et al. [17] localized homologous genes *TaDREB1* of bread wheat on the chromosomes of homeologous group 3 and revealed polymorphism by DNA sequences. Various single nucleotide polymorphisms (SNPs) in *DREB1* of wheat are associated with resistance to drought and salinity [18, 19]. In addition to bread wheat, *DREB* genes have been characterized in the related wild-growing species *Aegilops tauschii*, *Leymus chinensis* and *Elymus spicatus* [20-22].

The use of genetic engineering approaches permitted to demonstrate the role of *DREB* genes in enhancing resistance to abiotic stress. In particular, the gene of wild barley *Hordeum spontaneum* was inserted into the genome of *Paspalum notatum* [23]. In this and other similar experiments, the obtained transgenic plants differed from the control ones by increased resistance to salt and drought [24-27].

The use of the genetic potential of wild-growing species, closely related to wheat, can also be useful for its genetic improvement, including increasing resistance to biotic and abiotic factors. Evolutionally, many species were formed in extreme ecological and geographic conditions (drought and salinity) [28, 29]. Therefore, the genetically closest wild relatives can serve as a source of new genetic variants of *DREB* for stress-resistance selection.

Allopolyploid species, e.g. intermediate wheatgrass *Thinopyrum intermedium* (genomic constitution $J^rJ^{vs}St$, $2n = 6\times = 42$) and rush wheatgrass *Th. ponticum* (genomic constitution JJJ^sJ^s, $2n = 10x\times = 70$) are widely used in distant hybridization of wheat [29, 30]. They carry valuable genes of resistance to

salinity, drought and low temperatures [31, 32], as well as to pre-harvest sprouting [33]. Forms of wheat with the genetic material of wheatgrass have been developed, which have resistance to leaf rust [34-36], stem rust [37], fusariosis [38], wheat streak mosaic virus [39] and other diseases. Chromosomes of rush and intermediate wheatgrass are relatively easy to conjugate and exchange patches [40]. According to current representations, the donors of the subgenomes of these species are *Dasypyrum villosum* (V, $2n = 2\times = 14$), *Th. bessarabicum* (J^b, $2n = 2\times = 14$) and *Pseudoroegneria spicata* (St, $2n = 2x = 14$); the first two species are also widely used in distant wheat hybridization [41-43].

This article presents the DNA sequences of the *DREB1* orthologues, which were obtained for the first time in representatives of the genera *Thinopyrum*, *Dasypyrum*, *Pseudoroegneria*, and also the CAPS marker P18_*Fok*I was developed, which permits to distinguish the *TaDREB1* wheat gene and the gene of the listed species.

The purpose of this study was to sequence and analyze *DREB1* orthologues of wild species related to wheat and to create a PCR marker, which would allow distinguishing the *DREB1* gene of wild species and bread wheat.

Techniques. The authors used samples of *Thinopyrum bessarabicum* (Savul. & Rayss) Á. Löve (PI 531711), *Th. intermedium* (Host) Barkworth & D.R. Dewey (PI 401200), *Th. ponticum* (Podp.) Z.-W. Liu & R.-C. Wang (PI 508561), *Pseudoroegneria spicata* (Pursh) Á. Löve (PI 537371), *P. stipifolia* (Czern. ex Nevski) Á. Löve (PI 325181), *Dasypyrum villosum* (L.) Borbás (W6 21717), seeds of which were obtained from Germplasm Research International Network (GRIN, USA), and *Th. ponticum* (1158A/19), obtained from N.V. Tsitsin Main Botanical Garden (MBG) of the Russian Academy of Sciences (Moscow, Russia). *DREB1* gene mapping was performed on a set of bread wheat lines Chinese Spring supplemented with *Th. elongatum* chromosomes. Moreover, 10 samples of wheat-wheatgrass hybrids (WWH) containing wheatgrass and wheat chromatin were used from the collection of N.V. Tsitsin Main Botanical Garden of the RAS, the 5542, 2087, 548, 1674, 4082, ZP26, M3202, 4044, 4015 and M12. The bread wheat (*Triticum aestivum* L.) varieties Nemchinovskaya 24, Aivina, as well as bread wheat varieties Tulaikovskaya zolotistaya, Tulaikovskaya 10, Tulaikovskaya 100, Tulaikovskaya 110 with a substituted chromosome from intermediate wheatgrass 6J (6D) [35] served as the control ones.

The DNA was extracted from young leaves by the method of R. Bernatzky et al. [44]. In PCR, the primers P18F were used (5′-CCC AAC CCA AGT GAT AAT AAT CT-3′), P18R (5′-TTG TGC TCC TCA TGG GTA CTT-3′), P20F (5′-TCG TCC CTC TTC TCG CTC CAT-3′), P20R (5′-GCG GTT GCC CCA TTA GA CAT AG-3′), P21F (5′-CGG AAC CAC TCC CTC CAT CTC-3′), P21R (5′-CGG TTG CCC CAT TAG ACG TAA-3′), P22F (5′-CTG GCA CCT CCA TTG CCG CT-3′), P25F (5′-CTG GCA CCT CCA TTG CTG CC-3′), PRa (5′-AGT ACA TGA ACT CAA CGC ACA GGA CAA C-3′), developed by B. Wei et al. [17]. PCR was performed on a DNA Engine Tet-rad 2 (Bio-Rad, USA) by the following protocol: 5 min at 95 °C (1 cycle); 30-45 s at 95 °C, 30-60 s at 60 °C, 40-120 s at 72 °C (34 cycles); and 10 min at 72 °C (1 cycle). Samples were stored at 10 °C. The PCR products were separated in 1.5% agarose gel with TBE buffer at a field strength of 6 V/cm.

To clone the amplification products, they were purified using the GeneJET™ PCR Purification Kit (Fermentas, Lithuania) according to the manufacturer's instructions. Purified DNA was ligated into the pGEM®-T Easy vector (Promega, USA). The vector was transformed into *Escherichia coli* strain DH10B (Life Technologies, USA). Transformation was executed on the electroporator GenePulser (Bio-Rad, USA). Recombinant clones were detected by the

blue-white selection method.

Clones for sequencing were selected by PCR with primers M13 flanking the insertion site. Sequencing was performed on an ABI-3130XL device (Applied Biosystems, USA). The alignment of the nucleotide and amino acid sequences was performed with ClustalW2 program [45]. To obtain hypothetical amino acid sequences, the resource ExPASy [46] was used. The nuclear localization signal was found using the cNLS Mapper algorithm [47]. To determine the GCC-binding boxes and the AP2-binding domain, the Conserved Domain Database [48] was used.

The restriction endonuclease (Fok1) was selected based on the nucleotide alignment analysis of the region amplified with the primer P18. PCR products (10 μl) were restricted for 12 hours at 37 °C, and the restriction fragments were separated by electrophoresis in a 2% agarose gel.

Results. The involvement of the genetic resources of the tertiary pool of the tribe *Triticeae* plays an important role in the selective improvement of wheat. In this respect, wild-growing representatives of the genera *Thinopyrum*, *Dasypyrum*, *Pseudoroegneria* are considered promising. They are characterized by resistance to salt and drought, resistance to phytopathogens of viral and fungal origins, pests and by other economically significant characteristics [28, 31, 49]. *DREB1* genes of wild species closely related to wheat are important in wheat breeding for increased tolerance to salinity and drought.

As a result of cloning PCR products amplified with various primer combinations and subsequent sequencing, we obtained 30 unique nucleotide sequences for six examined species of wild cereals: *Th. bessarabicum* (Thbe1, Thbe2, Thbe3), *Th. intermedium* (Thin1, Thin2, Thin4, Thin5, Thin6, Thin7), *Th. ponticum* (Thpo1, Thpo2, Thpo3, Thpo4, Thpo5, Thpo6, Thpo7, Thpo8 – from the sample PI 508561; Thpo9, Thpo10, Thpo11, Thpo12, Thpo13, Thpo14, Thpo15, Thpo16 – from the sample 1158A/19), *P. spicata* (Pssp1, Pssp2), *P. stipifolia* (Psst) and *D. villosum* (Davi). All sequences were obtained with primers P18F/R, except Thbe1 (P20F + PRa is a full-length gene sequence), Thbe2, Thbe3, Thpo1, Thpo2, Thin1, Pssp1 and Davi (P21F + PRa).

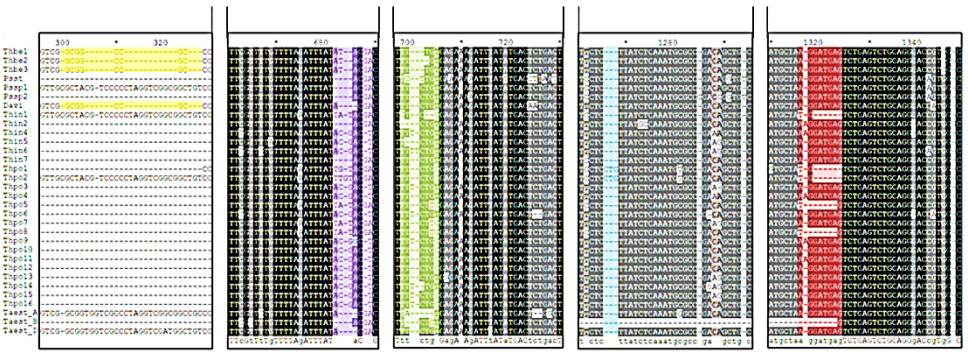

Fig. 1. Fragments of the aligned sequences of *DREB1* orthologous gene in *Thinopyrum bessarabicum* (lines 1-3), ***Pseudoroegneria stipifolia*** (line 4), ***P. spicata*** (lines 5-6), ***Dasypyrum villosum*** (line 7), ***Th. intermedium*** (lines 8-13), ***Th. ponticum*** (lines 14-29) **and *TaDREB* of three subgenomes of bread wheat** (three lower lines)**.** The largest indels are highlighted in color: four indels in positions 299-327 (highlighted in yellow); region 682-689 (highlighted in purple); region 698-705 (highlighted in green); region 1266-1268 (highlighted in blue); region 1316-1324 (highlighted in red).

The alignment of DNA sequences with *DREB1* genes of bread wheat revealed 92-98% homology between them. The main differences between the sequences of the species studied and the sequences of bread wheat were numerous SNPs and insertions/deletions (indels) (Fig. 1). The largest ones were four indels in the positions 299-327 in *Th. bessarabicum* (1 bp, 6 bp, 11 bp and 3 bp) and

the indel in *D. villosum* (see Fig. 1, highlighted in yellow), indels in all studied species in the positions 682-689 (see Fig. 1, highlighted in purple), 698-705 (highlighted in green), 1266-1268 (see Fig. 1, highlighted in blue) and 1316-1324 (see Fig. 1, highlighted in red). Despite a significant number of DNA sequences we obtained the polymorphism of the *DREB1* orthologous gene is not limited to the detected SNPs and indels, since even within a single specimen of a wild species there can be intrapopulation diversity.

The obtained DNA sequences were translated in silico into hypothetic sequences of amino acids (AA). None of them contained stop codons. All the predicted AA sequences have the same structural organization, which is characteristic of DREB protein. In the N-terminal domain, there is a nuclear localization signal (NLS). Near the AP2 DNA-binding domain, a conserved ST-rich region is located. The C-terminal domain is enriched with glutamic and aspartic acids, which indicates the presence of a transcriptional activation domain.

Fig. 2. Alignment of the hypothetical amino-acid sequence of the AP2 DNA-binding domain of DREB1 protein in *Thinopyrum bessarabicum* (lines 1-3), ***Pseudoroegneria stipifolia*** (line 4), ***P. spicata*** (lines 5-6), ***Dasypyrum villosum*** (line 7), ***Th. intermedium*** (lines 8-13), ***Th. ponticum*** (lines 14-29) **and subgenomes A, B and D of bread wheat** (three lower lines). The arrows indicate polymorphic amino acids in the AP2 domain; the amino acid residues that interact with the GCC box are highlighted in green, the replacement by one such amino acid residue is highlighted in red; asterisks indicate the amino acids that are important for DRE-specific binding.

An important role in the functioning of the DREB protein belongs to the AP2 DNA-binding domain; polymorphisms within this region can have a critical effect on the activity of the DREB protein. In all hypothetical AA sequences, there was an AP2 DNA-binding domain consisting of 59 amino acid residues (Fig. 2). The comparison of the AA sequences of the AP2 DNA-binding domain in the studied samples showed the presence of single amino acid polymorphisms (SAP). Since wild species are able to occupy different ecological and geographical niches and exhibit significant population diversity, it should be assumed that the SAP over the DREB1 protein may be wider than we have identified. In the sequences obtained by us all conserved amino acids of the AP2 domain were present which are characteristic of DREB proteins of cereals [50], with the probable exception of the Thpo11 sequence: in the position 25-27, the

specific WLG motif [25-27] was substituted by RLG (replacement of tryptophan with arginine) (see Fig. 2). The W(R) substitution that we revealed occurred in one of the seven conserved amino acids of the GCC-binding box in the AP2 domain. The presence of such a substitution in the AP2 domain may disorder protein configuration which, in turn, may affects the DNA-binding ability [51].

Comparative analysis of the DNA sequences of the *DREB1* gene of six wild species of cereals (*Th. intermedium*, *Th. ponticum*, *D. villosum*, *P. spicata*, *P. stipifolia*, *Th. bessarabicum*) with DNA sequences of the *DREB1* gene of bread wheat revealed SNPs which are characteristic for each species. This allowed the development of a PCR marker that can be used to identify most of the *DREB1* genes of the species which were studied in the genetic background of bread wheat.

We used the primers P18F/R, proposed by B. Wei et al. [17], to amplify the conserved region of the *TaDREB1* gene of bread wheat encoding the DNA-binding domain of AP2. By comparison of the nucleotide sequences of the *DREB1* orthologous genes obtained with a pair of primers P18F/R in the five examined species, the *Th. intermedium*, *Th. ponticum*, *D. villosum*, *P. spicata*, *Th. bessarabicum*, and the sequences of *DREB1* genes of bread wheat, three sites for FokI endonuclease restriction were identified (Fig. 3, highlighted in turquoise). The absence of one of the restriction sites and the presence of the other two were characteristic of most *DREB1* gene sequences in wild species, which distinguished them from the *DREB1* gene sequences of the subgenomes A and D of bread wheat. The *DREB1* gene of the subgenome B contained only one FokI restriction site; in addition, there was a large deletion (over 35 bp) in the same region. This difference allowed us to create CAPS (cleaved amplified polymorphic sequences) marker P18_FokI which in most cases can differentiate the *DREB1* orthologous genes of wheat and wild cereals.

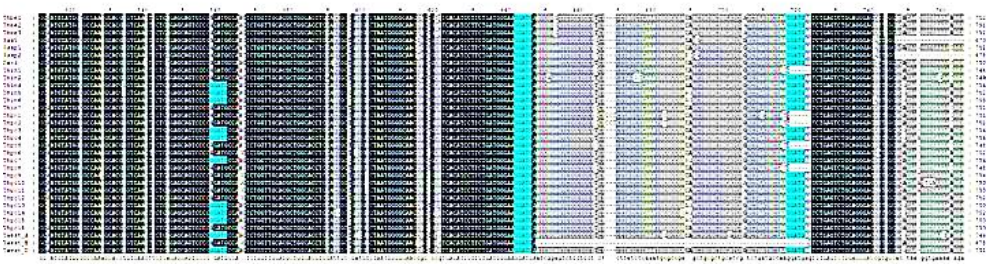

Fig. 3. Fragments of the aligned sequences of the *DREB1* orthologous gene in *Thinopyrum bessarabicum* (lines 1-3), ***Pseudoroegneria stipifolia*** (line 4), ***P. spicata*** (lines 5-6), ***Dasypyrum villosum*** (line 7), ***Th. intermedium*** (lines 8-13), ***Th. ponticum*** (lines 14-29) **and *TaDREB* of three subgenomes of bread wheat** (three lower lines). The turquoise color shows restriction sites for FokI endonuclease.

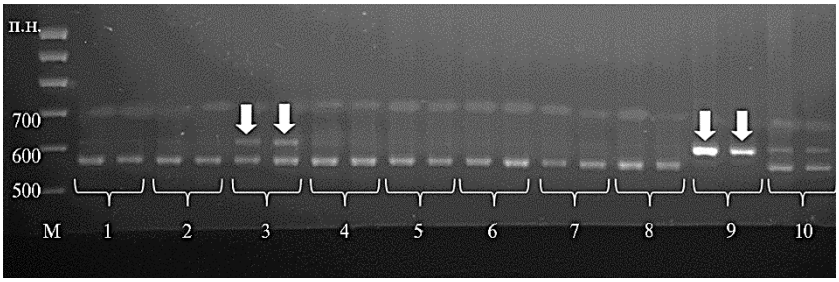

Fig. 4. An electrophoregram showing the results of the analysis of Chinese Spring (CS) **wheat lines with *Thinopyrum elongatum* chromosomes using the CAPS marker P18_FokI associated with the *DREB1* gene:** 1 — CS + 1J^e, 2 — CS + 2J^e, 3 — CS + 3J^e, 4 — CS + 4J^e, 5 — CS + 5J^e, 6 — CS + 6J^e, 7 — CS + 7J^e, 8 — *Triticum aestivum*, Aivina variety, 9 — *Th. bessarabicum*; M is a marker of DNA fragment length (GeneRuler 100 bp DNA Ladder, Thermo Fisher Scientific, USA). The arrow indicates an additional fragment of ~ 600 bp amplified from orthologous wheatgrass *DREB1* gene.

We established chromosomal localization of the *DREB1* gene in the genome J with the developed P18_FokI marker as a tool. For this, we used a series of wheat lines with alien chromosomes of tall wheatgrass *Th. elongatum* ($2n = 2\times = 14$, genome J^eJ^e). The control was wheatgrass *Th. Bessarabicum* carrying the genome J^bJ^b ($2n = 2\times = 14$), highly homologous to the genome of *Th. elongatum*. In collections of genetic resources noted as *Th. Elongatum* we revealed only representatives of the polyploid species *Th. ponticum* ($2n = 10\times = 70$, genomic composition $JJJJJJ^sJ^sJ^sJ^s$). These accessions were formerly called the Eastern European species of *Th. elongatum* [52, 53], which is a common thing for genetic collections of species with complicated botanical identification [54].

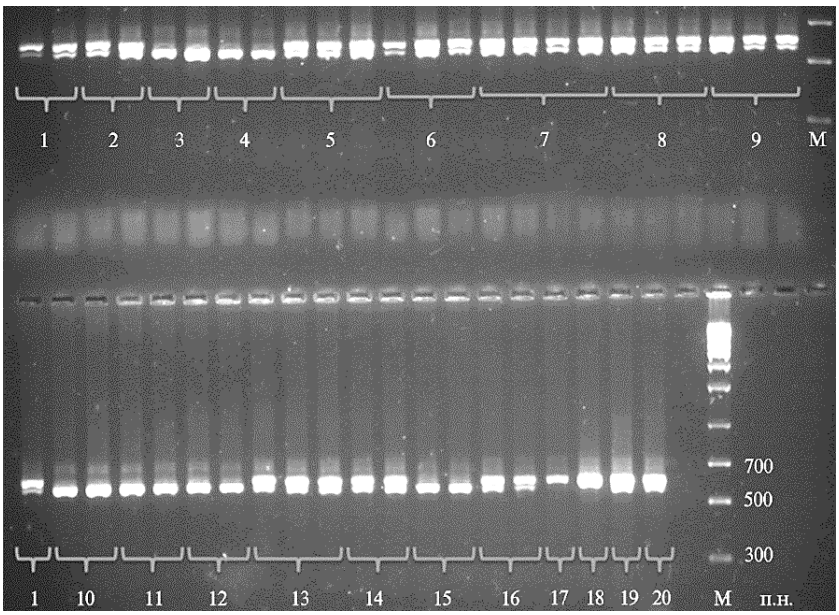

Fig. 5. Electrophoregram showing the results of analysis of wheat and wheatgrass hybrids and varieties using the CAPS marker P18_FokI for *DREB1* gene: 1 — 5542, 2 — 2087, 3 — Tulaikovskaya 110, 4 — Nemchinovskaya 24, 5 — 548, 6 — 1674, 7 — 4082, 8 — ZP26, 9 —— M3202, 10 — Aivina, 11 — Tulaikovskaya 10, 12 — Tulaikovskaya 100, 13 — 4044, 14 — 4015, 15 — Tulaikovskaya zolotistaya, 16 — M12, 17 — *Thinopyrum intermedium*, 18 — *Th. ponticum*, 19 — *Pseudoroegneria spicata*, 20 — *Th. intermedium*; M — marker of DNA fragment length (Fisher BioReagents™ exACTGene™ DNA Ladders, Thermo Fisher Scientific, USA).

With the marker P18_FokI, a fragment of about 570 bp in size was found in wheat and about 600 bp in wheatgrass *Thinopyrum bessarabicum* (Fig. 4, indicated by an arrow). In all bread wheat lines with chromosomes of tall wheatgrass *Th. elongatum*, a fragment of ~ 570 bp specific for wheat was found and only CS + 3E line with $3J^e$ chromosome had a fragment of ~600 bp specific for wheatgrass. That is, the DREB1 orthologous gene in *Th. elongatum* is located in the homeologous group 3, as in bread wheat, which confirms the correct work of the marker we elaborated.

To approbate the obtained marker P18_FokI, sequential PCR and restriction in 10 samples of wheatgrass hybrid was conducted. Using the marker P18_FokI, two fragments were identified in all the test samples of wheat and wheatgrass hybrids, pf ~ 570 bp and ~ 600 bp, that is, the samples carry the *DREB1* gene of both wheat and wheatgrass type (Fig. 5). All control samples of bread wheat, including varieties of bread wheat with a substituted chromosome from intermediate wheatgrass 6J(6D), i.e. Tulaikovskaya zolotistaya, Tulaikovskaya 10, Tulaikovskaya 100, Tulaikovskaya 110, a fragment of ~ 600 bp was absent. Hence, the fragment of ~ 600 bp of the wheatgrass type is specific for the

DREB1 gene localized in the homeologous group 3.

The marker-assisted selection increases the effectiveness of breeding. The use of molecular markers makes it possible to determine the valuable alleles in the splitting population with the least expenditures, to quickly and accurately identify any gene in case of introgression from the donor line to the recipient genome; it also provides a convenient tool for back-crossing [55]. With the help of the authors' CAPS marker P18_FokI associated with the *DREB1* orthologous gene it is possible to increase the efficiency of transferring this gene from wild cereals to bread wheat, to study the effect of the foreign *DREB1* gene on bread wheat resistance to drought, salinity and low temperatures, as well as in the long term to select valuable selection forms.

Thus, in this work we obtained DNA sequences of genes, orthologous to the *DREB1* wheat gene in the species *Thinopyrum intermedium, Th. ponticum, Th. bessarabicum*, *Dasypyrum villosum*, *Pseudoroegneria spicata* and *P. stipifolia*. The research on these nucleotide sequences revealed a polymorphism between the samples of the examined species, i.e. the presence of single nucleotide polymorphisms (SNPs) and indels. The analysis of the hypothetical amino acid sequences encoded by the detected *DREB1* orthologous genes showed conservativeness: only in one sequence there is a substitution of W(R) which is important for the functioning of the AP2 domain. A CAPS marker P18_FokI has been created, by means of which it is possible to distinguish the *DREB1* orthologous gene of the studied wild species from *DREB1* of bread wheat. Using the marker P18_FokI, the *DREB1* orthologous gene is mapped on the chromosome $3J^e$ of tall wheatgrass *Thinopyrum elongatum*. In 10 wheat and wheatgrass hybrids, there was the *DREB1* orthologous gene of a wheatgrass origin.

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RELATIONSHIP BETWEEN CYTOGENETIC CHARACTERISTICS AND MOLECULAR-GENETIC DIFFERENCES IN SPECIES OF THE GENUS *Rhododendron* L. WHEN INTRODUCED

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Abstract

Currently, woody plants attract special attention given the prospects of their involving in bio- and genomic technologies to address challenges of sustainable environment, biodiversity, food security and production of raw materials. Thence, studies of cytogenetic characteristics of woody plants are increasingly relevant. The change in a number of cytogenetic characteristics, in particular, mitotic activity, which may increase and decrease depending on the intensity of stress loads, an increase in the pathology of mitosis, etc., has been shown. However, attempts to identify the similarities and differences in cytogenetic characteristics in woody plants on the basis of the results of molecular genetic comparison weren't conducted yet. Sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal NA were used to generate a phylogenetic hypothesis for disjuncting of wood species of the genus *Aralia* (J. Wen, 2000), to specify of *Rhododendron* systematic state (T.V. Baranova et al., 2014) and other genera of the family *Ericaceae* (O. Schwery et al., 2015). Cluster analysis of nucleotide sequences and construction of the dendrogram were carried out using the ML (Maximum Likelihood, Nearest-Neighbor-Interchange) method in the MEGA software. Germination of *Rhododendron* seeds was carried out in Petri dishes at room temperature. Roots were stained with acetohematoxylin, rinsed with distilled water, and suppressed micro-preparations were prepared using Goyer's fluid. Nucleotide sequences of the ITS1-ITS2 spacer of the parent plants and cytogenetic parameters (mitotic activity, level and spectrum of pathological mitoses, number of cells with residual nucleoli in the metaphase-telophase mitosis stage) we obtained from seed progeny in four *Rhododendron* species introduced into the conditions of the Central Black Earth region of Russia. The identity of the nucleotide sequence of the spacer ITS1-ITS2 in species of the genus *Rhododendron* leads to their greater similarity in the aggregate of cytogenetic indices. However, there is no complete analogy of cytogenetic characteristics in the species studied that have the identical sequence ITS1-ITS2. On the basis of this comparison, it can be assumed that genetic similarity in the studied *Rhododendron* species causes the similarity of cytogenetic indices. According to mitotic activity in the root meristem of the seedlings, two groups can be distinguished among the seed progeny, i.e. with a high value of mitotic activity, namely *Rhododendron dauricum* (7.6±0.3 %) and *Rh. mucronulatum* (7.7±0.7 %), and with low value, namely *Rh. sichotense* (5.6±0.7 %) and *Rh. ledebourii* (6.1±0.6%). The greatest cytogenetic instability is noted in *Rh. ledebourii* (5.2±1.1 %, the level of pathologies of mitosis in this species is maximal), in three other species it was lower (from 3.5±0.5 % for *Rh. sichotense* to 1.6±0.4 % for *Rh. dauricum* mitosis pathologies). A higher level of cells with a residual nucleolus at the stage of metaphase—telophase mitosis indicates a greater intensity of synthetic processes associated with adaptation in conditions of introduction. For this indicator, we can distinguish two groups: i) *Rh. sichotense* (13.3±1.2 %) with a high level of cells with a residual nucleolus at the stage of metaphase—telophase of mitosis, and ii) *Rh. mucronulatum* (9.1±1.1 %), *Rh. dauricum* (10.2±1.0 %) and *Rh. ledebourii* (10.9±1.3 %) with low values. Despite the difference in cytogenetic

parameters in the seed offspring of the studied species, a cluster analysis of the totality of the characteristics of the course of mitosis and nucleolar activity made it possible to distinguish two groups: 1) *Rh. mucronulatum* and *Rh. dauricum*; 2) *Rh. ledebourii* and *Rh. sichotense*. The cytogenetic characteristics of the seed offspring of the species studied are species-specific.

Keywords: *Rhododendron* L., rhododendrons, seed progeny, introduced plants, cytogenetic characteristics, mitotic activity, cytogenetic abnormalities, mitotic pathologies, persistent nucleoli, ITS1-ITS2 sequences, cluster analysis

Woody plants attract more and more attention of researchers given the prospects of involving these biological objects in the sphere of application of bio- and genomic technologies for solving environmental problems, preserving biodiversity, food security and production of raw materials. Therefore, studies of the cytological and molecular and genetic bases of inheritance in such plants are relevant.

Currently, cytogenetic characteristics in Russia are widely studied in conifer species [1], especially in the representatives of the *Pinaceae* family [2-4]. Among deciduous plants, aboriginal species are studied, for example, *Betula pendula* [5-7] and *Quercus robur* [8], as well as introduced forms such as *Catalpa, Tilia* [9] and *Rhododendron* [10-12]. In woody plants, there is a change in the range of cytogenetic characteristics (in particular, mitotic activity, which may increase and decrease depending on the intensity of stress loads), as well as an increase in the frequency of pathologies of mitosis, etc. The cytogenetic indicators of seed progeny of woody plants are affected by chemical pollutants [5, 13], radiation contamination [14] and heavy metals [4, 15, 16]. Cytogenetic processes under stress conditions in seed progeny of many woody plants are similar, which raises questions about their species specificity (or non-specificity) and dependence on external factors. At the same time, attempts to reveal the similarity and differences in the cytogenetic features of woody plants by the results of molecular genetic comparison were not undertaken.

Sequences of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA were used to create a phylogenetic hypothesis for distinguishing species of woody plants of the genus *Aralia* [17], refining the systemic position of *Rhododendron* [18], and other genera of the *Ericaceae* family [19]. Assessment of the molecular and genetic similarity of species is carried out not only for the purpose of studying phylogeny (based on comparison of DNA sequences) [20-24] and biogeography [25] but also in the works on biotechnology [26, 27], bioengineering [28], for distinguishing genetic markers [29], confirming the hybrid status and differences of donor parent forms in ornamental hybrids, including *Rhododendron* species [30]. Thus, an ISSR marker was used to separate groups of the same species according to various characteristics, for example, by the change in the activity of enzymes in response to chemical stress [31].

Rhododendron species as an object of research were chosen due to the fact that now these plants are actively studied from the point of view of molecular systematics [20, 22, 31, 32], genetic diversity [34-36] and chemosystematics [37, 38], whereas information on their cytogenetic features is sketchy [39] and is mainly limited to the assessment of ploidy [10, 11]. Detailed studies on the cytology of *Rhododendron canadense* were conducted (*Rhododendron canadense* (L.) Torr.) [12]. In modern classification for the species *Rh. mucronulatum* Turcz., *Rh. dauricum* L., *Rh. ledebourii* Pojark. and *Rh. sichotense* Pojark, the subsection *Rhodorastrum* (Maxim.) Cullen, the section *Rhododendron*, and the subgenus *Rhododendron* [35, 40] are indicated. It is obvious that in order to establish genetic similarity and differences in the species of one subsection close in morphology, comparative studies of several groups of parameters are required to be conducted.

Reducing the number and the genetic diversity of rhododendrons in nat-

ural conditions also leads to a decline in the adaptive potential of plants; moreover, the difficulty in identifying the species of these beautifully flowering bushes by morphological features, in turn, makes it difficult to conserve the biodiversity of these species. Understanding the extent to which the molecular and genetic similarity causes cytogenetic reactions under introduction conditions will allow for a better understanding of the mechanisms of adaptation of plants to the habitat to maintain their biopotential under artificial conditions.

This paper is the first to analyze the ITS1-ITS2 sequences and compare these data and cytogenetic features of the *Dauricum* series of the subsection *Rhodorastrum* (Maxim.) Cullen (section *Rhododendron*, subgenus *Rhododendron*). As a result, species specificity of cytogenetic characteristics is revealed in the four examined rhododendron species when introduced under the conditions of Central Black Earth Region of Russia.

The aim of the research was to study the cytogenetic and molecular genetic features of rhododendron species under conditions, which are unusual for their natural growth.

Techniques. We used the seeds of bulk collection of four species of the genus *Rhododendron*, *Rh. mucronulatum* Turcz., *Rh. dauricum* L., *Rh. ledebourii* Pojark. and *Rh. sichotense* Pojark., introduced in the B.M. Kozo-Polyansky Botanical Garden of Voronezh State University (geographical coordinates: 39°22' of northern latitude, 51°40' of eastern longitude, height above sea level of 168.2 m). The age of the analyzed plants is 30-35 years. A sample consisted of 5 plants of each species.

DNA was extracted form plant leaves using a CTAB buffer (1.5 M NaCl, 20 mM Na_3-EDTA, 100 mM HEPES pH 5.3, 25 °C, 1.5% CTAB) using the PrimerDigital Oy protocol (http://primerdigital.com/dna.html). The ITS1-ITS2 site was amplified with primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [41] synthesized in Eurofins MWG, Inc. (Germany) (https://www.eurofinsgenomics.eu/). To amplify the fragments of ITS1-ITS2, a standard protocol for Taq DNA polymerase was used. The reaction was executed in 25 μl reaction mixture containing 25 ng of DNA, 1× DreamTaq buffer (with 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.3 μM of each primer and 1 unit of DreamTaq DNA polymerase (Thermo Fisher Scientific, Inc., USA), in the MasterCycler Gradient amplifier (Eppendorf AG, Germany): initial denaturation at 95 °C for 3 min; 20 cycles — 15 s at 95 °C, 60 s at 60 °C, 30 s at 72 °C; the final elongation is 5 min at 72 °C.

The amplified fragments were separated by electrophoresis in a 1.5% agarose gel (RESolute Wide Range, Biozym Scientific GmbH, Germany); the products were visualized with ethidium bromide. To determine the length of DNA fragments, a molecular weight marker was used (Gene-Ruler DNA Ladder Mix, #SM0331, Thermo Fisher Scientific, Inc., USA).

DNA fragments were extracted from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Inc., USA). Ligation of fragments of PCR products into the plasmid T-vector pGEM-T (Promega, Inc., USA) was performed according to the manufacturer's protocol. *Escherichia coli* cells of the strain JM109 were transformed with plasmid DNA (Promega, Inc., USA). Cells bearing a plasmid with an insert were detected by blue-white selection on medium with ampicillin (at a final concentration of 100 μg/ml), a chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 20 mg/ml) and isopropyl-β-D-1-thio-galacto-pyranoside (IPTG, 200 mg/ml). Colony testing for the presence of an inserted PCR products cloned in the vector was performed by PCR with universal pUC primers (M13, forward and reverse).

Sequencing of amplified DNA fragments was provided by the DNA se-

quencing laboratory of the Institute of Biotechnology (University of Helsinki, http://www.biocenter.helsin-ki.fi/bi/dnagen/sequencing_service.htm). The capillary sequencer 3730xl DNA Analyzer (Thermo Fisher Scientific, Inc., USA) was used. ITS1-ITS2 data were obtained for the species *Rh. mucronulatum* Turcz., *Rh. dauricum* L., *Rh. ledebourii* Pojark. and *Rh. sichotense* Pojark in growing under conditions of the Central Black Earth Region. Cluster analysis of the nucleotide sequences and construction of a dendrogram were performed by ML method (Maximum Likelihood, Nearest-Neighbor-Interchange) with MEGA v.6 software [23, 24].

For cytogenetic studies, rhododendron seeds were germinated in Petri dishes at a room temperature. When the roots were 0.5-1 cm long, they were fixed (at 9 am) in aceto-alcohol (a mixture of 96% ethyl alcohol and glacial acetic acid, 3:1), and stored in the refrigerator at +4 °C. Sprouts rootlets were macerated in 18% HCl solution at 60 °C for 1-2 min, then washed for 15 min in 45% acetic acid, stained with aceto-hematoxylin for 1-1.5 hours, rinsed with distilled water. Hoyer's medium was used for squashed preparations. Slides (20 for each species of rhododendron) were viewed with a LABOVAL-4 microscope (Carl Zeiss, Inc., Switzerland) at a total magnification of 40×1.5×10.

In each slide (one slide corresponds to one root and one sprout), about 500-700 cells were examined. A total of about 42500 cells of the studied species of the genus *Rhododendron* were examined. The following cytogenetic parameters were analyzed: mitotic activity (the mitotic index MI is the ratio of dividing cells to the total number of cells counted, %), the percentage of cells across mitosis stages, the frequency of mitotic abnormalities as the percentage of cells with disturbances from the total number of dividing cells, the proportion of cells with residual nucleoli at the metaphase-anaphase mitosis stage as the percentage of cells with residual nucleoli from the total number of cells during the stage). Mitotic abnormalities were classified by I.A. Alov [42].

Data were processed with Stadia v.7.0 software package (http://toptorrent.ws/soft-torrent/4463-camtasia-studio-70.html, TechSmith Corporation, USA). The procedure for data grouping and processing is described by A.P. Kulaichev [43]. To express each cytogenetic index, the mean value was used with an average error (M±SEM). Samples were compared in terms of the frequency of mitotic abnormalities and the proportion of cells with residual nucleoli by the Van der Waerden score criterion, since the analyzed traits do not comply with a normal distribution. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$. Cluster analysis was performed using the metric of the normalized Euclidean distance and the nearest neighbor classification strategy. The following cytogenetic indicators of seed progeny were included in the data matrix for the cluster analysis: MI calculated with regard to the cell number at the prophase stage (%), MI calculated without taking into account the cells at the prophase stage (%), the percentage of cells in the prophase, metaphase, and anatelophase, the frequency of mitotic abnormalities (%) and the proportion of cells with residual nucleoli at metaphase-telophase (%).

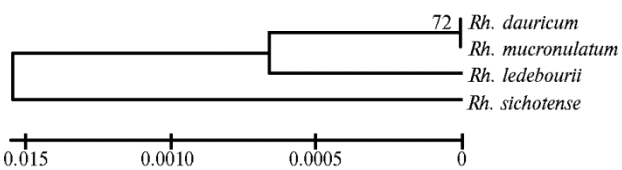

Fig. 1. Nucleotide differences in the ITS1-ITS2 sequence in species of the genus *Rhododendron* L. (Maximum Likelihood method, Nearest-Neighbor-Interchange, MEGA v.6).

Results. The data on analysis of ITS1-ITS2 sequences are presented on a dendrogram (Fig. 1). It is seen that *Rh. mucronulatum* and *Rh. dauricum* have an identical ITS1-ITS2 sequence. *Rh. ledebourii* differs from

Rh. mucronulatum and *Rh. dauricum* in 1 nucleotide of the ITS1-ITS2 sequences, *Rh. sichotense* and *Rh. mucronulatum*, *Rh. sichotense* and *Rh. dauricum* differ in 2 nucleotides. Differences between *Rh. ledebourii* and *Rh. sichotense* for the spacer ITS1-ITS2 is 3 nucleotides.

Tables 1 and 2 show cytogenetic indicators of the studied species of the genus *Rhododendron*.

Cell division is a highly canalized process [8]. In the species of the rhododendrons which were analyzed the parameters of mitosis varied (see Table 1).

1. Cytogenetic characterization of species of genus *Rhododendron* (*M*±SEM)

Species	MI, %		Cells, %		
	with account of prophases	without account of prophases	prophase	metaphase	ana-telophase
Rhododendron dauricum L.	7.6±0.3	3.9±0.2	48.9±2.0	13.3±1.4^{б}	41.7±2.3
Rh. mucronulatum Turcz.	7.7±0.7	4.9±0.7	37.5±1.9^{*в}	10.2±0.9**	51.9±1.4*
Rh. sichotense Pojark.	5.6±0.7^{*a}	3.0±0.4^{*a}	45.8±1.1	14.6±2.2	39.6±3.2^{**б}
Rh. ledebourii Pojark.	6.1±0.6*	3.8±0.4	37.9±1.9^{**г}	18.7±2.1*	43.4±1.7

N o t e. MI — mitotic index.

*, ** Differences with *Rhododendron dauricum* are statistically significant at $p < 0.05$ and $p < 0.01$.

^{a} — differences with *Rh. mucronulatum* are statistically significant at $p < 0.05$; ^{b} — differences with *Rh. mucronulatum* are statistically significant at $p < 0.01$; ^{c} — differences with *Rh. sichotense* are statistically significant at $p < 0.05$; ^{d} — differences with *Rh. sichotense* are statistically significant at $p < 0.01$.

2. Cytogenetic abnormalities in species of genus *Rhododendron* (*M*±SEM)

Species	Abnormal mitoses, %	Cells with residual nucleoli, %
Rhododendron dauricum L.	1.6±0.4	10.2±1.0
Rh. mucronulatum Turcz.	3.4±0.3	9.1±1.1
Rh. sichotense Pojark.	3.5±0.5	13.3±1.2^{a}
Rh. ledebourii Pojark.	5.2±1.1*	10.9±1.3^{в}

* Differences with *Rhododendron dauricum* are statistically significant at $p < 0.05$.

^{a} — differences with *Rh. mucronulatum* are statistically significant at $p < 0.05$; ^{b} — differences with *Rh. sichotense* are statistically significant at $p < 0.05$.

Thus, among the seed progeny, two groups can be distinguished in mitotic activity: the first is *Rhododendron dauricum* and *Rh. mucronulatum* with a high MI value in the root meristem of the seedlings (7.6±0.3% and 7.7±0.7%, respectively), and the second is *Rh. sichotense* and *Rh. ledebourii* with corresponding low indicators (5.6±0.7% and 6.1±0.6%). The analysis of cell distribution through the stages of mitosis showed that the examined species were grouped as follows. The number of cells in the prophase stage in *Rh. dauricum* (48.9±2.0%) and *Rh. sichotense* (45.8±1.1%) was significant, and in *Rh. mucronulatum* (37.5±1.9%) and *Rh. ledebourii* (37.9±1.9%), it was not high. An increased percentage of prophase cells indicates possible irregularities in the mitotic apparatus [44] and the activation of a checkpoint-control system for the integrity of the genetic material [45]. Such cytological responses can be explained by individual (species-specific) features of plants. *Rh. mucronulatum* and *Rh. dauricum*, which do not have differences in the ITS1-ITS2 sequence, are included in the group with high mitotic index values, estimated taking into account cells at the prophase stage. *Rh. ledebourii* and *Rh. sichotense*, differing from each other in the ITS1-ITS2 sequence, belong to the group with a lower mitotic index.

The number of cells in the metaphase of mitosis was maximal in *Rh. ledebourii* (18.7±2.1%) and *Rh. sichotense* (14.6±2.2%); the minimum was in *Rh. mucronulatum* (10.2±0.9%) and *Rh. dauricum* (13.3±1.4%). Delayed cells at the metaphase stage may indicate a violation of the spindle apparatus formation [42]. Grouping of species by the time of passage of the ana-telophase stage turned out to be different. The greatest proportion of cells at this stage is in *Rh. mucronulatum* (51.9±1.4%); in the other species, these values are significantly lower (see Table 1). An increase in the number of cells in the ana-telophase stage of mitosis indicates a violation of the formation of the cell wall [44].

The greatest cytogenetic instability is observed in *Rh. ledebourii* (5.2±1.1%, abnormalities of mitosis is maximal); in three other species, it was lower (from 3.5±0.5% in *Rh. sichotense* to 1.6±0.4% in *Rh. dauricum*) (see Table 2). It can be assumed that under the introduction conditions, the genetic system of *Rh. ledebourii* is the least adapted one. The pattern of irregularities was mainly represented by chromosome lagging in anaphase and metakinesis. Bridges were noted only in *Rh. mucronulatum* (25% of the total number of mitotic abnormalities) and *Rh. sichotense* (12% of the total number of mitotic abnormalities). A higher proportion of cells with a residual nucleolus at metaphase-telophase indicates a greater intensity of the synthetic processes [6] which are associated with the adaptation in the introduction conditions. According to this indicator, two groups were distinguished: the first one was formed by the species *Rh. sichotense* (13.3±1.2%) with a high percentage of cells with residual nucleolus in the metaphase-telophase stage of mitosis, the second one included *Rh. mucronulatum* (9.1±1.1%), *Rh. dauricum* (10.2±1.0%) and *Rh. ledebourii* (10.9±1.3%) with a smaller proportion of such abnormalities

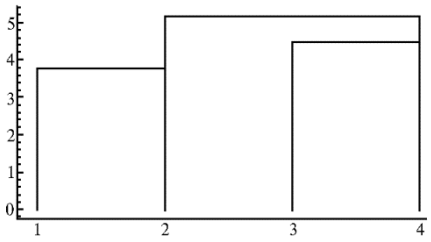

Fig. 2. Dendrogram of cluster distances between species of genus *Rhododendron* L. by cytogenetic characteristics: 1 — *Rh. dauricum*, 2 — *Rh. mucronulatum*, 3 — *Rh. sichotense*, 4 — *Rh. ledebourii* (the metric of the normalized Euclidean distance and the nearest neighbor classification strategy are used, the indicators included in the matrix of data see in the Techniques section).

Despite the difference in the cytogenetic indicators in the seed progeny of the studied species, a cluster analysis of the totality of characteristics of mitosis and nucleolar activity allowed us to distinguish two groups (Fig. 2): the first one is *Rh. mucronulatum* and *Rh. dauricum*, the second one is *Rh. ledebourii* and *Rh. sichotense*. The largest cluster distance is between the cytogenetic index totalities in *Rh. mucronulatum* and *Rh. sichotense*, the smallest ones between the species *Rh. mucronulatum* and *Rh. dauricum*. This agrees with the data we obtained in molecular studies which showed a similar grouping of these species. When introduced into the Central Black Earth Region, the parental plants of the examined species were in the same conditions (B.M. Kozo-Polyansky Botanical Garden of Voronezh State University); therefore, one can speak about the manifestation of the species-specificity of the cytogenetic indicators of their seed progeny. The data of our studies on the similarity of the cytogenetic characteristics of *Rhododendron* species agree with the results of M.B. Belousov et al. [37] on chemotaxonomy, morphology and anatomy, which showed the greatest morphological (secondary) similarity of the same species, explained by similar ecological conditions in the places of growth in the native habitat of species. It should be noted that, according to the molecular and cytogenetic characteristics, the species are grouped in a similar way. The cluster distance, estimated on the basis of studying the cytogenetic properties, between the species *Rh. mucronulatum* and *Rh. dauricum* is the smallest. These species have the same ITS1-ITS2 sequence; *Rh. ledebourii* differs from them by one nucleotide, *Rh. sichotense* by two nucleotides, which corresponds to a greater cluster distance between cytogenetic parameters. The difference between *Rh. ledebourii* and *Rh. sichotense* for the spacers ITS1-ITS2 is three nucleotides. However, the difference in the ITS sequence of 1-3 nucleotides does not play an important role in determining the molecular genetic characteristics. Thus, the similarity of the nucleotide sequence in the species of the genus *Rhododendron* determines their greater similarity according

to the totality of cytogenetic features.

Thus, the identity of the nucleotide sequence of the spacer ITS1-ITS2 in the species of genus *Rhododendron* of the *Dauricum* series leads to their greater similarity in the cumulative set of cytogenetic indicators. However, in the species having the identical ITS1-ITS2 sequence, cytogenetic characteristics do not completely coincide. Based on the comparison we have perforemed it can be assumed that genetic homogeneity in the studied members of the genus *Rhododendron* of the *Dauricum* series causes the similarity of cytogenetic properties. The cytogenetic characteristics of the seed progeny in the examined forms are species-specific.

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INDIRECT SHOOT ORGANOGENESIS OF SOYBEAN *Glycine max* (L.) Merr. FROM STEM SEGMENTS AND USE OF THE EXPLANTS FOR *Agrobacterium*-MEDIATED TRANSFORMATION

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Abstract

Soybean *Glycine max* (L.) Merr. is an important oil, food and fodder crop for human and animals fodder. Currently, soybean lines genetically modified for improved resistance to herbicides and pests and for reduced linolenic acid content are widely grown. More than 85 % of transgenic soybean plants are obtained using *Agrobacterium*-mediated transformation method. Developed *Agrobacterium*-mediated protocols are based on somatic embryogenesis and direct or indirect shoot organogenesis. Cotyledons, cotyledonary nodes, hypocotyl and epicotyl segments, immature or mature embryos serve as explants. Despite the large number of *Agrobacterium*-mediated protocols, stable transformation of soybeans is still not a routine procedure because it depends on the genotype. Surprisingly, the data on the use of soybean stem segments in genetic transformation is practically absent, although stem segments widely and efficiently serve as explants in the production of most transgenic monocotyledonous plants. Thus, the purpose of the study was to develop a protocol for shoot organogenesis from stem segments of soybean and their application as explants for the production of transgenic plants by *Agrobacterium*-mediated transformation. Stem segments of aseptic soybean seedlings of breeding lines 1476 and 1477 were used for callus induction and shoot organogenesis. The explants were cultured on four various MS-based growing media which differed in 6-benzylaminopurine (BA) concentrations (0.5 and 1.0 mg/l) in combination with i) 0.1 mg/l indole-3-acetic acid (IAA), or ii) 0.1 mg/l indole-3-acetic acid (IAA) and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2.4-D). It was shown that the studied soybeans genotypes differ significantly in morphogenetic ability. Out experiments confirmed that the addition of 2.4-D resulted in inhibition of shoot organogenesis in the both genotypes. It was found that 1 mg/l BA in combination with 0.1 mg/l IAA are the best growth regulators providing the highest frequency of indirect shoot organogenesis. As a result, an effective protocol of indirect shoot organogenesis from soybean stem segments of line 1476 seedlings was developed which ensures more than 50 % frequency of organogenesis. This protocol was applied in genetic transformation of soybean line 1476 by *Agrobacterium tumefaciens* strain AGL0 carrying the plasmid pCambia1381Z-pro-SmAMP1-771. By gradual selection on the induction medium supplemented with hygromycin B (1-10 mg/l), 8 independent lines of putative primary transformants were selected. PCR analysis confirmed the presence of the selective (*hpt*) and marker (*uidA*) genes in 4 independent transgenic lines. The transformation efficiencies calculated based on the results of PCR analysis was 2.0 %. These results indicate the successful involvement of stem segments as explants for genetic transformation of soybean.

Keywords: soybean, *Glycine max* (L.) Merr., in vitro culture, shoot organogenesis, *Agrobacterium*-mediated transformation

Soybean *Glycine max* (L.) Merr. is one of the most important food, in-

dustrial and fodder crops in the world. Soybean seeds contain about 40% of amino acid balanced protein, 20% of fat, as well as a large number of physiologically active substances, the vitamins, macro- and microelements and isoflavones [1]. According to the Food and Agriculture Organization of the United Nations (FAO), the global gross harvest of soybeans in 2014 amounted to more than 306.5 million tons with the cultivation in the territory of 117.5 million hectares, out of which Russia accounted for about 0.8% (2.3 million tons) from an area of 1.9 million hectares. At the same time, over 90% of soybean acreage in Russia is concentrated in the Far Eastern, Central and Southern Federal Districts. According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), genetically modified soybean lines, which have elevated resistance to herbicides and pests, as well as a reduced content of linolenic acid, were grown on the 78% of arable land in 2016 (91.4 million hectares), occupied by this crop in the world [2].

For the first time, two independent groups of researchers reported on the production of transgenic soybean plants in 1988 [3, 4]. To date, there are a large number of publications on this topic; however, despite the abundance of experimental data, the production of stable transgenic soybean plants has not become a routine procedure, since this process depends on the availability of effective protocols for shoot regeneration in vitro and under genetic transformation conditions. Over 85% of transgenic soybean plants are obtained by the method of agrobacterial transformation [4-8]. To introduce foreign genetic material into the soybean genome, direct methods are also used, i.e. bioballistic transformation [3, 9] and protoplast electroporation [10]. In recent years, the method of germ-line genetic transformation of soybean, in which elements of the plant's generative organs (germinating pollen, egg-cells, embryos and seeds) are used for introducing exogenous DNA, have become widespread [11, 12]. Each method of genetic transformation has its advantages and disadvantages. For bioballistic transformation, expensive consumables and equipment are required. Compared to direct methods of introducing exogenous DNA, the *Agrobacterium*-mediated method has a number of advantages: relative simplicity and cheapness, high competence of *Dicotyledoneae* plant cells to agrobacterial infection, the possibility of transferring large fragments of foreign genetic material given the low abundance nature of their integration in the genome.

Induction of morphogenesis in soybean tissue culture is a complex process, the regulation of which is executed at the cellular, tissue and organism levels [13]. The nature of morphogenesis (somatic embryogenesis or organogenesis), as well as its potential, are determined by the genotypic characteristics of the culture, the type and physiological age of the explant, the nutrient medium composition, physical factors, and many others factors [14, 15]. Thus, the research of C. Zhang et al. [16] demonstrated the functional role of the soybean transcription factor *GmESR1* in the regulation of genes responsible for the realization of the regeneration potential. It was suggested that differences in the ability to form somatic embryoids in vitro in soybean genotypes are most likely determined by the unequal content of endogenous auxins in cells and/or the degree of sensitivity to these hormones [17, 18].

Protocols for agrobacterial transformation of soybean with the use of somatic embryogenesis have been developed to date [19], although most often plants-regenerators are obtained by direct [7, 8, 20] or indirect [4-6, 9, 17-19] organogenesis using different types of explants, e.g. cotyledons [4, 17], hypocotyl [6, 15] and epicotyl segments [21], cotyledonary and leaf nodes [7, 8, 15, 20], immature [9, 14, 18, 19] and mature [5, 14] embryos. The selection of the basic composition of the nutrient medium, as well as the type and concentration of

growth regulators, is fundamentally important for the induction of the processes of morphogenesis in soybean tissue culture. Most often, explants are cultivated on media based on macro- and microelements according to Murashige and Skoog medium (MS) [5-8, 17, 18] or Gamborg medium [4, 15], supplemented by various growth regulators, particularly 6-benzylaminopurine (6-BAP) [4-6, 15, 20], thidiazuron [8, 14], 2,4-dichlorophenoxyacetic acid (2,4-D) [9, 17, 18] and indole-3-acetic acid (IAA) [8]. The positive effect of polyamines (spermidine) [5], as well as inhibitors of ethylene biosynthesis ($AgNO_3$) [15], on the increase in the frequency of shoot organogenesis in soybean was proved.

Available scientific publications show that segments of soybean stem are practically not used for genetic transformation, although such explants are effectively used in the genetic modification of most *Dicotyledoneae* plants. This type of explant can significantly reduce the amount of work for obtaining donor seedlings, which is especially important in case of limited seed material.

In this study, for the first time, experimental data on the model of two promising soybean breeding lines have been obtained, confirming the capacity of stem segments for shoot organogenesis in the culture in vitro, as well as their use as explants for genetic transformation by means of *Agrobacterium tumefaciens*.

The goal was to develop a protocol for the somatic organogenesis of shoots from stem segments in soybean tissue culture and their use as explants for the production of transgenic plants by the method of agrobacterial transformation.

Techniques. The initial plant material was soybean seeds *Glycine max* (L.) Merr. of two promising breeding lines (1476 and 1477), obtained at the All-Russian Scientific Research Institute of Leguminous and Cereal Crops (Orel Province, Russia). The seeds were surface sterilized for 10 min in 70% ethanol and then in 40% aqueous solution of sodium hypochlorite for 20 min, after which they were washed 3-4 times in sterile distilled water and germinated in culture vessels with a basic nutrient medium containing mineral components and vitamins according to MS medium [22] with sucrose (3%) and agar (0.8%). On days 12-14 of cultivation, 1.0-1.5-cm-long stem segments were isolated from aseptic donor seedlings. Then the stem segments were placed on the basic nutrient MS medium with the addition of various growth regulators to induce morphogenesis: MS_1 — 1 mg/l 6-BAP, 0.1 mg/l IAA; MS_2 — 1 mg/l 6-BAP, 0.5 mg/l 2,4-D, 0.1 mg/l IAA; MS_3 — 0.5 mg/l 6-BAP, 0.1 mg/l IAA; MS_4 — 0.5 mg/l 6-BAP, 0.5 mg/l 2,4-D, 0.1 mg/l IAA. Donor seedlings and explants were cultured in a climate chamber WLR-351H (Sanyo, Japan) at 18-21 °C, 4 klx illuminance and 16/8 h photoperiod (day/night). Passage to a fresh nutrient medium was performed every 14 days. On days 28 and 42 of culture, the frequency of shoot organogenesis was estimated to determine the variant of the nutrient medium providing the maximum yield of regenerants. The frequency of organogenesis, expressed as a percentage, was defined as the ratio of the number of stem segments, where at least one regenerated shoot originated, to the total number of explants. Each variant of the culture medium included at least 300 explants; the repetition was 3-fold.

For agrobacterial transformation, the previously obtained genetic construct pCambia1381Z-pro-SmAMP1-771 [23] was used; the plasmid pCambia1381Z-pro-SmAMP1-771 was transferred to the *A. tumefaciens* cells of the supervirulent strain AGL0 by the electroporation method [24].

Genetic transformation of plants was made by the method of co-cultivation of stem segments with a dilute suspension of *Agrobacterium*. Bacteria of AGL0 strain, bearing a genetic construct, were cultured on an orbital shaking incubator (180 rpm) for 12 hours at 28 °C in the dark in 20 ml of non-agar Luria-Bertani medium (LB) [25] supplemented with appropriate selective antibiot-

ics rifampicin (Sigma, USA) and kanamycin (JSC Biochemist, Russia) in concentrations of 25 and 50 mg/ml, respectively. The obtained agrobacterial culture was diluted with a non-agar medium MS to OD_{600} = 0.4-0.6. The optical density of the suspension was determined on a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The explants were incubated in a bacterial suspension for 40 min, then dried with sterile wipes from filter paper and transferred to Petri dishes with agarized MS medium. Co-culture of explants with *Agrobacterium* was executed in the dark at 18 °C for 48 hours. Treated stem segments were washed 5-6 times with a non-agar MS medium supplemented with antibiotic timentin (ticarcillin + clavulanic acid) (SmithKline Beecham Pharmaceuticals, UK) in a concentration of 300 mg/l to eliminate *Agrobacterium*. The explants were cultured on MS_1 nutrient medium supplemented with 300 mg/l of timentin and 1 mg/l of hygromycin B (PhytoTechnology Laboratories, USA) to select shoots, which are resistant to the selective antibiotic. In subsequent passages, the concentration of the selective antibiotic was gradually increased to 10 mg/l. The transgenic status of soybean shoots resistant to hygromycin B was confirmed by polymerase chain reaction (PCR).

Total DNA was isolated using a DNA-Extran-3 kit (ZAO Sintol, Russia) according to the manufacturer's instructions. The DNA concentration was determined on a NanoDrop 1000 spectrophotometer. The total genomic DNA preparations obtained from wild-type plants and the plasmid DNA of pCambia1381Z-pro-SmAMP1-771 vector were used as a negative and positive control, respectively. PCR to identify the *hpt*, *uidA* and *virE2* genes was conducted using specific primers on a MJ Mini™ Personal Thermal Cycler amplifier (Bio-Rad, USA) in the following modes: total denaturation for 3 min at 94 °C; 35 cycles of denaturation, annealing of the primer and elongation for 30 s (*hpt* and *virE2* genes) and 1.5 min (*uidA* gene) at temperatures of 94, 62 and 72 °C respectively; total elongation for 5 min at 72 °C. The primers for the *hpt* and *uidA* gene sequences were selected using VectorNTI (Thermo Fisher Scientific, USA). The 25 μl PCR reaction mixture contained 2.5 μl of 10× PCR buffer, 0.5 μl of a 10 mM dNTPs mixture, 1 μl of forward and reverse primers at a concentration of 10 pM each, 1 μl of Taq DNA polymerase (5 IU/μl), 17 μl of bidistilled water and 2 μl (~ 60 ng) of DNA. The amplification products were separated in an electrophoresis chamber (Hoeffer, USA) in 1% agarose gel with 1× TAE buffer with ethidium bromide (Helicon LLC, Russia). The amplified fragments were visualized on the transilluminator UVT-1 (CJSC Biokom, Russia); their sizes were estimated with the molecular weight marker Gene Ruler 1kb DNA Ladder (Fermentas, USA).

Statistical processing of the results was made using the parametric tests of Student, Fisher and Duncan (α = 0.05). Before making the two-way analysis of the variance the mean values of the shoot organogenesis frequency were recalculated using the angle-inverse function $\sqrt{X}$. The calculations were made using the statistical program AGROS (version 2.11).

Results. One of the most important conditions for the production of transgenic plants is the capacity of cultured organs and tissues for organogenesis of full-fledged fertile shoots. It should be taken into account that while performing agrobacterial transformation, the morphogenetic potential of cultured tissues, regardless of the type of the explant used, is significantly reduced. The reasons for this are direct infection with a pathogenic microorganism, the insertion of T-DNA into functionally important parts of the genome, the inhibitory effect of selective antibiotics, prolonged cultivation in vitro, as well as a number of other stress factors that generate the formation of an excessive amount of active oxygen forms [27, 28]. In this connection, the initial task of the present study was

to develop a protocol for shoot organogenesis from the stem segments of the used soybean breeding lines. Callus induction and organogenesis was executed on 4 variants of nutrient media composed according to MS and supplemented with various concentrations of 6-BAP in combination with IAA (MS_1, MS_3) or IAA and 2,4-D (MS_2, MS_4). As a result, on days 8-10 of culture of stem segments of both soybean genotypes on all nutrient media, there was an increase in the size of explants. By the end of the first passage, callus tissue was formed. Qualitative characteristics of the callus tissue formed, as well as the place of its formation on the explant, significantly depends on the composition of the nutrient medium. Thus, with the simultaneous presence of two growth regulators of the auxin type in the composition of the nutrient medium, regardless of the concentration of 6-BAP (MS_2, MS_4), a light yellow pithy callus was formed on the entire surface of the explant. Then, the cells of the upper layers of light yellow callus produced a non-morphogenic unstructured callus of white color which necrotized during culture. At the same time, when culturing stem segments on nutrient media MS_1 and MS_3, the formation of callus tissue occurres mainly at the edges of the explant. The callus has a yellow-green or light green color and a denser structure (Fig. 1, A). In the callus tissue of this type, meristematic foci were formed, from which shoot organogenesis originated (see Fig. 1, B).

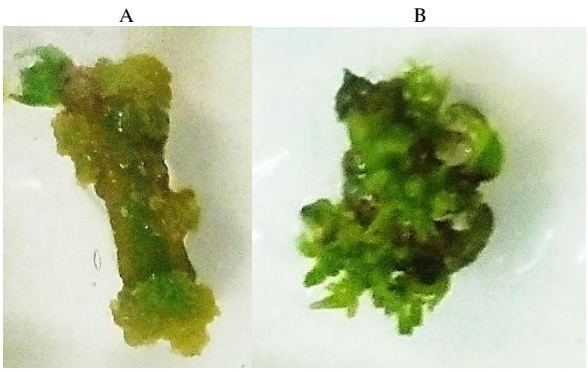

Fig. 2. Callus formation (A) **and mass shoot organogenesis** (B) **in culture of stem segments of soybean *Glycine max* (L.) Merr. line 1476 on Murashige and Skoog medium (MS_1) supplemented with 6-benzylaminopurine (1 mg/l) in combination with indole-3-acetic acid (0.1 mg/l).**

A two-way analysis of variance established the presence of significant differences at the 5% level of significance in terms of the frequency of shoot organogenesis between the studied genotypes and variants of nutrient media. In addition, the differences were also significant for the interaction of factors of the genotype × variant of the medium. The highest frequency of shoot organogenesis on days 28 and 42 was noted in the cultivation of soybean stem segments of the line 1476 on MS_1 nutrient media, containing 1 mg/l 6-BAP and 0.1 mg/l IAA. The frequency of organogenesis was 44.1% and 51.2%, respectively (Table 1). Reducing the concentration of 6-BAP in the nutrient medium (MS_3) led to a significant decrease in the frequency of shoot organogenesis in this genotype. Moreover, the addition of auxin 2,4-D to the nutrient medium inhibited the shoot organogenesis process. Similar results were noted with respect to the frequency of shoot organogenesis for the line 1477, except that this genotype was characterized by an extremely low

1. Frequency of organogenesis soybean *Glycine max* (L.) Merr. stem segments shoot as influenced by the genotype and composition of the nutrient medium

Nutrient medium	Organogenesis of shoots, %	
	day 28	day 42
	Line 1476	
MS_1	44.1^{e}	51.2^{g}
MS_2	20.8^{c}	35.1^{e}
MS_3	32.0^{d}	43.8^{f}
MS_4	14.5^{abc}	25.0^{cd}
	Line 1477	
MS_1	18.3^{bc}	27.0^{d}
MS_2	11.6^{a}	18.5^{b}
MS_3	13.5^{ab}	21.3^{bc}
MS_4	9.1^{a}	12.3^{a}

Note. See Techniques section for media. Differences between variants marked with at least one identical letter are statistically insignificant by the Duncan criterion ($\alpha = 0.05$).

capacity for morphogenesis in vitro. Thus, the frequency of shoot organogenesis on day 42 of explant culture on MS_1 medium did not exceed 27.0%. Thus, giving the low regeneration capacity of the line 1477, subsequent experiments on agrobacterial transformation with the use of this genotype seem inexpedient.

Within the framework of the present study, two independent experiments on the agrobacterial transformation of the soybean selection line 1476 were conducted. The genetic construct pCambia1381Z-pro-SmAMP1-771 [23] used for this purpose carries the selective gene *hpt* in T-DNA, which determines the resistance to hygromycin B, as well as a reporter gene *uidA* containing a modified intron of the castor catalase gene under the control of the 5′-deletion –771 bp of promoter *pro-SmAMP1* from *Stellaria media* (L.) (Fig. 2).

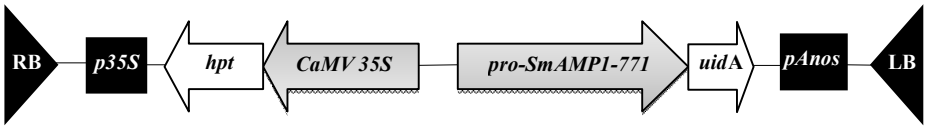

Fig. 2. Schematic representation of T-DNA of the genetic construct pCambia1381Z-pro-SmAMP1-771 used in experiments on the agrobacterial transformation of soybean *Glycine max* (L.) Merr.: RB, LB — respectively, the right and left flanking sequences of T-DNA, *CaMV 35S* and *p35S* — promoter and terminator of 35S RNA of cauliflower mosaic virus, *hpt* — hygromycin phosphotransferase gene of *Escherichia coli*, *pro-SmAMP1-771* — 5′-deletion variant (–771 bp) of *pro-SmAMP1* promoter from *Stellaria media*, *uid*A — reporter gene β-glucuronidase containing a modified intron of the castor catalase gene, *pAnos* — terminator of the nopaline synthase gene [23].

In total, 200 explants were inoculated while co-culture with a suspension of the *A. tumefaciens* AGL0 strain containing the plasmid pCambia1381Z-pro-SmAMP1-771. We used a strategy of gradual adaptation of explants to a selective agent, excluding shock and mass death (gradual increase in concentration in the selective environment of hygromycin B) which was successfully used in the agrobacterial transformation of tomato [29]. Despite the gradual increase in the concentration of hygromycin B in the selective medium MS_1, most of the stem segments (75.5%, or 151 explants) necrotized during the culture process. Moreover, bacterial contamination was observed for a part of the explants (36, or 18.0%). Necrotized and contaminated segments of the stem were excluded from the experiment. As a result, at the beginning of the third passage, the formation of light green callus-like tissue was observed for only 13 explants (6.5%). With an increase in the concentration of the selective agent in the nutrient medium, there was an increase in the size of the callus tissue. As a result, the frequency of callus formation on a selective nutrient medium supplemented with 10 mg/l of hygromycin B was 6.5%. However, only 8 of these 13 callus tissues (4.0% of the total number of explants) formed on a selective nutrient medium for the induction of morphogenesis were observed to form dense globular meristematic foci of green color, from which organogenesis of shoots subsequently originated. As a result, the frequency of organogenesis of shoots, resistant to hygromycin B, was 4.0%.

2. Nucleotide sequences of primers used in PCR to confirm the presence of *hpt*, *uidA* and *virE2* genes in hygromycin B resistant soybean *Glycine max* (L.) Merr. regenerants of breeding line 1476, and the expected size of amplicons

Gene	The nucleotide sequence of the primer (5′→3′)	The size of the amplicon, bp
hpt	F – TCTGATAGAGTTGGTCAAGACC	415
	R – CAAGGAATCGGTCAATACACTAC	
uidA	F – ATCGCGAAAACTGTGGAATTGATC	1628
	R – TTACCGCCAACGCGCAATATG	
VirE2 (26)	F – CGAATACATTCTCGTGCGTCAAACG	600
	R – TTTCGAGTCATGCATAATGCCTGAC	

Note. F, R — forward and reverse primers, respectively.

PCR analysis confirms the transgenic status of independent regenerants

resistant to hygromycin B. When amplified using specific primers (Table 2), for a sequence of the *hpt* selective gene, fragments corresponding to a positive control were obtained in 4 of the 8 regenerative plants (Fig. 3, A). Integration of the reporter gene *uidA* was established in all analyzed samples containing *hpt* gene (see Fig. 3, B). Also, all preparations of total genomic DNA in the studied samples were tested for the absence of the bacterial gene *VirE2* to exclude false positive results due to bacterial contamination (see Fig. 3, B). Thus, the efficiency of agrobacterial transformation of the soybean stem segments of the line 1476 when using the genetic construct pCambia1381Z-pro-SmAMP1-771 is 2.0%.

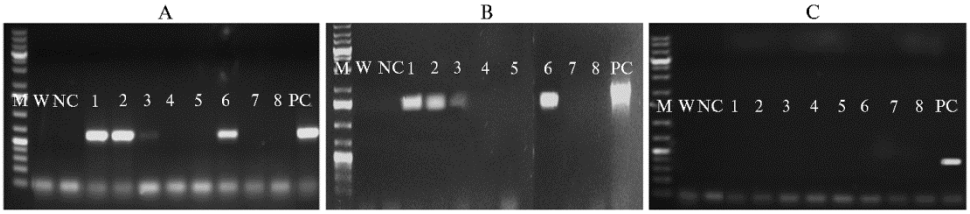

Fig. 3. Electrophoregrams of PCR products of *hpt* (A), *uidA* (B) and *VirE2* (C) genes in hygromycin B resistant regenerants of soybean *Glycine max* (L.) Merr. breeding line 1476: M — molecular weight marker (Gene Ruler 1kb DNA Ladder, Fermentas, USA), W — water, NC — negative control (total genomic DNA isolated from the 1476 soybean line), 1-8 — DNA of soybean regenerants resistant to hygromycin B, PC — positive control (plasmid DNA pCambia1381Z-pro-SmAMP1-771).

From PCR analysis, 50% of the regenerants resistant to hygromycin B are found to be so-called escapes. i.e. the plants adapted to exist on a selective medium with an antibiotic but not containing foreign DNA in the genome. In recent years, researchers have shown increased scientific and practical interest in this phenomenon. In particular, the escapes of fiber flax, which survived under the influence of stress factors after agrobacterial transformation, expanded the spectrum of genetic variability and served as a starting material for creating genotypes with an improved combination of selection characteristics [30].

Thus, as a result of the conducted studies, it is shown that the studied soybean breeding lines (1476 and 1477) differ significantly in their capacity for morphogenesis in vitro. It is experimentally confirmed that the addition of auxin 2,4-dichlorophenoxyacetic acid results in inhibition of shoot organogenesis. The growth regulators added to Murashige and Skoog nutrient medium and providing the maximum yield of regenerants are 6-benzylaminopurine (1 mg/l) in combination with indole-3-acetic acid (0.1 mg/l). For the soybean line 1476, an effective protocol is suggested for indirect somatic shoot organogenesis from stem segments with a frequency of over 50%. The protocol was used in subsequent experiments on genetic transformation performed by *Agrobacterium tumefaciens* AGL0 strain containing the plasmid pCambia1381Z-pro-SmAMP1-771. A stage-by-stage selection on a nutrient medium with increasing concentrations of hygromycin B (1-10 mg/l) resulted in eight independent lines. PCR method confirms the presence of the selective (*hpt*) and marker (*uidA*) genes in four of these lines. The effectiveness of agrobacterial transformation in our experiments is 2.0%. The obtained results testify to the successful application and prospects of using stem segments as explants for genetic soybean transformation.

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NUCLEAR DNA CONTENT IN RICE (*Oryza sativa* L.) REGENERANTS DERIVED FROM ANTHER CULTURE in vitro

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Abstract

Rice is an important food crop grown in the south of the Russian Far East. Therefore, breeding new varieties with high harvest and crop quality is relevant. Anther in vitro culture is successfully applied in breeding programs in rice-growing countries, including Russia. In anther in vitro culture, flow cytometry is applicable to select haploid, dihaploid and polyploid regenerants. Cytological studies show genome variations from haploids to hexaploids in plant tissue in vitro culture, and also chromosome changes which result in aneuploidy or endopolyploidy leding to an inconstant nuclear DNA content. In the work, we followed the aims i) to evaluated nuclear DNA content by flow cytometry in an androgenic rice regenerant population, and ii) to estimate the applicability of the combination of two approaches, the anther in vitro culture technique and flow cytometry, in rice breeding. A total of 1099 regenerants from in vitro anther culture of a single F_2 (UkrNIIS 3435 × Ukr 96) rice (*Oryza sativa* L. ssp. *japonica* Kato) hybrid plant were separated into four groups with regard to morphological features. Haploids were sterile plants with very small flowers, dihaploids were fertile plants, tetraploids were the plants with very few large seeds, an expressed keel and the ribbed floral scales. Also, there were the plants without seeds which flowers were normal in size but formed two or more sterile panicles. In the last group of the regenerants the plants died during early development. A total of 176 regeneranats were estimated by flow cytometry. It was revealed that nuclear DNA content varied greatly (*Cv* = 32 %) in the plants without seeds. This group seems to include plants with double set of chromosomes, triploids, tetraploids, and pentaploids. Additionally, in this group we found the regenerants with endopoyploidy since five of the plants had two nuclear DNA content peaks like those for haploids and diploids. In 23 plants nuclear DNA content approximated to dihaploid chromosome set and averaged 2.00 pg. Obviously, an aneuploidy characteristic of rice anther in vitro cultures could lead to aliquant changes in chromosome set in the regenerants, causing a loss of fertility. The dihaploid and tetraploid plants were low variable (*Cv* of 10.5 and 5.3 %) and had nuclear DNA content of 1.88 and 3.75 pg, respectively, whereas the haploids were high variable (C*v* = 29 %) with an average nuclear DNA amount of 0.89 pg. Our findings indicate that flow cytometry, together with production index, may be applied to reveal tetraploid regenerants and to remove haploids in rice breeding. That allows avoiding ex vitro trials of unpromising regenerants.

Keywords*:* *Oryza sativa* L., anther culture in vitro, flow cytometry, regenerant, haploid, dihaploid, tetraploid

The soil and climatic conditions of Russian Far East differ from the conditions of the South of Russia, where the main cultivation areas of rice are located. The nearest neighboring provinces of China, which have achieved significant success in rice breeding, use seeding technologies with a significant part of manual labor, which distinguishes them from the rice cultivation technology adopted in Russia [2]. Therefore, it is very difficult to borrow the varieties from the Western

regions of Russia and China for cultivation in the Primorsky Krai. In the State Register of Selective Breeding Results of the Russian Federation in the 12th zone, there are only rice varieties of Far Eastern breeders (http://www.gossort.com); therefore, the programs on the creation of rice varieties for the Far East of Russia should be continued.

To speed up selection and create new parental material, the anther culture is successfully used in vitro [3, 4]. Some varieties of rice in our country were selected by this method [5]. The biotechnology methods in rice breeding were also used in the Far East [6], including research to create varieties of rice using the anther culture in vitro; in particular, the basic elements of the technology of the androclinic haploidy for the Far Eastern varieties and hybrids of rice are optimized [7]. According to different estimates, 29-72% of rice regenerants obtained in the anther culture in vitro are productive dihaploids [3, 8-10]. They have a particular breeding value. Others (up to 60%) are seedless regenerants, often haploids which either are rejected or require manipulations to double their chromosome sets. Morphological features are mainly used to identify the type of the regenerant: haploid rice plants are characterized by smaller vegetative organs, increased bushiness, small flowers, and sterility [11]. Before identification, a routine procedure of growing the regenerants, bringing to the stage of flowering and ripening of seeds is carried out.

The method of flow cytometry in the study of plants has become widespread relatively recently [12]. In the anther culture in vitro, it is used mainly to separate the fractions of regenerants into haploid, dihaploid, triploid plants, etc. [13]. The data on a clear multiple increase in chromosome sets were presented; the peak value of every set is considered as a constant value [13-16]. Cytological studies of plants in cell and tissue culture in vitro indicate the existence of not only genomic variations from haploids to hexaploids [3, 4, 11, 17, 18] but also chromosomal changes leading to aneuploidy [3, 12, 18, 19] and endopolyploidy [12, 20]. In this case, the size of the genome cannot be a constant value.

In this paper, the parameters of the variability of the nuclear DNA content in different groups of regenerants are described first for the rice obtained in the anther culture in vitro (haploids, duplicated haploids, tetraploids, and non-haploid seedless forms).

The work objective was to characterize the population of rice regenerants obtained in the anther culture in vitro, according to the content of nuclear DNA by the flow cytometry method and to assess the effectiveness of the combination of these methods in breeding with rice.

Techniques. The hybrid F_2 (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) obtained in Primorskii Agricultural Research Institute was grown on the vegetative plot in 2014.

Before introduction to in vitro culture, rice anthers were exposed to low positive temperatures (5 °C) for 7 days by placing a panicle in the cylinder with water. Further on, the anthers were placed in the inductive nutrient solution N_6 [21] with 2,4-dichlorophenoxyacetic acid (2.0 mg/l) and cultured in the dark at a temperature of 25-27 °C to form calli with the size of 1-5 mm. For secondary differentiation of shoots, the calli were transferred in N_6 nutrient solution with sucrose (6%), 6-benzylaminopurine and kinetin (1 mg/l) and cultured at an illumination of 4000 lux, a temperature of 22-25 °C and a photoperiod 16/8 h. For rooting of regenerants, the Murashige and Skoog medium (MS) was used with the half mineral composition of macro salts in the variation by Yu.K. Goncharova [18].

Regenerants with a well-developed root system were planted in pots and kept growing in the conditions of a cultural room (4000 lux, a temperature of

22-25 °C and a photoperiod 16/8 h) till the formation of seeds. According to morphological characteristics, all regenerants were divided into five groups: haploids (sterile plants with very small flowers); dihaploids (plants with seeds); tetraploids (plants with few very large seeds, an expressed panicle and ribbing on the floral scales); plants with no seeds (formed flowers of normal size but did not form seeds on two or more panicles); plant, dead at the early stages of growth and development. The leaves were exposed to lyophilization and stored in a freezer at –80 °C.

The DNA content was measured by the flow cytometry method. From one to four haploid and dihaploid plants from each callus line were used, 52 plants with no seeds referred to the non-haploid group, and 10 tetraploids, the 176 plants altogether. Lyophil-dried leaves (1-2 cm^2) were graded by a blade in a Petri dish with 1 ml of chilled Tris-$MgCl_2$ buffer containing 0.2 M Tris-base, 4 mM $MgCl_2 \cdot 6H_2O$ (Russia) and 0.5% Triton X-100 with addition of β-mercaptoethanol (1 μl/ml) (Serva, Germany), 50 μg/ml of propidium iodide (Biotium, USA) and 50 μg/ml of ribonuclease (Sintol, Russia) [22]. The samples were filtered through the CellTrics nylon membrane with a pore size of 50 μm (Sysmex Europe GmbH, Germany). *Ficus benjamina* L. nuclei with the known DNA content of 2C = 1.07 pg [23] isolated with Tris-$MgCl_2$ buffer were used as an external standard. The mean value (*M*) of the standard peak was recorded three times per the day of the study; then, it was averaged for further calculations. The mean value (*M*) of the sample peak was recorded in singlicate at the same settings of the fluorescence-based cytometry device for the sample and the standard (the same voltage on the photomultiplier tube); the peaks with at least 1000 detectable particles were used. The fluorescence data of isolated nuclei were recorded on the fluorescence-based flow cytometry device Partec CyFlow PA (Partec GmbH, Germany) with a laser radiation source (λ = 532 nm). The signals were recorded in the logarithmic representation of the fluorescence results (the logarithmic scale) [24].

The data processing was done with Statistica 10.0 ("StatSoft, Inc., USA.) The mean values (*M*) and standard errors of means (±SEM) are presented in the tables. Histograms of the relative DNA content were constructed with Flowing Software 2 (Perttu Terho, Finland) at the standard settings with the determination of the number of events (nuclei), the coefficient of variation (*Cv*, %), the mean value (*M*) and the median peak (*Me*). To determine the significance of differences between the mean values of the nuclear DNA content in the groups, Student *t*-criterion was used; the correlation coefficient and the *t*-criterion were calculated at the significance level of 5%.

1. Callus formation and regeneration in the anther culture of F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in vitro

Indicator	Value
The number of inoculated anthers, pcs.	240
Calli formation, %	37.5
The number of planted calli, pcs.	90
Calli with regenerants, pcs./%	72/80
Calli with green regenerants, pcs./%	39.0/43.3
The number of green regenerants per callus, pcs.	14.9

Results. The frequency of callus formation in the anther culture of the studied hybrid was 37.5% (Table 1). Some anthers began to form callus very early, 18 days after inoculation. With the weekly transfer of callus aggregates to the regeneration medium for 6 weeks, up to five or six passages were obtained. All of them were considered in our experience as a single callus. However, on some passages of callus, for example, on the first and third ones, green buds were not formed sometimes, while green regenerants have been formed on others. At the same time, the haploidic regenerants only may be located at the callus of the

one passage; the doubled haploids only may be located on the other callus. The callus aggregates with different combinations of green regenerants were most often: dihaploids and haploids; dihaploids, tetraploids, and dead plants.

2. Characterization of groups of regenerants obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in anther culture in vitro

Indicator	Haploids	Dihaploids	Tetraploids	Plants with no seeds	Dead plants
Total, pcs.	348	494	10	58	189
The number of regenerants per callus, pcs.					
M	9.2	13.0	0.3	1.5	5.0
±SEM	12.1	26.5	0.7	4.0	6.6
Percent of the total number of regenerants	31.7	45.0	0.9	5.3	17.2
Total number of regenerants per callus, pcs.	45	126	3	22	32

The calli formed 1099 green regenerants (Table 2). After the determination of the nuclear DNA content, it was found that the three plants, referred to dihaploids, have the parameters of tetraploids (3.78; 3.83; 3.86). Two seedless plants with a small genome similar to haploids were also found (1.34; 1.05), and three haploids with the DNA content close to that of the diploid plants (2.13; 2.16; 2.05). To characterize the rice regenerant population, these plants were referred to tetraploids, haploids, and plants with no seeds correspondingly. The error in the reference of regenerants to the desired plants fraction according to the morphological characteristics was 4.5%.

3. Nuclear DNA content in the regenerants population obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in the anther culture in vitro

Indicator	Haploids	Dihaploids	Tetraploids	Seedless plants
The number of plants, pcs.	61	50	13	52
The DNA content, pg:				
M	0.887	1.881	3.752	3.046
±SEM	0.033	0.028	0.055	0.135
min	0.606	1.438	3.442	1.717
max	1.636	2.188	4.197	4.380
Cv, %	29.0	10.5	5.30	32.0
Relatively to the mean value of haploids		2.12	4.23	3.43

N o t e. *Cv* — the coefficient of variation; 1pg of DNA = 978 million bp [29]. The differences between the mean values of the nuclear DNA content in the groups are significant at p = 0.05.

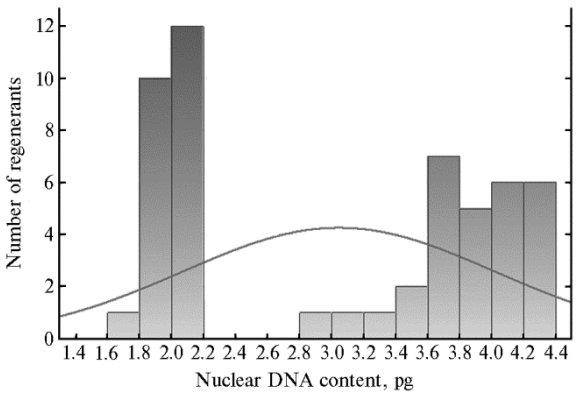

Fig. 1. Nuclear DNA content in seedless non-haploid regenerants obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in the anther culture in vitro The curve shows normal distribution.

The group of plants with no seeds was high variable in the DNA content in cell nuclei (*Cv* = 32%) (Table 3). This group probably includes plants with the double set of chromosomes, triploids, tetraploids, and pentaploids (Fig. 1). Endopolyploidy is observed in this group of regenerants, which is characterized by the peak of diploid nuclei (2×), the peak of the tetraploid nuclei (4×), combined with the G_2 peak of the mitosis stage of the diploid nuclei and the G_2 peak of the mitosis stage of the tetraploid nuclei (Fig. 2, C) [25, 26]. In the detection of the isolated nuclei, the five plants had double-vertex in the areas of haploids and diploids (see Fig. 2),

23 plants had the set of chromosomes close to that of the dihaploid regenerants (2.00 on the average). The phenomenon of aneuploidy, typical of the culture of rice anthers in vitro [18], led to the multiple changes in the chromosomal sets of regenerants, which did not allow plants to form seeds. In the groups of dihaploids and tetraploids, variability was insignificant (see Table 3). One endopolyploidy regenerant was found among dihaploids. In general, these results are comparable with the results of other authors, according to which the nuclear DNA content in the main set of chromosomes in the *O. sativa* rice varies from 0.91 up to 1.00 pg [27, 28].

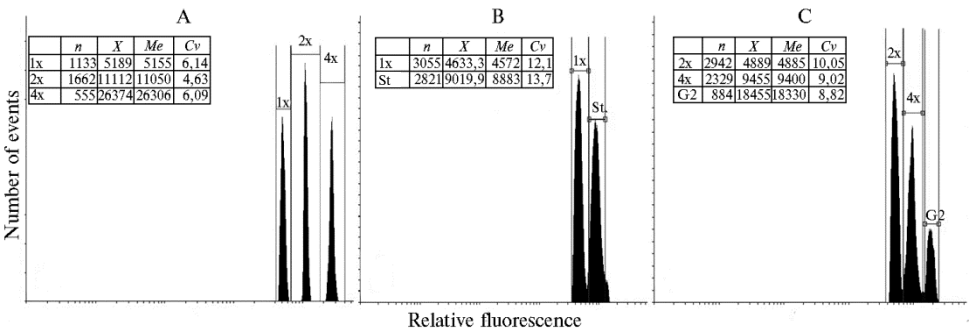

Fig. 2. Histograms constructed in the study of the ploidy and the relative DNA content among regenerants obtained from F_2 plants (UkrNIIS 3435 × Ukr 96) of rice (*Oryza sativa* L. ssp. *japonica* Kato) in the anther culture in vitro: A — consensus histogram of regenerants with 1×, 2×, and 4× ploidy, B — histogram containing regenerant peaks with 1× ploidy and the standard peak (St.), C — histogram of an endopolyploid regenerant; *n* — the number of events, *M* — the mean of the peak, *Me* — peak median, *Cv* — coefficient of variation, %.

The group of haploidic plants was highly variable in the nuclear DNA content (*Cv* = 29%). The maximum values of the haploids were higher than the minimum values of dihaploids. S.I. Maletskii et al. [29], by studying the variability of sugar beet plants (*Beta vulgaris* L.), also found higher epiplastomic and epigenetic instability in haploid genomes compared to dihaploids.

Haploid plants, derived from one callus line, differed habitually. First, strong, tall plants with large panicles and the significant number of sterile flowers were formed. The last formed plants were small with single flowers on a short panicle.

In the anther culture in vitro, the viability of haploids and dihaploids is expressed by the number of "harmful" genes, which they got as a result of meiosis [30]. The chromosomal changes occurring during the callus and regenerants cultivation in vitro [3, 18-20] lead to changes in the morphotype of plants and their viability. The plants that died in the early stages of development were probably haploids, the genotype of which contained a lot of lethals, semilethals, and sublethals [30]. The ratio of the average values of the nuclear DNA content in dihaploids and haploids was not divisible by two (see Table 3). This may indicate the loss of some parts of chromosomes in haploids indirectly during the cultivation, which led to the changes in the regenerants' morphotype.

We found no relationship between the duration of cultivation (up to 6 months) and the deviation from the average amount of the nuclear DNA in haploids (r = –0.09 at p = 0.05). The ratio of the average values of the nuclear DNA content in tetraploids and dihaploids was equal to two. Productive tetraploids, probably, arose by means of the multiple increase in the number of chromosomes in the basic set, but even in this case, they formed a small number of seeds, from one to five. According to the information provided by S.S. Guchenko (a personal communicaton), all the tetraploids of R_1 sprouted, developed, two of

them formed panicles but were sterile. Among seedless plants, 23 samples had the DNA content typical for tetraploids (see Fig. 1), but they turned out to be sterile. Low pollen fertility is associated with significant cytological changes in autotetraploid rice plants [31].

As is shown in this work and some foreign publications, the method of flow cytometry is accurate enough to identify aneuploid plants. However, the studied aneuploid plants mainly had a significant genome size with large chromosomes [22, 32]. Most of the fluorescence-based cytometry devices have the standard error of measurement equal to 2.5-5.0%. The average chromosome size for *O. sativa* is ~ 0.04 pg, which is within the limits of the standard error of fluorescence-based cytometry, whereas, for example, for *Triticum aestivum* L., the average size is ~ 0.82 pg, for *Lolium perenne* L. ~ 0.39 pg [33, 34]. Therefore, in addition to the flow cytometry technique, microscopy techniques should be used in the study of aneuploidy of plants with small genomes.

Thus, in the groups of sterile rice plants (haploids and seedless non-haploids) obtained in the anther culture in vitro, the content of the nuclear DNA is variable within considerable limits, while the fertile regenerants (dihaploids and tetraploids) have stable nuclear DNA content. The flow cytometry method together with the productivity assessment can be used in rice breeding to identify tetraploid regenerants, as well as for the purpose of haploid culling to exclude the stage of growing futile forms in ex vitro conditions.

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ADAPTIVENESS OF PROMISING LAVENDER AND LAVANDIN CULTIVARS UNDER in vitro CULTURE AND ex situ

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Abstract

Lavandula angustifolia Mill. and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel) are promising fragrant plants with medicinal, aromatic and ornamental properties. To obtain high quality healthy planting material, in vitro cultures of valuable cultivars Belyanka, Record (lavender) and Rabat, Snezhnyi Bars (lavandin) were derived. Obtained regenerants were cultured for 4-5 months on Murashige and Skoog medium with 0.3 mg/l kinetin, 0.025 mg/l NAA и 0.25 mg/l GA_3 in growth chamber at 25±1 °C under 16-h photoperiod and light intensity of 37.5 $\mu M \cdot m^{-2} \cdot s^{-1}$. Intact plants were studied during the growing season. In order to reveal plant morphogenetic capacity, biochemical stress indicators, indexes of photosynthetic activity, maximum fluorescence (F_m), stationary level of fluorescence (F_{st}) and water regime were determined. The proline content of lavender and lavandin plants grown ex situ was rather high (6.67-21.59 μg/g). In in vitro micro-plants, although there was considerable hydration of the plant tissues, the proline concentration was higher than that in the intact plants (8.24-35.72 μg/g). Intact lavender and lavendin plants accumulated high amounts of phenolic compounds (1033-1492 mg/100 g) and ascorbic acid (14.96-20.06 mg/100 g). In plants under controlled conditions, the concentration of phenolic compounds and ascorbic acid was lower (490-777 and 4.95-5.98 mg/100 g, respectively), which is caused by significant waterlogging of tissues and lack of stress. Regardless of the growing condition, the level of phenolic compounds was higher in the lavandin cultivars compared to lavandula plants. Open field cultivated plants were distinguished by high activity of catalase (18.13-36.97 g $O_2 \cdot g^{-1} \cdot min^{-1}$) and superoxide dismutase (12.55-14.82 a.u./g). Under the hydrothermal stress effect ex situ, relative photosynthetic activity and viability index indicated minor decrease in assimilation processes in lavender cultivars but was within vital limits. In in vitro culture, the catalase activity of lavender cultivars was higher than that of lavandin. At the same time, SOD and PPO activity of lavender micro-plants in vitro was lower than that of lavandin micro-plants. In open field cultivation, leaf tissue hydration of tested plants was 56-62 %, with greater part of bound water. In plants cultured in vitro, the rate of hydration was high (70-77 %), with the same trend of water fractional composition. Under the controlled conditions and nominal heterotrophic nutrition type, photosynthetic activity was 0.28-0.55 a.u. with the maximum in the Rabat cultivar. Values of chlorophyll fluorescence induction and vitality index indicated no photoinhibition. It was found out the lavandin cultivars had better capacity for a wide use under various conditions.

Keywords: *Lavandula* sp., biochemical indicators, photosynthetic activity, water regime, in vitro, ex situ

Lavandula (*Lavandula* L.) is a valuable essential-oil-bearing, aromatic, decorative and medicinal culture. The main cultivated essential-oil-bearing plants include true lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.). They contain essential oils used in medicine, perfume, cosmetics and food industry [1]. Phenolic compounds,

which have a wide range of physiological effects, were also found in plant raw materials [2]. Traditional vegetative reproduction of lavender and lavandin is a complex, long-lasting and not always effective process. A high-quality planting material of essential-oil-bearing plants can be obtained in vitro. Biotechnological methods allow obtaining in the shortest possible time a significant amount of healthy plants, which are genetically identical to the original species, variety or form, in case of a lack of starting material [3. 4]. To study the adaptive capabilities of the micro shoots grown in vitro, it is necessary to take into account the functioning of the antioxidant system, which includes low-molecular protecting compounds and specific antioxidant enzymes [5, 6].

The main protecting compounds of plants include proline, phenolic substances, and ascorbic acid. Proline is the source of energy, carbon and nitrogen in case of resource shortages caused by stress, and a decrease in the activity of synthesis enzymes [7]. Phenolic compounds and ascorbic acid are involved in the basic processes of plant cells activity, i.e. photosynthesis, respiration, protection from stress factors [8, 9]. Enzymes of the antioxidant system, i.e. superoxide dismutase (SOD), catalase (CAT), as well as polyphenol oxidase (PPO), bind excess amounts of reactive oxygen species (ROS), stop free radical chain reactions and thereby regulate the oxidative processes occurring in plants [10-12]. The activity of oxidation-reduction enzymes depends on the susceptibility of the organism to the stress factors and on the stage of plant development [13].

The photosynthetic activity and the vitality index are also sensitive parameters of the change in the functional state of plants. The amplitude and phase characteristics of the induction signal correlate with the physiological state of the tissue. The higher the speed and the amount of the change in optical parameters, the higher the functional activity of the plant [14]. The resistance of chlorophyll-containing tissues to excessive illumination is widely used in experimental biology as an integral criterion of the functional state of plants and adaptability to unfavorable environmental factors [15, 16]. The light-dark kinetics is discussed in connection with the response of photosystems I and II to changing conditions of cultivation [17, 18] and the effect of factors of abiogenic nature [19, 20].

Adaptation to new conditions of cultivation, namely the transfer of planting material from the open ground to the conditions of aseptic culture (in vitro), has a complex nature and is based on the lability and tolerance of biochemical and physiological parameters, the limits of which are determined by the genetic nature of the organism. Despite the high economic value of lavender and lavandin, data about the adaptive potential of this culture in vitro is insufficient. This report, for the first time, shows a comparative physiological and biochemical characteristics of promising lavender and lavandin varieties under various cultivation conditions.

The purpose of this paper is to identify the adaptive ability of valuable lavender and lavandin varieties in vitro and ex situ by determining their physiological and biochemical parameters.

Techniques. The study was conducted using valuable varieties of true lavender (Belyanka, Rekord) and lavandin (Rabat, Snezhnyy Bars) of the Nikitsky Botanical Garden (NBS-NSC, Crimea) from the gene pool collection. Intact plants in the phenological stage of technical maturity, grown ex situ on the NBS-NNC collection plots, as well as micro shoots cultured in vitro, were involved in physiological and biochemical studies. Samples were taken in the decades II and III of July 2016.

Apical meristems of auxiliary buds were used for in vitro tissue culture. Micro shoots were cultured for 4-5 months on a modified Murashige and Skoog medium with 0.3 mg/l kinetin, 0.025 mg/l α-naphthylacetic acid and 0.25 mg/l

gibberellic acid (Sigma, USA), 30 g/l sucrose and 8 g/l agar (Panreac, Spain). Explants in culture vessels were placed in an artificial climate chamber MLR-352-PE (Panasonic, Japan) at a temperature of 25±1 °C, a 16-hour photoperiod and a light intensity of 37.5 $\mu M \cdot m^{-2} \cdot s^{-1}$.

Biochemical indicators were determined by conventional methods: proline content was determined by the method of Chinard with ninhydrin reagent [21], the amount of phenolic substances was determined spectrophotometrically with Folin-Ciocalteu reagent (AppliChem GmbH, Germany) [22]. Calibration curves for proline content evaluation were constructed with L-proline (AppliChem GmbH, Germany), phenolic substances with gallic acid (Sigma, USA), flavonols with rutin (Sigma, USA). The amount of ascorbic acid was determined by iodometric titration [23], the catalase (CE 1.11.1.6) activity by the titrimetric method [24], polyphenol oxidase (CE 1.14.18.1) activity was measured in the presence of pyrocatechin (Sigma, USA) and p-phenylenediamine [25], superoxide dismutase (EC 1.15.1.1) by oxidation of quercetin (Sigma, USA) [26]. A spectrophotometer Evolution 220 UV/VIS (Thermo Fisher Scientific, USA) was used.

The total water content in the leaves, the fractional composition of water, and the water deficiency were evaluated as physiological criteria characterizing the water regime when cultivating plants in the open ground [27]. The parameters of photosynthetic activity were measured using a portable fluorometer (Institute of Cybernetics named after V.M. Glushkov of the National Academy of Sciences of Ukraine) [28]. The maximum (F_m) and stationary (F_{st}) fluorescence values after the darkness adaptation were recorded as components of Kautsky's fluorescence induction kinetics. The viability index and photosynthetic activity were calculated [15].

The experiments were arranged in 3-fold biological and 3-fold analytical replications. The data obtained was processed using Statistica 6.0 software (StatSoft, Inc., USA). The tables show the average values of the indicators (*M*) and their standard deviations (±SD). The significance of differences between the variants was evaluated by the arithmetic mean and variation coefficient at $p < 0.05$.

Results. The Rekord variety of true lavender was obtained by the method of inbreeding. The plants are large, 55-60 cm high, semi-spreading. The variety is mead-season, winter hardy, high-yielding and high oleic. The content of essential oil is 1.8-2.0% of the raw mass of the inflorescences. The main components are linalool (34.6%), linalyl acetate (31.2%) and 1.8-cineol (3.7%). The Belyanka variety is a recessive form of the Rekord variety, isolated by individual selection. The plants are compact, 50-55 cm high. The variety is early-season and low-yielding. The content of essential oil in the inflorescences is up to 1% of the raw weight. The main components are linalyl acetate (14.3%), linalool (63.7%), cineole (2.7%), camphor (2.1%). The Snezhnyy Bars variety is a recessive form of lavandin of the Pervenets variety of clonal selection. The bush is compact, 80-90 cm high. The variety is winter hardy, high-yielding. The yield is 75-85 c/ha, the yield of essential oil is 225-240 kg/ha, the content of essential oil is 3% of the raw biomass. The main components: linalool (41.8%), linalyl acetate (19.4%), terpineol (7.4%), camphor (4.9%). The Rabat variety is an allotriploid hybrid, obtained by the method of distant hybridization of true lavender with spike lavender. The plants are compact. The yield is 110-120 c/ha, the yield of essential oil is 341 kg/ha, the content of essential oil is 3.1% of the raw mass. The main components are linalool (36.7%), linalyl acetate (32.1%), camphor (5.6%), 1.8-cineol (3.7%).

In the decades II and III of July 2016, the average daily air temperature was 27.0 °C (maximum air temperature was 31.0 °C). Relative air humidity was 51%, the minimum was 47%. The temperature on the soil at the time of sample

collection reached 57.5 °C, at a depth of 10 cm was 30.0 °C. As per the instrumental determination of the soil moisture, the reserves of productive moisture in a one-meter layer of soil were up to 22 mm (14% of the lowest moisture capacity). The rainless period that preceded the date of the analysis lasted 18 days. During this period, 6 days with conditions corresponding to dry hot wind were observed (relative air humidity dropped to 40%, the average daily air temperature was above 25 °C, wind gusts reached 15 m/s, average values were 5-8 m/s).

In open ground, the proline content in lavender and lavandin plants has a variety-specific nature (Table 1).

1. The content of protective substances in the plants of lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) of different varieties (*M*±SD, 2016)

Variety	Conditions	Proline, μg/g	Ascorbic acid, mg/100 g	Phenolic compounds, mg/100 g
Belyanka	Ex situ	7.69±0.23	20.06±0.58	1033±26
	Cv, %	8.5	8.2	7.1
	In vitro	8.24±0.24	5.61±0.16	645±17
	Cv, %	8.2	8.1	7.5
Rekord	Ex situ	12.95±0.37	18.92±0.54	1181±31
	Cv, %	8.1	8.0	7.4
	In vitro	33.75±0.99	5.94±0.17	490±14
	Cv, %	8.3	8.1	8.1
Rabat	Ex situ	6.67±0.20	19.14±0.55	1305±34
	Cv, %	8.5	8.1	7.4
	In vitro	35.72±1.04	4.95±0.13	668±19
	Cv, %	8.2	7.4	8.0
Snezhnyy Bars	Ex situ	21.59±0.63	14.96±0.44	1492±40
	Cv, %	8.3	8.3	7.6
	In vitro	35.32±1.05	5.98±0.17	777±22
	Cv, %	8.4	8.0	8.0

N o t e. *Cv* — coefficient of variation at $p < 0.05$.

It is known that proline performs osmoregulatory functions and takes part in gene expression [29]. Proline content in plants grown in vitro, despite high water content tissues (56-62%), was significantly higher than in intact plants. This suggests that free proline has an effect on the growth and differentiation of lavender and lavandin cells. In vitro conditions are not stressful for plants because they are selected for optimal development of micro shoots and are characterized by constant temperature, humidity, and illumination.

Intact lavender and lavandin plants accumulated high amounts of phenolic compounds and ascorbic acid. An increase in the phenolic compounds, as a rule, is a response to the stress factors [30]. The content of phenolic compounds and ascorbic acid in the analyzed samples of the plants grown under controlled conditions was significantly lower, which is due to considerable water content in the tissues (56-62%) and the absence of stress. The content of phenolic compounds in lavandin varieties cultivated in the open ground was significantly greater than in lavender varieties.

Ex situ plants are characterized by high activity of catalase and superoxide dismutase (Table 2). In in vitro culture catalase activity in lavender varieties was 2 times higher than in lavandin. At the same time, the activity of SOD and PPO in lavender was lower than in lavandin. Comparative analysis showed that the minimum values of the catalase and polyphenol oxidase activity are typical for lavender and lavandin varieties grown in vitro. The decrease in enzyme activity is due to a high water content in tissues, a low content of ascorbic acid and phenolic compounds, and the absence of stress factors. The activity of SOD in lavandin varieties grown in the in vitro culture is similar to that in plants grown ex situ, and in lavender varieties in the in vitro culture, such activity was 50% lower than in intact plants.

2. Activity of oxidation-reduction enzymes in the plants of lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) of different varieties (*M*±SD, 2016)

Variety	Conditions	Catalase, g $O_2 \cdot g^{-1} \cdot min^{-1}$	SOD, c. u./g	PPO, c. u. $\cdot g^{-1} \cdot s^{-1}$
Belyanka	Ex situ	30.68±0.87	12.98±0.32	0.524±0.013
	Cv, %	8.0	6.9	7.1
	In vitro	7.65±0.19	5.62±0.14	0.103±0.002
	Cv, %	7.0	7.1	5.5
Rekord	Ex situ	18.13±0.45	13.60±0.33	0.628±0.016
	Cv, %	7.0	6.9	7.2
	In vitro	6.80±0.16	6.12±0.20	0.101±0.003
	Cv, %	6.7	9.2	8.4
Rabat	Ex situ	31.45±0.77	12.55±0.32	0.600±0.015
	Cv, %	6.9	7.2	7.1
	In vitro	3.68±0.09	12.43±0.31	0.112±0.003
	Cv, %	6.9	7.1	7.6
Snezhnyy Bars	Ex situ	36.97±0.92	14.82±0.38	0.377±0.008
	Cv, %	7.0	7.3	6.0
	In vitro	2.98±0.08	10.48±0.28	0.124±0.004
	Cv, %	7.6	7.6	9.1

N o t e. SOD — superoxide dismutase, PPO — polyphenol oxidase; c. u. — conditional units. *Cv* — coefficient of variation at $p < 0.05$.

When grown in the open ground, the water content in the leaves was 56-62% (Table 3), the proportion of bound water was 78-93% of its total content.

3. Parameters of the water regime and the relative quantum efficiency of the photosystem-2 in lavender (*Lavandula angustifolia* Mill.) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) of different varieties (*M*±SD, 2016)

Indicator	Conditions	Variety			
		Belyanka	Rekord	Rabat	Snezhnyy Bars
Total water content,%	Ex situ	61.1±3.0	57.9±2.5	56.3±4.8	62.3±2.1
	Cv, %	13.9	12.2	24.1	9.5
	In vitro	76.1±3.3	72.3±2.9	77.0±2.5	74.4±3.2
	Cv, %	12.3	11.4	9.2	12.2
Fraction of bound water, % of total water content	Ex situ	78.3±4.9	90.6±3.5	82.1±4.3	93.2±1.3
	Cv, %	17.7	10.9	14.8	3.9
	In vitro	69.5±4.1	58.1±2.2	68.3±4.8	49.4±6.1
	Cv, %	16.7	10.7	19.9	34.9
Water deficiency, %	Ex situ	26.9±1.4	24.8±2.9	23.1±2.9	29.1±1.2
	Cv, %	14.7	33.1	35.5	11.6
Relative photosynthetic activity, $(F_m-F_{st})/F_m$	In vitro	0.68±0.09	0.70±0.05	0.75±0.10	0.71±0.05
	Ex situ	0.28±0.10	0.45±0.05	0.55±0.08	0.45±0.09
Viability index, F_m/F_{st}	In vitro	2.61±0.50	2.51±0.61	3.18±0.52	2.94±0.70
	Cv, %	54.2	68.7	46.2	67.3
	Ex situ	1.41±0.03	1.71±0.12	2.36±0.37	2.00±0.36
	Cv, %	6.0	19.8	44.3	50.9

N o t e. F_m and F_{st} — the maximum and stationary values after darkness adaptation, respectively. *Cv* — coefficient of variation at $p < 0.05$.

After a long (18 days) drought period, the total water content in the vegetative organs decreased, while the proportion of bound water increased. The maximum water-retaining capacity was typical for tissues of vegetative organs of the Snezhnyy Bars and Rekord varieties due to the bound water fraction. The degree of water deficiency in the leaves of lavender and lavandin varieties varied from 23% to 29%. The water content in the leaves of in vitro micro shoots was higher in lavandin varieties (74-77%); there were no significant differences between varieties for this parameter. However, the smallest variability of water content in micro shoots during cultivation and the maximum ratio of bound and free water fraction maks it possible to highlight the Rabat and Belyanka varieties.

Changes in the water regime to a greater extent affected the photosynthetic activity of lavender varieties. They were characterized by the decrease in the relative quantum activity of photosystem II, photochemical reactions and the efficiency of energy capture by open reaction centers. The viability index of all studied varieties is within the standard, but in lavandin of the Rabat variety, it is sig-

nificantly higher.

The apical meristem in the in vitro conditions after 5-6 subculturings formed 2-5 micro shoots of 23-82 mm in height, each micro shoot had 10-26 leaves, the leaves were lanceolar, 9-15 mm in length. The micro shoots leaves showed high photosynthetic activity. When cultivating under controlled conditions in vitro and at a relatively heterotrophic nutrition, the viability index was also normal, its values were of a variety-specific nature. Parameters of the functional state of the studied in vitro plants indicate the absence of photoinhibition, the normal functioning of photosystems both during the operation of light-harvesting systems and at the moment of oxidation of electron donors in the reaction center of the photosystem II.

Thus, the content of phenolic compounds, ascorbic acid, and the activity of catalase, superoxide dismutase, and polyphenol oxidase are maximal in the plants of lavender and lavandin in the open ground. There are no significant differences between varieties of lavender and lavandin in terms of these parameters. The content of proline in the in vitro micro shoots is higher, and the content of phenolic compounds, ascorbic acid, and enzymatic activity is lower than in intact plants. Changes in the water regime of the studied varieties to a greater extent affect the photosynthetic activity of lavender varieties. The viability index is normal, there is no photoinhibition. The adaptive potential of lavandin varieties under different cultivation conditions is determined to be higher than that of lavender varieties.

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INTERSPECIFIC HYBRIDIZATION IN LAVANDIN (*Lavandula* × *intermedia* Emeric ex Loisel.) BREEDING FOR ESSENTIAL OIL QUALITY

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Abstract

Lavandin cultivars (*Lavandula* × *intermedia* Emeric ex Loisel.) are sterile interspecies hybrids *Lavandula angustifolia* Mill. × *L. latifolia* Medic. They are of great interest for the essential oil industry. Lavandin cultivars express 1.5-2-fold higher yields of raw biomass and essential oil production, as well as 4-fold higher essential oil yield per area unit as compared to the used lavender cultivars. World production of lavandin essential oil is 1200 thousand tons, and lavender oil production is only 200 thousand tons. However, the quality of lavandin essential oil is lower compared to lavender one because of camphor, 1,8-cineole and borneol significant amounts. Besides, it is impossible to use lavandin cultivars in further breeding works, as they are sterile. The main trend in lavandin breeding is to improve the essential oil quality by reducing unwanted components to a minimum. In order to create lavandin hybrids characterized by high essential oil quality we had first synthesized tetraploid forms of *L. angustifolia* and *L. latifolia* and then crossed these forms with each other. As the result, their sterility was overcome and amphidiploid hybrids were obtained. Those hybrids were further used to create new highly effective cultivars. Crossing between amphidiploid hybrid № 48 and lavender cultivars (Belyanka, Record, Prima) let us to select cross combinations and create lavandin cultivars with minimum amount of camphor, borneol and 1,8-cineol. It was revealed that obtained plants often demonstrated intermediate when compared to their parental forms. Thus, initial forms with high content of linalool and linalyl acetate and lower content of unwanted compounds should be used in breeding works. In cross combinations Amphidiploid 48 × Belyanka, Lavandin hybrids with high content of linalool (up to 68.8 %) were derived. Some hybrids had the content of borneol (up to 0.5 %), camphor (1.9 %) and 1,8-cineole (1.8 %) similar to that of parental forms. In cross combination Amphidiploid 48 × Prima hybrids were obtained with high content of linalool (up to 57.9 %) and linalyl acetate (up to 32.8 %) and low content of camphor (0.2 %), borneol (1.6 %) and 1,8-cineole (up to 0.9 %). The results of our investigations demonstrated that it is possible to create Lavandin hybrids with borneol amount lower than in the original forms. Camphor and 1,8-cineole content depression was not beyond the intermediate type of inheritance. We suppose that the extremely low content of 1,8-cineole and camphor in the lavender chemotypes selected for breeding corresponds to their lower biological limit of these metabolites. Hybrid plants (*L.* × *intermedia*) can only approximate these characteristics of *L. angustifolia*. Lavandin hybrids with the best essential oil composition have been obtained by crossings between the most closely related, from a biological point of view, chemotypes with dominant alleles of linalool and linalyl acetate and recessive alleles of camphor and 1,8-cineole in *L. angustifolia*, which is possible under creating Lavandin hybrids with two genomes of *L. angustifolia* and one genome of *L. latifolia*.

Keywords: allotriploid, Lavandin, *Lavandula* × *intermedia* Emeric ex Loisel., hybrid, essential oil composition

Selection of lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.), which

is promising for essential oil production, is associated with the production of interspecific hybrids F_1 at a diploid level using crossing of true lavender (*L. angustifolia* Mill.) with spike lavender (*L. latifolia* Medic.). The resulting hybrids are characterized by heterosis in terms of essential oil content and yield [1-3], which explains the interest in them. However, the quality of lavandin essential oil is lower than that of lavender essential oil due to the presence of components that have a negative effect on it, i.e. cineole, borneol, camphor, inherited from spike lavender. The studies on a more detailed characterization of the composition of interspecific hybrids essential oil are known [8, 9].

The main task in lavandin selection (along with an increase in the total yield of essential oil from 1 hectare) is a decrease in the amount of undesirable components in raw materials [10-12]. However, there is no theoretical justification for the selection of parental pairs, which makes it difficult to purposefully obtain hybrids with given properties. In our opinion, the creation of hybrid genotypes by means of distant hybridization involving the induced polyploid forms is a promising approach. This requires a preliminary study of patterns of traits inheritance in such combinations of crossing.

This report, for the first time, suggests a theoretical approach to the selection of parental pairs for lavandin crossing in the selection for improving the composition of essential oil. The results of obtaining induced amphidiploid forms of *L.* × *intermedia* and hybrid progeny based on them are presented and the patterns of target traits inheritance are analyzed.

The purpose of this study was to select parental forms, to develop a scheme for lavandin crossing when breeding for quality and to compare the manifestations of target traits in the hybrid genotypes and their parents.

Techniques. The following varieties were the initial parental forms of true lavender: Belyanka, of a linalool type with a total content of linalool and linalyl acetate about 80% and a predominance of linalool (up to 67%); Prima of a linalyl acetate-linalool type with a total content of these components about 80% and a predominance of linalyl acetate (up to 50%); Rekord with an approximately equal content of linalool and linalyl acetate, which in total are 70% of the essential oil. These lavender varieties are characterized by a relatively low content of camphor (not more than 1.9%), borneol (no more than 3.5%) and cineole (no more than 0.3%). Amphidiploid No. 48 was obtained by colchicination of sterile lavandin F_1 and refers to the linalool type with high linalool content (up to 62.5%), and a medium content of linalyl acetate (up to 12.9%), camphor (10.6%), cineole (3.4%) and borneol (5.8%).

The following combinations of crossing were used in the studies: Amphidiploid No. 48 × Belyanka, Amphidiploid No. 48 × Rekord, Amphidiploid No. 48 × Prima (Amphidiploid No. 48 was the female parent). Hybrids and the initial forms were grown under the same conditions on the collection sites of the Nikitsky Botanical Garden (Crimea). The paper presents average data for 2015-2017.

Cytological analysis of hybrids was carried out according to Z.P. Pausheva [18] using a Jenamed microscope (Carl Zeiss Jena GmbH, Germany, magnification ×900).

Mass fraction of essential oil was determined by 1-hour hydrodistillation according to Ginsberg (State Pharmacopoeia of the USSR. M., 1987) in fresh raw materials. The experiments were arranged in 3 replications. The composition of the essential oil was detected using a gas chromatograph 6890N (Agilent Technology, Inc., USA) with a mass spectrometric detector 5973N. The HP-1 column is 30 m long and the inner diameter is 0.25 mm. The thermostat temperature was programmed from 50 to 250 °C with a change rate of 4 °C/min.

The injector temperature was 250 °C, the carrier gas was helium, and the flow rate was 1 cm^3/min. The temperature of the transition line from the gas chromatograph to the detector was 230 °C, with the temperature of the ion source 200 °C. Electron ionization was carried out at 70 eV at m/z from 29 to 450 [19]. The essential oil components were identified by comparison with the library data of mass spectra NIST 05 (http://nistmassspeclibrary.com/) and WILEY2007 (http://www.sisweb.com/software/ms/wiley.htm) (total about 500000 mass spectra).

The software STATISTICA v.6.0 (StatSoft, Inc., USA) was used for statistical processing. Mean (M) and standard errors of means (±SEM) were calculated. The significance of differences between the variants was evaluated by the arithmetic mean and variation coefficient at $p < 0.05$.

Results. The initial parental forms have different chemotypes. The crossing of Amphidiploid No. 48 with the true lavender diploid resulted in the hybrid genotypes that have a somatic number of chromosomes $2n = 72$, include two genomes of *L. angustifolia* (AA) and one genome of *L. latifolia* (L), are allotriploids with the genomic composition AA-L. Their formation can be represented as follows (Fig. 1).

Amphidiploid		Diploid
$2n = 96$, AALL (metaphase I)		$2n = 48$, AA
24_{II} *L. angustifolia* + 24_{II} *L. latifolia*	×	24_{II} *L. angustifolia*
Gametes AL ($n = 48$)		Gametes A ($n = 24$)
$2n = 72$, AAL		

Fig. 1. Combination of crossing the amphidiploid (lavandin) with diploids of lavender *Lavandula* sp. used in hybridization for lavandin breeding.

A cytological study of F_1 hybrids showed that they are all germ-free and have a chromosome number $2n = 72$, that is, balanced 24-chromosome gametes of the true lavender and 48-chromosome Amphidiploid No. 48 participated in their formation. The study confirmed the hybrid nature of the plants obtained from crossing and showed that they are allotriploids. Thus, the studied hybrids (total 96 genotypes) do not differ in the number of chromosomes, which excludes the influence of the specified factor on the content and composition of the essential oil.

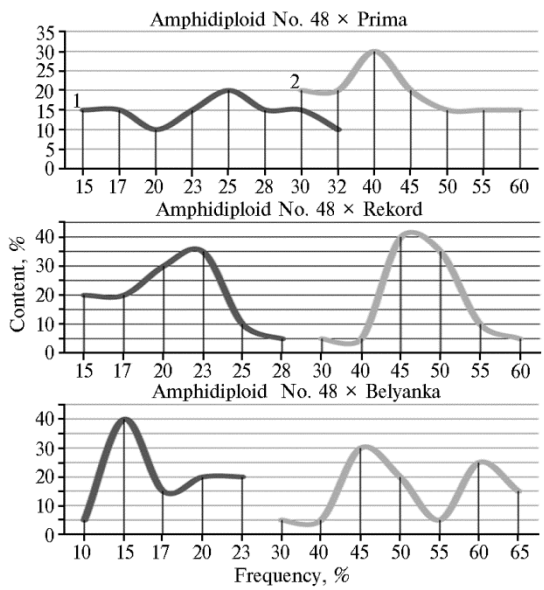

Fig. 2. Distribution of interspecies hybrids F_1 of lavender (*Lavandula angustifolia* + *L. latifolia*) × *L. angustifolia* according to the content of linalyl acetate (1) and linalool (2) in different combinations of crossing.

A significant increase in the variation of the essential oil content was observed in hybrids of all variants of crossing [13, 20]. Most of the hybrids inherited this trait for an intermediate type, with the mean values of the sample close to the high oleic forms, regardless of the type of crossing. The deviation of hybrids towards high oleic forms was observed when using the Rekord as a paternal component. Thus, the content of the essential oil is inherited towards the forms with a large manifestation of this trait with a certain influence of the paternal form [20].

When analyzing the patterns of variability and inheritance of the essential oil composition (Figs. 2-4), only its main components, linalool and linalyl acetate (see Fig. 2), as well as compounds that reduce its quality, i.e. cineole, camphor, and borneol (see Fig. 3), were considered.

When crossing Amphidiploid No. 48 and the true lavender varieties Rekord and Prima used as pollinators, the accumulation of linalool in hybrids is approximately the same. The content of linalyl acetate in all hybrids is characterized by an intermediate type of inheritance, and the average indicators are shifting toward the maternal form.

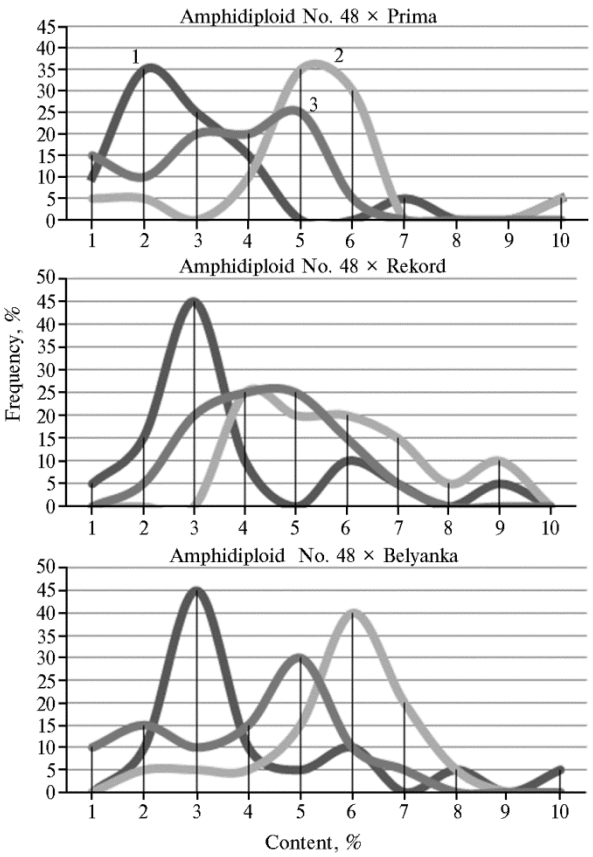

Fig. 3. Distribution of interspecific hybrids F_1 of lavender (*Lavandula angustifolia* + *L. latifolia*) × *L. angustifolia* according to the content of terpenes 1,8-cineole (1)**, camphor** (2) **and borneol** (3) **in different combinations of crossings.**

Inheritance of undesirable components was characterized by a more pronounced intermediate type. It was shown that only in a small number of plants, the content of borneol, cineole, and camphor is lower than in parental forms, but the depression is very small, 3%. These plants were identified in a crossing combination with the use of true lavender of the Belyanka variety as a paternal form (Table 1).

In general, the inheritance of the considered components in the used variants of crossing is predominantly intermediate, with a deviation towards the maternal line and a heterotic effect on the content of linalool in it.

1. Variation in the main components of essential oil in interspecific hybrids F_1 of lavender ♀(*Lavandula angustifolia* + *L. latifolia*) × ♂*L. angustifolia* obtained with Belyanka, as compared to the parents (M±SEM, n = 32, Nikitsky Botanical Garden, 2015-2017)

Component	Content, %	♂ Belyanka	♀ Amphidiploid No. 48	F_1
Linalool	Average	63.7±1.7	60.3±2.1	54.6±1.8
	Limits of variation	60.1-64.3	57.5-62.5	51.5-68.8
	Cv, %	4.5±0.5	6.8±0.9	15.1±1.4
Linalyl acetate	Average	13.9±0.7	9.4±0.9	10.4±0.8
	Limits of variation	9.8-15.4	7.5-12.9	9.7-16.2
	Cv, %	11.5±0.8	6.7±0.5	22.8±2.9
Cineole	Average	2.7±0.2	3.4±0.3	3.6±0.5
	Limits of variation	2.1-2.9	3.1-5.2	1.8-9.6
	Cv, %	2.2±0.1	6.5±0.4	57.2±4.9
Borneol	Average	1.8±0.3	5.8±0.3	3.6±0.4
	Limits of variation	1.6-2.1	4.7-7.2	0.5-6.8
	Cv, %	6.5±0.4	9.2±0.3	49.1±5.6

Continued Table 1

Camphor	Average	2.1±0.1	10.6±0.9	5.3±0.3
	Limits of variation	1.6-2.3	9.8-12.0	1.9-7.2
	Cv, %	7.5±0.2	8.7±0.8	26.8±2.1

N o t e. Cv — coefficient of variation at $p < 0.05$.

The crossing of the used chemotypes did not result in hybrids with high-quality essential oil. Only in the hybrid progeny of Amphidiploid No. 48 × Prima the content of linalyl acetate in a number of plants slightly exceeded the main commercial lavender variety Rekord (standard) (Table 2). When the Rekord, Prima and Belyanka were used as the paternal forms, a wide variation in the content of linalool (36.7-68.8%) was observed in hybrid plants.

2. Variation in the main components of oil in interspecific hybrids F_1 of lavender ♀(*Lavandula angustifolia* + *L. latifolia*) × ♂*L. angustifolia* with Rekord (standard) variety, as compared to parents (*M*±SEM, *n* = 32, Nikitsky Botanical Garden, 2015-2017)

Component	Content, %	♂ Rekord	♀ Amphidiploid No. 48	F_1
Linalool	Average	40.5±0.4	60.3±2.1	46.3±1.1
	Limits of variation	39.0-42.0	57.5-62.5	39.8-58.2
	Cv, %	8.2±0.7	6.8±0.9	10.4±0.8
Linalyl acetate	Average	32.2±0.2	9.4±0.9	19.7±0.7
	Limits of variation	30.0-34.0	7.5-12.9	15.3-28.6
	Cv, %	5.5±0.3	6.7±0.5	15.8±1.1
Cineole	Average	0.2±0.1	3.4±0.3	3.1±0.4
	Limits of variation	0.1-0.4	3.1-5.2	0.9-8.1
	Cv, %	2.5±0.1	6.5±0.4	60.8±5.8
Borneol	Average	3.5±0.2	5.8±0.3	3.9±0.3
	Limits of variation	2.0-4.0	4.7-7.2	1.7-6.8
	Cv, %	2.4±0.2	9.2±0.3	34.0±3.5
Camphor	Average	1.4±0.1	10.6±0.9	6.4±0.4
	Limits of variation	0.4-1.9	9.8-12.0	4.1-10.1
	Cv, %	4.1±0.2	8.7±0.8	20.7±2.1

N o t e. Cv — coefficient of variation at $p < 0.05$..

The same pattern of inheritance with intermediate values of the indicator was found in hybrids by accumulation of 1,8-cineole and camphor. In all crossings, the proportion of plants with a lower camphor content than in the standard was 60%. According to the amount of borneol, the depression was observed in 9% of hybrid progeny. Thus, the influence of the maternal form characteristics on the nature of essential oil composition inheritance is observed in all combinations of chemotypes.

Hybridization of linalool-linalyl acetate and linalool-camphor chemotypes (Table 3) made it possible to obtain hybrids in which the content of linalool is higher, the content of linalyl acetate is approximately equal, and the content of borneol is lower compared to those of commercial lavender variety Rekord. At the same time, the amount of 1,8-cineole in the resulting hybrids (the range of variation is 0.9-9.7%) is significantly higher than that in the standard variety Rekord (0.01-0.04%).

3. Variation in the main components of oil in interspecific hybrids F_1 of lavender ♀(*Lavandula angustifolia* + *L. latifolia*) × ♂*L. angustifolia* with Prima variety, as compared to parents (*M*±SEM, *n* = 32, Nikitsky Botanical Garden, 2015-2017)

Component	Content, %	♂ Prima	♀ Amphidiploid No. 48	F_1
Linalool	Average	32.8±0.4	60.3±2.1	46.8±1.6
	Limits of variation	31.0-33.0	57.5-62.5	36.7-57.9
	Cv, %	3.8±0.2	6.8±0.9	14.8±1.3
Linalyl acetate	Average	46.6±0.3	9.4±0.9	23.4±1.2
	Limits of variation	44.1-50.4	7.5-12.9	15.5-32.8
	Cv, %	9.8±0.5	6.7±0.5	22.3±1.9
Cineole	Average	0.3±0.1	3.4±0.3	2.7±0.5
	Limits of variation	0.2-0.4	3.1-5.2	0.9-9.7
	Cv, %	2.8±0.2	6.5±0.4	81.8±8.9

Continued Table 3

Borneol	Average	0.8±0.2	5.8±0.3	2.9±0.4
	Limits of variation	0.2-1.3	4.7-7.2	1.6-5.4
	Cv, %	3.4±0.4	9.2±0.3	55.6±7.1
Camphor	Average	0.4±0.9	10.6±0.9	5.2±0.4
	Limits of variation	0.1-0.9	9.8-12.0	0.2-6.8
	Cv, %	5.1±0.4	8.7±0.8	30.4±3.6

Примечание. *Cv* — коэффициент вариации при P < 0,05.

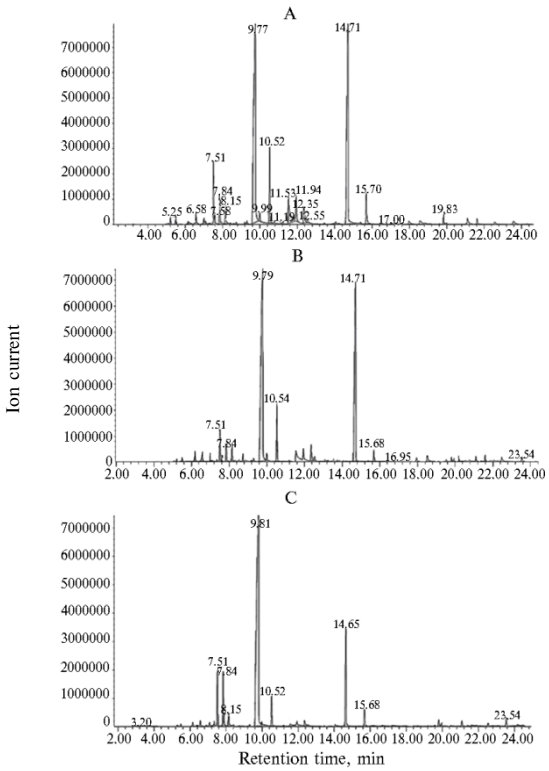

Fig. 4. Essential oils of interspecific hybrids F_1 of lavender (*Lavandula angustifolia* × *L. latifolia*) × *L. angustifolia* in crossing Amphidiploid No. 48 × Prima (A)**, Amphidiploid No. 48 × Rekord** (B) **and Amphidiploid No. 48 × Belyanka** (C) (Nikitsky Botanical Garden**,** 2015-2017). Peaks: 1,8-cineole (7.51), linalool (9.77-9.81), camphor (10.52-10.54), borneol (11.58), linalyl acetate (14.65-14.71) (a gas chromatograph/mass spectrometer 6890N, Agilent Technology, Inc., USA).

Hybrids from crossing with Belyanka, which has approximately the same linalool and linalyl acetate content as the parent plants (Amphidiploid No. 48) of the linalool chemotype, also show inheritance for these traits with a deviation towards the maternal Amphidiploid No. 48).

With respect to undesirable components, depression was observed in most hybrid genotypes in all combinations used. For example, there was a reduced amount of borneol (from 9 to 48%). However, none of the hybrids actually improved the performance of the standard in terms of the content of camphor and 1,8-cineole. At the same time, a rather significant part of progeny in the variant Amphidiploid No. 48 with Belyanka, on the contrary, showed a heterotic effect in these characteristics, especially in the content of 1,8-cineole.

The greater part of hybrids was characterized by a depression of linalool content in all variants of crossing, but some showed a heterotic effect, reaching 127.9-131.0%. When using Belyanka, the yield of plants with a heterotic effect was maximal and amounted to 18% (see Fig. 2). A small heterotic effect was also seen in the content of linalyl acetate, but only in combination Amphidiploid No. 48 × Prima and only in 3% of the hybrids obtained.

It should be noted that the combination Amphidiploid No. 48 × Prima gives a significant yield of hybrid plants with essential oil close to the standard Rekord variety in terms of quality, and 100% of the hybrids resulted from the crossing Amphidiploid No. 48 × Belyanka were better than Rekord variety in the linalool content.

By analyzing the obtained results, it is possible to make the following assumptions on the prospects of lavandin breeding for a certain content and composition of essential oil. The initial forms of true lavender and Amphidiploid No. 48 have a number of genetic features. Chemotypes of true lavender are characterized

by the presence of dominant alleles controlling the synthesis of linalool (L) and linalyl acetate (A), recessive alleles controlling the biosynthesis of cineole (c), borneol (b) and camphor (k). In Amphidiploid No. 48, the dominant alleles control the biosynthesis of linalool (L), borneol (B), and camphor (K). The biosynthesis of linalyl acetate is not characteristic of this clone and is controlled by a recessive allele (a).

In previous studies, it was shown that Amphidiploid No.48 as a donor of the feature of essential oil high yield should be used as a paternal form, since the essential oil content is inherited in the paternal line [13, 20]. At the same time, a high content of linalool and linalyl acetate can be achieved only if chemotypes of true lavender are used as a maternal form. This is especially important with regard to the selection for the content of linalyl acetate, the biosynthesis of which is not characteristic for Amphidiploid No. 48. Thus, the selection of lavandin allows obtaining a simultaneous combination of positive results in terms of both content and composition of essential oil. The foreign literature does not contain data on the evaluation of crossing combinations, and only gives a chemical analysis of the essential oil of lavandin varieties that have been already obtained [21-25]. Comparison of the results of the study on the composition of lavandin essential oil, obtained by us and by foreign researchers, shows that the hybrids we obtained exceed the foreign ones in oil quality [10, 11, 26-28].

The result of selection largely depends on the correct choice of chemotype pairs. In breeding for the content of essential oil, attention should be paid to the fact that the heterotic effect occurs at a certain degree of manifestation of this characteristic in the initial forms. Heterosis on essential oil content is most likely with its content of at least 5% of dry raw material for true lavender and 10% for Amphidiploid No. 48.

Amphidiploid No. 48, as well as the Belyanka variety, has dominant alleles that control linalool biosynthesis. As a consequence, heterosis on this component was observed in all combinations of crossing. The maximum content of linalool is proportional to its quantity in both initial forms, Belyanka and Amphidiploid No. 48. Heterosis on the content of linalyl acetate is rarer and occurs, obviously, only in case of the high content of both linalyl acetate and linalool in the maternal form. It should be noted that the inheritance of the high content of linalyl acetate and linalool cannot be combined, since high values for linalool are achieved when using Amphidiploid No. 48 as a maternal form, and for linalyl acetate when using it as a paternal form. This circumstance can be used in target selection for the content of one of these components.

Earlier, it was reported that the improvement of hybrids in the content of undesirable components 1,8-cineole, borneol and camphor of essential oil is more likely when using Amphidiploid No. 48 as a paternal form [13, 20]. However, only in the case of borneol it is possible to obtain hybrid plants with a depression of its content to a value that is lower than that in the initial forms. Depression in the content of 1,8-cineole and camphor does not exceed the limits of the intermediate type of inheritance. Thus, it can be assumed that the 1,8-cineole and camphor content in the chemotypes of true lavender selected for breeding corresponds to the extremely low amount of these compounds in members of the species. Hybrid plants are only able to be close to these values. When breeding lavandin for the high quality of essential oil, the chemotypes of amphidiploid with a low content of 1,8-cineole and camphor in combination with a high accumulation of linalyl acetate are most appropriate.

Thus, our experiments show that the highest content of essential oil and its best composition result from combinations of Amphidiploid No. 48 with closely related chemotypes of true lavender, bearing the dominant alleles of li-

nalyl acetate and linalool (Prima variety) or the dominant allele of linalool (Belyanka variety). These combinations produce hybrids with the maximum content of linalool and linalyl acetate. In addition, in this case there is the greatest yield of hybrids with heterosis on the desired traits. The study of the combining ability of parental pairs has shown that in order to obtain hybrids with a high quality of essential oil, target interspecific crossings are necessary of Amphidiploid No. 48 with Rekord and Prima varieties of *Lavandula angustifolia*. These result in allotriploids with two genomes of *L. angustifolia* and one genome of *L. latifolia*, in which the mass fraction of essential oil is up to 3.6% of wet weight of the raw material (or 10.25 for absolutely dry weight). The best combination of crossing is Amphidiploid No. 48 × Prima. It allows the creation of allotriploids with a high quality of essential oil containing up to 32.8% of linalyl acetate with a minimum amount of 1,8-cineole (3.7%) and camphor (5.6%). In addition, hybrids with high linalool content (up to 68.8%) for the essential oil industry are obtained in combination Amphidiploid No. 48 × Belyanka. Resultant hybrids with a linalool content up to 68.8% (Amphidiploid No. 48 × Belyanka) and linalyl acetate content up to 32.8% (Amphidiploid No. 48 × Prima) are identified.

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DYNAMICS OF THE PLANT COMMUNITY AND MICROBIOM OF CHRONO-SERIES OF POST-TECHNOLOGICAL SOIL IN LIMESTONE QUARRY IN THE CONDITIONS OF RECULTIVATION

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Abstract

Post-technogenic ecosystems represent informative natural models of initial pedogenesis and restoration under the abandoned and reclamation practices. These soils can be considered as natural experiments on restoration of microbial communities within the age row (time series or chronoseries). Investigation of different aged stages of soil formation on the spoil banks of the quarries give a possibility to obtain initial data on the rate and trends of the pedogenesis in various combinations of substrates and phytocenosis. Among the quarries of mineral substrates in North-West region, particular place is devoted to quarries for lime stone exploitation located on Izhora upland. The purpose of this study conducted in one of the largest limestone quarries of Leningrad region was to examine succession of plant and microbial communities. Species composition and vegetation cover were estimated for different plant communities within each ecotype of quarry. Also at the each plot the following characteristics of soil were measured: pH; organic carbon; soil basal respiration and substrate induced respiration; texture; CO_2 of carbonates and moisture content. Total soil DNA was exctracted using PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., USA), sequencing of v4, the variable region of 16S rRNA gene pDNA, was conducted using a third-generation GS Junior sequenator (Roshe, Switzerland). The results were processed using the QIIME software. To compare microbial communities, the alpha and beta diversity analyses were performed. Our results highlighted that the main difference between plant communities of different plots were due to position in the landscape, most similar communities colonize similar ecotypes. The microbial communities of the old (35 years or more) dumps are essentially specific, and communities of microorganisms of young (8-16 years) and middle-aged (28-30 years) dumps tend to group into separate clusters. As compared to young and middle age dumps, old dumps are characterized by a significant (6-8-fold) increase in the counts of *Micromonosporaceae* and *Sinobacteraceae* representatives. Communities of young dumps have 4.2 times more *Pseudomonas* and 3.8 times more *Micrococcaceae* members than the communities of old ones. The communities of wet terraces also differ from the microbiomes of the other ecotopes (dry terraces, the bases of the dumps), however, no significant differences could be identified. The main issue for successful soil reclamation is the restoration of the microbial community. It was shown that copiotrophes, the microorganisms adapted to high concentration of soil nutrients, dominate in the youngest soils. As microbial succession proceeded, oligotrophs which are involved in organic matter decomposition become dominating. It was established that the processes connected with transformation of organic matter became the main drivers of soil formation, which is especially important for initial stages of soil body restoration. Data on soil microbiomes and microbiomes of soil-vegetation complexes could be the most important tools for reclamation practices.

Keywords: primary succession of plant and microbial communities, limestone quarries, pedogenesis, metagenome, reclamation

The increase in the territories of disturbed lands, occurring as a result of the development and extraction of minerals, is a particular environmental problem. As a rule, anthropogenic landscapes are removed from the forest and agriculturally used areas, and the processes of soil and vegetation cover remediation are extremely slow [1]. The study of soil formation under impact of technogenesis is a theoretical basis for reclamation of disturbed lands and recovery of landscapes [2, 3]. Rendzina soils formed on the Izhora Plateau are characterized by high fertility and good drainage conditions, so these territories have been actively used in agriculture for a long time [4]. The high carbonate content in the dump rock results in the potential fertility of calcareous dumps, but the specificity of the composition of quarry-dumping complexes, as well as a relatively low content of nutrients and unfavorable physical properties of the substrate, creates difficulties in reclamation of these territories [5]. When selecting approach for reclamation, leveling the surface becomes quite a laborious and expensive task [3]. However, the complex terrain formed during the development of quarries provides a high variety of ecological niches, which are favorable for a large number of plant and animal species, including those valuable for natural conservation. Thus, in various conditions and geographical zones, it was found that abandoned quarries can increase biodiversity by acting as refugiums for rare species of plants and animals [6, 7].

The world practice of reclamation emphasizes the use of regeneration capabilities of natural ecosystems, where the biotic factors of the soil-forming process, the vegetation cover and associated soil microbiome play a critical role [8, 9]. The activity of microorganisms is directly related to the fertility and the provision of soil ecosystem functions, which include the balance of the nutrient elements cycle, as well as the ecological adaptation of plants to stressful conditions [10]. In addition, the high adaptive potential of soil microorganisms that are capable of adapting to any environmental changes in a short time and to use all available ecological niches allows the use of the microbiome structure as one of the most sensitive environmental indicators that mark different stages of soil remediation [11].

The spontaneous succession of plant communities in quarries with various substrates has been studied in sufficient detail. A significant part of domestic studies is related to various aspects of the succession vegetation cover [12-14], and the processes of pedogenesis have been analyzed sufficiently [15, 16]. However, complex observations on remediation of the soil and vegetation cover and the connection of these components remain insufficient. The majority of foreign papers, on the contrary, are focused on the conjugate development of the soil and vegetation cover after planting certain plant species, but not due to self-organized vegetation [17-20]. Traditionally, papers on the soil reclamation are focused on the study of vegetation cover and redistribution of soil organic matter [9, 15, 16]. The importance of a microbiome in ecosystem restoration processes has been underestimated for a long time. Over the recent decade, many publications [21-23] have been focused on the study of the microbiome of reclaimed soils and contain valuable information on the rates of biomass restoration and the activity of microbial communities in the chronoseries of soils in technogenic landscapes [24-26]. It has been shown that the structure and composition of microbiomes are determined by a combination of physical and agrochemical parameters (reserves of soil organic matter, total nitrogen, pH, cation exchange capacity), the values of which are significantly higher in reclaimed variants. Much attention is paid to the use of microbial communities as indicators of different stages of technogenic landscapes soil remediation [27-29]. At the same time, the diversity and structure of microbiomes in disturbed and reclaimed soils is still insufficiently studied. Only with the advent of metagenomic technologies, it became possible to effectively analyze and interpret the diversity of soil microbiota. Involvement in the study

on the soil microbiome diversity creates opportunities for the development of qualitatively new systems for accelerating and optimizing reclamation measures in disturbed areas. Recently, attention has been paid to the creation of microbial preparations to improve the quality of soils [30, 31].

A comprehensive study of the three main components of the ecosystem (soil, plant community and microbiome) in chronoseries of various ecotopes in conditions of reclamation and self-organized vegetation was carried out in the territory of one of the largest limestone quarries in the Leningrad Region for the first time. The taxonomic structure of the microbiome of the initial soils has been established both for quarries located in the Northwest and for the initial soils of Russia as a whole.

The purpose of the paper was to study the dynamics of phytocoenosis and soil microbiome in the process of succession on dumps of different ages at natural self-organized vegetation and reclamation of the limestone quarry.

Techniques. The study was carried out on a limestone quarry Pechurki (Slantsevsky District, Leningrad Region) during the spring and summer of 2016. Limestone mining was stopped here in 2014. First of all, the main ecotopes of the quarry, corresponding to the elementary soil areal, were determined. 12 test sites 25×25 m in size were set within each ecotope. The total plant cover and species composition of higher vascular plants, ground mosses and lichens, were evaluated on sites. Soil cuts were also set within each plot. Field descriptions of the soils were carried out on each of the open test pits, samples (700 g each) were taken from the pedogenic horizons to perform laboratory analyzes.

The following indicators were determined: substrate-induced respiration (SIR) of soil samples [32]; basal respiration according to the procedure for SIR [32], but in soil not enriched with substrate; the content of organic carbon in bichromate oxidation (I.V. Tyurin's method); pH of the aqueous suspension and saline pH (1:2.5 soil:solution); exchange and hydrolytic acidity; CO_2 carbonates content using the acidimetric method [33]; hygroscopic and maximum hygroscopic humidity, as well as total water capacity (moisture storage capacity) and the lowest water capacity using gravimetry method [34]; the density of soil and solid phase of soils, the structure and rockiness of soil using the method of dry screening; the grain-size composition using Kachinsky's pipette method with phosphate peptization of microaggregates [34]; the fraction-group composition of humus according to the scheme of I.V. Tyurin, modified by V.V. Ponomareva et al. [35].

The amount of carbon of the microbial biomass was calculated according to the formula of J.P.E. Anderson et al. [36]: C_{mic} (μg C/g soil) = SIR (μl CO_2/g soil · h^{-1}) × 40.04 + 0.37, where SIR is substrate-induced respiration. The microbial metabolic coefficient (specific respiration of microbial biomass) was determined as the ratio of basal respiration (BR) to the indicator C_{mic}: qCO_2 (μg CO_2-C/mg C_{mic} · h^{-1}) = BR/C_{mic}.

The similarity coefficient (the Sørensen-Czekanowski coefficient) was calculated according to the lists of higher plants species prepared for each site: $K = 2c/(a + b)$, where a is the number of species at the first site, b is the number of species at the second site, c is the number of common species for the first and second sites. The most typical coefficients of biodiversity were also determined for each site. The Simpson index (C) was calculated by the formula: $C = 1 - \sum(n_i/N)^2$, where n_i is an estimate of each species significance (projective cover), N is the sum of significance estimates. The Shannon index was calculated according to the formula: $H = -\sum n_i/N \times \log (n_i/N)$.

The method of forward selection with two limiting criteria [37] for reducing the type I error was used to evaluate the effect of environmental factors on the phytocenosis structure. The canonical correspondence analysis (CCA)

proposed by C.J.F. Ter Braak [38] was performed using only significant variables ($p < 0.05$) in order to determine the effect of environmental factors on the distribution of plant communities.

Soil samples were taken from a depth of 8-10 cm at sites No. 1-9. DNA was isolated from the samples (0.2 g) using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., USA) in accordance with the manufacturer's instructions. Purified DNA preparations (10-15 ng) were matrices in polymerase chain reaction (PCR) (temperature profile: 30 s at 95 °C, 30 s at 50 °C, 30 s at 72 °C; total 30 cycles) using En-cyclo DNA polymerase (Eurogen, Russia) and universal primers at the variable region v4 of the 16S rRNA gene: F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACTACVSGGGTATCTAAT). The oligonucleotide identifiers were introduced into the primers for each sample and the service sequences necessary for the pyrosequencing according to the Roche protocol (Switzerland). Samples preparation and sequencing were performed using GS Junior (Roche, Switzerland) in accordance with the manufacturer's recommendations. Demultiplexing, quality control of 16S rRNA gene sequences, isolation of OTU (operational taxonomic units, species analog), normalization of samples, taxonomic identification of OTU, calculation of α- and β-diversity indices were performed with QIIME software package (http://qiime.org/) using the default parameters [39, 40]. Libraries of 16S rRNA gene fragments were standardized according to the number of sequences in the smallest library.

Differences in the frequencies of microbial taxa between the test samples were determined by an accurate Fisher test, adjusted for multiple comparisons using the Benjamini-Hochberg procedure at a 5% significance level. In order to explain the variability in the composition of species of microbial communities under the influence of the environmental factor, the Principal Component Analysis (PCoA) was used. Calculations were carried out with Statistica 7 software (StatSoft, Inc., USA) and Microsoft Excel.

Results. Works on reclamation in the limestone quarry Pechurki began in 1970, during which pine seedlings were planted in some areas at different times. In this case, most of the quarry was left for self-organized vegetation. The quarry was characterized by a complex internal relief, which contributed to the formation of a relatively wide variety of soils. Most environmental factors were optimal for the development of vegetation (Table), except for high density and rockiness on the rocky bottom of the quarry. All sites showed an extremely heterogeneous distribution of fractions along the section and a significant amount of skeletal coarse clastic material with a relatively low content of fine soil. In general, the results of mesomorphological studies indicated a fairly high rate of pedogenic transformation of the substrate. Chemical, biochemical and physical weathering of carbonate rocks are the basic transformation processes of the mineral part of the soil. The high content of fine earth contributed to the intensive weathering of limestone fragments (except for the rocky bottom of the quarry). This, in turn, increased the water capacity of embryozem and its fertility, thereby ensuring the active development of plant communities that produce organic matter. Intensive decarbonization was in a significant decrease in the pH of the humus horizons in comparison with the rock.

Places for establishment of test sites were selected in such a manner as to avoid the border effect (the density and number of species increases at the joints of biocenoses which can lead to distorted results), that is, preferably in the central part of the phytocenosis (if available) or in the central part of the ecotope (in the absence of closed vegetation cover). The location of the test sites is shown in Figure 1. The key pedogenic processes in all areas were the accumulation of organic matter and the substrate oxidation. They led to the formation of an A-horizon with maximum power and the largest content of organic matter in

Characterization of soils formed in the limestone quarry Pechurki (Slantsevsky District, Leningrad Region, 2016)

A	B	C	D	E	F	G	H	I	J	K	L
1	O	0-4	5.02	0.00	8.16	0.85	0.07	0.07	3.225	0.021	13.1
	AY	4-33	4.40	0.06	6.79	–	0.03	0.03	1.695	0.018	
	C	33-48	5.72	0.10	1.19	–	0.02	0.02	1.186	0.017	
	[C]	48+	7.45	0.17	0.86	–	0.03	0.03	1.491	0.017	
2	O	0-3	5.26	0.00	1.69	0.76	0.11	0.12	5.060	0.022	30.5
	BF	3-13	6.35	0.24	5.73	–	0.04	0.04	2.103	0.019	
	C	13+	6.55	0.08	2.05	–	0.04	0.04	1.797	0.020	
3	O	0-7	6.36	0.10	3.27	0.78	0.04	0.05	2.205	0.018	6.9
	AY	7-15	5.86	0.10	7.85	–	0.02	0.03	1.491	0.015	
	G	15-36	6.43	0.07	0.65	–	0.06	0.07	3.021	0.020	
	G_{ox}	36-45	6.12	0.08	1.94	–	0.04	0.04	2.001	0.019	
	C	45	6.50	0.09	5.45	–	0.02	0.02	0.982	0.016	
4	AY	0-26	5.22	0.21	3.94	0.91	0.04	0.05	2.205	0.018	29.5
	AC	26+	6.34	0.16	6.32	–	0.02	0.02	0.982	0.016	
5	O	0-13	5.64	0.00	14.4	0.65	0.04	0.05	2.205	0.020	28.0
	AY	13-25	6.05	0.35	2.13	–	0.06	0.07	3.021	0.021	
	C1	25-37	5.76	0.08	0.67	–	0.02	0.03	1.390	0.018	
	$C2_{ox}$	37+	6.10	0.52	3.33	–	0.03	0.04	1.797	0.017	
6	O	0-4	6.57	0.00	24.81	–	0.06	0.08	2.817	0.022	13.8
	AY	4-28	6.53	0.13	13.25	0.76	0.04	0.04	2.001	0.018	
	C	28+	5.40	0.27	4.47	–	0.04	0.04	1.797	0.020	
7	O	0-7	5.98	0.00	8.24	–	0.07	0.08	3.429	0.019	4.5
	AY	7-9	6.90	0.00	9.46	0.68	0.03	0.04	1.899	0.017	
	C	9+	7.35	0.09	3.89	–	0.04	0.04	1.899	0.019	
8	C	0-5	6.70	0.52	3.24	–	0.05	0.05	2.307	0.020	80.0
9	O	0-3	5.96	0.00	8.80	0.57	0.07	0.07	3.327	0.021	22.4
	AC	3-14	6.70	0.36	3.75	–	0.04	0.04	2.103	0.019	
10	AY	0-18	6.50	0.04	12.64	0.62	0.02	0.11	4.669	0.005	22.4
	C	18+	6.50	0.07	12.50	–	0.04	0.08	3.726	0.010	
11	AC	0-3	6.00	0.91	16.00	–	0.03	0.08	3.621	0.009	80.0
	C	3+	6.30	1.03	15.58	–	0.02	0.09	3.831	0.005	
12	AY	0-25	6.15	0.23	17.68	0.8	0.02	0.10	4.250	0.005	30.0
	C	25+	6.11	0.22	10.00	–	0.02	0.07	3.097	0.006	

N o t e. A — site; B —horizon; C —power, cm; D — pH_{water}; E — CO_2, %; F — C_{org}, %; G — S_{ha}/C_{fa}; H — basal respiration, μg CO_2-C/g·h^{-1}; I —SIR (substrate-induced respiration), μg CO_2-C/g·h^{-1}; J — C_{mic}, μg C/g soil; K — qCO_2, μg CO_2-c/mg C_{mic}·h^{-1}; L — rockiness, %; C_{org} — the content of total organic carbon, C_{ha} — carbon content of humic acids, C_{fa} — carbon content of fulvic acids, C_{mic} — carbon content of microbial biomass, qCO_2 — metabolic coefficient. Dashes mean that the analysis was not carried out.

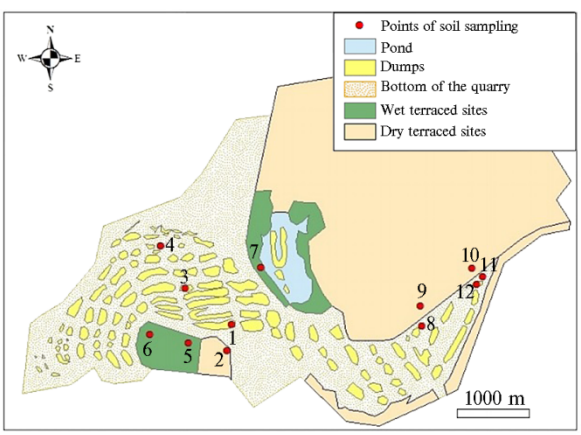

Fig. 1. The scheme of collection of soil samples in limestone quarry Pechurki: 1 — dump, rare vegetation (28 years old), 2 — dry terrace, small pine forest (16 years old), 3 — dump, rare vegetation (30 years old), 4 — dump, rare vegetation (8 years old), 5 — wet terrace, parvifoliate forest (35 years old), 6 — wet terrace, parvifoliate forest (35 years old), 7 — wet terrace, pleurocarpous moss pine forest (35 years old), 8, 11, 12 — base of the dump, rare vegetation (29 years old), 9, 10 — dry terrace, rare vegetation (29 years old) (Slantsevsky District, Leningrad Province, 2016).

accumulative ecotopes with optimal moisture conditions and physical parameters of the substrate. Differences between the upper organogenic horizons and the lower ones were increasing with the increase in the period of colonization. The primary substrate and the underlying horizons were characterized by higher acidity values (pH from 6 to 7.45) due to a large amount of primary minerals and the absence of organic matter. The content of organic carbon increased with the time of vegetation, while the pH decreased to 5.02. The most acidic reaction was observed on the positive elements of the relief (sections 1, 2, 4). The main dominants were *Populus tremula* and *Betula*

pendula.

The soils with a fulvate-humate type of humus prevailed, which was typical for the region. The increased proportion of humic acids is due to the carbonate substrate of the soil-forming rock. Five environmental factors that have the greatest influence on the distribution of vegetation have been highlighted by forward selection for inclusion in the model: the grain-size composition of the soil, rockiness, the content of physical clay, pH of water extract and moisture. These data are consistent with studies conducted in Canada [41]. All identified factors have a significant influence on the distribution of the plant community, but other sources note the great importance of the age of the dump [41-43].

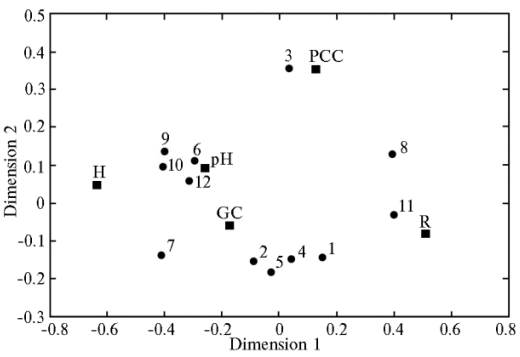

Fig. 2. Results of the canonical correspondence analysis of environmental factors and plant communities (non-metric multidimensional scales). Circles specify plant communities, PCC — physical clay content, H — humidity, GC — grain-size composition, R — rockiness (analysis with Statistica 7 software; quarry Pechurki, Slantsevsky District, Leningrad Province, 2016).

The results of the canonical correspondence analysis are shown in Figure 2. The first axis explains 65% variability, the second axis is for 23%. Sites with the maximum rockiness were colonized mainly by thinned plant groups with a predominance of *Ceratodon purpureus and Bryum pseudotriquetrum.* Many studies have confirmed that mosses and lichens are the first that colonize abandoned quarries [17, 44, 45]. Presumably, this is due to the fact that they are better adapted to adverse environmental conditions, and are also able to prepare a substrate for other plants [46-48]. Sites with a high proportion of clay fraction were colonized by grass spruce forest. Drained sandy loamy sites were occupied by various types of pine forests. Parvifoliate forests prevailed on the sites with a significant amount of skeletal material and a relatively low content of fine earth.

A total of 136 species of higher plants belonging to 106 genera, 49 families, 45 orders, 5 classes and 4 divisions were found in the quarry. In terms of the number of species, the *Fabaceae* and *Poaceae* families were predominant, each of them included 13 species (almost 10% of the diversity), as well as *Asteraceae* and *Rosaceae*, which included 11 species (8% each). A large number of *Fabaceae* family representatives are typical for disturbed habitats. *Asteraceae* and *Rosaceae* are the leading families of the flora of the Leningrad Region. There were 24 single-species families. A rather large number of species was in the *Orchidaceae* family, 9 species (6% of all species), which is due to the carbonate substrate of the quarry. A total of 22 types of mosses from 10 families and 11 epilithic lichens from 7 families, 75 species of algae and cyanobacteria from 38 genera and 29 families were also recorded. Representatives of all life forms of plants, common in the Leningrad REGION according to N.N. Tsvetlev, were found in the quarry [49]. There were all ecological groups in terms of requirements on soil richness: from oligotrophic plants to eutrophic and typical nitrophilous plants, as well as calciphile plants. All groups in terms of the water regime of soils have been identified, from xerophilous to hygrophytic plants. The number of higher vascular plants species on the sites varied from 14 to 39, depending on the ecotope. Fourteen protected species were found in different ecotopes. The maximum similarity of the species composition was observed at the rocky bottom of the quarry (the Sørensen-Czekanowski coefficient was 92%). The

most severe conditions for vegetation development were created here, so that communities were represented by a limited set of species. Sites with different positions in the relief were characterized by the smallest number of common species.

According to all the calculated indices, the smallest variety corresponded to the site where pine seedlings were planted on a flat surface (on a soft overburden) in 1970. Such biodiversity is typical for the pine forests of the Leningrad Province. The greatest variety, according to Shannon's index, corresponded to the recently recultivated site where the tree crowns had not yet closed, and marginal meadow species had not yet been displaced by typical forest species. By Simpson's index, the greatest variety was at the dumping ground of the quarry, where spontaneous succession without human intervention was taking place for more than 30 years. Biodiversity was reduced at flat sites under biological reclamation. Biodiversity has increased over time on sites, namely on dumps, where development was of spontaneous succession type. It is interesting that the development on heavily rocky sites (dumps of large fragments and rocky bottoms) was so slow that in 46 years no significant changes occurred. Apparently, this time is not enough for the natural transformation of the substrate by algae, lichens, and mosses. Our results are confirmed by the large body of literature data, which claims that spontaneous succession, in terms of the diversity of plant communities, is the best approach for partially managed land reclamation [50-53].

A microbiome is a link in the soil-plant system, which provides an ecological adaptation of the plant to stressful conditions of disturbed soils. In addition, microorganisms are able to utilize many chemical compounds by participating in the bioremediation of disturbed territories.

The content of microbial biomass in the samples studied ranged from 0.98 to 4.6 μg C/g of soil, while the values increased with the increase in the period of colonization and a tendency to a decrease in this indicator downward along the soil cross section was observed. According to published data [55, 56], the amount of microbial biomass can be considered as one of the indicators of organic matter accumulation and mineralization. Since basal respiration and the content of microbial biomass strongly depend on parameters such as humidity and temperature [57], a microbial metabolic coefficient, which refers to integral indicators of the biological state of soils, was calculated. Its values varied from 0.004 to 0.022 μg CO_2-C/mg $C_{mic} \cdot h^{-1}$. The obtained results indicate a reduced stability of microbial communities and the ineffective use of the organic substrate, especially at the first stages of quarry colonization. According to J. Frouz et al. [58], who actively studied the microbiological state of quarries in the Czech Republic, the index of soil respiration per unit of microbial biomass decreases with the increase in the period of colonization. However, according to the our data, it is difficult to identify the tendency of changes in microbiological indicators with age. Also, the authors note that the majority of microbiological indicators in 30-40-year-old communities were the same as in undisturbed communities.

The taxonomic analysis of microbiomes in our study showed that the dominant phylotype in all samples are *Proteobacteria* (55.7%), followed by *Actinobacteria* (17.0%), *Bacteroidetes* (10.3%), *Acidobacteria* (6.4%) and *Chloroflexi* (3.8%). The obtained results are consistent with the literature data: the listed phyla become dominant in the soils of disturbed habitats [25, 59, 60].

According to the results of the Principal Component Analysis (PCoA), the age of the dumps was the strongest factor (19.9% of the explained variability). The microbiomes of the old (35 years or more) dumps were distinctly isolated, while microorganism communities of young (8-16 years) and middle-aged (28-30 years) dumps tended to group into separate clusters (Fig. 3). Microbiomes

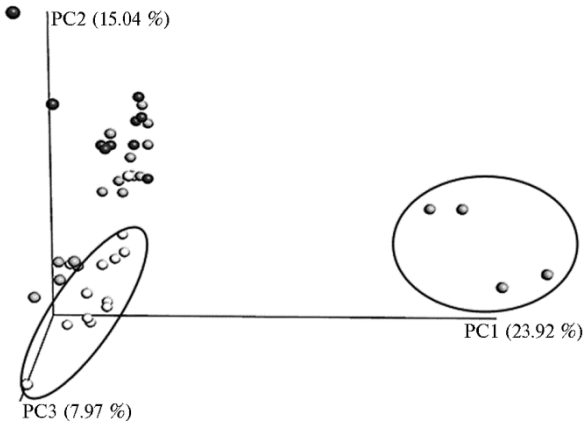

Fig. 3. The results of Principal Component Analysis (PCoA): white circles — old dumps (35 years old), gray circles — middle age dumps (28-30 years), black circles — young dumps (8-16 years). The highlighted areas specify groups of dumps of a certain age combined into clusters (the analysis was carried out with QIIME program; the Pechurki quarry, Slantsevsky District, Leningrad Province, 2016).

of the quarry bottom also formed a separate group, which was the result of special environmental conditions due to the rockiness of the substrate and thinned vegetation cover. The dominant taxa in young dumps were *Acinetobacter* (8.8% of the total community), *Micrococcaceae* (8%) and *Pseudomonas* (6%), while the number of these bacteria significantly decreased ($p < 0.05$) in the young > middle age > old dumps row. The *Acinetobacter* species have a comparatively wide ecological niche and can be found in habitats contaminated with hydrocarbons, activated sludge, sewage, and also in dumps with overburden grounds [61]. A high proportion of *Pseudomonadaceae* was recorded during the analysis of 6-year-old technosoils formed on dumps due to coal mining [62]. Representatives of *Micrococcaceae* (4.5%) and *Sphingomonadaceae* (1.4%) prevailed in the middle-aged dumps. The proportion of α-proteobacteria of the *Rhizobiales* order in the microbiome structure increased with the increase in the age of the dumps, mainly due to *Bradyrhizobiaceae* and *Hyphomicrobiaceae*. Statistically significant maxima of the proportion of these groups were in 35-year dumps — 5.0 and 2.5% of the sequences, respectively. It is known that the *Hyphomicrobiaceae* and *Bradyrhizobiaceae* bacteria play an important role in the processes of carbon and nitrogen transformations in soil [63]; many of them are often found in the rhizosphere of herbaceous plants and are part of the plant-growth-promoting (PGPR) group of bacteria. Old dumps were characterized by a significant (6-8-fold) increase in the proportion of *Micromonosporaceae* and *Sinobacteraceae* representatives in comparison with young and middle age dumps, and a significant increase in the proportion of *Chitinophagaceae* and *Cytophagaceae* bacteria in the microbiomes of 35 years dumps was also noted.

Wet terraces communities also differed somewhat from microbiomes of dry terraces and dump bases; however, no significant differences could be identified. Acidity (pH), which is one of the main soil factors and the main predictor of the microbial community composition [64-66], also did not have a significant effect on the composition of the microbiomes under study. Apparently, the formation of the specific structure of the microbial communities was the result of the combined effect of factors with their most favorable combination in 35-year-old dumps.

The results of a taxonomic analysis of the composition of dump microbiomes revealed a distinct change in the species composition of microorganisms with the increase in the age of dumps. Microorganisms that belong to the ecological group of copiotrophs, the presence of which in the soil marks the early stages of succession, prevailed in young dumps. According to the data of Y. Li et al. [25], the most important stage of microbiome recovery is between years 15 and years 20 after the beginning of the reclamation. In our studies, beginning with

middle-aged dumps (over 16 years old), oligotrophic groups of bacteria predominated in the community, including bacteria involved in the decomposition of complex biopolymers. The dominance of this group of microorganisms in the community can characterize the microbiocenosis transition to the climax stage.

Thus, the unfavorable grain-size composition becomes a limiting factor in the development of vegetation due to the high rockiness of rocky bottoms. The presence of areas with different physical parameters of the substrate, which are at different stages of vegetation, contributes to the development of a high diversity of plant communities. A change in the species composition of microorganisms is observed in the succession process, while the copiotrophic groups predominate in the early stages and the oligotrophic groups prevaile in the later stages. Monitoring of each site of the quarry is necessary in order to develop an optimal plan for territory reclamation and its further use for agricultural purposes since edaphic conditions can differ depending on the position in the relief and the impact on the ground. Territory reclamation for its further use for agricultural purposes requires expensive measures to flatten and level the high heterogeneity of the substrate. In terms of biodiversity, the best method for reclamation of carbonate quarries is the creation of favorable physical conditions of the substrate and further self-organized vegetation of the site.

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ESTIMATION OF THE OPTIMAL Cu CONTENT IN DIFFERENT SOIL TYPES BASED OF THE DYNAMIC MODEL FOR COPPER ACCUMULATION IN ABOVE GROUND PARTS AND ROOTS (ON THE EXAMPLE OF BARLEY *Hordeum vulgare* L. PLANTS)

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Abstract

Copper is one of the essential microelements for both animals and plants and plays an important role in a number of physiological processes. However, it becomes toxic to plants when entering high concentrations. The urgency of the work to determine the optimum and critical levels of copper content in soils, especially in the agricultural production, is caused by permanent technogenic pollution of soils with heavy metals. An increase in the content of copper in soils can cause changes in biochemical processes in plants, their morphology, and, ultimately, reduce productivity. The construction of complex dynamic models of heavy metals entering plants is not always justified, since most of the coefficients can be obtained only in laboratory experiments under conditions which are very different from natural ones. In our experiment, it was shown that it is possible to determine optimal and critical levels of soil contamination by heavy metals on the basis of an analysis of the dynamics of their accumulation in different parts of plants. Optimal and critical levels of contamination of two types of soils (sod-podzolic and chernozem) with copper were determined based on the analysis of the dynamics of Cu accumulation in the above-ground and root parts of barley plants (*Hordeum vulgare* L.) in vegetation trials. The concentration of copper in barley plants remains relatively constant throughout the IV-IX stages of organogenesis (20-60 days from the date of emergence). The Cu accumulation in the roots of barley plants linearly followed its content in the soil, while the accumulation rate in the shoots decreases with increasing copper content in the soil. The double excess of Cu accumulation in barley roots on sod-podzolic soil as compared to chernozem is probably due to agrochemical characteristics of soils. A function is proposed that reflects the dependence of the copper content in the shoots on its concentration in plant roots, which has the form of the sum of the exponential accumulation function and the linear elimination function due to the operation of the active molecular transport system Cu in plants: $Y = c \times X^a + b \times X \times (a - 1)^{-1}$. Approximation of the experimental data by this function made it possible to determine its coefficients: $a = 0.430 \pm 0.014$; $b = 0.020 \pm 0.005$; $c = 3.31 \pm 0.81$. Analysis of the dynamics of copper accumulation in the shoot and root parts of plants made it possible to determine the concentration at which, according to Becker's theory, a change takes place from the accumulative to the barrier type of metal accumulation, that is, the transition from increased accumulation of copper by a plant to protective mechanisms limiting the supply of metal. Optimum copper accumulation in barley plant was 7.6 mg/kg, with a total soil content of 3.5 and 6.9 mg kg for sod-podzolic soils and for chernozem, respectively. The calculated value of the «critical» concentration of copper in plant roots, at which its entry into the shoot due to passive transport and excretion due to active molecular transport mechanisms become equal, for barley is 650 mg/kg, and at this level the copper content in the shoot is 31 mg/kg. This level can be achieved with total soil Cu of 300 and 590 mg/kg for sod-podzolic soil and for chernozem, respectively.

Keywords: *Hordeum vulgare* L., barley, copper, sod-podzolic soil, chernozem, optimal level, critical level, dynamic model

Copper is one of the essential minor-nutrient elements. It plays an important role in a number of physiological processes [1] but becomes toxic to plants at high concentrations [2, 3]. In different soils, the total content of copper

is from 20 to 110 mg/kg. However, Cu concentration is much lower in soil solutions and varies from 30 to 241 μg/l [4-6]. Emissions of industrial enterprises, the constant use of various products based on copper, in particular, pesticides and fertilizers, can lead to the accumulation of copper in the soils of agriculturally used areas. Significant accumulation of Cu affects not only the soil microbiocenosis but also the physical properties of the soil [7]. Therefore, the study of the mechanism of Cu entry from soil into plants remains relevant, especially for agricultural plants.

It was established [8] that the interspecific differences in terms of copper accumulation in the plant-soil system can reach 9-fold values. According to Baker's heavy metals absorption model, the accumulative, barrier or indicative type of protective reactions can be formed in plants depending on the content of metals [8]. The nature of protective reactions is determined by the processes controlling the entry and distribution of Cu in the organs and tissues of plants. The entry of various metals ions from the soil solution and their distribution in the cells of roots, xylem, in the apoplast and cytosol of the above-ground parts of plants are carried out both passively (due to osmosis) and through active transport with the involvement transporters encoded by different genes [9]. In recent years, the greater focus has been placed on the molecular mechanisms of these processes. For *Arabidopsis thaliana*, the participation of ferric reductase oxidases of the FRO family in changing the form of copper oxidation (Cu/Cu^{2+}), affecting the absorption of copper by plant roots from the soil, has been described [10]. Also, *Arabidopsis* shows the important role of genes of the COPT family (copper transporter), products of which are localized in the plasmatic membrane of the root cells, in the entry of Cu from the rhizosphere [11]. It is assumed that members of the zinc-regulated transporters (ZRT) family and iron-regulated transport proteins (IRT) can indirectly participate in the absorption and transfer of Cu to *Arabidopsis thaliana* and *Medicago truncatula* [12-13].

Various Cu-transporting adenosine triphosphatases of the HMA family are involved in the transfer of both mono- and divalent copper cations from the root symplast to the plant xylem, and also from the cytoplasm to the vacuole of the cell. In the opposite direction, the specific transport of only copper (II) ions through the plasmalemma is probably carried out by proteins of the COPT subfamily of the CTR family, which are also responsible for the absorption of Cu in leaves and other above-ground parts of the plant [11, 12, 17, 18]. For *Arabidopsis* and rice, seven proteins of the COPT type, the genes of which were expressed in virtually all tissues of the root and shoot, have been identified [18, 19]. In *Brassica napus*, the balance of intensity of *HMA5* and *ZIP4* genes expression was established; the protein products of this genes are localized in the plasmalemma and provide, respectively, the transport of Cu ions from the cytosol to the apoplast and the entry of Cu into the cytosol [20]. It has been shown that in response to an increase in the concentration of Cu in the medium, the intensity of *HMA5* gene expression sharply increases, while *ZIP4* gene expression is completely blocked, which suggests that these genes may participate in the regulation of intracellular homeostasis of Cu in order to limit its accumulation up to lethal concentrations.

Usually, the Cu content is from 2 to 50 mg/kg dry weight, depending on the plant species. The value of 5-20 mg/kg is optimal for most plants; the symptoms of toxicity appear above this value, and the symptoms of deficiency appear below this value [21, 22]. Both the deficit and excess of Cu affect the physiological processes in plants and, ultimately, the productivity [23, 24].

Construction of complex dynamic patterns of heavy metals entering plants is not always justified, since most of the coefficients can be obtained only during model experiments, the conditions of which are very different from the

natural ones. Our experiment shows that it is possible to determine the optimal and critical levels of soil contamination by heavy metals on the basis of analysis of the accumulation dynamics in different parts of plants.

The purpose of the paper was to study the dynamics of copper accumulation in barley plants depending on its amount in the soil to assess the optimal and critical values of the content of this trace element.

Techniques. Vegetation experiments were performed on barley plants (*Hordeum vulgare* L.) of the Zazersky 85 variety. Plants were grown in vessels containing 4.5 kg of sod-podzol soil or leached heavy loamy chernozem. Agrochemical soil indicators, determined using the conventional methods [25], were pH_{KCl} 5.47 and 5.53, respectively; humus 1.7% and 4.8% (by Tyurin); exchange K_2O 64.7 and 134.3 mg/kg (by Maslova); mobile P_2O_5 805 and 214 mg/kg (by Kirsanov); hydrolytic acidity 2.7 and 3.0 mg-equ/100 g; the sum of the exchange bases 7.6 and 31.7 mg-equ/100 g (by Kappen). The total content of copper in soils was 3.8 and 9.1 mg/kg, which was used as a control. Prior to sowing, nutrients were added to the soil at the rate of $N_{200}P_{100}K_{100}$ mg/kg soil according to the active ingredient, Cu was added in the form of aqueous solutions of the nitric acid salt $Cu(NO_3)_2 \cdot 3H_2O$ up to 50, 100, 150 and 200 mg/kg for sod-podzol soil, 100, 150, 300 and 400 mg/kg – for heavy loamy chernozem. Barley was grown for 60 days from the date of the seedling to the milky stage. The sowing density is 13 plants on a vegetation vessel of 22 cm in diameter, 5 biological replications of the experiment.

The content of Cu in the above-ground biomass and plant roots was determined after 20, 30, 45 and 60 days from the date of seedlings (respectively, in the IV, V, VII and IX stages of organogenesis) [26]. During selection, plant roots were washed from the soil in distilled water. The experiments were performed in 3-fold biological and 2-fold analytical replications. The mass of the above-ground and root parts of the plants (for the air-dry state) was evaluated using the gravimetric method. The Cu content was determined by the atomic absorption method using the SpectrAA 250 Plus spectrometer (Varian, Inc., USA) as described [27]. Plant samples were mineralized with dry ashing according to RF State Standard GOST 26657-85.

The table and figures show the mean values (M) and their standard errors ($M \pm$SEM). The significance of differences with the control plants was established for the mean values using Student's t-test, for the variances using F-test at the significance level $p < 0.05$. For statistical data processing, we used Microsoft Office Excel 2003 and STATISTICA v.6 software package (StatSoft, Inc., USA, http://www.statsoft.com).

Results. The dynamics of copper accumulation in plant organs, depending on the metal content in the soils and the age of barley, is presented in the table. During the growth of plants, the amount of metal in the root and above-ground biomass changed insignificantly.

Accumulation of copper in barley (*Hordeum vulgare* L.) plants of the Zazersky 85 variety at different stages of organogenesis depending on the element content in soils ($M \pm$ SEM, greenhouse test)

Indicator	Introduced Cu, mg/kg	Organogenesis stage according to Kuperman			
		IV	V	VII	IX
	Sod-podzol sandy loam soil				
Cu content in the above-ground biomass, mg/kg	0	8.8±0.9	9.3±0.9	6.2±0.8	6.2±0.8
	50	18.6±1.2	23.0±1.2	18.8±1.0	21.6±1.1
	100	27.6±1.1	27.0±1.1	23.3±0.9	25.6±0.9
	150	29.1±1.9	31.0±1.9	23.7±1.6	25.6±1.7
	200	33.6±2.3	35.0±2.3	26.8±1.9	27.7±2.0
Cu content in roots, mg/kg	0	11.1±1.2	12.9±1.1	11.0±1.0	7.7±1.1
	50	94.2±5.7	84.2±5.3	109±5	92.6±5.3
	100	236±14	176±13	221±12	210±13

Table continued

	150	323±14	272±13	288±12	324±13
	200	517±18	489±17	439±15	474±17
	Leached heavy loam chernozem				
Cu content in the above-ground biomass, mg/kg	0	11.3±1.1	13.6±1.2	8.9±1.3	8.6±1.2
	100	19.5±1.6	23.6±1.8	16.9±1.8	16.3±1.7
	150	23.8±1.5	26.6±1.6	21.3±1.7	19.5±1.6
	300	25.2±1.3	29.5±1.5	24.7±1.5	23.0±1.5
	400	27.7±1.1	32.1±1.2	27.5±1.2	27.3±1.2
Cu content in roots, mg/kg	0	17.3±0.5	17.0±0.4	17.3±0.4	15.3±0.5
	100	67.0±8.2	81.2±6.4	104±7	81.0±7.6
	150	97.0±10	126±8	143±8	118±9
	300	223±8	224±6	252±7	246±7
	400	372±18	296±14	310±15	343±17

The analysis of the variance of data on the content of copper in the above-ground and root parts of the plants showed the dependence of the Cu amount in the above-ground parts on the physiological phase of development (for sod-podzol soil $F = 9.5$, for chernozem $F = 60.9$, $F_{0.05} = 3.49$) and did not identify such dependence for the roots (for sod-podzol soil $F = 1.98$, for chernozem $F = 0.58$, $F_{0.05} = 3.49$). The observed dependence is due to the increased accumulation of copper at the V stage of organogenesis and its decrease at later stages of plant development (see Table). However, in general, these changes do not exceed 20%.

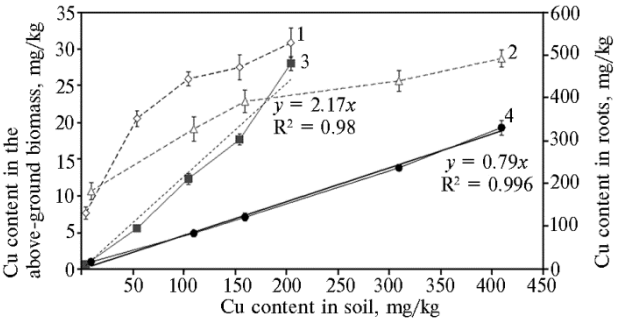

Fig. 1. Accumulation of Cu in barley (*Hordeum vulgare* L.) plants of the Zazersky 85 variety in the above-ground biomass on sod-podzol soil (1) **and chernozem** (2) **and in the roots on sod-podzol soil** (3) **and chernozem** (4) **depending on the element content in soils.** The average experimental values with an error are presented, dashed lines are equations and significance of linear approximation (greenhouse test).

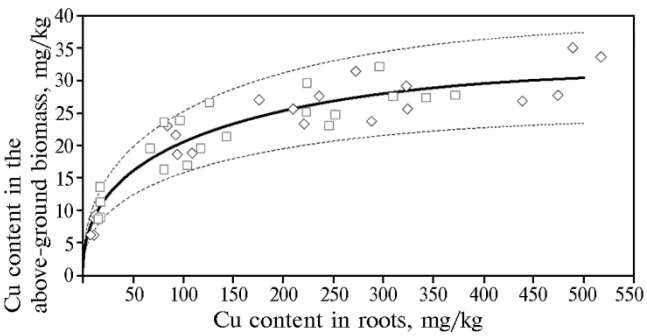

Fig. 2. Accumulation of Cu in barley plants (*Hordeum vulgare* L.) of the Zazersky 85 variety in the above-ground biomass, depending on the element content in the roots on sod-podzol soil (◇) and chernozem (□). The solid line is the approximation by the function (2), dashed lines – 95% confidence interval (greenhouse test).

Figure 1 shows the dynamics of copper accumulation in the above-ground and root parts of plants, depending on its content in soils of two types. The copper content in the roots had a definite linear dependence on the content of trace elements in the soil (the dashed lines in the figure represent the equations and the significance of linear approximation), while the accumulation rate in the above-ground biomass decreased with the increase in copper amount in the soil. A two-fold excess in the accumulation of Cu in barley roots on sod-podzol soil, compared with chernozem, is probably due to the agrochemical characteristics of soils and more solid fixation of copper in chernozem compared to sod-podzol soil. After the experiment was completed, the availability of copper from soils was determined

[25]. On average, in all variants with the introduction of copper into the substrate for sod-podzolic soil, the proportion of acid-soluble fraction (1 M HCl extraction) of the total copper content was 81±2%, of the total chernozem content 73±3%. The proportion of the mobile fraction (extraction with acetate-ammonium buffer, pH 4.8) was 43±4% and 7±2% for sod-podzol soil and chernozem, respectively.

Figure 2 shows Cu content in the above-ground biomass, depending on its content in the plant roots. It is obvious that the entry of Cu from the roots into the above-ground biomass is not due to the type of soil on which the plants were grown.

The entry of copper into the above-ground biomass from the roots of plants is regulated by the mechanisms of both passive (due to osmosis) and active transport with feedback, that is, the rate of Cu entry into the above-ground biomass from the plant roots should be proportional to the concentrations difference and inversely proportional to the copper content in the roots of plants:

$$\frac{\partial Y}{\partial X} = \frac{a \times Y - b \times X}{X}. \quad (1)$$

The solution of this equation is the following function:

$$Y = c \times X^a + \frac{b \times X}{a - 1}, \quad (2)$$

where Y is copper content in the above-ground biomass; X is the content of copper in the roots of plants; a, b, c are the coefficients.

The solid line (see Fig. 2) represents the calculated accumulation of copper in barley biomass at a = 0.430 ± 0.014; b = 0.020 ± 0.005; c = 3.31 ± 0.81. Dashed lines show 95% confidence interval of the approximation.

Since the coefficient is $a < 1$, so the function of copper content in the above-ground biomass of plants (2) summarizes both the entry of Cu from the roots and the reverse metal transport from the above-ground part to the root of the plants. In this case, the first term of the function ($c \times X^a$), characterizing the accumulation, completely coincides with the description of the regularity of copper entering barley plants established by R.D. Davis and P.H.T. Beckett [2, 3]. They showed that before reaching the copper concentration, which is critical for plants, the dependence of its accumulation in young barley plants is described by a linear function of the logarithms $\mathrm{Log}(y) = a + b \times \mathrm{Log}(x)$, where y is Cu content in plants, and x is the concentration in the nutrient solution.

The second part of the function (2) is provided by the active reverse transport system and corresponds to a direct linear dependence on the copper concentration. The linear dependence of gene expression activity, which provides transport of Cu in the plant, on its content was shown for *Brassica napus* [20]: in response to an increase in the concentration of Cu in the medium, the intensity of *HMA5* gene expression sharply increases, while *ZIP4* gene expression is completely blocked.

Mechanisms of heavy metals content regulation in plant organs and tissues form the corresponding type of protective reaction according to Becker's theory: in case of shortage of a trace element in the soil, a storage type of accumulation is observed, in case of excess − the barrier type of accumulation is observed. The indicator of the transition from one type of accumulation to another is the ratio of the metal content in the above-ground and root parts of the plant: if it is > 1, then the type is accumulative, if < 1, then the type is barrier [8]. Consequently, according to the dynamics of Cu accumulation in the above-ground and root parts of plants, it is possible to determine the concentration of Cu in the soil at which a change in the type of metal accumulation from the accumulative to the barrier takes place. This point will correspond to the copper content in the soil, which is optimal for plant nutrition:

$$\frac{Y}{X} = c \times X^{a-1} + \frac{b}{a-1} = 1\,, \tag{3}$$

$$X = \sqrt[a-1]{\frac{a-b-1}{c \times (a-1)}}\,. \tag{4}$$

The content of Cu in the roots and above-ground biomass of the plant, which is optimal for barley (7.6 mg/kg) is determined using the approximation coefficients obtained for the function (2). Based on the linear dynamics of copper accumulation in the roots (see Fig. 1), the total content of Cu in the soil, which is optimal for barley, is 3.5 and 6.9 mg/kg for sod-podzol soil and for chernozem, respectively.

The experiment used the quantities of Cu in soils that do not cause obvious toxic effects in barley. It can be assumed that when the critical Cu content in the roots is reached, its entry into the above-ground biomass due to passive transport and removal by means of active molecular mechanisms of transport will be equal:

$$\frac{\partial Y}{\partial X} = c \times a \times X^{a-1} + \frac{b}{a-1} = 0\,. \tag{5}$$

Based on this assumption, it is possible to calculate the critical accumulation of Cu in plant roots:

$$X = \sqrt[a-1]{\frac{b}{c \times a \times (1-a)}}\,. \tag{4}$$

The Cu content in the roots, which is critical for barley, 650 mg/kg, can be determined using the approximation coefficients obtained for the function (2). At this value, the content of copper in the above-ground biomass will reach 31 mg/kg. Accordingly, the total content of Cu, which is critical for barley, in sod-podzolic soil and chernozem is 300 and 590 mg/kg, respectively.

Thus, the intensity of copper accumulation in barley plants does not depend on the physiological phase of plant development and remains relatively constant throughout the IV-IX stages of organogenesis. The accumulation of Cu in the roots of barley plants is linear and determined by the content of copper in the soil. A twofold excess in the accumulation of Cu in barley roots on the sod-podzol soil in comparison with chernozem is probably due to the characteristics and buffer capacity of soils. The dependence of the copper content in the above-ground biomass on its amount in the roots is the sum of accumulation exponential function and removal linear function, which is due to the operation of the system of active molecular transport of Cu in plants. Based on the Becker theory (1981) and the analysis of the dynamics of copper accumulation in the above-ground and root parts of plants, the calculated metal content in biomass, which is optimal for barley, is 7.6 mg/kg. In the experiment, it corresponded to a total Cu content in the sod-podzol soil 3.5 mg/kg, in chernozem 6.9 mg/kg. The calculated value of the conditionally critical copper accumulation in the roots of plants, at which its entry into the above-ground biomass due to passive transport and removal by means of active molecular mechanisms of transport are equal, for barley is 650 mg/kg, while the amount of Cu in the above-ground part will be 31 mg/kg.

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MORPHOPHYSIOLOGICAL FEATURES OF WHEAT (*Triticum aestivum* L.) SEEDLINGS UPON EXPOSURE TO NICKEL NANOPARTICLES

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Abstract

The intensive development of nanotechnologies determines the need for the investigation on the patterns of biological impact of technogenic nanomaterials. The analysis of the researches reveals a wide range of toxicity manifestations when nanocompounds affect plants, which depends on the physical properties of the nanoparticles (dimensions, shape, catalytic activity, concentration). The relevance of the studies on the concentration effects of nanoparticles is due to the insufficient knowledge of their interaction with the plant cell, and, consequently, the need to determine the dose-effect relationship for each class of nanoparticles and various bio-objects. This paper presents the results of a comprehensive study of the effect of nickel nanoparticles (NP Ni^0, Δ50 = 5 nm in size) when used at different concentrations on growth, content of pigments, flavonoids and proline, photosynthesis and transpiration intensity of ten-day-aged wheat seedlings (*Triticum aestivum* L.). The calibrated seeds were pre-germinated for 2-3 days, up to the appearance of rootlets, in Petri dishes on filter paper impregnated with aquatic disperse systems of NP Ni^0 in concentrations of 0.01, 0.1, 1 and 10 mg/l. In the control, the seeds were germinated on distilled water. The germinated seeds were put into 500-millilitre vegetative pots for further growing in the aquatic dispersed NP Ni^0 systems of the above mentioned concentrations in the climate box until the 10-day age. Morphometric parameters assessed were the root length, the seedling height, the weight of the root and aboveground parts of the plants. To determine the content of photosynthetic pigments, flavonoids and proline, an average sample was collected from the leaves of 10 plants. The morphometric parameters under study depended on the doses of nickel nanoparticles in a disperse medium. NP Ni^0 at low concentrations (0.01 and 0.1 mg/l) did not change or stimulated growth, whereas at larger doses (1 and 10 mg/l) they suppressed the growth of roots and aboveground part of seedlings considerably. The root length decreased 2 times at 1 mg/l NP Ni^0 and 3 times at 10 mg/l NP Ni^0, the wet weight was 1.9 and 2.7 times lower, respectively, and the height declined 1.3 and 1.9 times. The content of chlorophyll a and b at 0.01 mg/l NP Ni^0 slightly increased and then decreased as the nanoparticle concentration increased, but no clear dose dependence was revealed. The amount of carotenoids gradually decreased with increasing NP Ni^0 concentration. The study of photosynthesis and transpiration showed a dose correlation of these indicators. NP Ni^0 at low concentrations (0.01 and 0.1 mg/l) increased the intensity of photosynthesis and transpiration significantly, at the concentration of 1 mg/l did not affect these processes, and at 10 mg/l concentration insignificantly suppressed these parameters. The amount of flavonoids decreased with increasing NP Ni^0 concentration; however, dose dependence was not observed. The lowest level of flavonoids, with a 75 % decrease, was at 0.1 mg/l NP Ni^0, and at 10 mg/l NP Ni^0 the amount of flavonoids decreased by 64 % as compared to the control. At the same time, the impact of nickel nanoparticles on wheat caused a rise in the level of proline from 22 to 130 %, with clear dose dependence on the nanoparticle concentration. Mass spectrometric studies revealed a significant accumulation of nanoparticles in plant organs, especially in the root system. In the roots of the experimental plants the nickel concentration was 50.89±1.67 μg/g per

dry weight, in the control plants this reached 3.8±0.15 μg/g. In the above-ground parts of plants the nickel concentration was an order of magnitude lower, 14.20±2.38 μg/g per dry weight for the test plants and 0.87±0.025 μg/g per dry weight for the control plants. Thus, our findings revealed the morphophysiological peculiarities of wheat seedlings grown on water dispersed systems of NP Ni^0 of 5 nm in size and showed a dependence of the majority of the studied parameters on NP Ni^0 concentration.

Keywords: *Triticum aestivum* L., nickel nanoparticles, accumulation, photosynthetic pigments, photosynthesis, transpiration, flavonoids, proline

Nanotechnologies are widely used in industry, medicine, and agriculture. However, some components of nanotechnology production are potentially dangerous for the environment, and their influence on biological objects has not been sufficiently studied [1]. The problem of the effect of nanoparticles on living organisms is related to the study of the mechanisms of their toxic effect and the cycle in nature. According to modern concepts, the complexity of interaction depends on the physical and chemical properties, the method of production, the size and structure of nanoparticles, as well as on the characteristics of biological objects, including plant species [2].

It is shown that substances in the form of nanoparticles have other properties and ability to penetrate into plants than the same substances in an ionic form [3]. Metal nanoparticles are characterized by excess surface energy and high reactivity; they actively enter the processes of aggregation and reactions with other chemical compounds [4]. In addition, when interacting with different cell structures and due to a prolonged action, nanoparticles can act as catalysts in reactions with the formation of both growth-promoting agents and inhibitors [5-6]. That is, plants make it possible to evaluate the specificity of nanoparticles action and their dose-dependent effects.

By now, the effect of TiO_2, Al_2O_3, Fe_2O_3, ZnO and CeO_2 nanoparticles on plant objects has been studied to a greater extent [7]. There is much less information on the action of nickel nanoparticles [8-10]. At the same time, there is a sufficient number of publications on the influence of nickel ions on the growth, development and physiological and biochemical parameters of plants [11, 12]. In terms of the production output of homogeneous metal powders with a high degree of purity, nickel nanoparticles are in the top five, along with iron, aluminum, copper and titanium nanoparticles [13]. They are widely used in medicine and biology [14, 15], are included in magnetic fluids and catalysts, are used to create high-speed optical devices [16, 17], and can also contaminate the environment during production, use, and disposal [18].

Nickel is considered an essential ultramicroelement for higher plants, since the activity of enzymes of various metabolic pathways, for example, urease, depends on its content. Low concentrations of nickel salts introduced into the nutrient solution have a positive effect on the growth and development of plants, including wheat [19]. Among heavy metals, nickel is highly toxic and causes significant failures in the structure and functioning of cells [20].

The present paper, for the first time, shows that the effect of low concentrations of nickel nanoparticles (Δ_{50} = 5 nm) in the range of 0.01-10 mg/l can cause significant changes in the structural and functional characteristics of wheat seedlings and mainly has a dose dependence.

The purpose of the study was to identify the morphological, physiological and biochemical features in wheat seedlings under the influence of nickel nanoparticles (NP Ni^0) at different concentrations.

Technuques. Ten-day seedlings of soft spring wheat (*Triticum aestivum* L.) of the Novosibirskaya 29 variety were used as the object of the study. The plants were grown under laboratory conditions in a climatic chamber (Labline Scientific Instruments, Poland) at a 12-hour photoperiod, a temperature of 23-

24 °C, and an illumination of 60 W/m^2. The experiment used seeds, the germination capacity of which was previously determined in accordance with RF State Standard GOST 12038-84 and was not less than 95%. The calibrated seeds were pre-germinated for 2-3 days (up to the appearance of rootlets) in disposable plastic Petri dishes with two layers of impregnated filtering paper. In the experiment, the suspensions of Ni^0 nanoparticles at a concentration of 0.01; 0.1; 1 and 10 mg/l were used, and in the control variant, distilled water was used. The germinated seeds were put into 500 ml vegetative pots, which were placed in the climatic chamber, for further growing until the 10-day age. In the experiment variants, suspensions of nickel nanoparticles of the concentrations indicated above were used for growing, and in the control variant, distilled water was used. Due to the aggregation of nanoparticles and the decrease in their concentration in free form, all the disperse systems in the vessels were replaced on a daily basis. Each vessel contained 25 plants; the experiment was carried out in 4 replications for each variant of the experiment.

Ni^0 nanoparticles were obtained using the method of laser ablation in distilled water from nickel bars (purity 99.95% by weight, grade Ni 3N5) (Girme", Russia). When the bar was exposed to radiation using an impulse Nd-YAG-laser LS-2134UTF (Lotis Tii, Belarus, Japan), ablation and spraying of the target material into the environment occurred. The thickness of the layer removed with one impulse was small and did not exceed a few tens of nanometers. Outside the target, the removed material was organized into nanoparticles [21]. According to the data obtained by transmission electron microscopy (Philips CM-12, Koninklijke Philips N.V., the Netherlands), the particle diameter was 2-12 nm with an average size of Δ_{50} = 5 nm and a specific surface area of 30 m^2/g. Necessary concentrations of disperse systems of NP Ni^0 were obtained by diluting the initial dispersion medium (DM) with distilled water followed by a 45-minute ultrasonic treatment at a frequency of 35 kHz in an ultrasonic bath (UZV-5.7/1 TTC, ZAO PKF Sapfir, Russia). The quantitative characteristics of nanoparticles absorption from the DM were determined by mass spectrometry with inductively coupled plasma in terms of Ni content in the samples of tissue from roots and above-ground parts (leaves + stem) of plants [22]. The roots before drying were washed twice with a 0.01% Na-EDTA solution, then were washed three times with distilled water in order to remove particles sorbed on the surface. Samples of roots and leaves dried to constant weight were ground in a porcelain mortar; then 0.1 g sample was taken for analysis. The samples were ashed in the microwave decomposition system Speedwave TM MWS-3+ (BERGHOF Products + Instruments GmbH, Germany) and analyzed using a mass spectrometer ELAN DRC-e (PerkinElmer, Inc., USA).

Morphometric parameters were evaluated according to the length of the root system and seedlings, the mass of the root and aboveground parts of the plants. The wet mass was determined by the standard weighing method. To assess the content of photosynthetic pigments, an average sample of 10 plants was formed (sample weight 0.4 g). The amount of chlorophylls and carotenoids was determined by spectrophotometry (spectrometer UV-1601PC, Shimadzu Corp., Japan) in alcohol extracts [23]. To measure the photosynthesis and transpiration rate, a portable infrared gas analyzer Li-6400 (LI-COR Biosciences, USA) with an open system was used, where a photodiode system (6400-02B LED) was used as an artificial light source, providing an illumination of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The temperature in the leaf chamber was maintained at 24 °C, the flow rate of CO_2 was 400 μmol/s, its content was 400 μmol/mol. Photosynthesis and transpiration rate was measured in the leaves of 10-day seedlings. The content of flavonoids was evaluated by spectrophotometry according to the reaction with alumi-

num chloride. The optical density of the solution was determined at $\lambda = 415$ nm using a UV-1601PC spectrophotometer (Shimadzu Corp., Japan). The number of flavonoids was calculated using a calibration curve constructed according to the routine (Sigma, Great Britain) [24]. The content of free proline in the shoots was evaluated using an acidic ninhydrin reagent by the method of L.S. Bates et al. [25].

Statistical processing of the data was performed using the Statistica 8 software (StatSoft, Inc., USA). The tables and figures show the arithmetic mean values (*M*) and their standard errors (±SEM) for morphological parameters from 100, for physiological-biochemical indicators and nickel accumulation from 4 biological replications. Differences were considered valid with an error probability of $p \leq 0.5$.

Results. A significant accumulation of nanoparticles in plant organs, especially in the root system, occurred at 10-day germination of wheat on a dispersion medium containing NP Ni^0 at a concentration of 10 mg/l. In the roots of the experimental plants, the nickel concentration was 50.89±1.67 μg/g per dry weight; in the control plants, this value was 3.8±0.15 μg/g. In the above-ground parts of plants, the nickel concentration was an order of magnitude lower: 14.20±2.38 μg/g dry weight in experimental plants, 0.87±0.025 μg/g dry weight in the control plants.

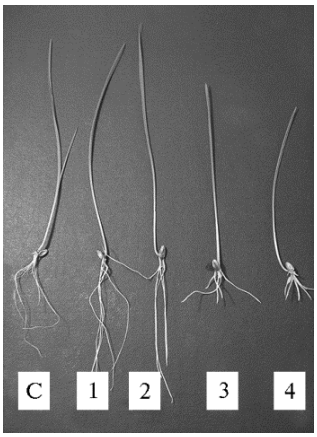

Fig. 1. 10-day seedlings of the soft spring wheat (*Triticum aestivum* L.) of the Novosibirskaya 29 variety under the action of Ni^0 nanoparticles at different concentrations: C — control; 1 — 0.01; 2 — 0.1; 3 — 1; 4 — 10 mg/l in the dispersion medium (laboratory test).

Accumulated nanoparticles caused visible changes in the morphometric parameters of the root system and the aboveground parts of the wheat seedlings (Fig. 1). The NP Ni^0 at concentrations of 0.01 and 0.1 mg/l did not change or even stimulated the growth processes, but the NP Ni^0 at higher concentrations (1 mg/l and 10 mg/l) significantly inhibited the growth of the roots and the aboveground parts (Table 1). The root length decreased by 2 times at 1 mg/l NP Ni^0 and by 3 times at 10 mg/l NP Ni^0, the wet weight decreased by 1.9 and 2.7 times, respectively, and the height decreased by 1.3 and 1.9 times.

D.F. Piccini et al. [11] also showed that the introduction of nickel nanoparticles of a size less than 100 nm at the concentration of 100 mg/kg had a toxic effect on the growth of the *Lepidium sativum* L. roots. The experiments on *Solanum lycopersicum* L. showed that nickel nanoparticles of 28 and 62 nm in size accumulate mainly in the roots, reduce the above-ground dry mass, and affect the Ca and K content in the leaves [9].

1. Morphometric parameters of the seedlings of the soft spring wheat (*Triticum aestivum* L.) of the Novosibirskaya 29 variety grown on a dispersion medium that contained nickel nanoparticles at different concentrations (*M*±SEM, laboratory test)

Concentration of nanoparticles, mg/l	Roots		Aboveground parts	
	length, cm	wet weight, mg	height, cm	wet weight, mg
Control	7.79±0.22	98.6±4.2	16.83±0.39	221.5±8.5
0.01	9.73±0.23*	129.7±6.4*	17.35±0.21	255.1±7.3*
0.1	8.33±0.16	112.6±8.2	17.02±0.19	233.1±10.2
1	4.12±0.07*	52.8±5.1*	12.89±0.28*	171.8±9.3*
10	2.56±0.07*	36.0±7.2*	8.03±0.34*	121.6±10.3*

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

Inhibition of root growth is one of the earliest responses to the action of

heavy metals [26]. This feature is widely used to assess the degree of their toxicity at different concentrations [27]. Protective mechanisms and barriers that operate at the level of cells and tissues of the root reduce the ingress of heavy metals into shoots; the result is the accumulation of heavy metals in the roots in significant amounts, which affects the development and formation of the root system [28, 29].

Accumulated NP Ni^0 had the effect not only on the growth parameters but also on the physiological and biochemical characteristics of the leaf apparatus of wheat seedlings. In particular, when cultivating seedlings on the dispersion medium containing NP Ni^0, the amount of chlorophylls a and b at a 0.01 mg/l concentration of NP Ni^0 increased insignificantly. With an increase in the nanoparticles concentration, a decrease in this indicator was observed. The content of chlorophylls decreased statistically significantly ($p < 0.05$) by 30% as compared to the control only at a 10 mg/l concentration of NP Ni^0 (Fig. 2). Similar changes were observed previously when the pigment complex of wheat was exposed to platinum nanoparticles [30]. The amount of carotenoids at 0.1 and 1 mg/l concentrations of NP Ni^0 decreased by 19-20%, and at 10 g/l concentration by 35% (see Fig. 2).

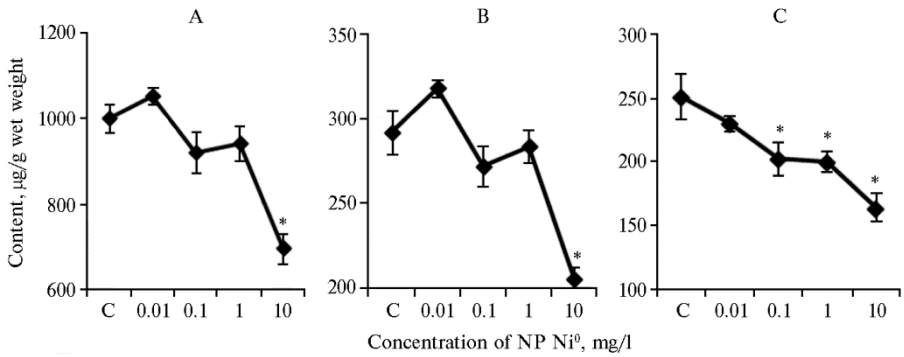

Fig. 2. The content of chlorophylls a (A) **and b** (B) **and the sum of carotenoids** (B) **in the leaves of the soft spring wheat (*Triticum aestivum* L.) of the Novosibirskaya 29 variety depending on the Ni^0 nanoparticles** (NP) **concentration in the dispersion medium** (laboratory test)
* Differences with control are statistically significant at $p \le 0.05$.

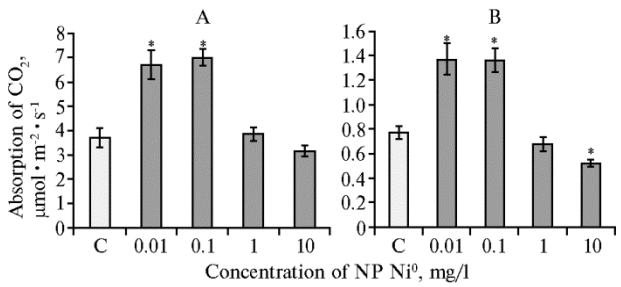

Fig. 3. Photosynthesis (A) **and transpiration** (B) **rate in the leaves of soft spring wheat (*Triticum aestivum* L.) of the Novosibirskaya 29 variety depending on the Ni^0 nanoparticles** (NP) **concentration in the dispersion medium** (laboratory test).
* Differences with control are statistically significant at $p \le 0.05$.

It is shown that the content of photosynthetic pigments decreases under the influence of most stress factors [19, 31]. For example, treatment of *T. vulgare* L. seeds with nickel nanoparticles of 57 nm in diameter with further 48-hour incubation using their solutions at concentrations of 0.0125-1 M caused a sharp decrease in the content of chlorophyll b in the leaves and led to an increase in the content of malonic dialdehyde in the root system of plants. This indicates the development of cytogenetic damage due to the oxidation of lipids in cell membranes. The dose dependence [10] was also observed.

The features of the photosynthetic pigments accumulation under the influence of Ni^0 NP as a whole were similar to the patterns of seedlings growth

(see Table 1). This is probably due to the strong correlation between photosynthesis and growth processes, which are regulated by metabolic and hormonal mechanisms [32].

A study of the integral functional characteristics of the leaf apparatus – photosynthesis and transpiration also revealed a dose-dependent effect. Nickel nanoparticles at low concentrations (0.01 and 0.1 mg/l) significantly ($p < 0.001$) increased the intensity of photosynthesis, at a 1 mg/l concentration did not change this indicator, and only at 10 mg/l concentration decreased it by 15% as compared to the control (Fig. 3, A). When measuring the intensity of transpiration, a similar dependence was found (see Fig. 3, B).

2. Biochemical parameters of the seedlings of soft spring wheat (*Triticum aestivum* L.) of the Novosibirskaya 29 variety grown on a dispersion medium that contained nickel nanoparticles at different concentrations (*M*±SEM, laboratory test)

Concentration of nanoparticles, mg/l	Sum of flavonoids		Proline content	
	μg/g of dry weight	to the control,%	mg/g of dry weight	to the control,%
Контроль	28.11±0.11	100	0.77±0.12	100
0.01	17.24±0.13*	61	0.95±0.09*	123
0.1	7.03±0.01*	25	1.15±0.21*	149
1	21.22±0.01*	75	1.28±0.25*	166
10	10.17±0.03*	36	1.79±0.26*	232

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

It is known that the stress-protective function under adverse effects is performed by flavonoids [33] and proline [34, 35], which are capable of binding metal ions with variable valency and thus limiting non-enzymatic free-radical processes.

In our experiments, the amount of flavonoids decreased with an increase in the concentration of NP Ni^0, but no clear dose dependence was observed (Table 2). The literature data on the change in the content of flavonoids under the influence of nanoparticles on plants are quite contradictory. Thus, when growing *Raphanus sativus* L. in the soil treated with cesium oxide nanoparticles at different concentrations, a considerable scatter of data in the variants of the experiment was observed, so the differences were not significant [36]. A decrease in the content of flavonoids in different organs of *Calendula officinalis* L. was observed under the influence of silver nanoparticles. The paper of C. Krishnaraj [38], on the contrary, shows a shift towards secondary metabolism and an increase in the content of flavonoids in *Bacopa monnieri* L. under the influence of silver nanoparticles.

At the same time, the effect of nickel nanoparticles led to an increase ($p < 0.05$) in the amount of proline in wheat leaves comparatively to the control. In this case, a dose dependence on the concentration of nanoparticles was observed (see Table 2), which agrees with the existing concept on the protector role of proline under stress [34].

Thus, this paper reveals the morpho-physiological peculiarities of wheat seedlings during their growing on aqueous disperse systems containing nickel nanoparticles ($\Delta_{50} = 5$ nm), with similar dependencies observed for the majority of the studied parameters (morphometric parameters, chlorophyll content, photosynthesis and transpiration rate), i.e. an increase at small concentrations of NP Ni^0 and a distinct decrease at higher concentrations of NP Ni^0. Among the compounds performing the protective function, a directly proportional increase in the content with an increase in the concentrations of NP Ni^0 is observed only in case of proline, while a decrease in the amount is shown for carotenoids and flavonoids. This suggests that, depending on the concentration, Ni^0 nanoparticles have a selective effect on the various metabolic processes. The obtained results can supplement the data on the justification of the permissible levels of contamination of plants and agrocoenosis by metal nanoparticles, and also be used to develop practical recommendations for diagnosing the negative impact of NP Ni^0 on plants.

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ROOT HABITUS AND PLANT PRODUCTIVITY OF SPRING BREAD WHEAT SYNTHETIC LINES IN WESTERN SIBERIA, AS CONNECTED WITH BREEDING FOR DROUGHT TOLERANCE

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Abstract

In Western Siberia, drought appears during the initial period of plant vegetation, and dryness in June and early July is increasing, as evidenced by the hydrothermal coefficients. Improvement of drought tolerance of wheat varieties is a breeding priority for ensuring crop stability over the years of warming and increasing frequency of dry years. This paper is the first our report of wide surveyed concerning the assessment of phenotypic differences in the main parameters of the root system between hexaploid synthetic wheat lines and their advantage over standard varieties due to the formation of the powerful root system penetrating into deep layers of the soil. The synthetic lines we studied in 2016-2017 in Western Siberia, were created in CIMMYT by crossing of durum wheat (*Triticum durum* Desf., genome AB) varieties Aisberg, Leucurum 84693, Ukr-Od 952.92, Ukr-Od 1530.94 (Odessa, Ukraine) and Pandur (Romania) with different entries of *Aegilops tauschii* Coss. (synonym *Ae. squarrosa,* genome D) from Caspian basin. Also, 15 synthetic wheat lines derived from Kyoto University (Japan) were also involved in studying. In total, we examines 126 lines of spring and winter types. Our screening revealed high variability of genotypes on the main parameters of root habitus in hybrid combinations with involving of different forms of the goat grass. The lines derived from hybrid combinations Aisberg/*Ae.sq.*(369), Ukr-Od 952.92/*Ae.sq.*(1031), Ukr-Od 1530.94/*Ae.sq.*(458) and Ukr-Od 1530.94/*Ae.sq.*(629) had high parameters of the root system development, i.e. the total root length was 73.9-141.1 cm, the root area was 16.6-25.3 cm^2, the number of root tips was 98-235, the root weight was 0.75-0.87 g. The lines with 5-6 germinal roots were mainly derived from the crosses when goat grass entries *Ae.sq.*(223) and *Ae.sq.*(310) of Gilan province, *Ae.sq.*(1031) of Zanjan province (Iran), and also *Ae.sq.*(409) from Dagestan (Russia) were the progenitors. The correlation coefficients between the main quantitative traits of plant productivity and the root system parameters calculated for the synthetic wheat lines showed that the plant height can be a marker for selecting genotypes with better parameters of root system, and therefore more drought-tolerant in Western Siberia. Synthetic lines No.No. 18, 28, 32, 38 of Aisberg/*Ae.sq.*(369), No. 37 of Ukr-Od 1530.94/*Ae.sq.*(310), No. 59 of Ukr-Od 30.94/*Ae.sq.*(1027), No. 61 of Pandur/*Ae.sq.*(409), and No. 36 of Aisberg/*Ae.sq.*(369)//Demir, selected for the elements of the spike productivity and a better root development may be involved in breeding for drought tolerance under conditions of Western Siberia.

Keywords: *Triticum durum* Desf., Aisberg, Leucurum 84693, Ukr-Od 952.92, Ukr-Od 1530.94, Pandur, *Aegilops tauschii* Coss., synthetic wheat, lines, parameters of the root system, drought tolerance

According to the information of FAO (Food and Agriculture Organization), the world population can reach 9-10 billion people by the middle of the 21st century. It will require the increase in the total yield of wheat from 650-700 million tons at present to about 1 billion tons (http://faostat.fao.org), for which the annual growth of grain wheat production is expected to be 2% in comparison

with the current 1.3%.

Targeted selection to increase the yield of commercial wheat varieties leads to the sharp decrease in their genetic diversity in resistance to abiotic stress factors. In the process of breeding, valuable adaptive alleles, accumulated for thousands of years in the local varieties of folk selection, were irretrievably lost [1].

Previously, a significant increase in the average minimum and maximum air temperature over the past 50 years in the Omsk Region was shown, and each second year was characterized by a shortage of precipitation during the vegetation period [2]. In the conditions of Western Siberia, drought is mainly manifested in the initial period of vegetation, and dryness in June—early July is increasing, as evidenced by changes in the hydrothermal coefficient. Due to the increasing frequency of dry years, increasing the drought resistance of wheat varieties becomes a priority for selection and the basis for increasing the yield of cultivated varieties in the Western Siberian region [3, 4].

Potential sources of drought hardiness genes are different kinds of goat grass growing in the arid regions. *Aegilops tauschii*, due to its wide adaptation in different ecogeographic zones (from Turkey in the West to Afghanistan and Central Asia in the East), is considered one of the donors of economically valuable genes for the expansion of the gene pool of soft wheat, which lost a wide polymorphism in the process of selection and cultivation [5-6]. About 400 samples of *Ae. tauschii*, found in the Caucasus region and the arid regions of Western Asia, were involved in the international program for Central Asia and Transcaucasia from the ICARDA (International Center of Agricultural Research) in the arid regions [7]. By now, CIMMYT (International Maize and Wheat Improvement Center) created about 1,300 synthetic hexaploids of spring and winter type, and 600 of them are based on *Ae. tauschii*. Numerous synthetic wheat lines have been successfully used in breeding and have proved their potential, in particular, when increasing resistance to biotic and abiotic stresses [8-11]. Increased drought resistance of synthetic wheat compared to varieties obtained by classical breeding methods was revealed by many researchers [12, 13]. In particular, it was noted that synthetic lines have larger habitus and longer root system length, which, with water deficiency, causes the formation of yield by 5-40% higher than that of soft wheat varieties [14]. The advantage of synthetic wheat and lines based on it is a better accumulation of dry weight of the roots and more productive use of moisture to increase the dry mass of the plant under water scarcity conditions due to better saturation of the soil with roots in the deep layers of the soil, whereas *Ae. tauschii* samples have higher indicators in the conditions of the optimal water supply of plants [15, 16].

Recent investigations have also proved a significant polymorphism of the synthetic wheat genome with *Ae. tauschii* on the morphology of the root system and drought resistance. The study of synthetic wheat lines with a lack of moisture in the soil revealed a close correlation between the water status of plants with biomass and root length [13, 17]. In some domestic works, it is noted that high productivity and drought resistance of plants are closely associated with a well-developed primary root system, due to which moisture from soil layers up to 1.5 m [18] is absorbed under drought conditions. The high heritability of the number of embryonic roots is proved, which indicates the possibility of a positive effect in the selection on this basis in breeding for drought hardiness [19].

QTLs that control the angle of the first root and the number of embryonic roots were revealed with the help of the mapping population of double wheat haploids. However, the relationship of genetic components with the physiological parameters of the water regime in dry conditions is not found [20, 21]. On the chromosomes 5B and 6D of spring wheat, QTLs, determining the signs

of water content, total root mass, and dry matter content in roots, were mapped. The coincidence of QTL localization, involved in the control of signs of water content and dry matter content in the roots, as well as water content, dry matter content in the roots and the water-holding capacity of the leaves, was noted; it indicates the relationship of physiological mechanisms that determine the water status of the above-ground part of the plant and the root system [22, 23].

Drought-resistant varieties created in the arid conditions are characterized by an increased length of roots and their total weight, which plays a crucial role in the formation of higher grain productivity with a moisture deficit [24-26].

It is also important to note that the previously obtained synthetic hexaploids did not exhibit significant polymorphism in the genome D; in this regard, the preliminary study and selection of samples of the *Ae. tauschii* is important for the creation of synthetic forms and hard wheat cultivars. The maximum genetic diversity of the *Ae. tauschii* forms, including rare and endemic ones, was found in the center of origin of this species. For example, the subspecies of *Ae. tauschii* ssp. *strangulata*, which is regarded as a potential donor of the genome D of *Triticum aestivum* [27, 28], grows in the geographically limited territory, i.e. in Transcaucasia (Armenia, Azerbaijan), as well as in the Northern provinces of Iran, the Golestan and Mazandaran [6, 29]. The greatest genetic diversity of the subspecies *Ae. tauschii* ssp. *tauschii* is concentrated on the South-West coast of the Caspian Sea, where the active forming process continues, which has great importance both for phylogenetic purposes and for practical breeding. In the NPGBI (National Plant Gene Bank of Iran), the collection of more than 180 samples of *Ae. tauschii*, growing in the territory of this country, is created; however, only 40 of them were used to create a collection of synthetics at the University of Kyoto [30-31]. In this regard, new synthetics from the University of Kyoto and CIMMYT, which were created by involving in the hybridization of unique samples of *Ae. tauschii* from the Caspian basin and drought-resistant hard wheat cultivars, represent a significant genetic resource for expanding the genotypic diversity of wheat in breeding for drought hardiness in the conditions of Western Siberia.

In this paper, we have revealed the breeding importance of synthetic wheat lines as sources for improving the characteristics of the root system in hybrid combinations involving different forms of goat grass and found that the height of plants can serve as a marker feature in the selection of genotypes with a better root system and, consequently, more drought-resistant in the conditions of Western Siberia.

The work objective is to analyze the morphometric parameters of the root system and the elements of productivity in the lines of hexaploid synthetic wheat for the selection of the parental material, promising for selection of drought hardiness of soft spring wheat in the conditions of Western Siberia.

Techniques. In the experimental field (the city of Omsk, 2016-2017), the synthetic lines were investigated; they were developed at CIMMYT by the hybridization of hard wheat cultivars Aisberg, Leucurum 84693, Ukr-Od 952.92, Ukr-Od 1530.94 (Odessa, Ukraine) and Randur, Romania (*Triticum durum* Desf., the AB genome) with various samples of *Aegilops tauschii* Coss. (synonym for *Ae. squarrosa*, *Ae.sq.*, genome D) from the Caspian basin area: from Iran *Ae.sq.*(310), *Ae.sq.*(369), *Ae.sq.*(629), *Ae.sq.*(1027), *Ae.sq.*(1031), the provinces Gilan, Zanjan, and Mazandaran; from Azerbaijan *Ae.sq.*(392), Shamakhi; from Russia *Ae.sq.*(409), Dagestan, of unknown origin *Ae.sq.*(458), *Ae.sq.*(511). Fifteen synthetic wheat lines obtained at the University of Kyoto (Japan) as a result of the hybridization of hard wheat cultivar Langdon (the USA) with *Ae. tauschii* samples were analyzed (Iran, Turkmenistan, Kyrgyzstan, India, China) [32, 33].

The *Ae. tauschii* samples (CIMMYT Germplasm Bank) referred to the subspecies of ssp. *tauschii*, var. typical (92 lines), ssp. *strangulate* (11 lines). A total of 126 lines of spring and winter type of development were investigated.

In 2016, 60 synthetic lines of spring type were sown in a single row of 1 m long; after every five numbers, the standards were alternately placed, No. 1 Pamyati Azieva (middle-early) and No. 2 Serebristaya (middle-late). The seeding rate was 25-30 grains per meter run. After harvesting, the structural analysis of the elements of productivity was carried out in the laboratory conditions. To analyze the root system development, 10 plants of each line and standards were dug from a depth of 25 cm; the root system was washed and scanned (Epson Expression 11000XL, Epson America, Inc., USA). In 2017, 47 lines of the winter type of development were investigated. Each of them was sown on the area of 1.4 m^2. The seeding rate was 25 g of grains per a working plot. After every 10 numbers, middle-early and middle-late standards No. 1 and No. 2 (the sorts of Pamyati Azieva and Serebristaya) were located alternately. The row width was 15 cm. Repetition was 4-fold, the placement of working plots in the experiment was randomized. To analyze the root system, 5 plants of each line and standards were dug from a depth of 25 cm in 4-fold repetition; the root system was washed and scanned as described above.

To process data on the root system state, the WinRhizo-2016 software package (Regent Instruments, Inc., Canada) was used. The following indicators were evaluated: root biomass, the number of embryonic roots, width, length, total length, root area, the average diameter of the root system, root volume, the number of root ends, the number of branches, the number of root intersections, fractal difference.

The statistical processing of the experimental data included the determination of the mean (*M*), standard error of the mean (±SEM), variation and correlation analyses. The comparison of synthetic lines and standards was carried out with the help of the analysis of variance; the reliability of differences was estimated by the smallest significant difference at the level of significance of 5% (LSD_{05}). The correlation coefficients (*r*) were calculated to determine the relationship between the studied features. To assess the significance of the correlation coefficient, table values of *r* were used at the significance levels $p = 0.05$ and $p = 0.01$. The correlation coefficients were considered reliable at $p < 0.05$. The indicators were calculated according to the description [34] using the Microsoft Excel statistical software application package and SPSS (PASW) Statistics 20.0 (IBM, USA).

Results. The weather conditions of the vegetation period of 2016 were characterized by high temperatures and lack of precipitation before the wheat sowing, which led to an acute early spring drought. For example, in May 2016, only 5 mm of precipitation fell, which was by 81% less than the average annual value (26 mm). In the first half of June, a large shortage of precipitation was observed as well. In the tillering phase, the common suppression of plants and reduction of growth processes were noted. In July, the moderately warm rainy weather, favorable for the development of plants, was the predominant one. In August, the weather was dry and warm. The maximum air temperature on some days in June, July, and August reached 32-35 °C. In 2017, an early summer drought, typical for the southern forest-steppe of Western Siberia, was observed. Since the 2nd ten-day period of June, for 3 weeks in critical periods of vegetation (the stooling stage and the formation of generative organs), the plants have experienced great stress due to atmospheric and soil drought. The hydrothermal coefficient in this period was 0.53, which characterizes the weather conditions as very dry. During the 2nd and 3rd ten-day periods of July, the conditions for plant growth and development were favorable in terms of water supply. In Au-

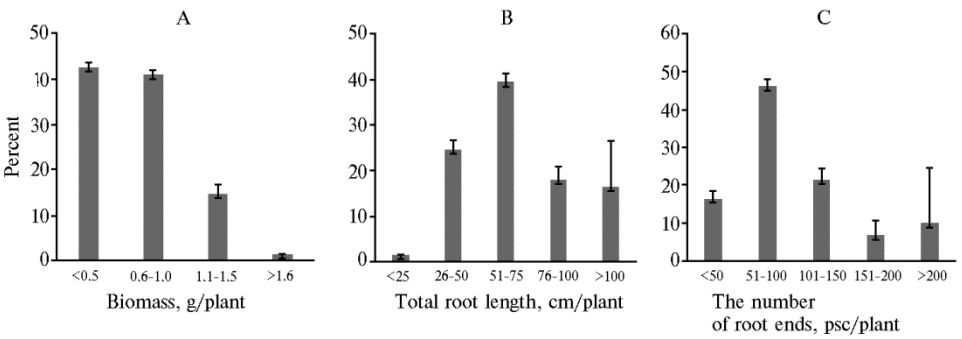
gust, the weather was dry and warm. The average daily air temperatures during the vegetation period slightly exceeded the average annual rates.

Significant polymorphism in the main parameters of the root system was observed in the lines obtained with the participation of different samples of *Ae. tauschii* (Table 1, Fig.). It is known that the D genome of *Ae. tauschii* has greater genetic variability compared to the genome of soft wheat.

1. The parameters of the root system among the studied synthetic wheat lines (at the average for hybrid combination, Omsk, 2016, microplot test)

Sample No. *Ae.sq.*, form	*n*	Perplant							
		A	B	C	D	E	F	G	H
Hard wheat cultivar Aisberg									
511	5	4	41.2	10.6	0.23	0.88	52	115	0.35
369	8	4	86.2	19.9	0.39	0.80	128	315	0.82
Hard wheat cultivar Leuc 84693									
409	1	4	51.1	11.3	0.21	0.78	39	85	0.38
Hard wheat cultivar Ukr-Od 952.92									
1031	3	5	73.9	16.6	0.32	0.83	98	233	0.75
Hard wheat cultivar Ukr-Od 1530.94									
310	3	5	66.2	13.6	0.24	0.73	89	160	0.56
392	3	4	52.8	11.5	0.21	0.75	69	142	0.40
458	3	4	78.1	16.9	0.31	0.78	114	221	0.85
629	3	4	141.1	25.3	0.38	0.62	235	356	0.87
1027	11	4	63.0	14.7	0.29	0.85	85	199	0.71
Hard wheat cultivar Pandur									
223	1	5	128.4	25.6	0.42	0.63	217	398	1.41
409	1	5	79.4	17.3	0.31	0.72	125	247	0.98
Hard wheat cultivar Langdon									
Forms from Japan	15	4	67.7	15.9	0.32	0.82	83	196	0.69
The synthetic line Aisberg/Ae. sq.(369)//Demir									
	1	6	128.2	28.4	0.52	0.73	151	329	1.06
Pamyati Azieva cultivar (middle-early standard)									
	1	4	50.9	13.9	0.33	0.97	73	155	0.63
Serebristaya cultivar (middle-late standard)									
	1	6	54.6	14.5	0.32	0.93	53	133	0.57
The variety range		3-6	15.3-213.2	5.1-35.9	0.15-0.67	0.56-1.36	28.5-366.8	37.5-624.4	0.19-1.85
LSD_{05}		0.22	2.56	0.58	0.01	0.03	3.78	10.1	0.10

Note. *n* — the number of lines in the hybrid combination of the spring type of development. A — the number of embryonic roots, pcs., B — the total length of the roots, cm; C — root area, cm^2; D — the volume of roots, cm^3; E — the average diameter of the roots, mm; F — the number of root ends, pcs.; G — the number of root branches, pcs.; H — root biomass, g.



The distribution of 47 studied wheat synthetic lines according to the root biomass (A)**, the total length of roots** (B) **and the number of root ends** (C) (Omsk, 2016, microplot test; vertical segments indicate the standard error of the mean).

The root biomass of some synthetics significantly ($p < 0.05$) exceeded the same indicator of standards by 0.4-0.9 g, and the total length of the roots exceeded 55-92 cm (Table 1). The increase in the root weight does not always increase their water absorption capacity; therefore, the main contribution to the productivity of plants is made by the total number of roots [12, 15].

In the combinations of Aisberg/*Ae.sq.*(369), Ukr-Od 952.92/*Ae.sq.*(1031), Ukr-Od 1530.94 92/*Ae.sq.*(458), Ukr-Od 1530.94/*Ae.sq.*(629), Ukr-Od 1530.94

92/*Ae.sq.*(1027), the representative differences between the synthetic lines and standards were identified by the following indicators: the total length of roots — 73.9-141.1 cm, roots area — 16.6-25.3 cm^2, the number of the root ends — 98-235 pieces, the root biomass — 0.75-0.87 g. In general, synthetics differed from the standards by a smaller diameter of roots (0.62-0.88 mm), which allowed them to extract moisture from the deeper layers of the soil. D. Eissenstat [35] notes that the smaller diameter of the root system reduces the nutrient costs of root formation and increases their area. The formation of embryo roots affects the productivity of varieties in the arid conditions and depends on the ecological origin of the variety. The primary root system has a strong influence on the growth and development of plants in the initial stages of ontogenesis, helping to survive in early drought [18, 19, 36].

The comparison of the number of embryonic roots of the studied lines of synthetics, created with the participation of different goat grass samples, has shown that 5-6-radicular samples are typical for the lines, obtained on the basis of the goat grass forms from Iran, i.e. *Ae.sq.*(223) and *Ae.sq.*(310) from the province of Gilan, and *Ae.sq.*(1031) from the province of Zanjanas well as the form from Dagestan *Ae.sq.*(409). The development of the primary root system was a moderately variable trait (*Cv* = 16.5%). Within the boundaries of each hybrid combination, a significant variation of the trait (from 3 to 6 roots), which indicates the possibility of selection, was observed.

2. Parameters of the root system among the best synthetic wheat lines on the spike productivity (at the average for hybrid combination, the city of Omsk, 2016-2017, microplot test)

№	Cultivar, line	A	B	C	D	E	F	G	H
18	Aisberg/*Ae.sq.*(369)	1.35^{a}	4.3	10.0^{ab}	109.3^{ab}	28.8^{ab}	384	768^{ab}	0,38
28	Aisberg/*Ae.sq.*(369)	1.40^{a}	3.3	11.3^{ab}	124.1^{ab}	32.3^{ab}	401	817^{ab}	$0,71^{ab}$
32	Aisberg/*Ae.sq.*(369)	1.39^{a}	3.2	12.1^{ab}	164.7^{ab}	41.9^{ab}	648^{ab}	1243^{ab}	$0,71^{ab}$
36	Aisberg/*Ae.sq.*(369)//Demir	1.52^{ab}	5.0^{ab}	11.6^{ab}	144.7^{ab}	35.3^{ab}	420	811^{ab}	$0,79^{ab}$
37	Ukr-Od 1530.94/*Ae.sq.*(310)	1.48^{ab}	5.5^{ab}	11.7^{ab}	151.2^{ab}	31.3^{ab}	579^{ab}	929^{ab}	$0,69^{ab}$
38	Aisberg/*Ae.sq.*(369)	1.30^{a}	3.6	11.2^{ab}	152.8^{ab}	40.2^{ab}	547^{ab}	1154^{ab}	0,59
59	Ukr-Od 1530.94/*Ae.sq.*(1027)	1.43^{ab}	4.1	11.2^{ab}	147.0^{ab}	38.9^{ab}	552^{ab}	1115^{ab}	0,66
61	Pandur/*Ae.sq.*(409)	1.49^{ab}	4.2	9.8	109.5^{a}	29.0^{ab}	701^{ab}	678	$0,72^{ab}$
Pamyati Azieva (middle-early standard)		1,21	4.3	9.2	93.4	19.4	380	601	0.46
Serebristaya (middle-late standard)		1,34	4.6	9.6	105.2	23.2	378	676	0.45
LSD_{05}		0,09	0.18	0.24	11.5	2.2	43.4	102.9	0.23

N o t e. A — the main spike mass, g; B — the number of embryonic roots, pcs.; C — the length of the largest root, cm; D — root area, cm^2; E — the number of root ends, pcs.; G — the number of root branches, pcs.; H — root biomass, g. The significant differences between lines and standards (LSD_{05}, $p < 0.05$) are marked with different Latin letters.

The weight of grain from the main spike is the most important feature in the assessment of the drought hardiness of varieties (Table 2). In studying this trait, we obtained the results that are consistent with the data from other researchers [18, 37]. In case of unfavorable moisture supply in the initial period of plant development in 2016 and in the conditions of early summer drought in 2017, the best synthetic forms had an advantage in the productivity of the main spike over the varieties of classical selection due to the formation of a powerful root system penetrating into the deeper layers of soil (10.0-12.1 cm), a larger total length (109.3-164.7 cm), a larger number of ends (384-701 pcs.) and branches (678-1,243 pcs.), a larger area (28.8-41.9 cm^2), and root biomass (0.59-0.79). As per root biomass (0.69-0.79 g), the following synthetics were distinguished: No. 28, 32 of Aisberg/*Ae.sq.*(369); No. 36 of Aisberg/*Ae.sq.*(369)//Demir; No. 37 of Ukr-Od 1530.94/*Ae.sq.*(310), and No. 61 of Pandur/*Ae.sq.*(409).

The length of the longest root significantly exceeded the standards for all lines except No. 61 Pandur/*Ae.sq.*(409). Lines with 5-6 embryonic roots, as a rule, were characterized by a greater total root length, increased productivity of the spike, and grain size. So, No. 36 Aisberg/*Ae.sq.*(369)//Demir and No. 37

Ukr-Od 1530.94/*Ae.sq.*(310) showed, at average over 2 years of research, the length of the roots of 145-150 cm, the productivity of the spike was 1.5 g and weight of 1000 grains of 44.1-45.8 g.

The most productive synthetics No. 32 Aisberg /*Ae.sq.*(369) and No. 61 Pandur/*Ae.sq.*(409), with the yield of 426-436 g/m^2 in 2017, had significantly high indicators of the area, number of ends, and root biomass. The results of the research indicate that just one or two backcrosses of the synthetic hexaploids with the best commercial varieties of wheat are enough for the allocation of the synthetic backcrossed lines with valuable introgressed components of plant productivity from *Ae. tauschii*.

For example, the line No. 36 obtained by crossing of the synthetic line Aisberg/*Ae.sq.*(369) with the cultivar of Turkish breeding Demir showed a noticeable improvement in the main agronomic indicators (productivity of the spike reached 1.52 g).

We calculated the correlation coefficients between the main quantitative characteristics of plants and the parameters of the root system of synthetic wheat lines (Table 3).

3. Correlation coefficients (*r*) between the indicators of root system development and some quantitative signs of productivity among 47 studied synthetic wheat lines (the city of Omsk, the average for 2016-2017, microplot test)

Trait	NER	RB	LLR	TLR	RA	RV	NRE	NRB
NER		0.12	0.28	0.34*	0.37*	0.37*	0.42**	0.41**
RB	0.12		0.26	0.26	0.31*	0.34*	0.10	0.17
LLR	0.28	0.26		0.88**	0.81**	0.71**	0.79**	0.79**
TLR	0.34*	0.26	0.88**		0.97**	0.89**	0.95**	0.97**
RA	0.37*	0.31*	0.81**	0.97**		0.97**	0.93**	0.97**
RV	0.37*	0.34*	0.71**	0.89**	0.97**		0.87**	0.92**
NRE	0.42**	0.10	0.79**	0.95**	0.93**	0.87**		0.98**
NRB	0.41**	0.17	0.79**	0.97**	0.97**	0.92**	0.98**	
PH	0.37*	0.18	0.56**	0.66**	0.67**	0.64**	0.68**	0.66**
PTC	0.35*	0.24	–0.28	–0.34*	–0.31*	–0.28	–0.39**	–0.36*
NGS	0.18	0.33*	0.46**	0.55**	0.55**	0.51**	0.50**	0.52**
GWS	0.32*	0.22	0.62**	0.73**	0.71**	0.66**	0.74**	0.72**
GWP	0.36*	0.28	0.47**	0.56**	0.55**	0.52**	0.57**	0.56**
1000GM	0.38*	0.11	0.58**	0.67**	0.63**	0.57**	0.75**	0.69**

N o t e. NER — the number of embryonic roots, RB — root biomass, LLR — the length of the longest root, TLR — the total length of roots, RA — roots area, RV — roots volume, NRE — the number of root ends, NRB — the number of root branches; PH — plant height, PTC — productive tilling capacity, NGS — the number of grains per spike, GWS — grain weight from a spike, GWP — grain weight from a plant, 1000GM — 1000 grains mass.
*, ** The critical value of *r* is 0.30 and 0.39, respectively.

M. Reynolds et al. showed [15] that the increase of root biomass does not affect the improvement of the water supply of plants in conditions of water deficit. The authors have found a weak positive relationship between the basic elements of spike productivity with the number of embryonic roots, and the biomass of roots (*r* varies from 0.18 to 0.38); therefore, it can be assumed that the main mechanism of adaptation of synthetic lines to drought under the conditions of Western Siberia is enhanced root growth in length, which allows plants to extract moisture from deeper soil layers.

For the length of the largest root and the total length of the roots, the authors found an average positive relationship with the main elements of productivity (r = 0.47-0.73). This information corresponds to the previously published results by the foreign researchers [38-40].

The synthetic wheat lines were characterized by the formation of a greater total length and a greater number of root branches. In the conditions of the early summer drought in 2017, the most productive lines No. 32 Aisberg/*Ae.sq.*(369) and No. 61 Pandur/*Ae.sq.*(409) showed better adaptation to drought due to the maximum total length of roots (232-239 cm), and the line

No. 38 Ukr-Od 1530.94/*Ae.sq.*(310) had the most developed root system (diameter 5.6 cm) at a depth of 18-20 cm.

Plant height had correlation coefficients higher than the average value for the following traits: for root length r = 0.56-0.66; for area and volume of roots, the number of ends and branches of roots r = 0.64-0.68. The revealed positive correlation indicates that the height of plants can serve as a marker sign in the selection of plants with good indicators of the formation of the root system and, consequently, more drought-resistant in conditions of Western Siberia. It should be noted that the opposite results were obtained in the United States in the study of the contribution of the root system to the drought resistance of spring wheat [41].

The length of the largest root and the total length of roots were poorly correlated with biomass (r = 0.26), because the inverse relationship between the diameter of roots and their length (r = –0.39) was found; it allowed roots with a smaller diameter to penetrate into the soil to a greater depth. The unproductively large outflow of assimilates to the formation of excessive root biomass can lead to the decrease in plant productivity and grain yield per unit of the area. According to the paper by B. Ehdaie et al. [42], the formation of a larger area of roots due to the spread of lateral roots in the horizontal direction increases the absorption capacity of the root system, which favorably affects the productivity of plants.

V. Nazem and A. Arzani [43] during the investigation of morphological features of the synthetic hexaploids, noted less intense leaf color, from light to gray-green with a well-developed wax coating, which also contributes to optimal stomatal regulation due to less heating of the leaf surface and reducing of the moisture consumption for transpiration. It can be assumed that the architectonics of the root system, in particular, the formation of a larger number of ends and branches, as well as the maximum development in the length of the main roots of a smaller diameter, maintain the positive water status of the synthetic lines in conditions of water scarcity. The obtained results show the relationship of the elements of productivity with the parameters of the root system, which can improve the efficiency of selection and indicate the prospects in the use of synthetic wheat lines in breeding for drought resistance.

Thus, combinations Aisberg/*Ae.sq.*(369), Ukr-Od 952.92/*Ae.sq.*(1031), Ukr-Od 1530.94 92/*Ae.sq.*(458), Ukr-Od 1530.94/*Ae.sq.*(629), Ukr-Od 1530.94 92/*Ae.sq.*(1027) show a significant polymorphism in the features of the root system development; it allows selection of the parental material for breeding for drought resistance. Standard varieties of spring wheat compared to the best synthetic lines have less root biomass and lower basic indicators of the root system biometric parameters. The positive correlation between the development of roots system and the height of plants is revealed; therefore, the height of the plant can be a marker trait in the selection of genotypes with an effective root system. The lines No. 18, 28, 32, 38 Aisberg/*Ae.sq.*(369), No. 37 Ukr-Od 1530.94/*Ae.sq.*(310), No. 59 Ukr-Od 30.94/*Ae.sq.*(1027), No. 61 Padur/*Ae.sq.*(409), and No. 36 Aisberg/*Ae.sq.*(369)//Demir, distinguished by the elements of the spike productivity and the parameters of the root system, can serve as parental material for inclusion in breeding programs to improve the drought hardiness of wheat varieties in different regions of Russia.

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DYNAMIC OF PROLINE, PIGMENT CONTENTS, WATER FRACTIONS IN APPLE (*Malus domestica* Borkh.) FOLIAGE UNDER TEMPERATURE DROUGHT STRESS AND PROTECTION MEASURES

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Abstract

The hydrothermal factors and the high summer solar radiation in the South of Russia actualize the experimental studies and search for objective expressly determined quantitative indicators to evaluate functional state of fruit plants and their seasonal variability in agrocenoses. The main purpose of this work was to confirm the hypothesis of the possibility to use the amino acid proline content as a criterion for rapid assessing effects of abiotic stress intensity (soil and air drought, intensity of solar radiation) on perennial fruit plants in agrocenosis. On the example of apples (*Malus domestica* Borkh.) varieties Prikubanskoe and Aidared we identified the regulatory functions of special fertilizers in combination with growth regulator Novosil to improve adaptive properties of the apple trees. For the rapid determination of the free amino acid proline content in apple leaves we used capillary electrophoresis in the Kapel 103P device (Lumeks, Russia). To analyze tolerance of the apple trees against the summer period stressors, we used the weight method, determined the content of free and bound water in leaves, and also assessed content of chlorophyll (a + b) and carotenoids in leaves using a UNICO 2800 spectrophotometer (United Products & Instruments, USA). In the course of 4 year-studying (2009-2012) from May to August, it was shown that the leaf level of bound water increased while free forms of water declined. The leaf treatment with fertilizers together with growth regulator Novosil led to an increase in the content of the bound water in the leaves as compared to the control, which indicates an increase in the resistance of the apple tree to stress factors of the summer time. In July and August, at maximum adverse hydrothermal factors, the free proline content in the shoot leaves of apple plants became higher compared to the values under normal environmental conditions (decade III of May). Proline contents annually increased 1.4-2.9 times in July compared to May which is due to a lack of moisture, extremely high air temperatures and excessive insolation. Our data showed that the use of aqueous solutions of special fertilizers in combination with the growth regulator Novosil contributed to a decrease in the proline amount in apple leaves in July and August as compared to the control and led to an increase in plant resistance to stressors of the summer period, that is also consistent with the dynamics of the bound water we found. The conducted researches attest the possibility of using expressly estimated free proline content in apple shoots leaves as an important criterion for assessing resistance to drought and anthropogenic factors in perennial fruit plants in commercial orchards.

Keywords: *Malus domestica* Borkh., apple, environmental stress factors, the dynamics of proline content, pigments, fertilizer, growth regulator, adaptation

Ensuring sustainability of bio-technological systems is closely connected with water and temperature stress intensity in the cycle of seasonal development of plants [1-3]. The importance of such studies in the south of Russia is due to extremely high air temperatures against the background of dry hot winds and lack of precipitation during the summer period [4, 5]. In perennial fruit crops, this leads to disrupted bud set, prefloration, differentiation of flower parts and,

as a result, the productivity losses [6]. Therefore, it is important to quickly identify physiological and metabolic changes in plants at the intensity of the extreme factor and to use the agro-engineering measures necessary to stabilize biological processes [7, 8]. This requires objective quantitative indicators of the crops condition and their seasonal variability in conditions of fruit agrocoenosis.

Different aspects of diagnosing functional status of agricultural plants influenced by abiotic factors are regularly covered in scientific publications of domestic [9-11] and foreign authors [12-14]. The effect of temperature stress, mineral nutrition deficiency, water and salt stresses on citrus fruit crops in the conditions of Israel and the Republic of South Africa was evaluated in accordance with changes in the content of amino acids and other biochemical indicators [12, 13]. Data are published on the effect of drought on the accumulation of proline in the leaves of 3-year-old apple trees [14], on the enzymatic activity of the leaves of young apple trees as an indicator of drought resistance [15], on the diagnosis of changes in the activity of the oxidoreductase enzyme in apple skin at high-temperature summer stress and excess of solar radiation [16]. An increase in the amount of proline and soluble sugars with a decrease in the content of soluble protein in the leaves of alycha under soil drought is shown [17]. Pot trials under soil drought conditions showed a decrease in the amount of chlorophyll and water in leaves of plum seedlings and an increase in proline content [18]. The dynamics of proline and chlorophyll content in the European olive leaves under irrigation with industrial wastewater in Jordan [19] and the effect of rhizospheric drought on the accumulation of proline and primary metabolites in apple leaves in vitro were reported [20].

Our paper is the first to report data of field trials which show seasonal changes in the leaf level of proline, an osmoprotector increasing cell resistance to dehydration, in apple shoots under the soil climatic conditions of southern Russia. In preliminary studies, the effect of aqueous solutions of fertilizers and growth regulating chemicals on the productivity of apple plants in the region was determined [21]. It is shown that changes in the ratio of water fractions and in the content of photosynthetic pigments of leaves serve as the estimates of plant physiological state under the effect of physical stress.

The purpose of the study was to confirm the hypothesis of the possibility to use proline amino acid for rapid assessment of the resistance of perennial fruit plants to soil and air drought, as well as identify the regulatory effect of leaf treatment with fertilizers in combination with the biologically active substance Novosil.

Techniques. Apple tree (*Malus domestica* Borkh.) plants of Prikubanskoe and Aidared winter ripening varieties (commercial plantations of the experimental farm Tsentralnoye, Krasnodar, 2009-2012) were studied. During the experiment, weather observation was conducted to describe in detail the conditions of the seasonal development of plants in the summer during the differentiation of reproductive buds (III-IV stages of organogenesis), as well as after reduction of excessive productivity due to the fall of flowers, ovaries and fruits (X-XII stages of organogenesis). Trees were treated with 0.5% aqueous solutions of complex nutrient salts of the Aquarin series (OAO Buysky Chemical Plant, Russia). Fertilizers $N_{18}P_{18}K_{18}Mg_1S_{1.5}$ were used during the first half of the vegetation period, $N_{12}P_{12}K_{35}Mg_2S_{0.7}$ during the second half. The composition included trace elements Fe, Cu, Zn, Mn, Mo, B in the chelate form. A natural polyfunctional growth regulator Novosil (Biokhimzashchita, Russia) at a concentration of 0.2% was introduced into the aqueous solutions of fertilizers. The active ingredient is triterpenic acids produced from needles of Siberian fir. In the control (without fertilizers and the growth regulator) and experimental variants,

there were 6 trees. The analysis was conducted in 3 analytical replications.

The content of proline free amino acid in leaves was determined using the method of capillary electrophoresis in a modification for the analysis of plant samples [22, 23]. The method is based on the separation of charged components of complex mixtures, which allows analyzing ionic and neutral components in the plant material with high speed and accuracy. Super high frequency (SHF) extraction of proline from the plant material, free from external contaminants, was performed on an SHF extractor-mineralizer Minotavr (Lumeks, Russia). An average sample (not less than 15 healthy leaves, 1.0 g of cuttings) was placed in the SHF mineralizer container with the addition of 25 ml of a 10% ethyl alcohol aqueous solution; then extraction was carried out for 10 min in the decomposition mode without pressure, after this the container was removed and cooled. The resulting extract was quantitatively transferred to a 25 ml measuring flask using a 10% aqueous solution of alcohol. The extract was analyzed in a capillary electrophoresis system Capel-103R (Lumeks, Russia) at a voltage of 17 kV, applied current of 30±5 A, analysis time of 12 min, when the sample was injected under a pressure of 30 mbar for 5 s. With the help of the instrument software, using an electropherogram, the mass concentration of the components was calculated according to the established calibration characteristics.

The content of chlorophylls (a + b) and carotenoids in the leaves was determined with a spectral method using a Unico 2800 spectrophotometer (United Products & Instruments, USA) [24]. The water content in the leaves and the content of free and bound forms of water were analyzed by the weight method [25]. All parameters were studied in May and July-August.

Statistical analysis was carried by F.A. Volkov [26]. The calculations were performed using the Microsoft Office 2010 software package. Each year, the significance of the difference between the analyzed indicators at 5% significance level was estimated, with calculation of the arithmetic mean (M), variance (σ^2), standard deviation ($\pm$SD), the coefficient of variation (Cv) and sampling error (μ).

Results. In 2010, the greatest intensity of hydrothermal factors was observed during the summer months. In different years, the maximum air temperatures in July and August were 37.3-38.5 °C, and the temperature on the surface of the soil reached 63.2-64.5 °C. The minimum values of the relative humidity of air were 26-42%.

Leaves of apple trees shoots are not only a source of nutrient substrates and plastic equivalents but also the center of active regulation of vital processes of the perennial woody plant, the metabolite composition of which varies depending on environmental conditions. By analyzing the dynamics of the photosynthetic pigments content, it was determined that the effect of high air temperatures during the summer period against the background of a decrease in the amount of atmospheric precipitation in July and August compared with May caused a decrease in the content of chlorophyll (a + b) by 10-16%, carotenoids by 4-18%. The use of aqueous solutions of special fertilizers reduced the loss of chlorophyll (a + b) by 3.0-7.0%, carotenoids by 1.5-5.0% at an average. The decrease in chlorophyll losses at the use of fertilizers was significant in 2010 and 2011: LSD_{05} was 0.02 mg/g of dry matter; deviation from the standard 0.01 mg/g of dry matter and the experiment accuracy ($S_{x\%}$) was 0.54 and 2.45%, respectively. The decrease in the content of carotenoids was significant (in the years of observation, LSD_{05} was 0.03, 0.02, 0.03 mg/g of dry matter), deviation from the standard, respectively, was 0.01; 0.02; 0.01 mg/g of dry matter and the experiment accuracy was 1.42; 4.73; 4.62%.

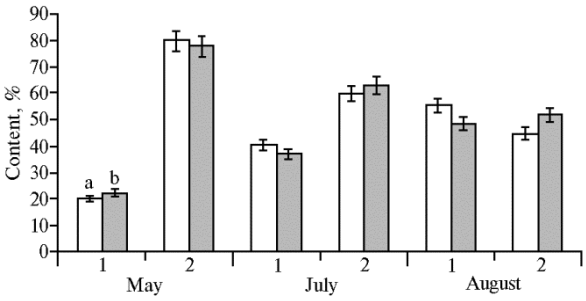

Fig. 1. Dynamics of free (1) **and bound** (2) **water content in leaves of apple (*Malus domestica* Borkh.) tree shoots on average for the Prikubanskoe and Aidared varieties:** a — control (without treatment), b — leaves fertilizing using aqueous solutions of fertilizers with the addition of the growth regulator Novosil (experimental farm Tsentralnoye, Krasnodar, 2009-2012).

The observed changes in the content of pigments at maximum intensity of hydrothermal factors are associated with the dynamics of the water fractional composition in the leaves. Such functional shifts in apple plants, typical for the state of stress, were noted by other researchers [8, 18].

From May to August, at a decrease in the total content of moisture in the leaves by 7-9%, the amount of free water increased 2.0-2.8 times and the amount of its bound form decreased. Under the effect of aqueous solutions of fertilizers and the growth regulator Novosil, the water-retaining capacity of apple tree leaves in summer slightly increased in comparison with the control (without treatment) (Fig. 1).

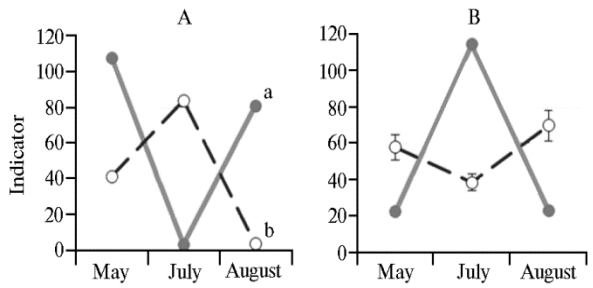

Fig. 2. Precipitations (A) **and the proline content** (B) **in leaves of apple (*Malus domestica* Borkh.) tree shoots on average for the Prikubanskoe and Aidared varieties:** a — 2011, b — 2012 (experimental farm Tsentralnoye, Krasnodar, 2009-2012).

When changing the humidity and air temperature, an increase in the free proline content was observed, which agrees with the previously published data [9, 11, 14]. In July 2011, in comparison with May, the proline content in the leaves of the studied apple varieties increased by 5 times, and in August 2012 increased by 20.8% and was inversely proportional to the amount of precipitation (Fig. 2). The proline content in leaves in May at an average air temperature of 13.8-24.8 °C and precipitation of 36.9-67.2 mm did not exceed 22.6-57.6 mg/kg.

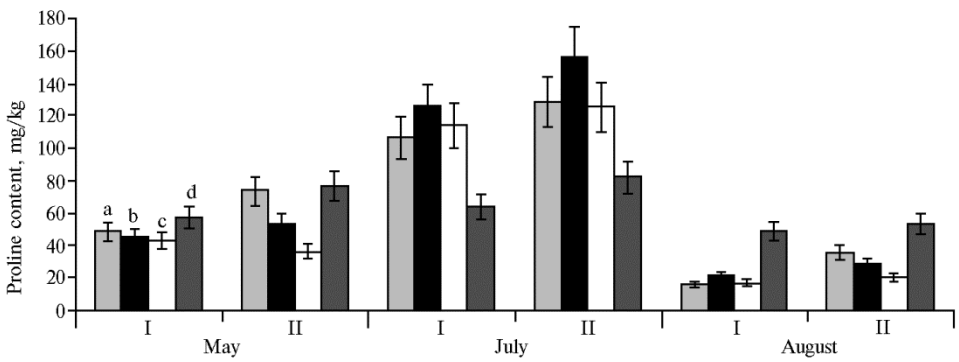

Fig. 3. The proline content in leaves of apple (*Malus domestica* Borkh.) tree shoots in the control (without treatment) (I) **and with the use of aqueous solutions of fertilizers with the addition of the growth regulator Novosil** (II) **on average for the Prikubanskoe and Aidared varieties:** a — 2009, b — 2010, c — 2011, d — 2012 (experimental farm Tsentralnoye, Krasnodar, 2009-2012).

The change in the free proline content in plants (including apple trees) under the conditions of hydrothermal factors intensity is consistent with the data of the studies of other authors [27-29].

The effect of aqueous solutions of special fertilizers used in the summer allowed reducing the content of proline slightly, which, perhaps, is associated with a weakening of stress (Fig. 3).

Thus, a decrease in the pigments content in apple tree leaves and a change in the water regime during the summer period indicated a significant effect of external conditions on the functional stability of perennial fruit plants in the seasonal development cycle. At stress (lack of moisture, extremely high air temperatures), an increase in the free proline content as an indicator of resistance to drought is observed in leaves of shoots. The use of aqueous solutions of special fertilizers in combination with the growth regulator Novosil partially contributes to a decrease in the intensity of stress, which is confirmed by a decrease in the proline accumulation in the leaves. At moderate air temperatures and regular precipitation, treatment of leaves does not have a significant effect on the proline content. As per the reduction of this indicator, the third decade of August can be considered as a period of plant reparation after stress. Our study indicates the dynamics of free proline content to be considered as an important criterion for assessing drought resistance and anthropogenic impact on perennial fruit plants in anagrocenosis.

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FACTORS AFFECTING *Alternaria* APPEARANCE IN GRAINS IN EUROPEAN RUSSIA

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Abstract

Infection of cereal seeds induced by fungi of the genus *Alternaria* is very common. There are different opinions about the damage of *Alternaria* spp. probably because of the fact that these fungi are not a homogenous group. It is rational and available at this moment to identify *Alternaria* fungi isolated from the grain samples up to the section level only. Members of two sections, *Alternaria* and *Infectoriae*, are the most widespread. Our work was aimed to reveal impact of a number of factors on *Alternaria* infection rate in cereal grain using relatively extensive data. We studied significance of the crop, its cultivar, predecessor, region, district, season, infestation by other *Alternaria* species and by fungal species of some another genera. During this research, 422 grain samples of wheat, barley, sorghum and maize collected in 2010-2012 in seven regions of European part of Russia were analyzed. The majority of samples represented wheat grain from Stavropolskii Kray and Krasnodarskii Kray (southern Russia regions). Meteorological factors played the principal role in infection. A district in different years and neighbouring districts during one growing season were characterized by highly divergent ($p < 0,001$) *Alternaria* infection rate of wheat and barley grain. In *Alternaria*, infection rate ranged from 7.0 to 71.5 % for sect. *Alternaria*, and from 8.6 to 74.0 % for sect. *Infectoriae*. Grain of all wheat and barley cultivars were similarly greatly infected by *Alternaria* fungi (p was equal to 0.6-0.9 for section *Alternaria* and 0.1-0.5 for section *Infectoriae*). As it was previously shown, rye and oat usually are infected as much as wheat and barley. Maize has several tough and thick husk leaves around the cob that likely protect the grain against *Alternaria* infection. We did not observe a significant impact of predecessors (maize, sunflower, sugar beet, winter wheat and fallow) on the infection rate in the wheat grain samples. Correlation of *A.* sect. *Alternaria* infection of wheat and barley grain with appearance of *Bipolaris sorokiniana* and *Pyrenophora* spp. was negative and moderate ($r = –0.69$ and –0.61, respectively). The same pattern was found for coinfection of *Alternaria* sect. *Infectoriae* with *Bipolaris sorokiniana* ($r = –0.64$) and *Pyrenophora* spp. ($r = –0.61$). However, species of *Alternaria* sect. *Alternaria* did not significantly affect infestation of wheat and barley by *Alternaria* sect. *Infectoriae* fungi, and vice versa. Difference between germinability of seeds infected by *Alternaria* and those free from *Alternaria* fungi was statistically insignificant. On average, the fungal contamination of germinable and ungerminable seeds differed by –1.6 % within *Alternaria* sect. and by +2.1 % within *Infectoriae* sect.

Keywords: *Alternaria alternata, A. tenuissima, A. infectoria*, grain infection rate, germinability, wheat, barley

The fungi of the *Alternaria* genus can be found in plant seeds, including crops, very often and everywhere. The contamination of grain with these micromycetes in some cases reaches almost 100%; it may adversely affect the quality of seeds, food, and feed grain [1]. The study of cereal *Alternaria* blights have been conducted in Russia for more than 100 years, but it is still relevant. The conflicting views on the etiology and harmfulness of diseases, traditionally associated with the fungi of the *Alternaria* genus, exist. For example, in early works, it was report-

ed that the seeds infected with *Alternaria* are well developed, fertile, and give rise to healthy plants [2-3]. Further on, different descriptions of the internal infection of seeds that promoted the development of root rot, weak the sprouts or leading to the death of plants, appeared [4, 5].

One of the most probable causes of conflicting data is the diversity of fungi of the *Alternaria* genus, which is not always taken into account adequately. Among the species of *Alternaria* found in the grain, saprotrophic and weakly phytopathogenic ones, the species that produce phyto- and mycotoxins, and do not synthesize them, exist [1]. Despite the obvious need for correct identification, it is often carried out either inaccurately (only to the genus) or with errors due to some objective difficulties. In general, there are more than 50 specific epithets of the *Alternaria* genus, denoting fungi related to the contamination of cereals, but only about 10 specific names are legitimate and used [6]. The so-called small-sporous species mentioned most often are *A. alternata*, *A. arborescens*, *A. tenuissima* and other species of the *A. infectoria* geni group [7-9]. The *Alternaria* genus, including about 300 species, has been divided into 27 sections recently [10-12] as a result of a thorough revision. *A. alternata*, *A. arborescens*, and *A. tenuissima* species are similar phylogenetically and placed in one *Alternaria* section, featuring in a total of about 60 species [13], many of which are so similar in molecular genetic markers that they should be combined into 10-15 species obviously [12, 14]. The species of *A. infectoria* are in the *Infectoriae* section. Within the sections, species are differentiated by morphological features primarily, which remains controversial [15, 16]. In other words, only differentiation of grain infecting *Alternaria* spp. on the representatives of the *Alternaria* and *Infectoriae* sections can be considered a sustainable one. In solving most applied tasks, identification to the section is sufficient, as the sections combine species that are generally similar in their environmental properties [11]. The species of the *Alternaria* and *Infectoriae* sections are typical components of the grain microbiota on all continents where crops are grown [1, 8, 17, 18]. In the European Russia in wheat, the representatives of both sections are found with about equal frequency (the infection rate for *A. tenuissima* is of 2-56%, for the species of the *A. infectoria* complex 2 to 72%). In the Far East, *A. tenuissima* was almost the only species in wheat representing the genus (29-76%) [1, 18].

The harm caused by these fungi is connected with contamination by mycotoxins, dangerous to humans and animals [19, 20]. The species of *Alternaria* can synthesize toxic metabolites. The spectrum of myco- and phytotoxins secreted by them is almost identical and depends more on the strain than on the species of fungus [9, 21]. Most of *A. tenuissima* strains isolated from cereals and other hosts produce several toxins of different chemical nature in different combinations [7, 9, 22]: alternariol, alternariol monomethyl ether, altenuene, tentoxin, tenuazonic acid, altertoxins I, II, and III. Some isolates of the *Infectoriae* section can produce the same toxins as the species of the *Alternaria* section but in small quantities [23, 24]. Most of the section species are characterized by metabolites which are the structural analogs of several mycotoxins [9, 25, 26], but the biological activity of these substances has not been studied.

Great practical importance of grain Alternaria blight determines the necessity of studying the influence of the external conditions on the infection rate of grain with fungi of the *Alternaria* genus. It should be taken into account that depending on the environmental conditions, representatives of different *Alternaria* sections may have different effects on grain quality. There is not so much information about the influence of different factors on the contamination of grain by the *Alternaria* species. Thus, the role of abiotic factors contributing to the development of the black embryo was reported. The appearance of such an

embryo and Alternaria blight of grain are associated with the exposure to similar abiotic factors in the period of flowering—ripening, e.g. heavy precipitation, long-term dew, abundant watering, high air humidity [27-31], to a lesser extent extreme temperatures [32, 33]. Many studies have suggested that the black embryo is also caused by the *Alternaria* species, but they have not been identified, and such a relationship has not been shown, so the interpretation of the results is difficult. Most often, the correlation between symptoms and *Alternaria* contamination is absent [34].

One can make a few assumptions (null hypotheses) about what phenomena should affect the frequency of occurrence of *Alternaria* spp. in cereal crops seeds. It is obvious that weather and climatic factors can influence the development of grain Alternaria blight. In this case, the difference in weather conditions in different areas during one season and in one area in case of the change of seasons should lead to different average contamination in different regions in one year and in one area in different years. All of the above-mentioned *Alternaria* species are not a substrate-specialized; therefore, the species and variety of plants should not affect the contamination. Maize can be a single exception which has dense cob shells, which can prevent the penetration of the *Alternaria* infection in caryopsis. As the source of infection is the remains of any plants, including weeds and wild vegetation, the forecrop and crop rotation should not affect the development of Alternaria blight.

The question of the impact of another species of the same genus or other genera on the contamination with one of the *Alternaria* species is of particular interest. The fungi of the *Alternaria* genus penetrate only into the surface layers of the seed and occupy it not completely, and the presence of several strains and species of *Alternaria* and other fungi in different parts of the seed seems quite probable.

This paper presents the first results of the hypothesis tests that the average grain contamination by the *Alternaria* species does not differ significantly for different crops, varieties, and forecrops, but is different when comparing different regions and seasons.

The work objective is to determine the relationship between the contamination of cereal crops seeds by the representatives of two sections of the *Alternaria* genus and germination, plant species, growing conditions in the European part of Russia.

Techniques. The grains of wheat, barley, sorgo, and maize (422 samples from the harvest of 2010-2012) were received from 7 regions of the European part of Russia for the analysis. Most of the samples were wheat seeds from the South of the country — from Stavropol and Krasnodar Krai (2010, 2011 and 2012) (31, 53, 38 and 65, 64, 72 samples, respectively). In these regions, grain was selected in 22-28 areas.

Grain contamination with *Alternaria* spp. in the regions was compared at 48 samples of barley from 6 regions and 161 samples of wheat from 8 regions, in the regions − at the samples from Stavropol and Krasnodar Krai. Comparison by areas was performed in 3-fold repetition (harvest 2010-2012), by regions only once (harvest 2012). The selection of every year contains 16-72 samples of wheat of different varieties from 5-15 areas of each region. The area was represented by at least 3 samples from different farms.

The influence of conditions during the year was assessed on 72 wheat samples from 6 areas of Stavropol and Krasnodar Krai (harvest of 2010-2012, 3-6 samples from every area in one year).

To compare contamination of crops, 48 samples of barley and 76 samples of wheat (harvest 2012) from 6 regions of Russia, 5 samples of sorgo from

Samara Region (2012), 5 samples of maize from Lipetsk Region (2012) and 6 samples of maize from Krasnodar Krai (2011) were used. The influence of the variety was studied on 131 samples of wheat (harvest 2011 and 2012), representing 16 varieties from different areas of Stavropol and Krasnodar Krai.

During the study of the forecrop role, 74 samples of wheat of harvest 2010 from Krasnodar and Stavropol Krai were evaluated, grown after maize and sunflower (both regions), sugar beet (Krasnodar Krai), winter wheat, and fallow (Stavropol Krai).

The frequency of joint infection by the representatives of different *Alternaria* sections was determined using 235 samples of wheat and barley of harvest 2012 obtained from different regions. To assess the relationship between the contaminations with various dark-colored hyphomycetes, the same sets of grain 48 samples of barley harvest 2012 from 6 regions were used where infection by *Alternaria* species of *Alternaria* section and *Infectoriae* section was 3-80% and 0-50%, respectively, infection by *Bipolaris sorokiniana* was 0-66%, and by *Pyrenophora* spp. 0-37%.

The correlation of germination after contamination, considering it separately for viable and not viable seeds, was examined at 11 samples of 8 varieties of wheat of harvest 2011 from 6 districts of the Stavropol Krai.

Before all the tests, the seeds, for surface sterilization, were placed in 1% sodium hypochlorite solution for 2 min with constant stirring, rinsed with sterile water three times and transferred in the Petri dishes (10 seeds per a dish 90 mm in diameter) with potato-sucrose agar (PSA) [35], in which 0.002% Triton X-100 was added to slow the growth of fungi. Cultures were incubated during 7-10 days at 22 °C. Fungi of *Alternaria*, *Pyrenophora*, and *Bipolaris* genera were identified by direct scanning of colonies and by sporulation (the top-light microscope Stemi 2000C, Carl Zeiss AG, Germany, ×50 magnification). In case of impossibility of unambiguous identification, the colonies were individually deselected in Petri dishes with potato-carrot agar (PCA) [6] and incubated under 12/12 h photoperiod (the fluorescent lamps of 1000-2000 lux), identifying the habitus of sporulation and morphology of conidia [6, 35].

Mean values (M), mean errors (±SEM), statistical significance (p) by Fisher criterion (F-criterion) and the correlation coefficient (r) were calculated with Statistica 6.1 (StatSoft, Inc., USA).

Results. For further averaging and comparison, 9 selections were formed from the common pool (Table 1). Every selection (grain of one crop/crop variety of one year in one region/area) included 3-11 samples.

1. Characterization of cereal crop seed saplings used in assessing the impact of various factors on the contamination by fungi of *Alternaria* genus

Factor	Crop	Year	N	Sampling with regard to		
				regions	areas	cultivars
Region	Wheat	2012	161	8		
	Barley	2012	48	6		
Area	Wheat	2010	31	1 (Stavropol Krai)	16	24
		2011	53		18	25
		2012	38		11	25
		2010	65	1 (Krasnodar Krai)	24	26
		2011	64		16	22
		2012	72		15	28
Season	Wheat	2010-2012	72	2 (Stavropol and Krasnodar Krai)	6	
Crop	Wheat	2012	76	6		
	Barley	2012	48	6		
	Sorgo	2012	5	1 (Samara Region)		
	Maize	2012	5	1 (Lipetsk Region)		
	Maize	2011	6	1 (Krasnodar Krai)		

Continued Table 1

Variety	Wheat	2011	25	1 (Stavropol Krai)		5
		2012	9			3
		2011	43	1 (Krasnodar Krai)		6
		2012	55			11
Forecrop	Wheat	2010	65	1 (Stavropol Krai)		
			31	1 (Krasnodar Krai)		
Alternaria species interinfluence	Wheat, barley	2012	235	10		
Bipolaris and *Pyrenophora* species influence	Barley	2012	48	6		
Germination	Wheat	2011	11	1 (Stavropol Krai)	6	8
Total sampling	Wheat, barley, sorgo, maize	2010-2012	422	7	> 60	

N o t e. *N* — the number of samples. Gaps in the table mean that these characteristics were not essential for the experiment and were not taken into account during selection.

By the example of 8 regions, it was shown that regional features significantly affect the contamination of wheat and barley grain by the *Alternaria* species (Tables 2, 3). At the same time, the contamination by the representatives of different sections may vary several times depending on the region.

2. Average contamination of wheat and barley seeds with fungi of the *Alternaria* genus in different regions of the European part of Russia (harvest 2012)

Region	Wheat					Barley				
	N	Alt	±SEM	Inf	±SEM	*N*	Alt	±SEM	Inf	±SEM
Stavropol Krai	38	46.0	2.5	30.6	2.0	11	49.9	8.0	23.5	5.0
Krasnodar Krai	79	59.0	1.4	24.6	1.2					
Lipetsk Region	13	31.4	3.0	44.2	2.5	9	64.7	6.0	22.7	2.0
Kursk Region	10	38.8	4.4	38.2	2.7	9	39.1	6.5	17.1	3.6
Voronezh Region	6	47.7	9.0	38.2	3.8	8	34.9	4.1	35.4	4.5
The Republic of North Ossetia	6	46.2	9.2	5.5	2.7					
Leningrad Region	5	13.0	4.3	10.4	4.6	5	12.2	2.2	18.6	3.7
Tambov Region	4	37.0	5.8	42.3	4.1	6	37.7	7.7	29.7	8.0

N o t e. N — the number of samples; Alt — mean contamination (*M*) by *Alternaria* section species; Inf — mean contamination (*M*) by *Infectoriae* section species; ±SEM — error of the mean. Gaps in the table mean the absence of data.

3. Statistical significance (p) **of the combination of factors affecting the contamination of wheat and barley by fungi of *Alternaria* genus in Stavropol and Krasnodar Krai in different years**

Factor	Sample formation parameter	*Alternaria* section	*Infectoriae* section
Region	Wheat	< 0.001	< 0.001
	Barley	0.001	0.027
Area	Stavropol Krai, 2010	0.927	0.487
	Krasnodar Krai, 2010	0.748	0.917
	Stavropol Krai, 2011	< 0.001	< 0.001
	Krasnodar Krai, 2011	0.041	0.122
	Stavropol Krai, 2012	< 0.001	< 0.001
	Krasnodar Krai, 2012	< 0.001	< 0.001
Season (year)		< 0.001	0.012
Crop (wheat/barley)		0.975	0.304
Wheat cultivar	Stavropol Krai, 2011	0.872	0.109
	Krasnodar Krai, 2011	0.678	0.508
	Stavropol Krai, 2012	0.806	0.515
	Krasnodar Krai, 2012	0.595	0.272
Forecrop	Stavropol Krai, 2010	0.699	0.506
	Krasnodar Krai, 2010	0.493	0.723
Contamination:			
Bipolaris sorokiniana		< 0.001	< 0.001
Pyrenophora spp.		< 0.001	< 0.001
Alternaria section species		–	0.090
Infectoriae section species		0.090	–
Germination	Crop (wheat)	0.846	0.637

N o t e. Dashes in the table indicate that the selection included samples of different years from several regions.

Despite the significant fluctuations from year to year, when comparing the indicators for Stavropol and Krasnodar Krai, it is clear that, at average, in

the first case, the contamination by the *Alternaria* section members is lower, and by *Infectoriae* section is higher. The exception was in 2010 when the contamination of wheat with the species of the *Alternaria* section in both regions was low and did not differ significantly.

4. Average contamination (%) of wheat seeds by fungi of *Alternaria* genus in some areas of Stavropol and Krasnodar Krai in the years of examination

Area	*Alternaria* section			*Infectoriae* section		
	2010	2011	2012	2010	2011	2012
Stavropol Krai						
Georgievskii	15.3	30.7	31.8	43.3	56.7	20.0
Izobilnenskii	14.3	50.0	48.0	34.7	42.8	44.3
Novoaleksandrovskii	13.8	60.2	52.5	25.3	31.7	33.3
Predgornii	15.0	37.8	70.0	32.7	46.8	24.0
Average per Stavropol Krai	15.1	47.4	46.0	34.2	42.6	30.6
±SEM	1.4	2.0	2.5	3.0	2.1	2.0
min	10.3	17.3	22.3	25.3	29.4	14.2
max	15.3	60.2	70.0	43.3	74.0	45.0
Krasnodar Krai						
Kanevskii	9.0	52.2	52.0	20.8	48.5	33.0
Novokubanskii	16.5	56.5	58.3	19.8	29.3	31.0
Average per Krasnodar Krai	12.1	58.3	59.1	24.3	35.2	23.8
±SEM	0.9	1.5	1.4	1.6	1.1	1.2
min	7.0	17.3	38.8	14.0	29.4	8.6
max	19.0	60.2	71.5	28.3	74.0	38.0

5. Average contamination (%) of seeds by fungi of *Alternaria* genus in Stavropol and Krasnodar Krai for different wheat cultivars in the years of examination

Variety	*N* (*n*)	*Alternaria* section		*Infectoriae* section	
		M	±SEM	*M*	±SEM
Stavropol Krai, 2011					
Batko	6 (6)	50.0	5.4	44.7	4.5
Nota	6 (5)	45.8	5.6	47.1	4.1
Tanya	7 (4)	53.7	3.8	34.3	4.4
Evklid	3 (3)	50.4	7.9	28.5	2.2
Yunona	3 (3)	48.6	9.7	43.0	9.2
Stavropol Krai, 2012					
Batko	3 (2)	44.7	5.2	41.7	6.3
Viktoria Odesskaya	3 (3)	50.7	9.7	21.7	9.2
Grom	3 (3)	49.7	4.1	31.7	9.2
Krasnodar Krai, 2011					
Gratsiya	4 (4)	56.3	4.1	36.9	3.2
Irishka	7 (6)	65.3	3.8	29.0	1.2
Krasnodarskaya 99	3 (3)	53.1	3.2	39.1	3.2
Moskvich	12 (7)	61.3	2.7	33.7	4.4
Sila	4 (3)	59.3	4.3	33.4	2.0
Tanya	13 (10)	62.0	6.5	34.5	4.7
Krasnodar Krai, 2012					
Batko	4 (3)	63.8	5.7	29.0	6.1
Vassa	11 (6)	61.5	2.1	18.1	3.1
Grom	4 (4)	51.8	3.6	25.5	3.5
Delta	3 (2)	71.3	3.7	32.7	1.9
Irishka	3 (3)	49.3	9.8	16.3	8.2
Krasnodarskaya 99	3 (3)	57.3	6.7	16.7	2.7
Lebed	4 (4)	56.8	5.4	25.5	4.3
Liga	4 (3)	62.8	6.8	21.8	6.7
Moskvich	3 (3)	64.3	6.7	30.3	3.8
Nota	9 (6)	58.4	5.3	26.3	3.7
Tanya	7 (5)	58.9	5.9	28.3	4.4

Note. *N* — the number of samples, *n* — the number of areas.

The contamination of wheat seeds with the *Alternaria* genus species by areas varied in different years (Table 4). In Stavropol and Krasnodar Krai, the difference for the species of both sections in the areas was unreliable in 2010, but reliable in 2012 (see Table 3). In 2011, the influence of the area as a factor was statistically insignificant only for the *Infectoriae* section members in Krasnodar Krai. Area features were not reproduced from year to year. So, in one area, this figure could be one of the highest in the region for 2 years, but it decreased

in the 3rd year. In other words, contamination depends more on the weather conditions than on climatic or other physical and geographical factors.

A survey in 6 areas of two regions within 3 years (see Table 4) showes that the average contamination by species of *Alternaria* section in the area may vary almost 6 times over the years. The impact of the year, as expected, is significant (see Table 3). The contamination of seeds by the members of *Infectoriae* section varied less, 2.3-fold, but the difference was statistically significant.

The comparison of wheat and barley seed contamination by *Alternaria* genus fungi in 6 regions showed no significant difference (see Tables 2, 3). Maize grains were affected much less than others. The contamination of samples from Krasnodar Krai (6 pcs., 2011) and Lipetsk Region (5 pcs., 2012) by the species of *Alternaria* section was 0.2-0.4% (the *Infectoriae* section species were not revealed). At the same time, wheat infection in the same regions in 2011 and 2012 reached 32-59 and 24-44%, respectively. The contamination of 5 samples of grain sorgo from Samara Region was 29.2% at average for *Alternaria* section, and 0.6% for *Infectoriae* section.

The differences in wheat varieties by contamination by both *Alternaria* sections were insignificant in 2011 and 2012 (Tables 3, 5).

6. Average contamination (%) of wheat seeds by fungi of *Alternaria* genus with different forecrops (harvest 2010)

Forecrop	*N*	*Alternaria* section		*Infectoriae* section	
		M	±SEM	*M*	±SEM
Krasnodar Krai					
Maize	21	13.4	1.8	25.5	3.4
Sunflower	24	10.9	1.3	23.5	2.6
Sugarbeet	7	11.3	2.2	27.4	3.6
Others	13	12.5	1.5	22.2	2.9
Stavropol Krai					
Maize	3	15.0	1.1	24.0	4.0
Sunflower	3	11.7	2.0	31.7	1.2
Fallow	7	18.4	3.6	40.0	6.8
Winter wheat	9	16.3	3.1	38.4	6.3
Others	9	14.5	2.7	37.1	4.4

Note. *N* — the number of samples.

Contamination of wheat by *Alternaria* species does not significantly depend on the forecrop (Table 6). The comparison of the samples of wheat harvest 2010, grown after maize, sunflower, and other forecrops, does not reveal a significant difference for *Alternaria* and *Infectoriae* sections and in both Krasnodar and Stavropol Krai (see Table 3).

In case of high contamination with fungi of the *Alternaria* genus and other dark-colored hyphomycetes, their joint growth from one seed was often observed. The relationship of *Alternaria* infection with the presence of *Bipolaris sorokiniana* and *Pyrenophora* spp. was negative and moderate, $r = -0.69$ and -0.61, respectively, for *Alternaria* section, and $r = -0.64$ and -0.61 for *Infectoriae* section, at low significance levels (see Table 3). In wheat and barley, the fungi of *Alternaria* genus (*Alternaria* section) and *Fusarium* genus (*F. langsethiae*, *F. tricinctum*, and *F. graminearum*) were found in the same caryopsis. Sometimes, the intensive growth of *Alternaria* and the weak growth of *Fusarium* from the same caryopsis was observed. However, in most cases, *Fusarium* fungi grew faster and suppressed the growth of *Alternaria*, often covering the colony with its mycelium (reliable identification of *Alternaria* was often impossible). In exceptional cases, the colonies of *Fusarium* sp. and *Alternaria* of *Infectoriae* section or *Fusarium* sp. and two *Alternaria* isolates from different sections grew from the same caryopsis simultaneously.

Two *Alternaria* isolates, belonging to different sections, were separated

from some grains. If one imagines that these species do not compete with each other, being in the same seed, the frequency of their joint meeting should be equal to the product of the frequency of occurrence of each. However, the species of *Alternaria* section usually start growth and sporulation earlier and do it more intensively than the species of the *Infectoriae* section. Therefore, in some cases of the joint growth, the colonies of the *Infectoriae* species are probably unnoticed. In other words, the use of traditional assessment methods makes the observed rate of co-infection slightly lower than it is expected in theory. For 235 samples of wheat and barley (harvest 2012) from different regions, the product of the frequency of occurrence of the representatives of every section separately was calculated. The expected frequency of co-infection was 2 times lower than the actual proportion of seeds with co-infection (0.12 vs. 0.06). To understand whether there is an antagonistic effect, two correlation coefficients were calculated. For the expected and the actual percentage of the seeds with joint infection, it was high ($r = 0.81$, $p < 0.001$), and the relationship between the infection with the species of sections A*lternaria* and *Infectoriae* was weak ($r = 0.31$, $p > 0.090$). It does not confirm the positive or negative influence of the representatives of different sections on each other.

The relationship of germination and contamination of *Alternaria* spp. turned out to be unreliable (see Table 3). At average, the contamination of germinated and non-germinated seeds differed by –1.6% for the representatives of *Alternaria* section and by +2.1% for *Infectoriae* section, which was less than the standard error of the average.

The group of small-sporous species of *Alternaria* is cosmopolitical. They are found on all continents, on various plant substrates, in natural and agrophytocenoses, in the street and indoor air [36]. These fungi are common on cereals in different parts of the world. The species of *Alternaria* section infect grain often and everywhere [1] and are very plastic eurybiontic fungi. The species of *Infectoriae* section are also common in grain in many regions of the planet, but almost never occur, for example, in the South of the Far East. Our data show that in the European part of Russia, the types of both sections are extremely common in all regions. The average contamination varies from region to region and from year to year. Within the boundaries of the region in different areas, this indicator may vary greatly or may be similar in relation to a particular year. The differences between the areas of seed contamination proved to be significant only in 2011 and 2012. The reason is that in 2010, the average contamination with fungi of the *Alternaria* genus was lower than in subsequent years. This was especially true for the *Alternaria* section (the figure in 2010 in the two most studied regions is about 3 times lower than in 2011 and 2012). The absence of significant differences in 2010 might also be associated with a smaller number of the analyzed samples from Stavropol Krai. These trends indicate that the leading factors affecting the contamination of grain by *Alternaria* are the weather conditions.

Different crops were contaminated with small-sporous *Alternaria* almost equally. Wheat and barley were infected greatly. Previously, it was shown that rye and oat are equally susceptible to the infection [1, 37]. Maize was infected in the least degree. Resistant cultivars were not revealed. Resistance to some necrotrophic weakly specialized species, if detected, has a quantitative character. In the case of widely specialized *Alternaria* species, which do not actually infect the seed but only colonize its covers, the detection of resistant varieties is highly improbable. These fungi can develop and persist on any plant residues and many plants, including weeds. It means that the sources of inoculum are abundant in almost all phytocenoses. Despite the relatively large size, the *Alternaria* spores can spread in the air. Therefore, a large number of *Alternaria* conidia are found almost every-

where in the boreal non-arid zone. Forecrops and crop rotation, as expected, have little effect on the *Alternaria* contamination due to aerogenicity rather than the soil origin of the infection and because almost any plant residues, including those outside the field, could be a sufficiently productive source of spores.

In the review by F. Culshaw et al. [38], the facts demonstrating the lack of influence of *Alternaria* spp. on the size and germination of caryopsis are considered. The authors have also shown the lack of relationship between the infection rate and laboratory germinating capacity. It is known that the infection occurs after flowering, and the embryo is not affected by the *Alternaria* mycelium. The saprotrophic nature of the *Alternaria* species associated with cereal crops is manifested in the fact that only the seed coat is affected. Therefore, the embryo non-infected by *Alternaria* can germinate and, under favorable conditions for the plant, will not be infected by these fungi growing from the seed coat.

Specific weather conditions (temperature, humidity, precipitation) during the vegetation season and some peculiarities of the agricultural technology (crop density, irrigation, fungicides) should be included in the number of underinvestigated factors affecting the spread of fungi of the *Alternaria* genus in grain crops. The influence of the *Alternaria* species on field germination under different weather and edaphic parameters is still an important issue.

Thus, regardless of the variety, wheat, barley, rye, and oat grains are about equally infected with fungi of *Alternaria* genus from *Alternaria* and *Infectoriae* sections. Maize is infected in a less degree. The significant influence of the forecrop (maize, sunflower, sugar beet, fallow, winter wheat) on the proportion of the infected seeds of wheat is not found. Weather and climatic conditions play a decisive role in grain contamination. We revealed negative correlation between the contamination of barley grain by *Alternaria* species and some other fungi (*Bipolaris sorokiniana* and *Pyrenophora* spp.) and the lack of a reliable relationship between the contamination of wheat and barley seeds by species from both sections. The inconsistency of the results of previous studies may be the consequence of incorrect attributing species (with regard at least the section).

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THE FORMATION OF EMBRYONIC INFLORESCENCES AND REALIZATION OF PRODUCTIVITY POTENTIAL OF COMMERCIAL GRAPE VARIETIES IN THE TEMPERATE CONTINENTAL CLIMATE OF SOUTHERN RUSSIA

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Abstract

In the Russian Federation, the main production of grapes is concentrated in the southern regions, in unstable weather conditions of a temperate continental climate. When the weather conditions deviate from the optimal values for the grape plants, they experience stress. Embryonic inflorescences are thr most sensitive to abnormal air temperatures in winter, during forced rest of plants. After wintering, the plant loses part of the inflorescence, and the expected harvest of grapes decreases. A degree of realization of the potential of economic productivity is on the average 60 %. In these conditions, the problem of preserving embryonic inflorescences during the wintering of plants, increasing the degree of realization of the potential for economic productivity in varieties of *V. vinifera* grapes is topical in the Russian viticulture. The aim of this work is to reveal the degree of setting and preservation of embryonic inflorescences during wintering and the level of realization of the productivity potential in grape varieties of different eco-geographical origin. The work was carried out in the vineyards of the Krasnodar Territory with the use of field and laboratory methods on varieties Ekaterinodarskii in the zone of covering viticulture (CJSC Novokubanskoye) and the European-Asian species *Vitis vinifera* L. — *Convar orientalis* Negr. (eastern group), *Convar occidentalis* Negr. (Western European group), *Convar pontica* Negr. (group of the Black Sea coast), as well as interspecific and intraspecific hybrids in the zone of unguided viticulture on the ampelographic collection (the city of Anapa). Minimum air temperature is the main limiting factor which impacts on grape productivity potential under environmental conditions of the Southern Russia. The dependence of embryonic inflorescence initiation and yield formation on the winter minimum air temperatures was determined on the 72 of grape varieties of different ecogeographic origin. Intergroup hybrids of the *V. vinifera* (88 %), grape varieties of the *orientalis* group (85 %), *occidentalis* group (80 %), *pontica* group (74 %) and interspecific hybrids (60 %) showed the greatest destruction of wintering buds and the smallest preservation of embryonic harvest of technical grades when the minimum temperature reduced to critical values (–27 °C). Table grape cultivars of *occidentalis* group were the most affected by cold (83 %), the *orientalis* cultivars were less affected (80 %) followed by intergroup hybrids (78 %), interspecific hybrids (68 %), and *pontica* plants (65 %). The productivity level after wintering depended closely on the number of surviving embryonic inflorescence at the beginning of vegetation ($r = 0.77$). A total of 25 % varieties studied realized their productivity traits inefficiently (only to 50 %), 35 % of the showed 50-60 % of potential productivity, the next group of 27.5 % showed a moderate efficiency (60-70 %), and only 12.5 % of the varieties ensured high (over 70 %) of productivity potential. As a result of the survey, the varieties were selected for practical use and breeding programs with a satisfactory and high adaptability. These are Pinot Franc (60 %), Aligote (61 %), Codreanka (62 %), Pearl of the Hall (63 %), Saperavi (64 %), Chardonnay (64 %), Kunlean (64 %), Bianca (65 %), Kutuzovsky (66 %), Cardinal (68 %), Sauvignon (68 %), Firstborn Magarach (69 %), Riesling (74 %), Alan (74 %), Traminer (75 %), Krasnostop Anap (83 %), and Citron Magarach (86 %).

Keywords: grapes, environment, adaptivity, embryonic inflorescences, growth, wintering, productivity

Grape fruiting is preceded by the period consisting of the successive

stages in the small (annual) cycle of the plant ontogenesis. The main stages are setting and differentiation of embryonic inflorescences in the buds of wintering eyes in the year before fruiting; differentiation and wintering of embryonic inflorescences; predifferentiation of embryonic inflorescences in the buds (eyes) after wintering; growth of inflorescences and flowering; growth and ripening. Every stage has its specific functions, the realization of which subsequently determines the economic productivity of grape [1].

It was found that setting and differentiation of embryonic inflorescences, as well as crop formation stages, are variable and depends on the biological peculiarities of cultivars, the environmental conditions of plants habitation, and the anthropogenic factors. In the environmental conditions of the Crimea, the Cardinal, Moldova, and Italiya varieties have setting of first generative sprouts before flowering, with the second one after flowering [2]. Differentiation of inflorescences continues during the rest period at positive air temperatures [3, 4]. The biggest and most developed embryonic inflorescences are located in the eyes of the middle part of the shoot [5-7]. The formation of the embryonic fertility in the wintering buds is closely related to the origin of grape varieties [8-11]. The phytosanitary condition of plants has a significant impact on the embryonic fertility. Even middle development of anthracnose and oidium means that shoots fertility is significantly reduced [8]. Abiotic environmental factors significantly affect the productivity of grapes at all stages of setting and harvest formation [12, 13].

The main production of grape in the Russian Federation is concentrated in the southern regions, in the conditions of a temperate continental climate. Unlike in Europe, the vineyards are affected by weather anomalies here. The greatest damage to viticulture is caused by minimum temperatures during the period of induced dormancy. If the weather conditions deviate from the optimal once every five years, the plants experience stress, the damages of reproductive organs are observed, the growth processes and the stability of fruiting are disturbed, energy is spent on their recovery. The degree of realization of the economic productivity potential of is 60% on average [14]. The main reason for poor fruiting is the low adaptability of most of the varieties used in unstable weather conditions [15], the damage of the main and additional buds by frost [16, 17]. If during the rest period, the damage of the generative organs and the decrease in productivity because of the minimum air temperatures are often observed, then, during the vegetation, a similar reaction of grape plants to high-temperature and water stresses occurs [18].

To increase the grape productivity, the awakening of buds is activated by special techniques [19, 20]. Another approaches are agrotechnical regulations, including the load with wintering eyes [21, 22], ring-barking to realize the potential productivity of buds [23], and breeding for stress-resistant varieties [24, 25]. The fruitfulness of the buds, depending on soil composition in the vineyards [26], and the influence of the plantations age on the productivity of grapes are also accounted [27].

The study of embryonic inflorescences setting and their wintering safety in terms of grape plant productivity potential is still fragmented. This paper is the first report about death rate of shoot buds and stability of grape varieties of different eco-geographical origin during wintering as influenced by extremal minimum air temperatures in a temperate continental climate of the south of Russia.

The aim of the research is to determine the dependence of the setting, the preservation of embryonic inflorescences and the degree of the potential realization of economic productivity among the grape varieties of *V. vinifera* on

minimum air temperatures in the period of forced dormancy of plants in the sever climate conditions.

Techniques. In the study we used grape variety Ekaterinodarskii (ZAO Novokubanskoe, Krasnodar Krai) and plants of European-Asian species *Vitis vinifera* L. from the Russian ampelographic collection (the city of Anapa, Krasnodar Krai) of different eco-geographical origin, i.e. *Convar orientalis* Negr. (Eastern group), *Convar occidentalis* Negr. (Western European group), *Convar pontica* Negr. (Black Sea coast group), as well as interspecies and intraspecies hybrids. Plantings of 1997 were grafted and fruiting. Observations were carried out in 1997-2012, including 2006, 2010, and 2012 with the abnormal minimum air temperatures in winter. Common field and laboratory tests were used [28].

Embryonic fertility was determined using stereoscopic microscopy (MBS-10, Opticheskie pribory, St. Petersburg, Russia) during deep (physiological) dormancy by viewing the central buds of wintering eyes on typical shootsvselected diagonally from the site of every variety [2]. The character and degree of buds damage by low temperatures during wintering were assessed visually on the longitudinal section of wintering eyes. Healthy buds had a bright green color on the section; the dead ones were dark brown or black.

The development of shoots was determined by direct calculation during active regrowth period (in May). To assess the productivity (yield) of grape bushes, grape clusters at ripening (in September) were cut, counted and weighed. The degree of realization of productivity potential was calculated as the ratio of the average to the maximum possible yield under favorable conditions.

The correlation analysis of parameters characterizing viability of wintered buds and development of shoots and grape clusters was carried out, regression equations were calculated.

Results. The investigation was carried out in the places of the greatest concentration of commercial grape plantations in the agroecological conditions of the South of Russia. In the years of setting and subsequent differentiation of inflorescences, weather conditions, in general, except for abnormal phenomena, were favorable for physiological, biochemical, and growth processes, as well as the formation of the grape harvest.

The optimum temperature for the beginning of blooming, formation, and differentiation of embryonic inflorescences is 25-30 °C, the sum of active temperatures is 380 °C. In case of a drop in the temperature to 15-16 °C [30], blooming is slowing down, the growth of the pollen tube stops. In Krasnodar Krai, blooming takes place most often in the first decade of June. In this and subsequent periods, the temperature conditions do not exceed the optimal values. Over the last 37 years, the average daily temperature in early June in the Black Sea open-earth grape growing area in Anapa was 19.1 °C on average, in the central cover-earth area (ZAO Novokubanskoe) was 19.3 °C. The maximum temperature during this period in Anapa went up to 32.0 °C, in ZAO Novokubanskoe to 34 °C, the minimal temperature fell respectively to 8.0 and 2.0 °C. In the period of inflorescence differentiation from the second decade of June and until the physiological dormancy (in September), the temperature in Anapa was 21.8 °C on average, in ZAO Novokubanskoe 21.2 °C. In these conditions, embryonic inflorescences of the Aligote variety were set for 89% of eyes at average, with 92% for Moldova, 94% for Ekaterinodarskii and 97% for Podarok Magaracha.

The viability of embryonic inflorescences of wintering buds is the important for the full realization of the economic productivity of grapes. Abnormal minimum air temperatures during wintering has the strongest impact on embry-

onic inflorescences and productivity of grape varieties in the coming period of plant vegetation. After wintering, as a rule, the plant loses some of the inflorescences, and the expected harvest of grape is reduced.

The assessment of the state of eyes after wintering showed a close dependence of the preservation of grape reproductive organs on the temperature regime ($r = 0.8$). With the gradual decrease in temperature, the percent of the dead eyes increased. According to the information from the curve trend line, the frost-resistant variety Ekaterinodarskii had the critical destruction of the eyes (50%) in winter at temperatures –23... –24 °C (Fig. 1).

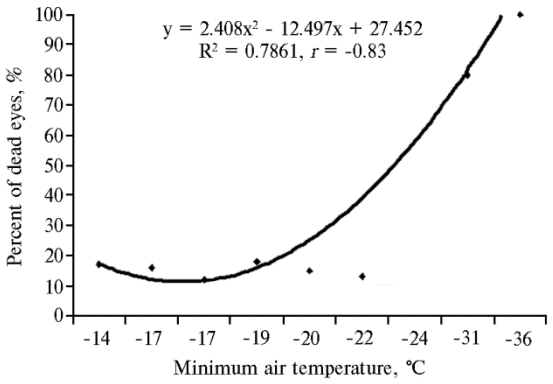

Fig. 1. The percent of dead buds in grape (European-Amur hybrid of *V. vinifera* × *V. amurensis*) Ekaterinodarskii variety in relation to the minimum temperature (ZAO Novokubanskoe, Krasnodar Krai, 2004-2012).

The decrease in air temperature to critical values in January 2006 (the city of Anapa) gave the opportunity to trace the effect of stressful wintering conditions on the safety of the eyes on grape fruit shoots in a large number of varieties. In case of the decrease in the temperature up to –27 °C, numerous damages of eyes in the group of technical varieties have been detected. The greatest death had the intergroup hybrids of *V. vinifera* (88%), then followed *Convar orientalis* Negr. (85%), *Convar occidentalis* Negr. (80%), *Convar pontica* Negr. (74%), and intraspecific hybrids (60%). In the group of table varieties, the Western European ones (83%) were the most affected, the Eastern and Eastern Mediterranean ones (80%), intergroup hybrids (78%), interspecific hybrids (68%) were less affected, and the varieties of the Black Sea basin group were the least damaged (65%).

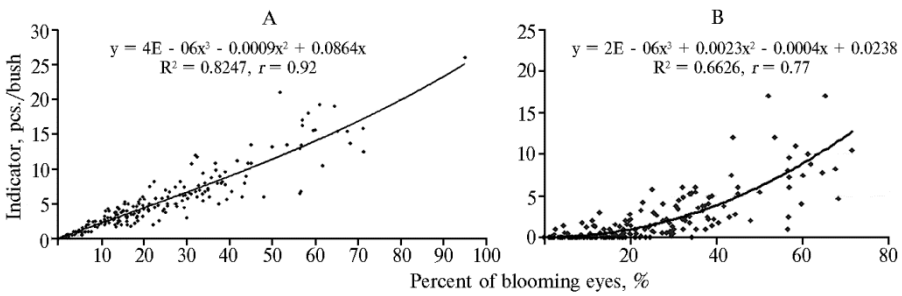

Fig. 2. The number of green shoots (A) **and formed clusters** (B) **in relation to the resistance of grape (*Vitis vinifera* L.) varieties to frost (–27 °C)** (the ampelographic collection, Krasnodar Krai, the city of Anapa, 2006).

The low-temperature stress and the death of embryonic inflorescences affected the development of shoots and the productivity of grape bushes. Varieties with high adaptability had more developed green shoots and formed full clusters of grapes. The dependence of the regrowth of green shoots on the adaptability of varieties (in the proportion of surviving eyes) was high, $r = 0.92$ (Fig. 2). Similarly, the increase in grape productivity with the increase in the adaptability of varieties was observed. With the increase in the resistance of varieties to frost, the number of clusters on the bushes is increased as well, $r = 0.77$ (see Fig. 2).

1. Resistance of grape (*Vitis vinifera* L.) varieties to frost (–20 °C) in relation to their origin (the ampelographic collection, Krasnodar Krai, Anapa, 2010)

Origin	Percent of blooming eyes										Total	
	0-20		21-40		41-60		61-80		81-100			
	The number of varieties											
	pcs	%	pcs	%	pcs	%	pcs	%	pcs	%	pcs	%
Interspecies hybrids	0	0	4	5	12	15	33	40	33	40	82	100
Intraspecific hybrids	0	0	10	10	17	17	40	40	34	33	101	100
Convar occidentalis Negr.	0	0	0	0	0	0	2	50	2	50	4	100
Convar orientalis Negr.	1	3	1	3	6	16	19	51	10	27	37	100
Convar pontica Negr.	0	0	3	15	0	0	11	55	6	30	20	100
Unknown origin	0	0	4	8	10	20	20	39	17	33	51	100
Total	1	0,3	22	7,5	45	15	125	42	102	35	295	100

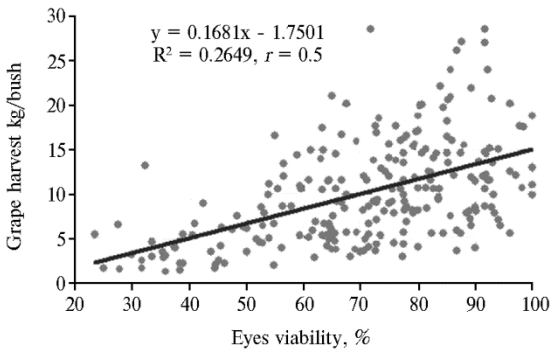

Fig. 3. The dependence of the grape yield (*Vitis vinifera* L.) on buds safety after wintering (–17 °C) (the ampelographic collection, Krasnodar Krai, the city of Anapa, 2010).

In 2010, at the decrease in the temperature up to –20 °C, the identified patterns were preserved, in general. Among interspecies hybrids, the death of eyes was insignificant (Table 1). The greatest damage was noted among the varieties of the Eastern ecological and geographical group.

The degree of the realization of potential productivity after wintering is in close dependence on the number of survived embryonic inflorescences to the beginning of vegetation. The correlation coefficient in the group of table varieties (240 pcs.) after stressful wintering in 2010 (–17 °C) showed a high dependence of the grape productivity on the safety of the eyes and was 0.513 (Fig. 3).

2. Realization of potential productivity (%) among grape (*Vitis vinifera* L.) varieties in the agro-ecological conditions of the Krasnodar Krai (the ampelographic collection, the city of Anapa, 1997-2006)

Variety	Indicator	Variety	Indicator
Biruintsa	36	Podarok Magaracha	58
Lyana	38	Pino Blanc	59
Dekabrskii	45	Viorika	59
Muscat Ottonel	46	Pino Franc	60
Rannii Magaracha	46	Aligote	61
Karaburnu	46	Codreanka	62
Strashenskii	46	Zhemchug Zala	63
Sukholimanskii	48	Saperavi	64
Vostorg	48	Chardonnay	64
Muscat Amber	48	Kunlean	64
Muscat Bessarabian	51	Bianca	65
Muller Thurgau	51	Kutuzovskii	66
Shasla	53	Cardinal	68
Italiya	54	Sauvignon	68
Tsimlyanskii chernii	55	Pervenets Magaracha	69
Krasnostop	56	Riesling	74
Kaberne	56	Alan	74
Moldova	58	Traminer	75
Rkatsiteli	58	Krasnostop Anapskii	83
Avgustin	58	Tsitronnyi Magaracha	86

The degree of realization of potential productivity varied greatly among the most popular varieties (36-86%). From their total number, 25% realized the potential of economic productivity ineffectively, up to 50%, 35% had 50-60%,

the next group of varieties (27.5%) was characterized by moderate productivity, 60-70%, and only 12.5% of varieties had a high rate, more than 70% (Table 2).

The degree of realization of potential productivity varied greatly among the most popular varieties (36-86%). From their total number, 25% realized the potential of economic productivity ineffectively, up to 50%, 35% had 50-60%, the next group of varieties (27.5%) was characterized by moderate productivity, 60-70%, and only 12.5% of varieties had a high rate, more than 70% (Table 2).

Thus, the natural soil-climatic conditions of the South of Russia are in general favorable for the formation of a good harvest in all periods of the small (annual) cycle of the grape ontogenesis. However, the periods of deep (physiologic) and induced dormancy are the exceptions. The embryonic inflorescences formed in the buds of the wintering eyes at this time are subjected to the destructive effect of minimal air temperatures. When the temperature drops to the critical values –27 °C, the greatest destruction of the wintering eyes and the lowest preservation of embryonic inflorescences among technical varieties are among the intergroup hybrids of *V. vinifera* (88%), and among table varieties in those of *occidentalis* group (83%). The productivity of varieties after wintering is in close dependence ($r = 0.77$) on the number of survived embryonic inflorescences to the beginning of vegetation. Preservation of embryonic inflorescences during wintering, exploitation of grape varieties highly adaptive to unstable weather and climatic conditions should be recommended to increase the sustainability and productivity of the ampelocenosis in the moderate continental conditions of the South of Russia.

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SOME FEATURES OF EMBRYOLOGY OF *Rosa spinosissima* L., *R. canina* L. AND *R.* × *damascena* Mill. INTACT AND VIRUS-INFECTED PLANTS

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Abstract

Due to high decorative and economically valuable traits, roses have long been used in garden and park construction, and as medicinal and aromatic raw materials. Selection carried out with roses for many years, has resulted in more than 30 thousand cultivars. To create new forms and varieties, it is necessary to in-depth know species biology, including developments of generative elements. This paper shows the study of male and female generative structure formation in two wild species of *Rosa* genus, *Rosa spinosissima* L. and *R. canina* L., naturally grown on the north-eastern slope of Mount Chatyr-Dag, and in two *R.* × *damascena* Mill. cultivars, which may be involved in breeding as parental plants. As a result, we have determined the main embryological types of generative sphere formation in these species. It has been shown that the anthers are four-nested with two thecas, the wall of microsporangium is formed centripetal. A formed wall of microsporangium consists of epidermis, endothecium, 2-3 middle layers and tapetum, which is a derivative of a secondary parietal tissue. A tetrad of microspores is formed simultaneously. Differentiation mitosis in microspores leads to the formation of 2 celled pollen grains. Mature pollen grains are 2 celled with 3 pores. Microsporangium wall of the mature anther is represented by the epidermis covered with cuticle, fibrous endothecium and remnants of a middle layer. Morphogenicity and viability of mature pollen of these species and varieties have been established (abnormal pollen grains can be up to 30 %). Pollen sizes were measured. The diameter of pollen grains varies from 19 to 30 microns. The influence of viral infection on the formation of pollen grains has been shown, which causes an increase in the number of pollen abnormalities, changes the pollen grain size and ultimately leads to pollen grain degeneration. Defective pollen grains can be up to 50 %. Gynoecium of rose consists of many free carpels, each contains 1-2 ovules. Rosa ovule is anatropous, bitegmal, crassinucellate. Multicellular hypostase with thick-walled cells is formed in chalasa area. Micropyle is formed by an internal integument, which in its development is ahead of the outer one. Archesporium is polycelled and differentiated in the subepidermal layer of ovule. As a result of meiosis, linear tetrads of megaspores are mostly formed, of which chalasal or epichalasal megaspores were functionally active to derive embryo sacs of Polygonum-type. Mature embryo sacs are 8 nucleate and 7 celled. Egg apparatus consists of egg and 2 synergids, antipods are represented by three cells. Majority of embryo sacs gradually are obliterated, and only one of them matures. The studied species of genus *Rosa* are entomophilous plants pollinated by insects. It has been concluded that in selection, while choosing parental forms for hybridization, attention should be paid not only to the viability of their generative structures, but also to plant viral infection, since the presence of pathogens has led to a significant decrease in reproductive ability.

Keywords: *Rosa* sp., male and female generative structures, viral infection, reproductive ability.

Due to the variety of forms, abundant and long flowering, the rose has long been used in garden and park construction. The first information about the cultivation of roses was found for 4 thousand years BC; later, more than 30 thousand cultivars with decorative [1-3] and valuable medicinal characteristics [4-7] were created. Nowadays, the range of roses resistant to environmental stress factors, diseases, and pests is expanding [8-10]. Cytological and genetic investigations

have shown the possibility to obtain polyploid forms [11-13]; the in vitro culture method has been widely used for the cultivation and reproduction of valuable genotypes of roses in recent decades [14-17]. It is important to mention that different species of the *Rosa* genus [18-20] can be used as medicinal plants.

The *Rosa* genus has a wide geographic range, is widely studied and actively used in hybridization [21-24]. The genus includes 10 sections, including *Pimpinellifoliae* and *Caninae*, the representatives of which, along with others, serve as the original forms of garden varieties [6, 25-28].

The active development of industry and agriculture, the increase in air and soil pollution often lead to abnormal development of the generative sphere of plants; therefore, when conducting breeding, careful selection of parental forms is necessary. In this paper, the authors have presented the results of the investigation of the processes of male and female gametophyte formation in two prospective initial forms used in roses selection, *Rosa spinosissima* L. and *R. canina* L., and two high-yielding varieties of essential-oil-bearing rose (*R.*× *damascena* Mill.), Dzhalita and Festivalnaya. In addition, the quality of pollen among visually healthy and virus-infected plants was assessed.

The aim was to identify the features of the development and construction of generative structures for the representatives of the three species from the *Rosa* genus in normal and viral infection.

Techniques. Collection of samples from *Rosa spinosissima* and *R. canina* for making permanent preparations and observation of blooming were carried out in Crimea in 2016 under natural vegetation of the studied species in different populations on the North-Eastern slope of the Chatyr-Dag mountain at the altitude of 750-868 m above the sea level. The biomaterial of Dzhalita and Festivalnaya cultivars (*R.* × *damascena*) was taken in the collection of Nikitsky botanical garden.

To make permanent cytoembryological preparations, buds at different stages of development were fixed in the Carnoy mixture containing 96% aqueous solution of ethyl alcohol, chloroform, and glacial acetic acid in the 6:3:1 ratio. The material was dehydrated with butyl alcohol and xylene and then enclosed in paraffin [34, 35]. Paraffin serial sections with the thickness of 10-12 microns were prepared with a rotary microtome MRTU (Russia). The preparations were stained with methyl green-pironin with additional staining with Alcian blue [38] and viewed (light microscopes Jenaval and AxioScope A. 1, Carl Zeiss Microscopy GmbH, Germany). PowerShot A550 digital camera (Canon, Inc., Malaysia) and AxioCamERc5s image analysis system (Carl Zeiss Microscopy GmbH, Germany) were used for documentation.

Temporary preparations of average pollen samples colored by 1% acetoorcein were used for cyto-morphological characterization of pollen grains of *R. canina*, *R. spinosissima*, and *R.* × *damascena*. Morphologically normal and defective grains were counted. The first group includes the grains with homogeneous coloring, a clearly expressed core of the vegetative cell, and the generative cell. Pollen grains with visual signs of cytoplasm and nuclei destruction of vegetative and generative cells were considered defective. Their percent was determined as a ratio of the number of pollen grains with abnomalities to the total number of the recorded pollen grains for every species or variety. The diameter of pollen grains was measured on permanent preparations of average pollen samples [37] (AxioVision Rel.4.8 software, Carl Zeiss Microscopy GmbH, Germany).

Statistically significant differences in the proportion of defective pollen grains from anthers of *R.* × *damascena* with visual signs of infection and asymptomatic ones were determined by φ-angle transformation of Fischer (F_φ)

if $p < 0.05$ [36]. Data processing, with descriptive statistics and comparison of samples on the diameter of plants pollen grains with and without viral infection, was carried out with Statistica 6.0 (StatSoft Inc., USA), using Student's *t*-criterion. The arithmetic mean values (*M*) and standard errors of means (±SEM) are presented.

Results. Rosa spinosissima L. (syn. *Rosa pimpinellifolia* L.) is a shrub up to 2 m with prickly shoots. Buzzes are of different sizes, thin, straight, usually extended at the base. Leaves are with 5-11 folioles and naked stipules. Flowers are single, on long pedicels, sepals are simple, up to 15 mm long, remaining with fruits. Corollas are white, up to 5 cm in diameter, petals are sinuate, hypanthia are globular, baculums form a large head of the stigma. Fruits are up to 15 mm long, spherical or flattened-spherical, ripened, of dark color (up to black). Despite the rather extensive geographic range, the population size of *R. spinosissima* is reduced, so it was listed in the Red Books of some regions, such as Kursk Region and the Republic of Khakassia [29, 30]. *R. spinosissima* is the ancestor of many varieties, in particular, the extensive group of Scott's roses, which are known since 1600 and, according to modern ideas, belong to the Hybrid Spinosissima class [31]. In the past, many authors examined *R. spinosissima* and *R. pimpinellifolia* as different species, but molecular studies of samples from natural populations of Great Britain have shown that they are of one species [10]. Rose varieties selected on the basis of *R. spinosissima* are resistant to diseases and frost [32]. *Rosa canina* L. is a deciduous shrub, reaching a height of 2.0-2.5 m. Shoots are thick, arched curved. Buzzes are rare, sickle-shaped, with a very short base. Flowers are single or 3-5 flowers collected in the apical cymose inflorescence. The corolla is white or bright pink, with a diameter of 5-8 cm; sepals are broad-lanceolate, with abundant pinnate appendages, bend back after flowering and fall off long before fruits ripening [33]. The fruits are smooth, glossy, orange-red with many pilose seeds. *Rosa* × *damascena* is a hybrid form of *R. gallica* and *R. moschata*. DNA analysis also showed the presence of the third species, *R. fedtschenkoana,* in the genealogy of this hybrid. The Dzhalita variety is a shrub reaching a height of 2 m; flowers are pink with a lilac hue, 6-7 cm in diameter with soft orange-yellow petals, have a delicate aroma. The Festivalnaya variety is a shrub up to 170 cm of height, with pink or pale pink flowers, 5-6 cm in diameter, with a strong aroma.

All the studied wild species and varieties are entomophilous plants, pollen of which is carried by insects. As a result of effective processes of pollination and fertilization, aggregate accessory fruits formed by stone fruits enclosed in fleshy hypanthiums, bright color of which attracts birds, develop. The rose blooms in May-June in the Crimea. Flowers are actinomorphic, ambisexual, 5-membered, *R. spinosissima* and Dzhalita and Festivalnaya have fragrant flowers, *R. canina* flowers are odorless. The androecium is represented by a great number of stamens, located in circles. First, the outer stamens were opened, then the inner ones. Anthers are 4-locular, 2-sided. The placentoid juts out inside the microsporangium. The microsporangium wall is formed centripetal. First, the archesporial cell differentiated in the subepidermal layer, which formed the primary parietal and sporogenous cells by fission. The latter forms multi-layer sporogenous tissue. The parietal cell forms secondary parietal cells by division, one of which produces tapetum, and the other, continuing to divide, gives endothecium and middle layers. The formed microsporangium wall consists of the epidermis, endothecium, 2-3 middle layers, and tapetum (Fig. 1, B). After all divisions in the sporogenous tissue, the cells form a separate structure, surrounded by deposited callose, and microsporocytes appear in which meiosis and simultaneous formation of the microspore tetrad occur. The arrangement of micro-

spores in the tetrad, like in species of *Malus* genus [35], is tetrahedral and isobilateral (see Fig. 1, C, D).

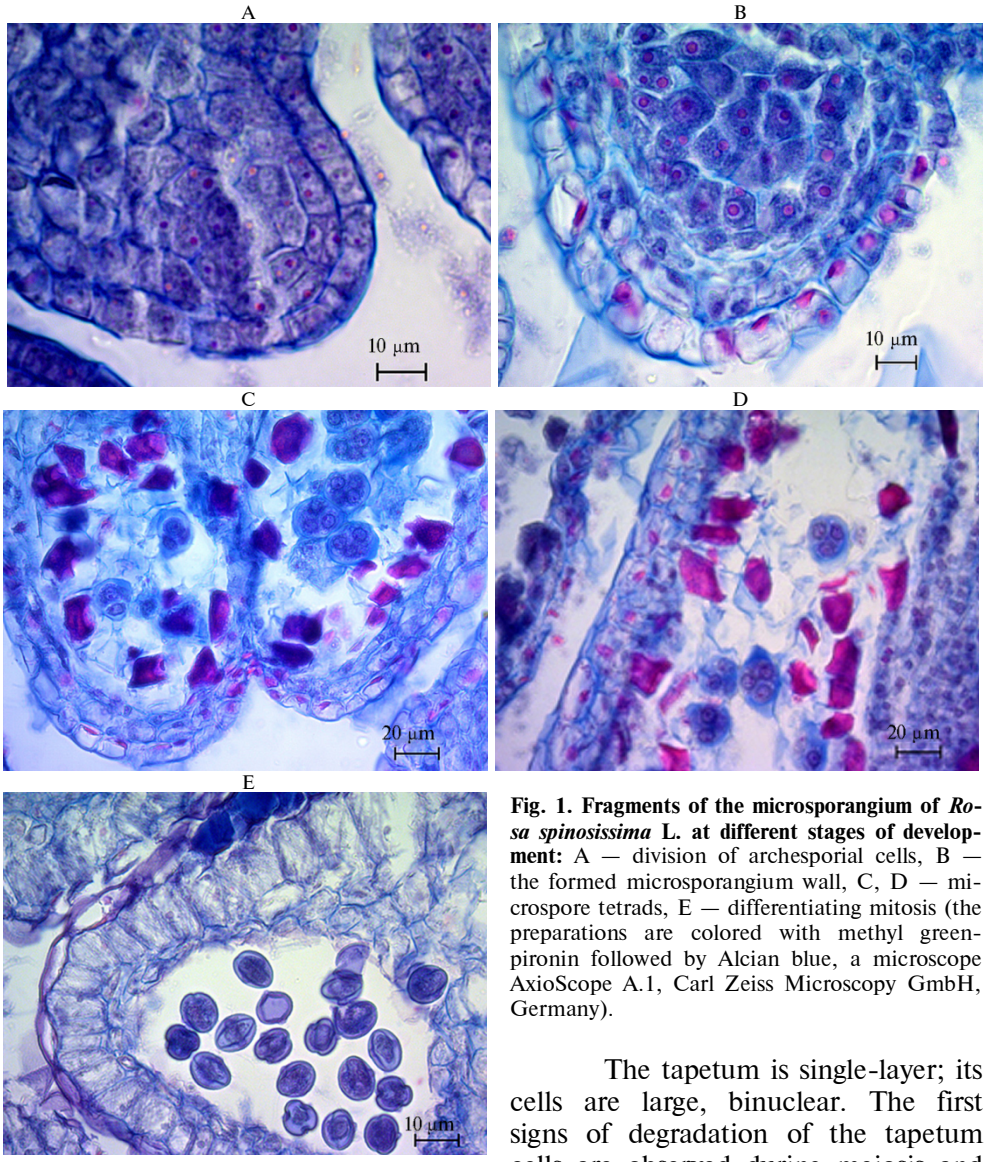

Fig. 1. Fragments of the microsporangium of *Rosa spinosissima* L. at different stages of development: A — division of archesporial cells, B — the formed microsporangium wall, C, D — microspore tetrads, E — differentiating mitosis (the preparations are colored with methyl green-pironin followed by Alcian blue, a microscope AxioScope A.1, Carl Zeiss Microscopy GmbH, Germany).

The tapetum is single-layer; its cells are large, binuclear. The first signs of degradation of the tapetum cells are observed during meiosis and formation of microspores tetrad, and their full lysis occurs during formation of pollen grains. The cells of the middle layer, adjacent to tapetum, degenerate, the cells of the second layer become flat (see Fig. 1, E). Outer stamens outgrew inner ones. When the outer anthers contained 2-cell pollen grains, tetrad decay was observed in the inner ones. At the stage of differentiating mitosis, the microsporangium wall consists of epidermis covered with cuticle, endothecium with fibrous thickenings on the walls of cells, and flattened cells of the middle layer (see Fig. 1, E).

The microsporangium wall of the mature anther contains epidermis covered with cuticle, fibrous endothecium and the remnants of the middle layer (Fig. 2, A, B). Mature pollen grains are 2-cell; most of them were morphologically normal, but some were defective. Among *R. canina* the number of defective pollen grains could be up to 30%, among *R. spinosissima* approximately 15% (Table 1). Male germ cells formed in pollen tubes when they germinated through

the tissues of the baculum.

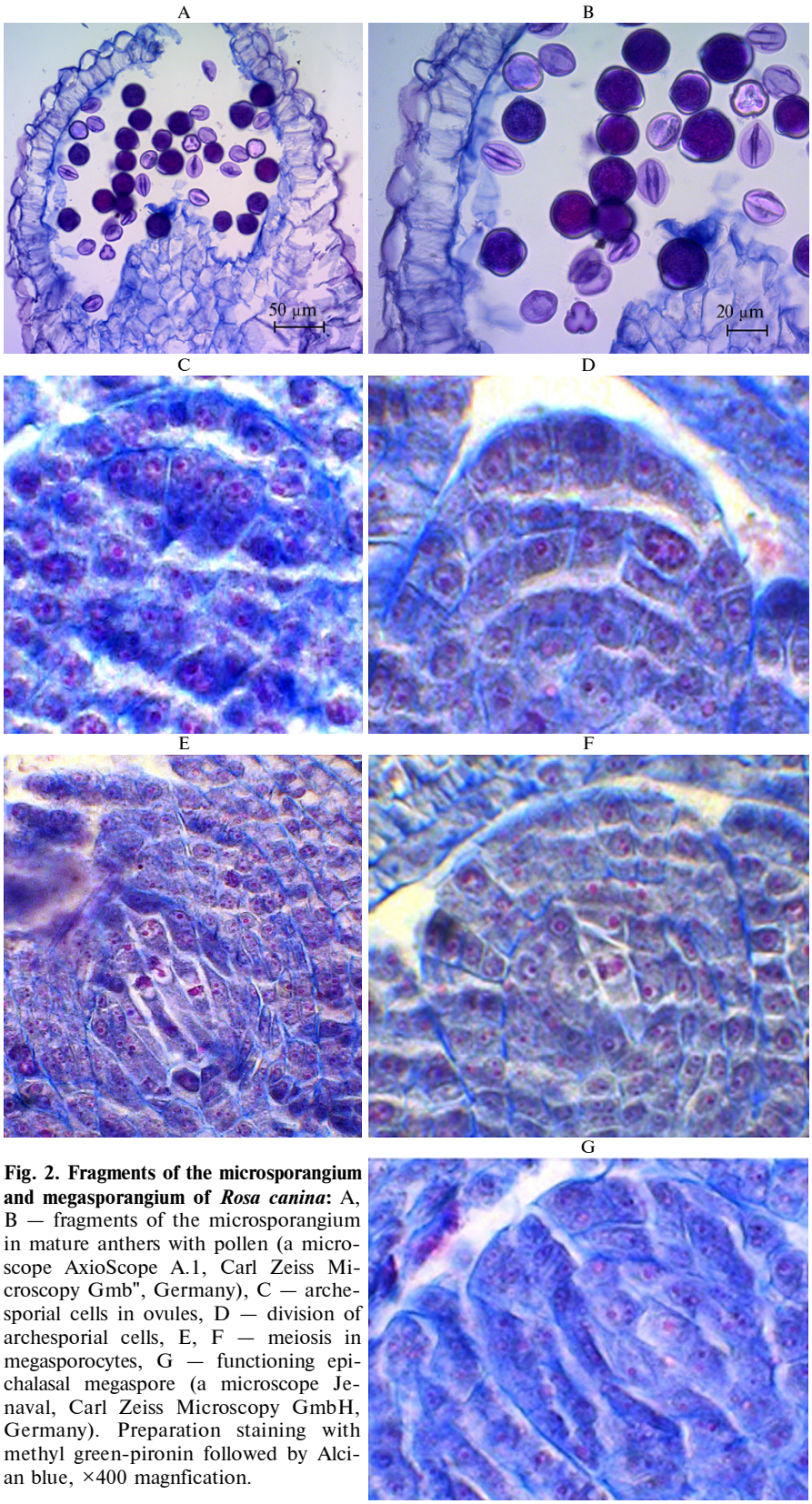

Fig. 2. Fragments of the microsporangium and megasporangium of *Rosa canina*: A, B — fragments of the microsporangium in mature anthers with pollen (a microscope AxioScope A.1, Carl Zeiss Microscopy Gmb", Germany), C — archesporial cells in ovules, D — division of archesporial cells, E, F — meiosis in megasporocytes, G — functioning epichalasal megaspore (a microscope Jenaval, Carl Zeiss Microscopy GmbH, Germany). Preparation staining with methyl green-pironin followed by Alcian blue, ×400 magnfication.

1. Pollen formation in different cultivars of roses with a viral infection and without its symptoms (the Republic of Crimea, 2016)

Species, cultivar	Total number of PG, pcs	Morphologically normal PG, pcs	Defective PG		F_φ
			Total, pcs	%	
Rosa canina	3195	2230	1065	31.4	
R. spinosissima	2390	1050	340	14.3	
R. × *damascena*:					
Dzhalita					
no symptoms	1030	730	300	29.0	13.64
infection	3260	1880	1380	42.3*	
Festivalnaya					
no symptoms	1240	870	370	29.8	204.39
infection	2400	1100	1300	54.1*	

N o t e. PG — pollen grains, F — the value of the Fisher criterion obtained with the use of the method φ [36]. Gaps mean that this indicator was not determined.
* Differences between the number of morphologically normal pollen grains when infected with viruses and without symptoms of infection are statistically significant at $p < 0.05$.

Rose gynoecium consisted of many free carpels, containing 1-2 ovules per each. The pistil is straight; the baculum is with a cephalated stigma. The ovule is quite large, anatropous, two-sided, crassinucellate. The multicellular hypostasis with thick-walled cells formed in the chalazal zone. The micropyle was formed by the internal tunicle, which in its development was ahead of the external.

Rosa sp. has multicellular archesporium as well as many other species of *Rosaceae* [40]. As a result of the archesporium division, parietal and sporogenic cells are formed (see Fig. 2, C, D), the latter is transformed into a megasporocyte, and the nucellus develops from the parietal cell. As a result of meiosis (see Fig. 2, E, F), ordered tetrads of megaspores are formed. Chalazal or epichalasal megaspores usually were the functioning ones (see Fig. 2, F), from which 7-cell 8-nuclear polygonum-type embryo sac developed. Several embryo sacs could be in the ovule, but only one developed to the mature condition.

Fig. 3. The leaves of essential-oil-bearing rose (*Rosa damascena*) of the Dzhalita (A) **and Festivalnaya** (B) **varieties with symptoms of viral infection (discoloration and wrinkles)** (the Republic of Crimea, Nikitsky botanical garden, 2016).

Biogenic and abiogenic stress factors, including virus damage, affect the physiological and biochemical parameters of plants, which violate the metabolic balance and affect the anatomical and morphological characteristics. Changes in leaf blade traits, such as spotting, wrinkling, and dwarfism, are used for visual assessment of plant infection and its viability in general [41-44]. The state of the male gametophyte, characterized by the morphological maturity of pollen grains,

is one of the most accessible and effective signs, giving an idea of the degree of stress effects of various factors on the plant and its viability [43, 44]. Previously, the diagnosis of viral pathogens in the plant material of essential-oil-bearing rose (*R.* × *damascena*) revealed that most of the selected material (up to 70%) was affected by viral pathogens of different origin [45]. The external manifestation of virus infection is most noticeable on the leaves and flowers with wrinkles, interveinal chlorosis, and spotting of different nature (Fig. 3), which was the result of changes in the state of their internal structures.

2. Morphometric parameters of pollen grains of *Rosa canina* and *R.* × *damascena* with a viral infection and without its symptoms (the Republic of Crimea, 2016)

Species, cultivar	Plant condition	*M*±SEM, μm	min-max, μm	σ	*Cv*, %	*t*
R. canina	No symptoms	30.88±0.18	24.18-38.45	2.41	7.80	26.140
	Infection	22.91±0.25	19.16-39.92	2.37	10.34*	
R. × *damascena:*						
Dzhalita	No symptoms	24.69±0.11	20.18-30.62	1.70	6.89	6.430
	Infection	25.76±0.13	21.21-35.12	2.01	7.08*	
Festivalnaya	No symptoms	24.56±0.11	20.40-31.65	1.76	7.17	0.096
	Infection	24.55±0.10	19.91-30.25	1.62	6.60	

N o t e. *M* —the arithmetic mean, SEM — standard error of the mean, min-max — minimum and maximum values of the selection, σ — the mean square deviation, *Cv* — coefficient of variation, *t* — Student criterion at $n = 130$.
* Differences in the diameter of the pollen grains of the average samples when infected with viruses and without symptoms of infection are statistically significant at $p < 0.05$.

Morphometric analysis of the male generative structures of the virus-infected and visually clean plant of essential-oil-bearing rose varieties, and *R. canina* showed that infection leads to the increase in the number of abnormal pollen grains and change in their sizes (see Tables 1, 2). Among plants without symptoms of infection, more morphologically normal pollen grains were formed, and among plants with signs of infection, the proportion of abnormal and defective pollen grains was increased, which may indicate potential violations in the genesis of particular elements of the flower.

Thus, according to the main embryological features, *Rosa spinosissima* and *R. canina*, as well as the studied varieties of *R.* × *damascena*, are similar to other members of *Rosaceae* family and especially of *Rosoideae* subfamily. They are characterized by the centripetal development of the microsporangium wall, the simultaneous type of formation of microspore tetrad, 2-cell pollen grains, multicellular archesporium, and the formation of several embryo sacs in the ovule, most of which obliterate at different stages of development. The viability of the generative structures of the species presented in this paper can provide a normal reproductive process, which is very important for their use in selection in order to obtain stress-resistant varieties. During selection of the initial forms for hybridization, it is necessary to pay attention not only to the viability of the generative sphere of plants but also to their infection because the presence of pathogens in the plant leads to the decrease in its reproductive ability.

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KEY METHODOLOGICAL FEATURES OF TUBULIN CYTOSKELETON STUDIES IN NODULES OF LEGUME PLANTS

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Abstract

The discovery of microtubules in plants, as well as their subsequent study, was made possible by the methods of electron microscopy. Further, methods for visualizing the cytoskeleton in a plant cell were actively developed using immunolocalization combined with laser scanning confocal microscopy (K. Celler et al., 2016). All the above-listed methods involve the fixation of the analyzed biological material. It should be noted that the tubulin cytoskeleton is an extremely dynamic structure; therefore, techniques of microtubule visualization in living plant cells using fluorescent proteins have been actively developed in recent years (K. Celler et al., 2016). Nevertheless, immunohistochemical analysis is still an essential method (J. Dyachok et al., 2016). First of all, this is due to the fact that in vivo observations are limited to plant cells of the surface layers (root hairs, epidermis) (F.M. Perrine-Walker et al., 2014; J. Dyachok et al., 2016). Moreover, for many plant species, the size of their organs is much larger than that of *Arabidopsis thaliana*, which makes it impossible to analyze changes in the organization of the cytoskeleton in vivo (J. Dyachok et al., 2016). Another limiting factor is that for several plant species, transformation protocols have not yet been developed or are very difficult, including the pea (*Pisum sativum* L.) (A. Iantcheva et al., 2013). Therefore, the optimization of fixation protocol of plant material remains relevant for effective immunohistochemical analysis of the tubulin cytoskeleton. In our study, it was shown that this optimization is required when new legume species are studied. For instance, the protocol for pea nodule fixation developed by us required changes when applied to the nodules of *Medicago truncatula*. Moreover, modifications in the protocol for fixation may even be necessary when examining different mutants in the symbiotic genes of a plant species, because such mutations can exert a strong influence on the physicochemical properties of the nodule tissues. Therefore, we used various fixation protocols for the wild-type line of *M. truncatula* A17 and its mutants *dnf1-1*, *efd-1* and TR3 (*ipd3*). It has also been shown that the preparation of sections of fixed nodules using a microtome with a vibrating blade can significantly improve the preservation of the structure of the tubulin cytoskeleton as compared to the use of fixed specimens embedded in Steedman's wax and subsequent sectioning using a rotary microtome. It was found that the age of the nodules is also an important factor in the visualization of the tubulin cytoskeleton. To compare the patterns of tubulin cytoskeleton in different cell types, quantitative analysis is required. We found that the MicroFilament Analyzer (E. Jacques et al., 2013) with additional scripts seemed well suited for checking the frequency of microtubules with a given orientation.

Keywords: legume-rhizobial symbiosis, microtubules, immunolocalization, *Pisum sativum*, *Medicago truncatula*, quantitative analysis, MicroFilament Analyzer.

The study of the tubulin cytoskeleton organization began with the use of electron microscopy methods, which were later supplemented with immunohistochemical analysis using fluorescence and laser scanning confocal microscopy [1]. All these methods involve the fixation of biological material. At the same

time, the tubulin cytoskeleton is a dynamic structure; therefore, approaches based on the study of microtubules with the use of fluorescent proteins in vivo [1] have become widespread. Nevertheless, immunolocalization of microtubules in plant cells remains in demand [2]. This is due to the fact that live imaging of microtubules was limited to the surface layers of cells (root hairs, epidermis); the size of many organs of the plants exceeds that of *Arabidopsis thaliana* [2]. In addition, there are methodological difficulties in the transformation of many plants [2]. For example, the protocol for effective transformation of pea has not been developed yet [3]. It also should be noted that fixation allows visualizing the organization of cytoskeleton elements, which cannot always be detected with the use of fluorescent proteins in live imaging observations [4].

Studies of the tubulin cytoskeleton in symbiotic nodules also began with the use of electron microscopy [5]. In the following years, the organization of microtubules in root hairs during the formation of infection threads, as well as the primordium of the symbiotic nodule in *Medicago sativa* and *M. truncatula*, was identified using immunolocalization and fluorescence and laser scanning confocal microscopy [6]. Later, the rearrangement of the tubulin cytoskeleton during the formation and growth of the infection thread in the root hairs of *M. truncatula* and *Lotus japonicus* was studied using fluorescent proteins in vivo [7-10].

The immunohistochemical methods and wide-field fluorescence microscopy or laser scanning confocal microscopy, as well as electron transmission microscopy, were used in studies of the tubulin cytoskeleton in nodules of different species of legumes. As a result, microtubule patterns were detected in mature nodules of *M. truncatula* [11], *Glycine max* [12], *Pisum sativum* [13], and *Lupinus albus* [14]. Nevertheless, these studies contain no detailed description of the three-dimensional organization of microtubules, especially around infection threads and infection droplets [15].

The previous studies on the structural organization of microtubules in mature nodules describe one approach, in which thick sections were obtained manually, and the tubulin cytoskeleton was fixed and visualized [12-14]. Another approach involved fixation of the nodules followed by embedding in Steedman's wax [16], preparation of serial sections, which after rehydration were used for immunolocalization [6].

In the present study, for the first time the authors present an improved method for nodule fixation, compare the possibility of preparing sections with the use of embedding medium and sections of fixed nodules obtained using a microtome with a vibrating blade, optimize the immunolocalization method and the method of quantitative analysis of the tubulin cytoskeleton.

The purpose of the study was to develop an approach for studying the tubulin cytoskeleton in the nodule of legumes.

Description of the method. Optimization of the method for nodule fixation. To study the organization of the tubulin cytoskeleton in pea nodules, a modified method for fixing the maize roots was used [17]. A new method for fixing symbiotic nodules is proposed [18]. The pea nodules were incubated for 7 min under vacuum and 15 min without a vacuum in a fixing solution (3% formaldehyde, Ted Pella, USA, 0.25% glutaraldehyde, Ted Pella, USA, 0.3% Tween 20, Sigma-Aldrich, USA, 0.3% Triton X-100, Helicon, Russia) with $^1/_3$ concentration of MTSB buffer, containing 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (AMRESCO Inc., USA), 5 mM $MgSO_4$ and 5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (GERBU Biotechnik GmbH, Germany), pH 6.9; the procedure was repeated 6–7 times. After this, the nodules were allowed for a night at 4 °C.

Optimization of the method for embedding into Steedman's wax. After fixation, the nodules were washed in $^1/_3$ MTSB buffer 3 times for 20 min and embedded in Steedman's wax [16] according to M. Stumpe et al. [19] with modifications. The material was dehydrated in a gradient ethanol series: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 96%. The processing time for the first four concentrations was 10, 20, 30 and 40 min, respectively, for subsequent concentrations 50 min. Staining of the samples with 0.1% toluidine blue in ethanol was performed overnight. After washing 2 times (1 h, 96% ethanol), the nodules were passed through increasing concentrations of Steedman's wax (10, 20, 35, 50, 65 and 80%) mixed with ethanol (40 °C, 2 h of incubation at each stage). The plant material was embedded in 100% Steedman's wax at 40 °C for a night, and then placed into fresh 100% Steedman's wax, after 2 h of incubation in which the nodules were put into molds and filled with 100% Steedman's wax. The resulting blocks with nodules were kept at 4 °C for 30 min, then serial sections with a thickness of 16 μm were prepared using a rotary microtome HM360 (Microm, Germany).

Thick (50 μm) sections without embedding in the medium were also used instead of embedding nodules in Steedman's wax. For this, nodules after fixation were washed in $^1/_3$ MTSB, enclosed in blocks of 3% agarose (Helicon, Russia), and then serial 50 μm thick sections were prepared using a microtome with a vibrating blade HM650V (Microm, Germany), after which the sections were washed in $^1/_3$ MTSB.

Optimization of the immunolocalization method. The corresponding protocols described by F. Baluška et al. [17] and M. Stumpe et al. [19] were modified for this purpose. The obtained sections were placed on silanized glasses coated with egg white and spread by the addition of distilled water. After the sections were dried at room temperature, the embedding medium was removed with treatment in 96% ethanol for 10 min for 3 times, then in 70 and 40% ethanol for 10 min with a final incubation in $^1/_3$ MTSB for 15 min for 2 times. To prevent nonspecific binding, the sections were incubated in a blocking solution (5% bovine serum albumin (BSA), 0.5% normal goat serum (Sigma-Aldrich, USA), 0.2% cold water fish skin gelatin (Sigma-Aldrich, USA) in $^1/_3$ MTSB) for 30 min at 28 °C and in acetylated BSA solution (Sigma-Aldrich, USA) (2 mg/ml) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 30 min at 28 °C. To visualize the tubulin, the sections were incubated overnight at 4 °C with mouse monoclonal primary α-tubulin antibodies (clone DM1A) (Sigma-Aldrich, USA) at a dilution of 1:1000 in 1% BSA in TBS. The sections were washed 5 times for 10 min in TBS buffer, blocked in a solution of 5% BSA in TBS at 28 °C and incubated with secondary goat antibodies against mouse γ-globulin conjugated with Alexa Fluor 488 (Life Technologies, USA), at a dilution of 1:500 in TBS at 28 °C for 90 min. The sections were then washed 3 times for 10 min in TBS, stained with propidium iodide (0.5 μg/ml) for 7 min to visualize the nuclei and bacteria, after that washed again 3 times for 10 min in TBS and mounted antifade reagent ProLong Gold® (Thermo Fisher Scientific, USA) under coverslip.

The pattern of microtubules in the nodule cells was studied using laser scanning confocal microscopes LSM510 META and LSM780 (Carl Zeiss, Germany). Spatial organization of microtubules in different types of nodule cells was analyzed using the 3D reconstruction of the obtained images (ZEN software, Carl Zeiss, Germany) [18].

Analysis of the tubulin cytoskeleton in the nodules of legumes with various fixation methods. Microtubules were detected in all types of cells in the nodule of pea (*P. sativum*), but fragmentation and

depolymerization of microtubules were often observed (Fig. 1, A, B). To improve the quality of microtubule, thick (50 μm) sections without embedding in the medium were used instead of embedding nodules in Steedman's wax. This method allowed avoiding long incubation in ethanol and stages of embedding in Steedman's wax.

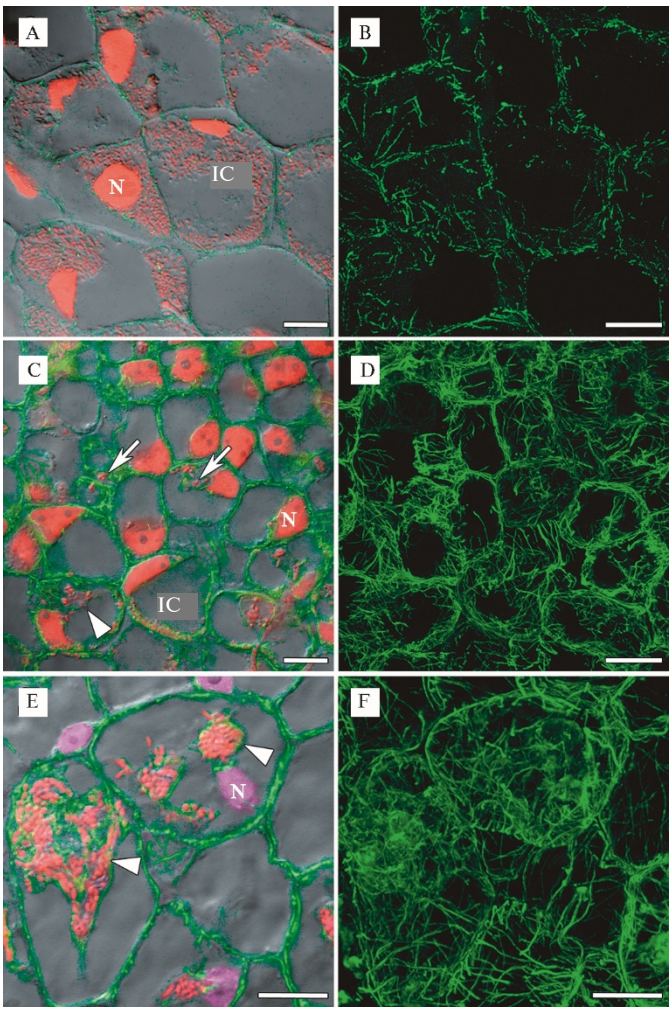

Fig. 1. Immunolocalization of the tubulin cytoskeleton in symbiotic nodules of pea (*Pisum sativum* L.) of the wild-type SGE (A-E) **and the mutant *efd-1 Medicago truncatula* Gaertn.** (B, D). The sections were obtained by embedding in Steedman's wax (A, B) or by immersion in 3% agarose (C-F); confocal laser scanning microscopy (microscopes LSM510 META and LSM780, Carl Zeiss, Germany). Microtubules are fragmented (A, B), cortical and endoplasmic microtubules clearly identified (C-F).

A-F: immunolocalization of tubulin, green channel;
A, C: staining of DNA nuclei and bacteria with propidium iodide, red channel;
E: staining of DNA nuclei with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI), magenta channel; localization of red fluorescent protein (RFP) — visualization of bacteria, red channel. Merge of a single optical section, differential interference contrast and the maximum intensity projections of optical sections in the red and green channels (A, C), green, red and magenta channels (E). The maximum intensity projections of optical sections in the green channel (B, D, F).

Legend: N — nucleus, IC — infected cell; the arrow points to the infection thread, the tip of the triangle — infection droplet. The scale bar is 10 μm.

The use of a modified method on thick sections of fixed material made it possible to visualize the tubulin cytoskeleton with high quality. In this case, both cortical and endoplasmic microtubules were clearly identified (see Fig. 1, C, D). Using this approach, a detailed analysis of the tubulin cytoskeleton in cells of various histological zones of nodules was carried out in pea of wild-type SGE [20], Sprint-2 [21], and mutants in the symbiotic genes SGEFix$^-$-1 (*sym40*), SGEFix$^-$-2 (*sym33*) [22], Sprint-2Fix$^-$ (*sym31*) [23], as well as the study of microtubules organization around various symbiotic structures, like the infection threads, infection droplets and symbiosomes [18].

However, the developed method did not allow visualization of the tubulin cytoskeleton with high quality when studying the organization of microtubules in symbiotic nodules of *M. truncatula*. In the meristem zone, diffuse fluo-

rescence of tubulin was observed, which may be due to poor permeability of *M. truncatula* cells upon fixation. To increase the permeability, 10% dimethyl sulfoxide (DMSO) was included into the fixing solution composition and the time for air pumping was increased to 30 min, the nodules were left without vacuum for 10 min, the whole procedure was repeated 3 times.

Several genotypes were used in the analysis of the tubulin cytoskeleton organization in the nodules of *M. truncatula*: the wild-type line A17 and corresponding mutant lines *dnf1-1* [24], *efd-1* [25] and TR3 (*ipd3*) [26, 27]. The fixation modification described above was suitable for nodules of the A17 and *dnf1-1* lines. At the same time, nodules of the TR3 (*ipd3*) and *efd-1* lines after fixing became too softened to prepare sections. Therefore, the nodules *efd-1* and TR3 *(ipd3)* were fixed without addition of DMSO, and the concentrations of Tween 20 and Triton X-100 were reduced to 0.05% in the fixing solution for TR3 (*ipd3*) nodules. The influence of the concentration of the buffer solution salts, on the basis of which the fixing solution was prepared, was also noted. During fixation a cell compression was observed for *M. truncatula* nodules of all genotypes. Therefore, the nodules of A17 and *dnf1-1* lines were fixed using $^1/_6$ MTSB buffer, and the nodules of TR3 (*ipd3*) and *efd-1* – with $^1/_{10}$ MTSB. As a result, a high quality of visualization of the tubulin cytoskeleton was achieved for all the studied genotypes of *M. truncatula* [18] (Fig. 1, E, F).

An important factor in the visualization of the tubulin cytoskeleton is the age of the analyzed nodules. Microtubules were preserved better in 2-week-old nodules of pea and *M. truncatula* than in 4-week-old nodules.

Analysis of the tubulin cytoskeleton in cells from different histological zones of the nodule identified different patterns of microtubules [18]. Quantitative analysis was used to confirm the differences in the observed patterns.

Quantitative analysis of the tubulin cytoskeleton. The ImageJ software was used for this purpose [28, 29]. The Angle Tool allows creating a line over the analyzed image, after which a table containing the values of inclination angles of such lines is formed. This method proved to be extremely inconvenient by the reason that not the whole cell is processed, but only one line and, therefore, obtaining data for a large number of images with differently oriented microtubules in nodules takes a long time. Later, the authors used an ImageJ plug-in FibrilTool [30], which was used to determine the average direction in microtubules orientation in an arbitrarily selected area in the image. The applied approach significantly reduces the analysis time of similarly oriented microtubules, but in case of their disordered orientation (for example, in nitrogen-fixing cells of the symbiotic nodule), the researcher receives data on the average direction, which does not provide all the necessary information. The authors also used an OrientedJ plug-in [31], which allows obtaining the distribution of microtubule orientations on the entire image, but it becomes impossible to separate the data on the orientation of the elements in different cells. The undoubted advantages of the OrientedJ plug-in include the possibility to obtain a "distribution map" of microtubules according to the frequency, depending on the orientation angle, each of which corresponds to the color.

The best option was the use of the MicroFilament Analyzer [32], the functions of which allow analyzing the distribution of microtubules in several cells selected on the image either manually or automatically. Microtubule segments with an angle of 0°, then 1° and so on until 179° were detected in the image. As a result, a table containing the angles and coordinates of each line was created, and a graph of the angles frequency distribution was obtained.

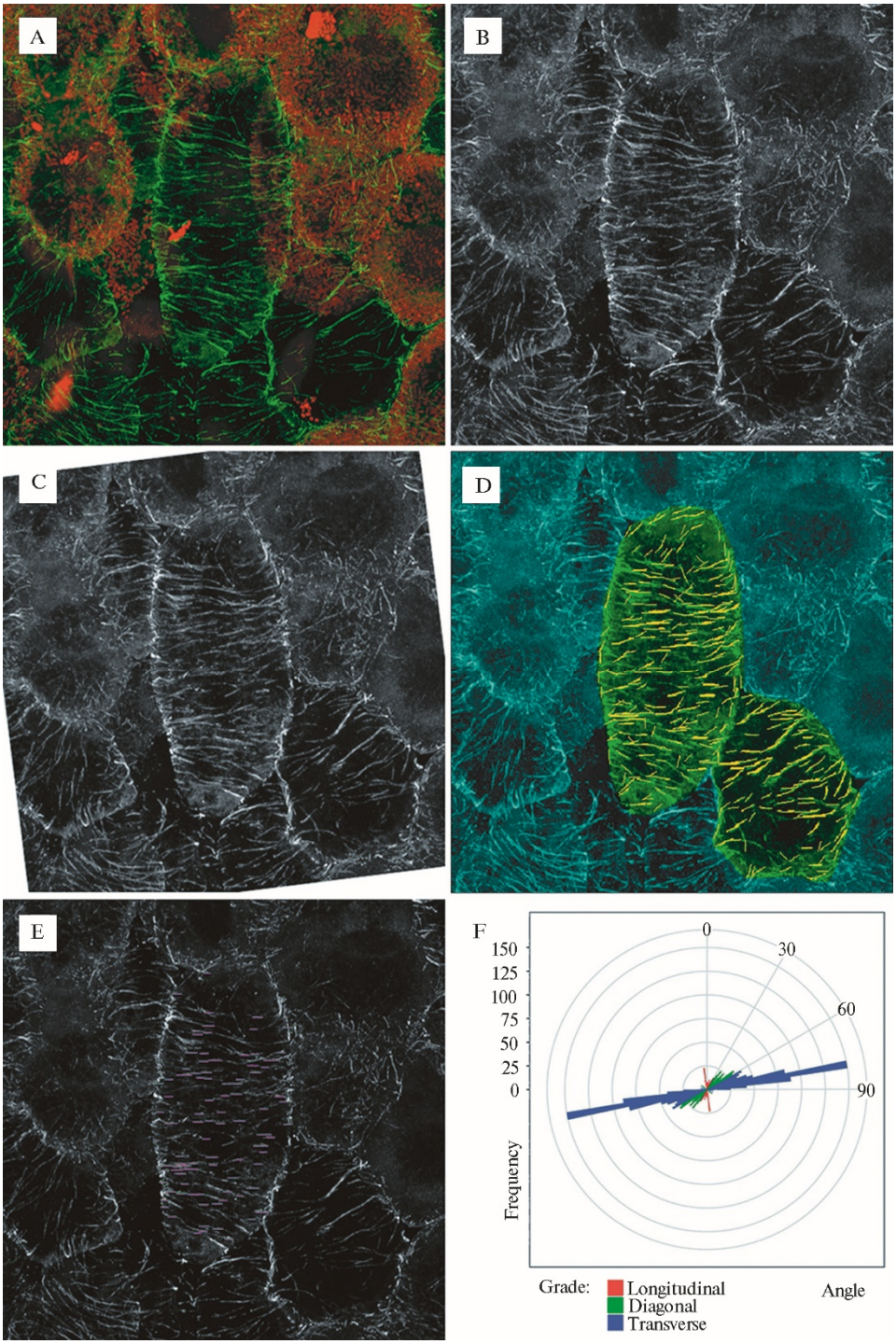

Fig. 2. Quantitative analysis of microtubules in an uninfected cell of a nitrogen-fixing nodule of pea (*Pisum sativum* L.) the wild-type SGE. Confocal laser scanning microscopy (LSM780, Carl Zeiss, Germany).

A: immunolocalization of tubulin, green channel, staining of DNA nuclei and bacteria with propidium iodide, red channel; the maximum intensity projection of optical sections in the red and green channels. B: green channel, converted into grayscale. C: rotated image, in which the longitudinal axis of the cell is oriented vertically. D: analysis in the MicroFilament Analyzer, the cells analyzed are highlighted with green, the microtubules detected by the program are highlighted with yellow. E: all microtubules with an angle of 90° (relative to the longitudinal axis of the cell), identified by the MicroFilament Analyzer in the cell under study. F: distribution of microtubules detected

in the cell according to the magnitude of the angle.

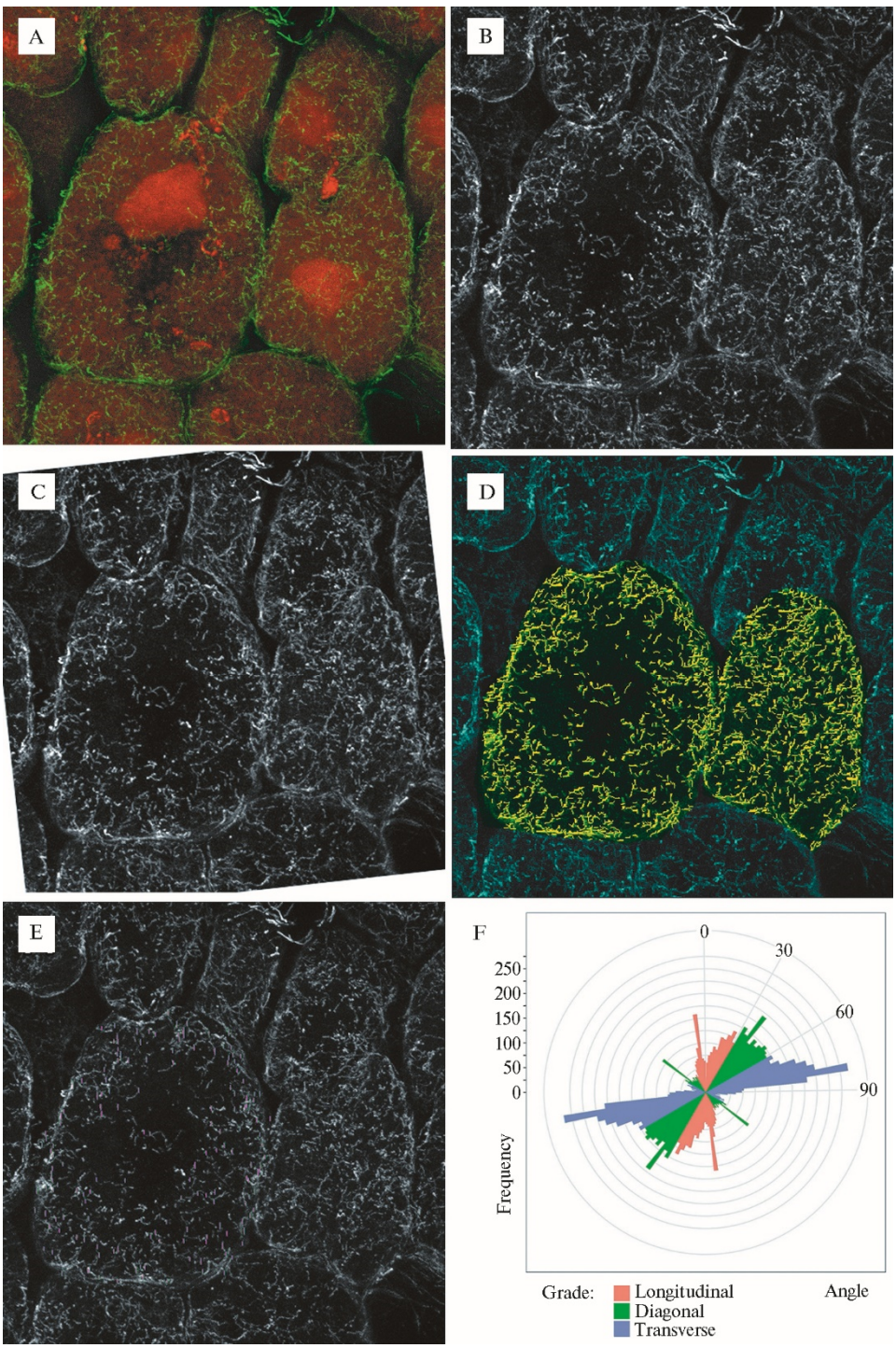

Fig. 3. Quantitative analysis of microtubules in an infected cell of a nitrogen-fixing nodule of pea (*Pisum sativum* L.) the wild-type SGE. Confocal laser scanning microscopy (LSM510 META, Carl Zeiss, Germany).

A: immunolocalization of tubulin, green channel, staining of DNA nuclei and bacteria with propidium iodide, red channel; the maximum intensity projection of optical sections in the red and green channels. B: green channel, converted into grayscale. C: rotated image, in which the longitudinal axis of the cell is oriented vertically. D: analysis in the MicroFilament Analyzer, the cells analyzed are highlighted with green, the microtubules detected by the program are highlighted with

yellow. E: all microtubules with an angle of 0° (relative to the longitudinal axis of the cell), identified by the MicroFilament Analyzer in the cell under study. F: distribution of microtubules detected in the cell according to the magnitude of the angle.

The original RAW images were converted in the the maximum intensity projections. However, when using full-size images (1024×1024 pixels), microtubule detection was difficult. The image size was reduced using bicubic interpolation to 512Ч512 pixels. Then a channel corresponding to a tubulin marked with antibodies was selected, and then was saved as a gray-scale image. These operations were carried out using ImageJ, because it allows applying the above operations on a variety of images at once due to the built-in Macro Language. The resulting images were further analyzed using the MicroFilament Analyzer. Since incorrectly detected segments led to an increase in the frequency of occurrence of certain angles, it was necessary to minimize the number of such segments. To do this, an R script was prepared, which selected segments with a given angle (angles) from the table and using ImageMagick program applied the corresponding lines on a copy of the initial image. This is how the accuracy of certain angles detection by the MicroFilament Analyzer was evaluated and, if necessary, the options of this program were adjusted.

To construct graphs that represent the distribution of segments with certain angles in the cell, an R script that uses the package ggplot2 was written [33]. This script was used to process the spreadsheet obtained in the MicroFilament Analyzer. Angles were recalculated in such a way that the zero angle was located vertically on the graph, and the values of the angles increased clockwise. Half of the angles, not reaching the full circle, were added and the detected angles were divided into three grades: longitudinal, diagonal and transverse.

The developed approach was used for the quantitative analysis of the tubulin cytoskeleton (microtubule distribution) in uninfected (Fig. 2) and nitrogen-fixing (Fig. 3) cells of the pea nodule. The obtained results confirmed the previously assumed (using visual analysis) differences in microtubule patterns in cells of different types in a symbiotic pea nodule.

Thus, the obtained results clearly demonstrate that the study of the tubulin cytoskeleton with the use of immunolocalization in the nodules of legumes is characterized by a number of features. The critical factor is the composition of the fixing solution, which must be adapted for each species of the studied legumes and for the symbiotic mutants of these species. The necessary condition is a rejection of embedding media and obtaining the sections of fixed nodules using a microtome with a vibrating blade, which significantly increases the stability of the tubulin cytoskeleton structure. The age of the nodules being analyzed is also important. Therefore, when starting to analyze the tubulin cytoskeleton organization, it should be understood that such study is a non-trivial task that requires a critical approach to the use of previously developed methods.

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NONDESTRUCTIVE LEAF AREA AND FRESH WEIGHT ESTIMATION FOR *Taraxacum kok-saghyz* Rodin AND THEIR SAMPLING NUMBER

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Abstract

Kok-saghyz (*Taraxacum kok-saghyz* Rodin), Russian dandelion, is a perennial plant widely recognized as one of the most promising sources of natural rubber. The works to utilize natural rubber are underway in the United States, China and Western Europe (Germany, Spain, Czech Republic and the Netherlands). The aim of this study was to determine nondestructive models for estimating leaf area and fresh weight of Russian dandelion plants. Regression analyses were performed between leaf area, fresh weight, leaf length, and leaf width in two hundred and fifty leaf samples collected during different growth stages of Russian dandelion plants. Data from another fifty leaves were used for validating the proposed models. Regression analyses were performed among ten data groups with different numbers of data randomly selected from the total three hundred leaves data set to determine the smallest sampling number for applying the final models correctly. The model for estimating leaf area (LA) is: $LA = 6226.424 + 26.31L + 545.334W - 313.993L^{0.5} - 3138.047W^{0.5} - 0.009L^2 - -3.86W^2 + 0.057LW$, with R^2 and RMSE values of 0.818 and 168.29, respectively. The model for estimating leaf fresh weight (FW) is: $FW = 1125.572 - 24.857L + 233.070W + 0.055LW + 276.956L^{0.5} - - 1264.466W^{0.5} + 0.067L^2 - 1.964W^2$, with R^2 and RMSE values of 0.735 and 87.84, respectively. At least ten leaf samples are required when applying the two models. Determining transformed forms of leaf dimensions that are linearly related to leaf area and fresh weight, and integrating all of them into one equation maybe a better solution for establishing models to estimate leaf area and fresh weight of plant species, particularly those with higher variation among individual leaves.

Keywords: *Taraxacum kok-saghyz* Rodin, leaf length, leaf width, estimation model, regression analysis

Russian dandelion (*Taraxacum kok-saghyz* Rodin) is a perennial herbaceous plant,_rare and endemic Eastern Tien Shan species, growing on depleted and saline soils, The plants were studied as a local rubber plant in 1930-1940 and is now widely recognized as one of the most promising sources of natural raw materials for rubber production [1, 2]. The plant may contain up to more than two hundred leaves, which form a basal rosette above the root. Leaf shape is narrow obovate or oblanceolate with entire or undulate margins, and without a petiole [3]. It is well known that leaf area plays an important role in plant growth analysis. Leaf area and leaf weight measurements are necessary to estimate leaf area index [4-6], photosynthesis rates, light interception, water and nutrient use and crop growth [7-9]. Due to these leaf characteristics, it is diffi-

cult to monitor the aboveground growth status of Russian dandelion plants directly; therefore, it is necessary to develop indirect methods to estimate the leaf area and fresh weight of the plant.

Among various methodological approaches for estimating leaf area and fresh weight, indirect and nondestructive estimating methods have been widely applied for their inexpensive, rapid and simple features [6, 10]. Additionally, indirect methods enable researchers to measure leaf area and fresh weight on the same plants during the plant growth period. This may reduce variability in experiments [11-14]. In nondestructive methods, leaf area and weight are usually estimated by traits such as leaf length, leaf width, growing degree days, and petiole length. The proposed models for estimating leaf area and weight are dependent on the growth traits and leaf shape of the plant. Amongst these investigations, those which correlated leaf length and width with leaf area and fresh weight are most common [6, 15-18].

Although Russian dandelion has been studied for decades, leaf area and weight prediction models have not been developed for this plant until this paper.

Therefore, the objective of this study was to develop a reliable model for leaf area and fresh weight estimates of Russian dandelion, based on leaf dimensions, which can be applied in studies of the plant.

Techniques. Russian dandelion plants were grown in Harbin City, China in June, 2014. The experimental site is situated at N45°34'59.9", E126°34'18.8". The climate is temperate continental monsoon with a mean annual temperature of 4.2 °C and a mean annual precipitation of 532 mm. Standard crop management practices were followed in the experiment.

Russian dandelion leaf morphology varied among different plants. Two months after the seedlings were planted, fifty representative and integrated leaves were randomly selected at 14-day intervals from the plot. Each leaf sample was measured for fresh weight on an electronic balance, and the length and maximum width of the leaves were measured with a simple ruler. Length was measured from the lamina tip to the point of intersection of the lamina and stem. Width of the leaves was measured from tip to tip between the widest lamina. Leaf fresh weight was measured to ten percent of a milligram. Leaf length and width was measured to the nearest millimeter on a linear scale. Thereafter, all leaves were arrayed and marked with a serial number on white paper with a ruler as a standard measurement, covered with a white plastic film to flatten them. Photographs were taken and presented in JPG format. The leaf samples were then dried at 80°C till constant weight and measured for dry weight. Photographs were viewed using ImageJ software (version 1.48) and leaf area for each leaf was calculated using this software. Detailed procedures were followed according to the method described by H.M. Easlon and A.J. Bloom [19]. A total of two hundred and fifty leaves were measured for leaf area, fresh weight, leaf width, and leaf length in the preliminary calibration experiment.

The relationships between leaf area and fresh weight as dependent variables, and leaf length and leaf width as independent variables were determined using regression analysis on data from two hundred and fifty leaves. Independent variables were transformed into other forms (square, square root, exponent, etc.) to establish linear relationships with leaf area and fresh weight. Therefore, scatter-plot matrixes were established using OriginPro software (version 9.0) (OriginLab Corporation, USA) to find linear relationships between leaf area, fresh weight and transformed leaf length and width forms. The model equations were then developed based on scatter-plot matrixes using SPSS (Statistical Product and Service Solutions, version 19.0) software (IBM, USA). Equations with the highest coefficients of determination (R^2) were used in the estimations. Both esti-

mated (*Sim.Yi*) and measured (*Obs.Yi*) leaf areas and fresh weights were compared by testing the significance of the regression equation and the degree of goodness of fit (R^2) between them. The final model was selected based on the combination of the highest R^2 and the lowest root mean square error (RMSE) [4]. Calculation of RMSE as follows (*n* for number of measurements, $n = 250$):

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(Sim.Yi - Obs.Yi)^2}{n}}.$$

To validate selected models, fifty leaves of Russian dandelion were selected randomly from the plot in October, 2015. Observed values of leaf length, width, area and fresh weight were determined as described as above. Simulated values of leaf area and fresh weight were obtained and compared with the observed values through correlation analysis. The slope and intercept of the model were tested to determine whether they were different from the slope and intercept of the 1:1 correspondence line at 0.05 level [20]. Regression analyses were conducted using SPSS (version 19.0) software. To determine the smallest sampling number of the proposed model, a hypothesis was proposed. Ten groups, with same number (sampling number) of leaf data selected randomly from tree hundreds leaves data set, were established. Estimated values of leaf area and fresh weight were calculated through the proposed model using the ten groups of leaf data. Linear regressions were performed between estimated values and measured values. If all the significant values of linear regression equations were smaller than 0.05, the sampling number was appropriate. Ten groups of fifty individual leaves were tested initially and if all significant values of the linear regression equations were smaller than 0.05, then ten groups of twenty five leaves data were tested next. The process could continue until the smallest sampling number was found.

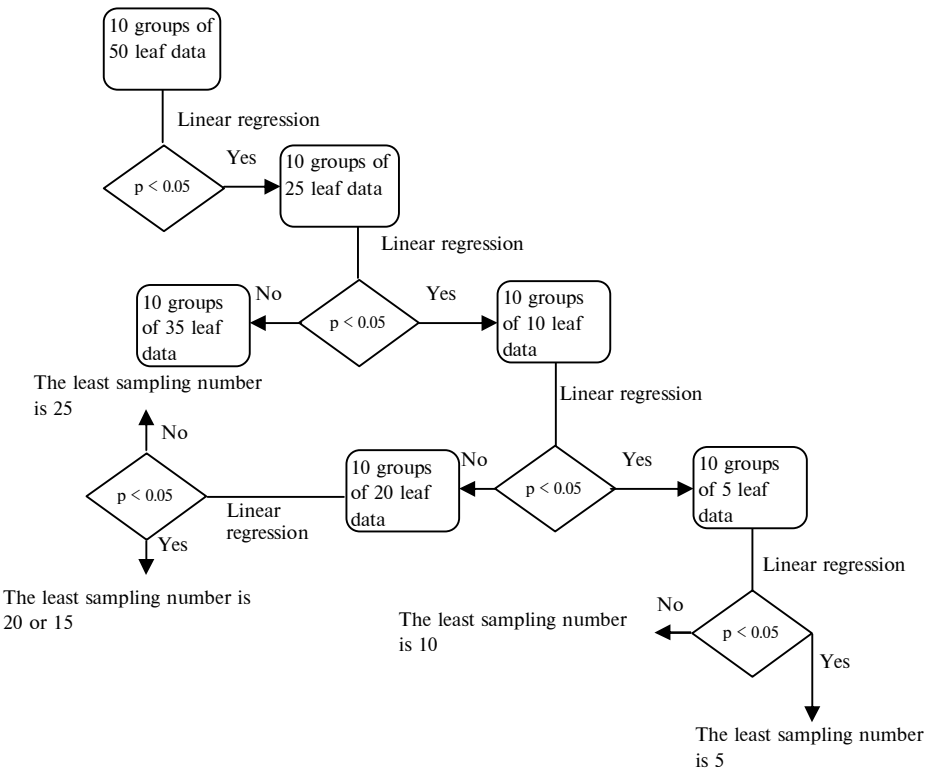

Fig. 1. Road map of determining sampling number of the proposed model for Russian dandelion (*Taraxacum kok-saghyz* Rodin) leaf nondestructive biometry.

Results. Evaluation of leaf morphology [21-24] and the development of adequate models [25] are relevant for many plants. The procedure we used to estimate the sampling number of Russian dandelion was as shown (Fig. 1).

Leaf area models established and validated. Variability of the leaf

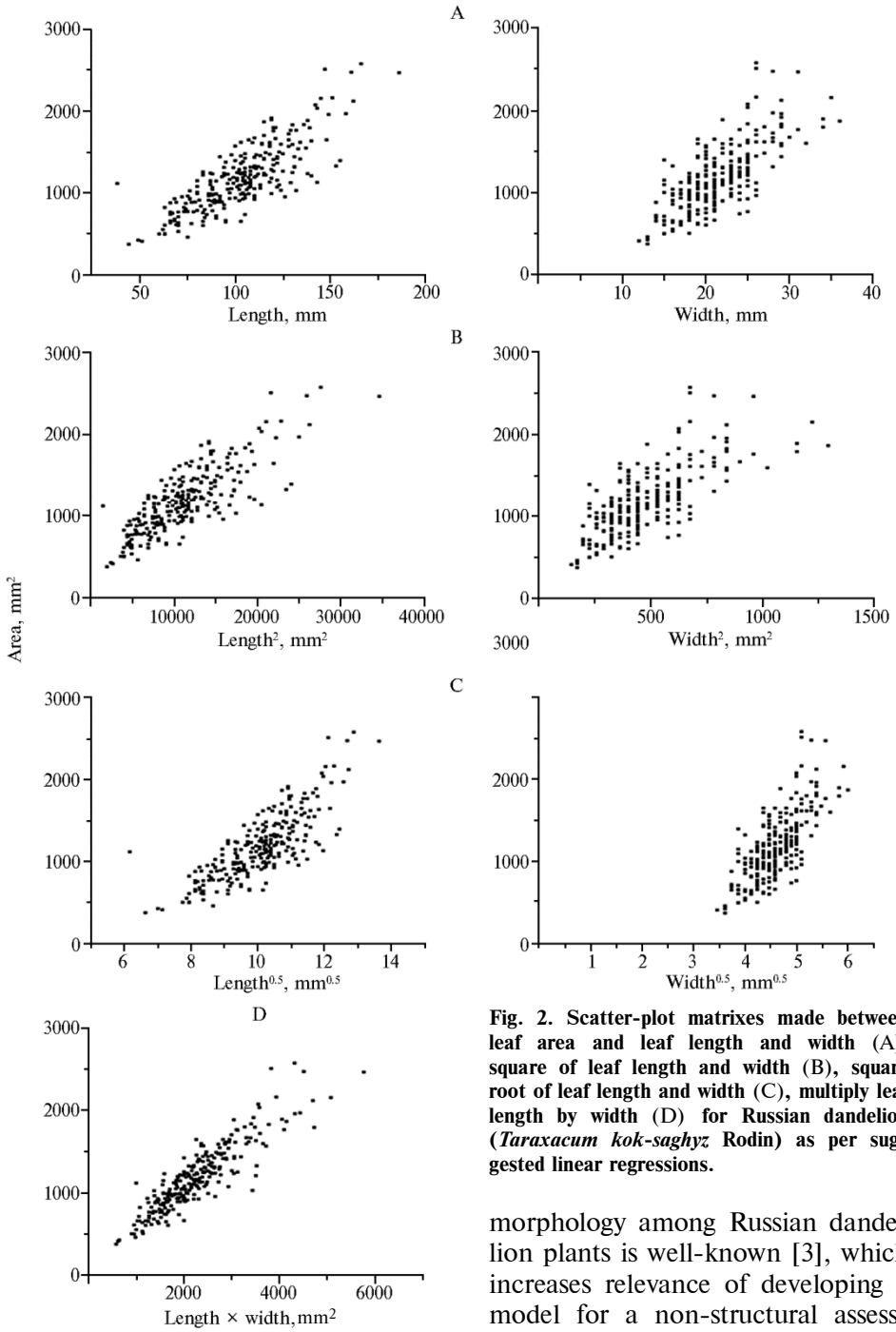

Fig. 2. Scatter-plot matrixes made between leaf area and leaf length and width (A)**, square of leaf length and width** (B)**, square root of leaf length and width** (C)**, multiply leaf length by width** (D) **for Russian dandelion (*Taraxacum kok-saghyz* Rodin) as per suggested linear regressions.**

morphology among Russian dandelion plants is well-known [3], which increases relevance of developing a model for a non-structural assessment of biometric indicators of the leaves.

Leaf area was linearly related to leaf length and leaf width (A), square root of leaf length and width (B), square root of leaf and width (C) and value of leaf length multiplied by width (D). Other transformed forms of leaf length and

width were not linearly related to leaf area. Relative equations Leaf area was linearly related to leaf length and leaf width (A), square root of leaf length and width (B), square root of leaf and width (C) and value of leaf length multiplied by width (D). Other transformed forms of leaf length and width were not linearly related to leaf area. Relative equations ($LA = a + bL + cW$; $LA = a + bL^2 + cW^2$; $LA = a + bL^{0.5} + cW^{0.5}$; $LA = a + bLW$) were established one by one (Table 1). It was found that when two different transformed forms of leaf length and width which are linearly related to leaf area, were included in one equation, the R^2 value increased and RMSE value would decreased, except in equation 8. When three or four different variables (equation 9 and 10, see Table 1) were included, R^2 values were the highest, namely 0.818. However, equation 10 had a lower RMSE value than equation 9.

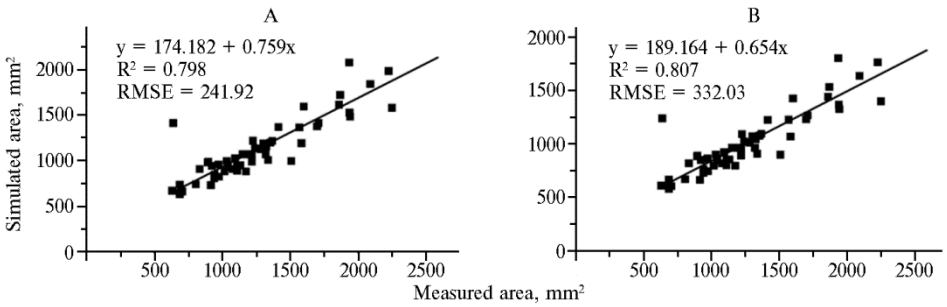

Fig. 3. Validation of the measured vs. estimated values of Russian dandelion (*Taraxacum kok-saghyz* Rodin) single leaf using model 9 (A) **and model 10** (B)**.** Solid line represents linear regression line.

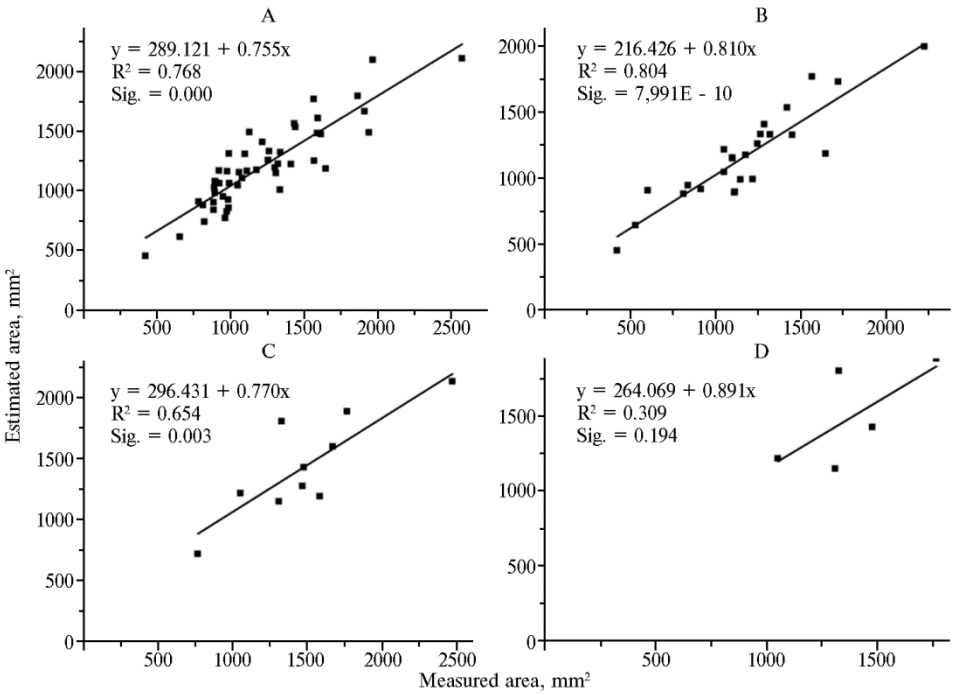

Fig. 4. Sampling number validation of proposed leaf area model for Russian dandelion (*Taraxacum kok-saghyz* Rodin) (one example): fifty leaves (A), twenty five leaves (B), ten leaves (C) and five leaves (D) validation, Sig. stands for significance level.

Validation experiments demonstrated that both leaf areas estimated using model 9 and model 10 were very similar to the measured value of leaf area. The linear regression for the relationship between both measured and estimated values were the same to the 1:1 line at 0.05 level (Fig. 2 and Fig. 3). Model 10 had

1. Estimated models for leaf area of Russian dandelion (*Taraxacum kok-saghyz* Rodin)

No	Variables	Regression models	Constant								R^2	RMSE
			a	b	c	d	e	f	g	h		
(1)	L, W	$LA = a + bL + cW$	−744.204	9.962	42.043						0.810	171.96
(2)	LW	$LA = a + bLW$	225.999	0.424							0.805	174.30
(3)	L^2, W^2	$LA = a + bL^2 + cW^2$	222.184	0.047	0.907						0.810	171.87
(4)	$L^{0,5}$, $W^{0,5}$	$LA = a + bL^{0.5} + cW^{0.5}$	−2609.279	195.109	396.179						0.795	178.54
(5)	L, W, LW	$LA = a + bL + cW + dLW$	−356.135	6.274	23.674	0.171					0.813	170.74
(6)	L, W, L^2, W^2	$LA = a + bL + cW + dL^2 + eW^2$	−392.679	2.062	46.133	0.037	0.287				0.816	169.20
(7)	L, W, $L^{0,5}$, $W^{0,5}$	$LA = a + bL + cW + dL^{0.5} + e\ W^{0.5}$	711.166	25.248	38.795	−306.976	29.071				0.816	169.00
(8)	$L^{0,5}$, $W^{0,5}$, LW	$LA = a + b(L^{0.5}W^{0.5}) + cLW$	−220.583	18.881	0.231						0.808	172.92
(9)	L, W, L^2, W^2, $L^{0,5}$, $W^{0,5}$	$LA = a + bL + cW + dL^{0.5} + eW^{0.5} + fL^2 + gW^2$	6321.276	21.902	579.001	−248.841	−3346.881	0.002	−3.984		0.818	168.34
(10)	L, W, L^2, W^2, $L^{0,5}$, $W^{0,5}$, LW	$LA = a + bL + cW + dL^{0.5} + eW^{0.5} + fL^2 + gW^2 + hLW$	6226.424	26.310	545.334	−313.993	−3138.047	−0.009	−3.866	0.057	0.818	168.29

N o t e. L is leaf length, W is leaf width, LA is leaf area.

2. Estimated models for leaf fresh weight of Russian dandelion (*Taraxacum kok-saghyz* Rodin)

No	Variables	Regression models	Constant								R^2	RMSE
			a	b	c	d	e	f	g	h		
(1)	L, W	$FW = a + bL + cW$	−304.693	4.243	15.378						0.699	93.69
(2)	LW	$FW = a + bLW$	74.630	0.172							0.703	93.00
(3)	L^2, W^2	$FW = a + bL^2 + cW^2$	76.556	0.021	0.319						0.717	90.83
(4)	$L^{0.5}$, $W^{0.5}$	$FW = a + bL^{0.5} + cW^{0.5}$	−1041.233	81.679	147.543						0.678	96.85
(5)	L, W, LW	$FW = a + bL + cW + dLW$	29.784	1.064	−0.455	0.147					0.709	92.03
(6)	LW, L^2, W^2	$FW = a + bLW + cL^2 + dW^2$	77.149	0.048	0.016	0.205					0.718	90.74
(7)	L, W, L^2, W^2, $L^{0.5}$, $W^{0.5}$	$FW = a + bL + cW + dLW + eL^2 + fW^2$	−45.050	−4.630	32.631	0.086	0.033	−0.587			0.734	88.11
(9)	L, W, L^2, W^2, $L^{0.5}$, $W^{0.5}$, LW	$FW = a + bL + cW + dLW + eL^{0.5} + fW^{0.5} + gL^2 + hW^2$	1125.572	−24.857	233.070	0.055	276.956	−1264.466	0.067	−1.964	0.735	87.84

N o t e. L is leaf length, W is leaf width, FW is leaf fresh weight.

a higher R^2 value than that of model 9 and was selected as the final model.

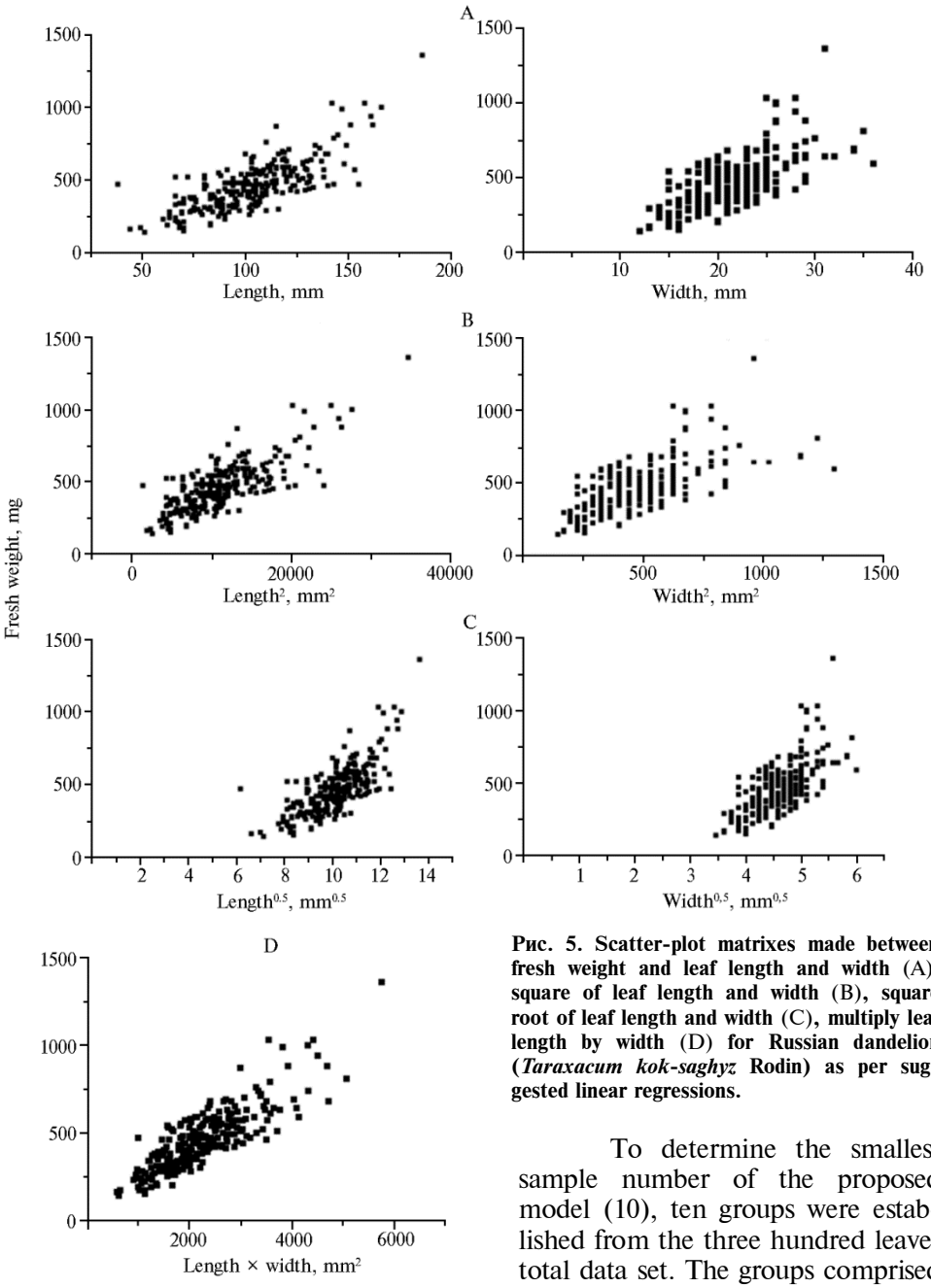

Рис. 5. Scatter-plot matrixes made between fresh weight and leaf length and width (A)**, square of leaf length and width** (B)**, square root of leaf length and width** (C)**, multiply leaf length by width** (D) **for Russian dandelion (*Taraxacum kok-saghyz* Rodin) as per suggested linear regressions.**

To determine the smallest sample number of the proposed model (10), ten groups were established from the three hundred leaves total data set. The groups comprised fifty, twenty five, ten and five leaves data. Linear regression analyses were performed between estimated values and measured values. The results indicated that all significant values of linear regression equations established from ten groups of fifty, twenty five and ten leaves data were smaller than 0.05. However, some significant values of linear regression equations established from ten groups of five leaves data were bigger than 0.05 (Fig. 4). This suggests that the smallest sampling number required is ten when the proposed model (10) is applied.

Leaf fresh weight model established and validated. Scatter-plot matrix figures (Fig. 5) indicated that leaf length, width and other three transformed forms, namely (length, width), ($length^2$, $width^2$), ($length^{0.5}$, $width^{0.5}$),

length multiplied by width, were linearly related to leaf fresh weight. When all the four forms were included in one equation, the final regression equation with the highest R^2 and the lowest RMSE values was established (Table 2). The final simulated equation is: $FW = 1125.572 - 24.857L + 233.070W + 0.055LW + + 276.956L^{0.5} - 1264.466W^{0.5} + 0.067L^2 - 1.964W^2$.

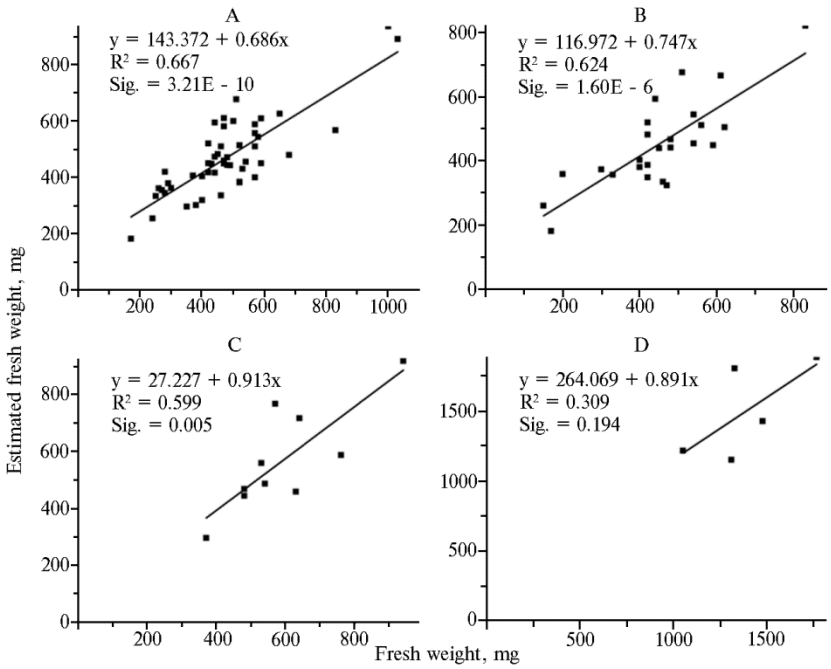

Fig. 6. Sampling number validation of proposed fresh weight model for Russian dandelion (*Taraxacum kok-saghyz* Rodin) (one example): fifty leaves(A), twenty five leaves(B), ten leaves(C) and five leaves (D) validation. Sig. stands for significance level.

Regression analysis to determine the minimum sampling (Fig. 6) showed results similar to the leaf area (at least 10 leaves).

Thus, four transformed forms of leaf length and width, i.e. (length, width), ($length^2$, $width^2$), ($length^{0.5}$, $width^{0.5}$), (length × width), were found to be approximately linearly related to leaf area and fresh weight. When the four transformed forms were integrated in one equation, regression models were established for the estimation of leaf area and fresh weight of Russian dandelion, without the destruction of leaves. The equation for estimating leaf area is: $LA = 6226.424 + 26.31L + + 545.334W - 313.993L^{0.5} - 3138.047W^{0.5} - 0.009L^2 - 3.86W^2 + 0.057LW$, R^2 and RMSE values for the model are 0.818 and 168.29 respectively. At least ten leaves are required when the model is applied. The equation for estimating leaf fresh weight is: $FW = 1125.572 - 24.857L + 233.070W + 0.055LW + 276.956L^{0.5} - - 1264.466W^{0.5} + 0.067L^2 - 1.964W^2$, R^2 and RMSE value for the model are 0.735 and 87.84 respectively. At least ten leaves are required when this model is applied. The proposed models need to be validated in other varieties of Russian dandelion in the future. For plants species with higher variation among individual leaves, determining transformed forms of leaf dimensions that are linearly related to leaf area and fresh weight and integrating all of them into one equation maybe a better solution.

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