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PHYTOPLASMA DISEASES: A REVIEW OF 50 YEAR HISTORY AND CURRENT ADVANCES

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Abstract

Phytoplasma diseases were known long before the discovery of their agent. Since the early 1930s in the former Soviet Union the infectious nature of the disease known under the name of "stolbur of tomato" has been recognized. Attempts were made to find vectors of the disease (I.K. Korachewski, 1934; V.L. Ryzhkov et al., 1934). In 1945 it was found that planthopper *Hyalesthes obsoletus* can be the disease vector (K. Sukhov et al., 1946). Searching for an infectious agent of plant diseases with symptoms of dwarfism, yellowing, damaged leaves and generative organs, which was unable to grow on artificial nutrient media was unsuccessfully conducted for several decades in our country and abroad. The discovery was made only in 1967 by Japanese researchers (Y. Doi et al., 1967). The causative agent of the disease, the unknown earlier phytopathogen from *Mollicutes* class, was similar to mycoplasma pathogens of animals. Later it was found that the life cycle of phytoplasmas is associated with the phloem cells of the plants, in which they multiply, and with vector insects of *Hemiptera* order which are feeding plant juice and able not only to support the phytoplasma reproduction in their body, but even transmit phytoplasma to the offspring. Phytoplasmas, like other *Mollicutes*, have no cell wall; they have a minimal genome known to cellular organisms, which causes their obligate parasitism. In the ex-USSR, phytoplasma disease was extensively studied by electron microscopy, immunological methods, and phytoplasma were successfully cultured on artificial nutrient media. Three decades later Italian researchers managed to culture the infective agent on artificial media and confirmed its belonging to phytoplasma by DNA sequencing (A. Bertaccini et al., 2010; N. Contaldo et al., 2012, 2013). In 1990s, a great step forward was made due to molecular methods of phytoplasma diagnosis and study. Phytoplasmas' taxonomy was developed based on the conservative 16S ribosomal RNA gene and further elaborated with the involvement of other genes having both highly and less conserved sequences (I.-M. Lee et al., 1993; B. Schneider et al., 1993, 1997; I.-M. Lee et al. 1998, 2010; M. Martini et al., 2007). The next important step was a discovery of virulence factors of phytoplasma affecting host plants and making them more attractive to insect vectors naturally involved in the spread of phytoplasmas. In recent years, a lot of genomic data has been obtained for various phytoplasmas; attention is paid to elucidate phytoplasma metabolism which is important to understand the host—pathogen—vector interactions (K. Oshima et al., 2004; X. Bai et al., 2006; A. Hoshi et al., 2009; A. Sugio et al., 2011; A. MacLean et al., 2011; K. Sugawara et al., 2013; Z. Orlovskis et al., 2016). In Russia, molecular methods have allowed the researchers to reveal the phytoplasma nature of a group of diseases with unclear etiology that gives the key to control of these widespread and harmful diseases. Prevention is the primary means of controlling phytoplasma diseases, including the use of healthy planting material, resistant varieties, methods aimed at spatial isolation from sources of infection, weed eradication, and the use of biopreparation and bioagents capable of producing tetracycline antibiotics.

Keywords: phytoplasma, phytoplasma diseases, yellows, witches'-broom, stolbur, phytoplasma vectors, phytoplasma taxonomy, phytoplasma marker genes

In 2017, there were several anniversaries in phytopathology: the 125th

anniversary of the discovery of viruses by D.I. Ivanovsky, the 110th anniversary of the organization of the Phytopathological Laboratory in Russia by A.A. Yachevsky and 50 years from the discovery of phytoplasmas and viroids; what is more, the diseases caused by both these pathogens were known and were considered viral long before their agents were detected. Phytopathogens, which were named phytoplasma in 1994, were discovered in 1967 by Japanese virologists. This discovery could have happened 10 years earlier in the USA, but for unfortunate reasons, it did not happen. In the phloem of plants affected by yellows-like diseases (witches' broom, dwarfism), bacterial polymorphic bodies resembling mycoplasma, which is the pathogen of bovine pleuropneumonia (pleuropneumonia like-organisms, PPLO = PLT) were detected, not viruses [1]. They were also detected in vectors of the disease — leafhoppers (family *Cicadellidae*), and later in jumping plant lices or psyllids (family *Psyllidae*).

In the former USSR, long before the discovery of phytoplasmas, plant diseases such as stolbur, yellows, alfalfa witches' broom, potato and wood witches' broom, grain dwarfism were known. Most often, due to the similarity of symptoms, they were referred to as a virus, but sometimes they were considered non-infectious. The revolutionary discovery by D.I. Ivanovsky in 1892 of the microorganism that causes a mosaic disease of tobacco was followed by a series of discoveries of the so-called filterable viruses, the pathogens of humans and animals, but in phytopathology, virological studies were continued only in the 1920s after A.A. Yachevsky visited the USA. He described a number of viral diseases of potato (*Solanum tuberosum* L.), among which in 1926 was witches' broom [2]. Until the 1930s, studies of viral diseases were limited to describing virus-like symptoms in plants of different species in different regions and comparing them with the diseases described in foreign reviews. In the 1930s and 1940s, the first virology laboratories were organized and data were obtained on the infectivity of a number of diseases considered viral, including the stolbur, and cereal pupation, which were classified as yellows.

For the first time, a disease called Stolbur (from Ukrainian “stovbur” — a “trunk” or “stem”) was observed on tomatoes with symptoms of fruit lignification in the late 1920s. This word later became an international term for phytoplasmas belonging to the 16SrXII group. In 1934, I.K. Korachevsky described the characteristic stolbur symptoms on tomatoes [3]. The infectious nature of the disease was proven by grafting a tomato with stolbur on healthy plant [4]. The disease was not transmitted by seeds or by inoculation of the juice in the tissue of a healthy plant. At the same time, there were cases of the rapid spread of stolbur, leading to a massive infection of plantations. I.K. Korachevsky tried to find a vector of the disease among insects, but the test of aphids, thrips, bugs, and some species of leafhoppers did not give results; therefore, the cause was attributed to the effects of various abiotic factors on the physiology of tomatoes. This was a significant step back in understanding the nature of stolbur [5].

In 1945, K.S. Sukhov and A.M. Vovk, realizing that the environmental hypotheses of the disease causes lead the wrong way, began to persistently look for a vector. They identified the entire species composition of the insects of the *Hemiptera* order, the *Auchenorrhyncha* (or *Cicadinea*) suborder, which visited tomatoes. These were insects from the families *Cixiidae* (2 species), *Delphacidae* (3 species), *Aphrophoridae* (spittlebugs, 1 species), *Cicadellidae* (leafhoppers, 11 species). In total, 17 species of *Cicadinea* and several rarely encountered species were tested (without determining the species). It was possible to identify only one vector species, the *Hyalesthes obsoletus* (Sign.) from the *Cixiidae* family (planthoppers) [6]. In subsequent years, these data were confirmed [7, 8]. The discovery of Soviet scientists was an important step in explaining the epidemiol-

ogy of widespread and harmful diseases of economically significant crops in Eurasia, caused by the stolbur phytoplasmas.

For the first time, infection of potatoes presumably with stolbur was recorded in the Crimea in 1935 [9]. In 1940, it was shown that potatoes can be infected by inoculation [10]. The first outbreaks of stolbur on potatoes were recorded in 1943 in Kyrgyzstan and in 1944 in the Moscow Province [11]. Since that time, the term "stolbur" has been widely used to describe diseases with characteristic symptoms of yellowing and redness of the lamina and growth retardation [12]. In 1945, from 40 to 70% of the potato plantations in the Krasnodar Territory suffered from stolbur. The disease caused a serious decrease in crop yields and led to catastrophic economic losses [12]. The disease had a serious economic impact in other regions of the Russian Federation, especially in the Volga Region [7], as well as in the Union republics of the former USSR: in Crimea [4, 5, 13, 14], in Ukraine [15, 16], in Moldova [17], Armenia [18], Georgia [19, 20], Azerbaijan [21, 22] and in the Central Asian republics [23-25].

In Georgia, a new species of tomato stolbur vector was discovered, the planthopper *Hyalesthes mlokosiewiczi* Sign. (*Cixiidae* family) [26]. Its larvae, nymphs, and adults captured on stolbur-infected corn bindweed plants (*Convolvulus arvensis* L.) were transferred under isolators to healthy tomatoes, where these insects fed for some time, and 21-23 days after feeding, the first symptoms of infection appeared [20, 26].

For a long time in the domestic phytopathological literature, there has been a discussion about the ecological or fungal nature of the stolbur wilting of potatoes Even at present, for the first time encountering the stolbur wilting of potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.) or eggplant (*Solanum melongena* L.), phytopathologists associate it with the damage made exclusively by the fungi of the genus *Fusarium, Rhizoctonia, Verticillium or Colletotrichum* [27]. This is explained by the fact that phytoplasma infected plants of vegetable and grain crops are more susceptible to damage root rot and vascular wilting pathogens, as well as to fungal leaf spots caused, for example, by Alternaria and Cladosporium [28-30].

In 1955, A.M. Vovk and G.S. Nikiforova made the first attempt in the USSR to determine the size and shape of the stolbur pathogen in the juice of a diseased tomato using electron microscopy [31].

In 1890-1900, the main cause of peach tree decline and dieback in the USA, Delaware, was the yellows. In the early 1930s, L.O. Kunkel found the disease vector, leafhopper *Macropsis trimaculata* (Fitch) [32]. Studying the ecology of the vector, during electron microscopic examination of *M. trimaculata* and fixed vessel sections of peach, which showed symptoms of the yellows, A. Hartzell found plasma-like bodies and inclusions. However, because of their lability, specific morphology and differences from the phytoviruses, he failed to identify the pathogen [33, 34].

The American phytovirologist K. Maramorosch injected leafhoppers *Macrosteles fascifrons* Stal. with juice of the aster plant diseased by aster yellows, as well as an extract from leafhopper-vector, and showed the possibility to reproduce pathogen in both the plant and the vector [35, 36]. In one experiment, antibiotics (penicillin, streptomycin, and tetracycline) were added to the extracts. The experimenter was convinced that they should not affect infection, since it was thought that the infectious agent was a virus. However, in the case of tetracycline use, infection did not occur. Finding none explanation for this fact, the author attributed it to the effect of high temperatures in the greenhouse. The experiment was not repeated.

In 1966, K. Maramorosch visited the laboratory of the famous virologist

and immunologist W. Henle in Philadelphia. He brought with him electron microscopic photographs of the salivary glands of leafhoppers. The electron microscopy expert T. Hummeler, who worked at the same institute, after looking at these photographs, drew the author's attention to the presence of structures similar to mycoplasmas. However, K. Maramorosch did not attach any importance to this, because he was not familiar with the work on the successful cultivation of *Mycoplasma pneumonia*, did not know what the word "mycoplasma" means, and did not even take an interest in this [37, 38].

In 1967, during the annual meeting of the Japanese Society of Phytopathologists, E. Shikata from the University of Hokkaido, a former employee of K. Maramorosch, studied the abstract of the article by Y. Doi and colleagues, which considered mycoplasma-like organisms found in the mulberry (genus *Morus*) with symptoms of dwarfism. E. Shikata suspected that such microorganisms were in electron microscopic photographs of the aster yellows pathogen made by him in 1954, when he worked in the laboratory of K. Maramorosch, and asked Maramorosch to send him the photoplates. Photographic negatives were found, but because of the absence of viruses on them, they were not sent on the request [37].

A key role in the recognition of phytoplasmas was played by a veterinarian from the University of Tokyo K. Koshimizu. After studying electron micrographs taken by Y. Doi in 1967, he discovered the similarity of the structures visible on them to mycoplasmas and suggested testing the effect of tetracycline on trees. Y. Doi did not leave this information unaddressed. At the suggestion of Y. Doi, his manager H. Asuyama instructed his other employee T. Ishii to conduct an experiment on healing a diseased young plant of mulberry with tetracycline, which was done with a positive result. As a result, three reports were presented at the annual meeting in Sapporo (Japan), which marked the discovery of mycoplasma-like organisms in plants, later known as "phytoplasmas" [39-41].

In May 1968, the first paper by J. Giannotti et al. [42] on determining the mycoplasma-like organisms in forest apple tree with signs of proliferation appeared in France. The researchers did not make references to the publications of Japanese scientists, presenting their work as a pioneer paper. Later J. Giannotti published data on the cultivation of mycoplasma-like organisms on artificial nutrient media. However, other scientists, in particular, J. Bovй and R. Davis, failed to repeat the cultivation of mycoplasma-like organisms. An attempt to cultivate phytoplasmas in the laboratory of K. Maramorosch was also unsuccessful due to the formation of pseudo-colonies formed with an excess of horse serum [43]. Several unsuccessful attempts made in different laboratories led to the adoption by the international committee of mycoplasmologists of the postulate that it is impossible to cultivate mycoplasmas on artificial nutrient media. However, later K. Maramorosch expressed the hope that the cooperation of phytoplasmologists with other microbiologists will eventually lead to the possibility of cultivating these microorganisms [37].

After the discovery of phytoplasmas, active electron microscopic studies of plant yellows pathogens and their tetracycline therapy began worldwide. The number of papers focused on plant mycoplasmas began to increase progressively: in 1967, four papers were published, in 1968 — 29, in 1969 — 61, in 1970 — 90, and by 1974, the number of detected cases of phytoplasma diseases reached 50. In the USSR, the study of mycoplasma-like diseases was conducted at the Institute of Microbiology of the USSR Academy of Sciences, at the Zabolotny Ukrainian Institute of Microbiology and Virology, at the All-Union Scientific Research Institute for Plant Protection (VIZR), where the laboratory of viral and mycoplasmal diseases was headed by Professor Yu. I. Vlasov, the follower of

Professor K.S. Sukhov, and at some other research institutes.

The purpose of the research carried out at VIZR was to study the patterns of phytoplasma diseases spreading in biocenoses and the development of methods to control them. The classical scheme of the circulation of the stolbur pathogen in nature was described earlier by K S. Sukhov and A. M. Vovk (1949) in the Krasnodar Territory. They noted the natural-focal nature of the disease. Subsequently, corrections and additions were made to this scheme, mainly relating to the species composition of vectors and infection reservoirs [44, 45]. In addition to the planthoppers *Hyalesthes obsoletus*, the disease is spread by meadow froghoppers *Phylaenus spumarius* L. (family *Aphrophoridae*, spittlebugs), leafhoppers *Aphrodes bicinctus* Schrank and *Cicadella viridis* L. (family Cicadellidae), as well as planthopper *Pentastiridius leporinus* L. [46]. Leafhoppers get an infection, feeding on infected perennial plants, i.e. bindweed *Convolvulus arvensis* L., *Goebelia alopecuroides* (L.) Bunge, Canada thistle *Cirsium arvense* (L.) Scop., etc. The migration of leafhoppers from weed and wild plants to crops occurs when the nutritional conditions of insects in a natural focus become unfavorable, for example, when wild plants dry out under the conditions of dry hot weather.

In the 1970s-1990s, the prevalence of stolbur on tomatoes in some seasons reached 50-60%.in the Astrakhan and Volgograd Regions, in the North Caucasus, as well as in Armenia and Uzbekistan. In addition, witches' broom was common on potatoes (*Solanum tuberosum* L.) and alfalfa (*Medicбgo satнva* L.), and phyllody on clovers (*Trifolium* L.). Alfalfa witches' broom was often found in Kazakhstan, Kyrgyzstan, Uzbekistan, and the Volga Region. As a result of the studies of these diseases, carried out by VIZR personnel, republican institutes for plant protection and other institutions, the list of vectors and reservoirs of infection was expanded, the properties of the pathogen were studied, measures to prevent and control diseases were substantiated, taking into account their natural focal nature, practical guidelines were published [47, 48].

In the 1970s-1980s, the laboratory of viral and mycoplasma diseases of VIZR actively cooperated with many institutes in the USSR and other countries. In the joint work, experts from Armenia, Georgia, and Ukraine were engaged. In 1981-1985 the productive cooperation was with the expert from Institut national de la recherche agronomique (INRA, Paris, France) Dr. J. Giannotti. French scientists were interested in a rich collection of plant samples infected by phytoplasmas collected in different regions of the USSR (Astrakhan, Volgograd, Armenia, Uzbekistan) from tomatoes, potatoes, eggplants, and other crops. Electron microscopy revealed the presence of an infectious agent belonging to the *Mollicutes* class. French researchers suggested using artificial nutrient media to study the microbiological properties of pathogens.

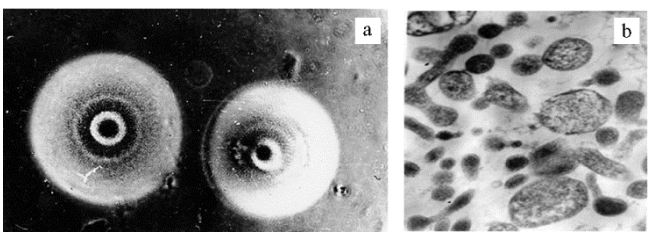

Fig. 1. Growth of stolbur pathogen on an artificial nutrient medium as a "fried egg" type colonies (×480) (a)**; the pathogen of tomato stolbur on a section** (electron microscopy, ×20,000) (b). Photo by L.N. Samsonova [44].

It has been already indicated that after several unsuccessful attempts to culture phytoplasmas on artificial nutrient media, their non-culturability began to be considered as an irrefutable fact. However, in the 1980s, VIZR actively engaged in the culture of microorganisms of the *Mollicutes* class (Fig. 1). Microorganisms isolated on media were serologically related to *Acholeplasma laidlawii* and *Spiroplasma citri*. Now it is difficult to say whether they belonged to phyto-

plasmas, but at that time researchers were sure of this and developed complex nutrient media for their culture [49, 50]. These were the first works of Soviet scientists on the possibility of phytoplasma culturing confirmed in the 21st century by Italian phytoplasmologists by molecular genetic methods.

Beginning from 2010, papers began to appear in Italy that showed the possibility of achieving the growth of phytoplasmas from various 16Sr groups on special commercial media using fragments of shoots of periwincle *Catharanthus roseus* (L.) G. Don. The growth of phytoplasma colonies on agar usually occurs within 2-5 days, although a relatively long pre-incubation in a liquid medium is required. Under equal conditions, phytoplasmas and mycoplasmas form morphologically similar colonies of 0.1-0.2 mm in size. However, quantitative indicators cannot be a differential characteristic, since they can vary widely and depend on the species, the strain of mycoplasma, the medium composition, the temperature and time of incubation, etc. Polymerase chain reaction (PCR) revealed the presence of phytoplasma DNA in cultured microorganisms used as a source of DNA matrix. Identification using restriction fragment length polymorphism analysis (RFLP) and direct amplicon sequencing also confirmed that it is a phytoplasma [51-53].

Until the 1990s, a precise definition of the taxonomic identity of a pathogen causing the stolbur or similar diseases was not possible. In the early 1990s, molecular identification methods appeared. Using 16S rRNA gene, specific oligonucleotide primers were developed, which allowed PCR amplification of the phytoplasmas' 16S rDNA fragments in a wide range of host plants infected by phytoplasmas [54, 55]. It has become possible to determine, differentiate and classify phytoplasmas by RFLP analysis. PCR-amplified phytoplasma 16S rDNA fragments are exposed to restriction endonucleases (each separately), resulting in DNA fragments of different length, which are then separated by electrophoresis in polyacrylamide gel and compared with published restriction maps. The length of the fragments and their number depend on the number of restriction sites in the amplicon molecule for the endonuclease used. Recently, amplicons are more often exposed to direct sequencing and further virtual in silico (computer) cleavage and separation of DNA fragments. A new phytoplasma classification system was developed based on differences in the primary structure of DNA encoding the 16S rRNA gene [54-56].

During the next two decades, phytoplasmas have been found in many plants under diseases of unknown etiology with characteristic yellowing symptoms. The development of virtual RFLP analysis (computer simulated RFLP) allows analyzing a large number of 16S rRNA gene sequences of phytoplasmas deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and greatly facilitates the ability to update their list [57]. Currently, this list contains about 50 groups and over 100 subgroups of phytoplasmas 16S rRNA (16Sr) [58]. It was shown that 16Sr groups correspond to phylogenetic clades established by the method of phylogenetic analysis of the 16S rRNA gene full-length sequence, which indicates the validity of the classification based on RFLP analysis (Fig. 2).

Since 2006 (first within the framework of the Russian-American cooperation on the project of the International Science and Technology Center — ISTC, now —Astana, Republic of Kazakhstan, and then on the Russian programs), the All-Russian Research Institute of Phytopathology has continued to identify phytoplasmas using PCR/RFLP analysis. Phytoplasmic diseases affecting potatoes were monitored in eight economic regions of the Russian Federation: North, North-West, Central, Central Black Earth, North Caucasus, Ural, and West-Siberian. During 7 years of research, more than 1,200 samples with phytoplasma infection symptoms have been tested. Phytoplasmas belonging to five

16Sr groups and eight subgroups have been identified: 16SrI-B, 16SrI-C, 16SrI-P, 16SrII-A, 16SrIII-B, 16SrVI-A, 16SrVI-C, and 16SrXII-A. It is shown that symptoms like stolbur on potatoes can cause both phytoplasma of the stolbur group (16SrXII-A) and phytoplasmas belonging to other groups (16SrI, 16SrIII, 16SrVI) [60].

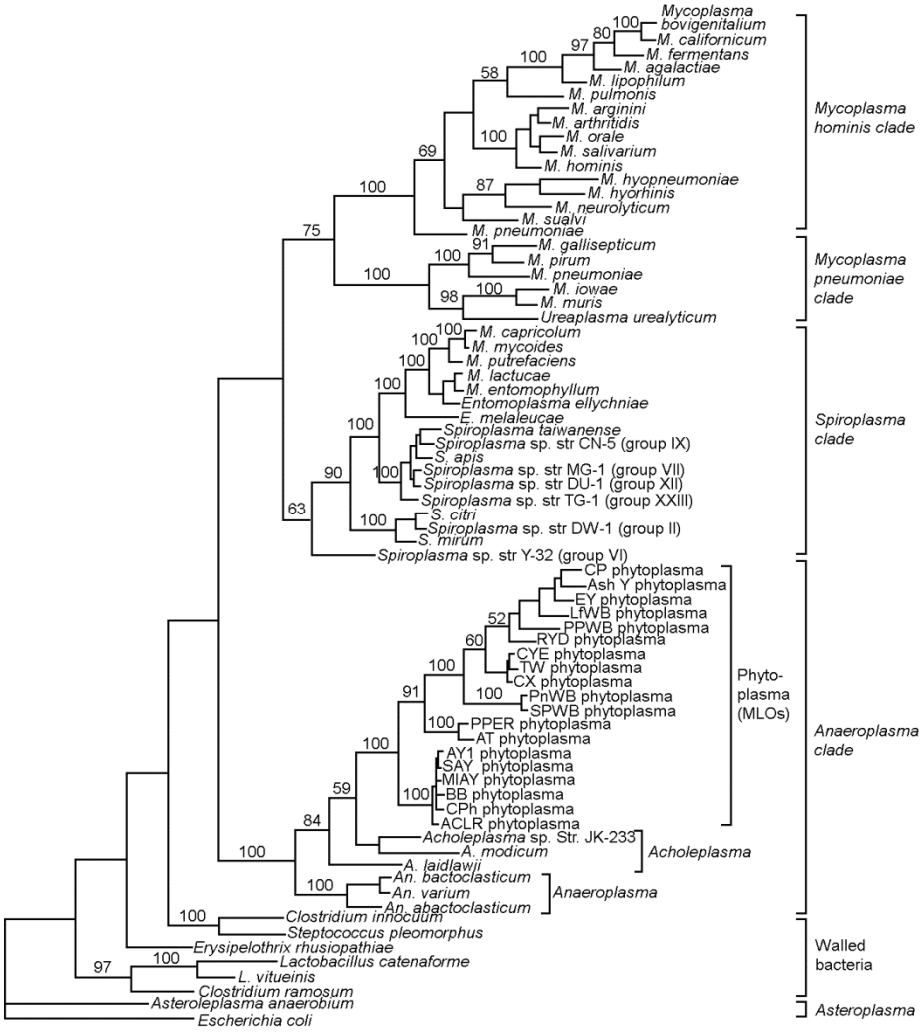

Fig. 2. Phylogenetic tree built using the parsimony analysis based on the full-lenght 16S rRNA gene sequence for 46 members of the *Mollicutes* class and a few representatives of bacteria with cell wall. *Escherichia coli* is used as an external group. The branches length is proportional to the number of changes in the sequence. The bootstrap analysis values for the internal node are indicated on the branches of the tree [59].

Monitoring of phytoplasmic legume diseases in four Russian regions (Northern, Central, Volga and West Siberian) showed that the majority of infected clover plants had typical symptoms of Clover phyllody (CPh), Clover yellow edge (CYE), and Clover proliferation (CP) diseases. These same diseases occurred on plants of other genera and species, but their symptoms could vary significantly. Infected alfalfa showed the typical symptoms of witches' broom. In total, phytoplasmas belonging to four groups and six subgroups were identified on legumes, with the phytoplasmas of Clover yellow edge (16SrIII-B) and Clover phyllody (16SrI-C) being more common; stolbur subgroups (16SrXII-A) and clover proliferation (16SrVI-A) were less often; in a few cases phytoplasmas of

the 16SrI-B and 16SrIII-F subgroups were identified [61]. In addition to potatoes and legumes, phytoplasma was isolated from cultivated and wild plants of more than 30 families, among which, along with grass, were shrubs and woody species. The most rarely encountered groups of phytoplasmas include phytoplasma of the Peanut witches' broom (PnWB) (16SrII), isolated from potatoes and wormwood (*Artemisia vulgaris* L.) in 2009; phytoplasma of the Elm yellows (EY) group (16SrV) isolated from large-leaved elm tree (*Ulmus laevis* Pall), and phytoplasma of the Apple proliferation (AP) 'group (16SrX) isolated from pears (*Pyrus communis* L.) [62]. More than 20 species of insects from the order *Hemiptera*, collected in the Moscow region, have been tested for several years for the phytoplasma carrier state. In eight species of leafhoppers and three species of spittlebugs (*Aphrophoridae*), phytoplasmas of 16SrI (16SrI-B, 16SrI-C, and 16SrI-P subgroups), 16SrIII (16SrIII-O subgroup), 16SrVI and 16SrXII-A groups were found. *Euscelis incisus* Krs., *Macrosteles laevis* Rib. and *Aphrodes bicinctus* Schrk. leafhoppers prevailed [60, 61]. In the Volga Region, *Dictyophara europaea* L. was identified as a carrier of phytoplasma of the 16SrIX group, planthoppers *Hyalesthes obsoletus* Sign. and *Pentastiridius leporinus* L. as carriers of phytoplasma of the 16SrXII-A subgroup and *Psammotettix striatus* L. was infected with the phytoplasma of the group 16SrIII. All the listed insect species of the *Hemiptera* can be potential vectors of phytoplasmas in the central region of Russia and in the Volga Region [62].

Only the phloem-feeding species, mainly from the suborder *Cicadinea*, families *Aphrophoridae* (froghopper), *Cicadellidae* (leafhoppers), *Membracidae* (treehoppers), *Cixiidae*, *Delphacidae* and *Dictyopharidae* (planthoppers), and suborder *Sternorrhyncha*, family *Psillidae* (jumping plant lice) are capable of phytoplasma transfer. It is assumed that the specificity of the vector and phytoplasma connection is determined by the interaction of the main antigenic protein (Amp) of the phytoplasma membrane and the insect microfilament complex, which determines the transition of phytoplasma through the stylet to the intestine, and then to the hemolymph and salivary gland in which phytoplasma multiplies and reaches an infectious titer. This period is called latent. Thus, the majority of *Cicadinea* from the *Cixiidae* family *(Hyalesthes obsoletus* Signoret*, Pentastiridius leporinus* Linnaeus*, Cixius wagneri* China*, Reptalus panzeri* Lцw*,* etc.) have a specific connection with the stolbur group phytoplasmas (16SrXII), and the incubation period can be 20 days.

Fourteen sequences of Russian isolates of potato phytoplasma (EU333397, EU333398, EU333400, EU344884, KP864663-KP864669, KP864672-KP864675) and 42 sequences of legume phytoplasma (KX773491-KX773530, KY587524, and KY587525) were deposited in the GenBank database (Fig. 3) [60, 61].

IRPCM Phytoplasma/Spiroplasma Working Team — Phytoplasma Taxonomy Group (2004) recommended the term *Candidatus* Phytoplasma for naming new types of 16S rRNA gene that has less than 97.5% similarity to the previously described *Ca*. Phytoplasma. Due to the high conservation of 16S rDNA, many biologically and ecologically different strains of phytoplasma, which could be considered as new taxa, are not considered them given this criterion. In this case, to determine the species, additional unique biological properties should be taken into account, such as the specificity of the antibodies, the range of host plants, specific vectors, and molecular criteria.

Another household gene *sec*Y, encoding the translocation of a ribosomal subunit, is also successfully used as a marker to identify more distinctions within groups and subgroups of phytoplasmas. It can be used to differentiate genetically close, but ecologically different strains that cannot be distinguished by analyzing the 16S rRNA gene [67]. RFLP analysis of amplified fragments containing the

sequence of the phytoplasma *groEL* gene, encoding the heat shock proteins from the HSP60 family, made it possible to differentiate eight different strains previously attributed by the ribosomal classification to one 16SrI-B subgroup [68]. The sequence of the 16S-23S rRNA intergenic region has also been used to differentiate phytoplasmas [69, 70]. The indicator of the genetic diversity of phytoplasmas is significantly increased by analyzing *vmp1* and *stamp* genes encoding phytoplasma membrane proteins [71, 72].

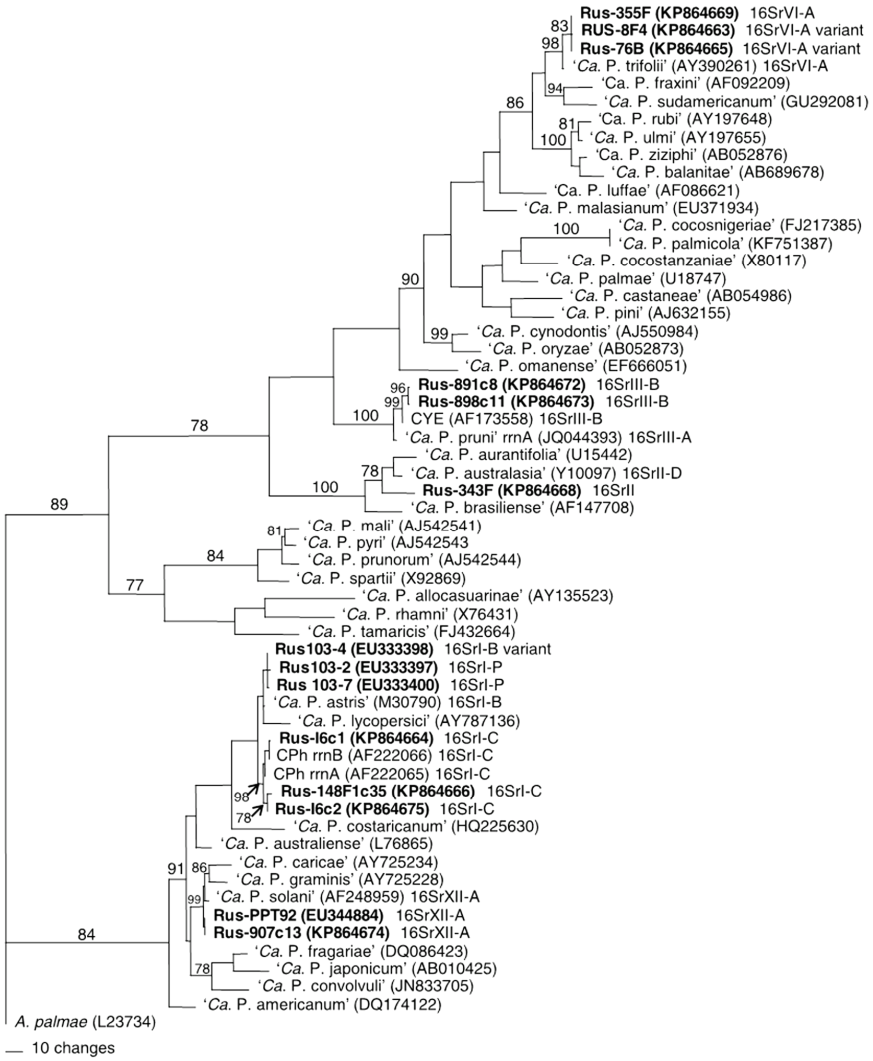

Fig. 3. Phylogenetic tree constructed using the parsimony analysis based on a partial sequence of the 16S rRNA gene for Russian potato phytoplasma strains of the *Candidatus* Phytoplasma species. *Acholeplasma palmae* was used as an external group for the tree rooting. The branches length is proportional to the number of changes in the sequence. The bootstrap analysis values are shown on the main branches of the tree. In the brackets, there are numbers under which sequences are deposited in GenBank. Numbers of Russian isolates are in bold [60].

In 2004, the full genome was sequenced in the *Candidatus* Phytoplasma asteris strain OY-M. Its size was 800 thousand bps. This was a minimum set of genes necessary for the existence of an obligate intracellular parasite. As it turned out, even less metabolic functions are encoded in the phytoplasma genome than in mycoplasmas. In addition, the pentose phosphate cycle and ATP synthase subunits, which were thought to be necessary for life, were not found in the studied phytoplasma. This may be the result of intracellular parasitism in a nutri-

ent-rich environment [73]. *Acholeplasmas* and phytoplasmas differ from mycoplasmas and spiroplasmas in that the UGA triplet serves as stop codon, whereas in the rest of prokaryotes, including most *Mollicutes*, this triplet encodes the amino acid tryptophan.

Like many plant pathogens, phytoplasmas produce virulence factors (i.e. effectors) that interfere with the host's normal life processes, changing them in favor of the pathogen. The first such effector protein described, the "tengu-su inducer" (TENGU), was isolated from onions (*Allium* sp.) infected with phytoplasma which caused yellowing [74]. This protein is transported via the phloem into other cells, including cells of the apical and axillary meristem, and causes characteristic symptoms, the witches' broom and dwarfism. The N-terminus of TENGU contains an 11 amino acid signal peptide which is cleaved *in vivo* during proteolysis by plant serine protease. It is assumed that this fragment at the N-terminus of the protein directly induces the development of the observed symptoms [75].

After 2 years, a report appeared on deciphering the genome of another phytoplasma strain which is the pathogen of the witches' broom of lettuce (*Lactuca sativa* L.). While the first strain belonged to the 16SrI-B subgroup, the second belonged to the 16SrI-A subgroup. This is the largest subgroup causing more than 100 economically significant diseases [76]. It was shown that strain AY-WB uses at least two protein effectors (SAP54 and SAP11) to affect the host plant, making the plant more suitable for colonization by insect vectors. The spread of phytoplasmas in nature [77-79] depends entirely on them.

A genome sequence was constructed for four phytoplasmas belonging to the 16SrIII group (X-disease) — the MA strain that causes the witches' broom of cranberries *Vaccinium* subgen. *Oxycoccus* (Hill) A. Gray, the JR1 strain that causes the phyllody of clover (*Trifolium* L.) in Italy, phytoplasmas that induce branching of poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch), and phytoplasma of euphorbia yellows (*Euphorbia antiquorum* L.). All four strains, despite their belonging to different subgroups of the 16SrIII group, had similar genomes and included a highly conservative portion (the DNA sequence identity was 92-98% for 500 bps) and small strain-specific regions. The genes encoding functional proteins that provided interaction with the host plant (membrane transport, proteases, DNA methylases, effectors, etc.) differed from each other and from strains of other species [80].

German researcher M. Kube compared four phytoplasmas, the OY and AY-WB strains of *Candidatus* Phytoplasma *asteris*, as well as *Ca.* P. *australiense* and *Ca.* P. *mali*. A complete set of genes required for glycolysis was found in all phytoplasmas except for *Ca.* P. *mali* [81]; therefore, the issue of the alternative way of obtaining ATP arose. Phytoplasmas do not have a set of genes for sterol biosynthesis, tricarboxylic acid cycle, phosphotransferase, *de novo* nucleotide synthesis, and amino acid synthesis. This explains the need for localization of the pathogen in sieve tubes, since it is their juice that contains the necessary metabolites. Although its composition varies depending on the type of plant, it always has a large amount of carbohydrates. Despite the difference in genome size, the set of functional proteins is the same for all phytoplasmas [82].

In recent years, methods for diagnostic of phytoplasma diseases have been developed, the phytoplasma taxonomy continues to be improved, a database has been developed, which expands the possibilities of studying the alleged virulence factors, and a lot of information has been obtained about the organization of the genomes of various phytoplasmas [83-86]. The basis for controlling phytoplasmic diseases are prevention methods: obtaining healthy planting material, the use of resistant varieties and agricultural methods that determine resistance to both the

pathogen and its vectors, spatial isolation from sources of infection, the destruction of infectious reservoirs and vectors, the use of biopreparations and bio-control agents capable of producing antibiotic substances of the tetracycline group.

Thus, over the 50 years that have passed since the discovery of the causative agents of phytoplasma diseases, significant progress has been made in studying these pathogens: the list of hosts-plants and vectors of diseases has been extended, the genetic aspects of harmfulness have been studied and continue to be studied, the papers considering the metabolism of phytoplasmas have appeared, which is important for understanding the host—pathogen—vector interaction. The study of phytoplasmic diseases has confirmed their wide distribution and catastrophic harmfulness. Monitoring studies conducted in Russia in the last decade indicate that phytoplasmas affect various cultures, which requires the deeper experimental researches and joint efforts of virologists and experts related to agriculture and forestry.

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MULTIPLEX PCR-BASED IDENTIFICATION OF POTATO GENOTYPES AS DONORS IN BREEDING FOR RESISTANCE TO DISEASES AND PESTS

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Abstract

The breeding of potatoes with the traditional technology of hybridization and selection of individual plants is a time-consuming process. The use of DNA markers linked to genes underlying resistance to diseases and pests can significantly improve the efficiency of the selection of valuable genotypes in the early stages of breeding process. The aims of the work were i) screening of potato genetic resources from the VIR collection (Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg) for the presence of genes encoding resistance to cyst-forming nematodes, potato wart, potato viruses X and Y (PVX and PVY) by the multiplex PCR method; ii) evaluation of the effectiveness of molecular markers for the identification of potato genotypes resistant to the golden nematode, potato wart and PVY. A total of 90 accessions from the VIR collection were studied, among them the cultivated potatoes from two subspecies, the *S. tuberosum* subsp. *chiloense* (native varieties of Chile) and *S. tuberosum* subsp. *tuberosum* (breeding varieties), as well as hybrid clones have been distinguished as sources and donors of potato resistance to pathogens of the economically significant or quarantine diseases. In this work, several molecular markers that were early recommended for the identification of potato genes responsible for the resistance to cyst nematodes, Y and X viruses, and potato wart were first used for the multiplex PCR analysis of genetically diverse material. Ten markers used were TG 689, 57 R, N 195 of *H1* gene and Gro1-4-1 of *Gro1-4* gene (resistance to the golden nematode *Globodera rostochiensis* pathotypes Ro1, Ro4), marker Gpa 2-2 of *Gpa2* gene (resistance to the pale nematode *G. pallida* pathotype Pa2), RYSC3 marker of Ry_{adg} gene, Ry 186 marker of Ry_{chc} gene and YES3-3A marker of Ry_{sto} gene (all genes provide immunity to the potato virus Y), the PVX marker of the *Rx* gene (immunity to potato virus X) and the NL 25 marker of the *Sen1* for resistance to potato wart caused by *Synchytrium endobioticum* (Schilb.) Percival. The PCR screening results were matched with the phenotypic characteristics of the test potato genotypes for resistance to the golden nematode, wart and potato virus Y. Multiplex PCR analysis allowed us to identify potato genotypes with several (up to five) resistance genes, including those providing resistance to the nematode *G. rostochiensis* patotype Ro1, *S. endobioticum* patotype 1 and potato virus Y. A significant association was established between the molecular markers linked to the *H1* gene and the resistance of potato genotypes to the nematode *G. rostochiensis* Ro1 (r_A = 0.59, r_s = 0.72-0.79), and between the marker N L25 of *Sen1* gene and potato resistance to wart (r_A = 0.62). No association was detected between Ry_{adg} and Ry_{sto} molecular markers and plant resistance to potato virus Y due to a large number of tested resistant potato genotypes which possibly carry unknown immunity genes.

Keywords: potato, *Solanum* ssp., interspecific hybrids, DNA markers, marker assisted selection, potato wart, *Synchytrium endobioticum*, nematodes, *Globodera rostochiensis* Ro1, potato virus Y

Potato varieties are created by hybridization of pre-selected parental forms the progeny of which has a high probability of the genotypes with an optimal combination of the necessary traits [1, 2]. High heterozygosity and tetraploid nature of the forms involved in crossing (varieties and interspecific hybrids) causes a phenotypic diversity of F_1 hybrids. Segregants (potentially new varieties of potato) are evaluated according to 40-50 characters of production and quality, as well as resistance to adverse abiotic and biotic factors [3-5]. The conventional potato breeding scheme is based on an annual phenotypic assessment and selection of the best genotypes. Individual genotypes are retained by vegetative propagation in the form of clones the number of which gradually decreases with a simultaneous increase in the number of laboratory and field tests. The duration of selection from the first stage, which is related to visual assessment and selection in the F_1, until the transfer of promising clones to the state variety testing is at least 10 years [3, 4, 6]. Improvement of this process via advanced molecular techniques and the creation of new breeding technologies is one of the current priorities.

Many valuable breeding traits of potato, including resistance to pathogens and pests, e.g. buckeye rot (pathogen *Phytophthora infestans* Mont. de Bary), Potato Virus X (PVX), Potato Virus Y (PVY), Potato Virus S (PVS), Potato Leaf Roll Virus (PLRV), cyst nematodes, potato wart caused by *Synchytrium endobioticum* (Schilb.) Perc., have monogenic nature [1]. Molecular markers linked to *Rpi*-resistance to buckeye rot, the Ry_{sto}, Ry_{adg} and Ry_{chc} genes, which control immunity to PVY, markers of the *Rx1* gene, which control immunity to PVX, the *H1* and *Gro1-4* genes for resistance to the golden nematode *Globodera rostochiensis* (Wollenweber) Behrens and the *Sen1* gene for resistance to the potato wart, can be an effective tool for breeding intensification. Their use to identify valuable genotypes, including forms with several resistance genes, can significantly improve the efficiency of selection in the early steps of breeding [7-9]. The list of DNA markers that are linked to resistance genes or are fragments of potato genes for resistance to pests is constantly expanding [10-12]. A new approach to the use of DNA markers is the development of multiplex PCR analysis technology for the simultaneous testing of varieties and breeding lines for several genes that control resistance to viruses, nematodes, and buckeye rot [9, 13, 14].

Screening of varieties and parental forms for the presence of disease and pest resistance genes is important for the development of potato breeding [15-17]. A permanent source of valuable germplasm for breeders from Russia and neighboring countries is the potatoes collection of Vavilov All-Russian Institute of Plant Genetic Resources (VIR) [18-20]. Molecular genetic characterization of potatoes interspecific hybrids, which are provided to breeders as sources and donors of pest resistance traits, will allow more reasonable selection of parental pairs for crossing.

In the present paper, Chilean aboriginal potato varieties were first characterized using DNA markers of *H1*, *Gro1-4*, *Gpa2*, Ry_{sto}, Ry_{adg}, Ry_{chc}, *Rx1*, and *Sen1* genes. The results of simultaneous testing of clones of interspecific hybrids and potato varieties for several genes were obtained for the first time. The effectiveness of multiplex PCR was evaluated by comparing the results of DNA analysis of collection samples with their phenotypic characteristics for resistance to the golden nematode, potato wart, and Potato Virus Y.

The goal of the study was the molecular screening of varieties and potato breeding material from the collection of Vavilov All-Russian Institute of Plant Genetic Resources (VIR) to identify forms with genes for resistance to cyst

nematodes, potato wart, potato viruses X and Y using multiplex PCR analysis technology, as well as an assessment of effectiveness of the PCR test.

Techniques. A total of 90 accessions from the VIR potatoes collection were studied, which represent the species *Solanum tuberosum* L. and hybrid clones isolated according to a complex of breeding and economically valuable traits in the progeny resulting from interspecific crosses with wild-growing and cultivated species of the *Petota* Dumort. section of the *Solanum* L. genus. The sample studied included 9 forms of the subspecies *S. tuberosum* subsp. *chiloense* (A.DC.) Kostina (indigenous varieties from Chile) and 14 forms of the subspecies *S. tuberosum* subsp. *tuberosum* (5 domestic and 9 foreign varieties). The remaining 67 analyzed forms were clones selected in the progeny of various combinations resulting from cross-breeding or self-pollination of hybrids created on the basis of wild-growing and cultivated species, varieties or breeding lines of potatoes. They included 12 clones created in 1990-1997 by K.Z. Budin at VIR, 3 clones obtained in 2008 by V.A. Kolobayev at the All-Russian Research Institute of Plant Protection, and 52 clones selected in 1999-2011 at VIR. Among the hybrid clones, 22 were two-species hybrids, including 16 selected in combinations of *S. tuberosum* with wild-growing relatives: the endemic Bolivian species *S. alandiae* Card. (12 clones), Mexican species *S. stoloniferum* Schlecht. (2 clones) or widespread in South America *S. chacoense* Bitt. (2 clones). Another 6 clones represented progeny resulting from crossing wild-growing species *S. okadae* Hawkes et Hjerting and *S. chacoense*. The three-species hybrids included 21 clones obtained by crossing samples of cultivated potato species *S. tuberosum*, *S. andigenum* Juz. et Buk. and *S. rybinii* Juz. et Buk. or *S. phureja* Juz. et Buk. The complex multi-species hybrids included 24 clones; in their creation, varieties, breeding lines, and species of cultivated and wild potatoes (*S. acaule* Bitt., *S. stoloniferum*, *S. bulbocastanum* Dun., *S. microdontum* Bitt., *S. polytrichon* Rydb., *S. spegazzinii* Bitt. and *S. vernei* Bitt. et Wittm) were crossed.

Multiplex PCR analysis was performed to identify eight genes that control potato resistance to the most dangerous pathogens, including cyst nematodes golden nematode *G. rostochiensis* pathotype Ro1, Ro4 (*H1*, *Gro1-4* genes) and pale nematode *Globodera pallida* (Stone) Behrens pathotype Pa2 (*Gpa2* gene); PVY (Ry_{sto}, Ry_{adg} and Ry_{chc} genes) and PVX (*Rx1* gene), and potato wart pathogen *S. endobioticum* pathotype 1 (*Sen1* gene). DNA was isolated from leaves of the collection potato plants (the experimental field of the VIR Pushkin laboratories, St. Petersburg—Pushkin).

To improve the analysis efficiency, eight markers with similar amplicons sizes (RYSC3, Ry 186, YES3-3A, TG 689, 57 R, N 195, Gro 1-4-1 and Gpa2-2) were combined into one multiplex reaction. For the internal positive multiplex control, the length of the analyzed fragments in the Golubizna, Kolobok, Uladar, and Belosnezhka varieties were used as standards. Two more markers with larger amplicon sizes (NL 25 and PVX) were combined into another reaction using the length of the analyzed Meteor fragments for their control. Amplification products were sequenced (a Nanofor 05 sequencer, Institute of Analytical Instrumentation RAS, Russia). Direct primers were marked using 6FAM or 5R6G fluorescent dyes (Sintol LLC, Russia).

The amplification reaction of 8 multiplex PCR markers was performed (Applied Biosystems 2720 Thermal Cycler, ThermoFisher Scientific, USA) according to the following program: 10 min at 94 °C (1 cycle); 30 s at 94 °C, 30 s at 68 °C, 1 min at 72 °C (5 cycles); 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C (35 cycles); 30 s at 94 °C, 5 min at 72 °C (1 cycle). The amplification reaction of two markers with larger sizes was performed using the same instrument according to the same program, but with a 30 s increase in the elongation time on

all cycles.

The resistance of *S. tuberosum* and hybrid clones to PVY was assessed by the method of artificial infection with subsequent ELISA diagnosis of viral infection. Information about the resistance of indigenous varieties from Chile was found in the paper by Kostina [22]. Data on the resistance of breeding varieties to the nematode *G. rostochiensis* (pathotype Ro1) and potato wart *S. endobioticum* (pathotype 1) were based on the characteristics of domestic varieties included in the State Register of Breeding Achievements and foreign varieties presented in the database (http://www.europotato.org). The resistance of breeding clones to the nematode was assessed in laboratory experiments at All-Russian Research Institute of Plant Protection (VIZR, St. Petersburg—Pushkin), and resistance to the potato wart was assessed during laboratory experiments at Lorch All-Russian Research Institute of Potato Farming (VNIIKH, Moscow Province) [23].

The relationship between the identified resistance of varieties and hybrid clones to PVY, nematode or potato wart, and detected DNA markers of R-genes was determined using the χ^2 test. The H_0-hypothesis was rejected if $\chi^2_F \geq \chi^2_{st} = 10.83$ for the significance level $\alpha = 0.1\%$. The strength of the relationship between the resistance of the studied potato samples to PVY, nematode or potato wart, and the detected DNA markers of R-genes was evaluated using the association coefficient r_A. The significance of the association coefficient was determined by the Student's *t*-test. The H_0-hypothesis was rejected if $t_F \geq t_{st}$ for significance level $\alpha = 0.1\%$ [24].

Results. We screened 90 potato samples using 10 DNA markers recommended for the detection of R-genes that control the resistance of varieties and selection clones to different types of cyst nematodes, potato viruses X and Y and potato wart (Table 1).

1. *R*-genes and DNA markers used for molecular screening of potato samples (*Solanum* L.) from the collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR)

Gene	Chromosome	Trait	DNA marker (specific fragment)	Reference
Ry adg	11	Immunity to Potato Virus Y (PVY)	RYSC3 (321 bp)	[25]
Ry sto	12	Immunity to PVY	YES3-3A (341 bp)	[26]
Ry chc	7	Immunity to PVY	Ry 186 (587 bp)	[13]
H1	5	Resistance to *Globodera rostochiensis* pathotypes Ro1, Ro4	TG 689 (141 bp)	[27]
H1	5	Resistance to *G. rostochiensis* pathotypes Ro1, Ro4	57 R (452 bp)	[28]
H1	5	Resistance to *G. rostochiensis* pathotypes Ro1, Ro4	N 195 (337 bp)	[29]
Gro1-4	7	Resistance to *G. rostochiensis* pathotypes Ro1, Ro4	Gro 1-4-1 (602 bp)	[29]
Gpa2	12	Resistance to *G. pallida* pathotype Pa2	Gpa 2-2 (452 bp)	[29]
Rx	12	Immunity to potato virus X	PVX (1230 bp)	[13]
Sen 1	11	Resistance to potato wart (*Synchytrium endobioticum*)	NL 25 (1400 bp)	[30]

Screening using multiplex PCR identified genotypes with markers of the *H1* and *Gpa2* genes that control resistance to different types of cyst nematodes, the *Sen1* gene, which provides resistance to potato wart, and the *Rx* and Ry_{adg}, Ry_{sto} genes that provide immunity to Potato Viruses X and Y (Table 2). The Gro1-4-1 markers of the *Gro1-4* gene (another gene for resistance to the golden nematode *G. rostochiensis* pathotypes Ro1, Ro4) and Ry 186 of the Ry_{chc} gene (resistance to Potato Virus Y) were not detected in the studied sample.

The frequency of detecting DNA markers of genes for resistance to harmful organisms was different (see Table 2). The largest number of such markers in one genotype was detected in commercial potato varieties. Markers of five genes, the Ry_{sto}, *Rx*, *Sen1*, *Gpa2,* and *H1*, were detected in the varieties Meteor (Lorch VNIIKH) and Nur-Alem (Kazakhstan). Markers of *Sen1* and *H1* genes were detected in Nayada and Nevsky varieties, *Sen1* and Ry_{adg} in Valeriy and

2. Frequency of genotypes with DNA markers of *R*-genes for resistance to pathogens in potato samples (*Solanum* L.) of different origin

Pedigree-based group	Number of genotypes (total $n = 90$)	Frequency of gene markers							
		H1			*Gpa2*	*Sen 1*	Ry_{adg}	Ry_{sto}	*Rx*
		TG 689	57 R	N 195	Gpa 2-2	NL 25	RYSC3	YES3-3A	PVX
Indigenous Chilean varieties (*S. tuberosum* subsp. *chiloense*)	9	0	0.22	0	0.11	0.44	0.11	0	0
Bred varieties (*S. tuberosum* subsp. *tuberosum*)	14	0.14	0.36	0.14	0.14	0.64	0.21	0.14	0.14
Hybrids (*S. tuberosum*, *S. alandiae*)	12	0.25	0.58	0.58	0	0.42	0	0	0
Hybrids (*S. tuberosum*, *S. stoloniferum*)	2	0	0	0	0	0	0	0	0
Hybrids (*S. tuberosum, S. chacoense*)	2	1.0	1.0	1.0	0	0.50	0	0.50	0
Hybrids (*S. okadae*, *S. chacoense*)	6	0	1.0	1.0	0	0	1.0	0	0
Hybrids (*S. tuberosum*, *S. andigenum*, *S. rybinii* or *S. phureja*)	21	0.33	0.33	0.33	0	0.57	0	0.09	0
Complex multispecies hybrids	24	0.25	0.17	0.17	0.08	0.21	0.04	0	0

Udacha varieties. Among the 9 studied Chilean indigenous varieties, Frutilla had markers of Ry_{adg} and *Gpa2* genes, Amarilla redonda of *Sen1* and *H1* (57 R) genes, Magellanes and Negra of *Sen1* gene, and Negra var. infectum had the marker 57 R of *H1* gene. It should be noted that not only commercial potatoes varieties but also Chilean indigenous varieties had markers of *H1*, Ry_{adg}, and *Gpa2* genes. The source of these genes in breeding varieties is the highland Andean potato which is considered by Russian botanists as an independent species *Solanum andigenum* Juz. et Buk., and by foreign researchers as subspecies *S. tuberosum* [22]. Isolated area and a clear difference in morphological and biological characteristics from Chilean potatoes are the main arguments for determining the species status of *S. andigenum*. A possible reason for the detection of DNA markers of *S. andigenum* genes in Chilean potato samples in the VIR collection may be reproduction of some samples of this collection through seed reproduction. In this case, the progeny resulting from free (uncontrolled) self-pollination may have some genotypes of hybrid origin.

Markers of the *H1* gene were detected with a high frequency in hybrid clones selected in the first generation of *S. tuberosum* hybrids or backcrossings with an endemic Bolivian species *S. alandiae*. The marker *Sen1* was also detected in two genotypes (24-2 and 135-2-2006) of this group. Markers of the *H1* and *Sen1* genes were detected in four clones the pedigree of which contained the cultivated species *S. tuberosum*, *S. andigenum,* and *S. rybinii* or *S. phureja*, and clone 94-5 (two-species hybrid resulting from crossing Bobr × *S. chacoense*). Markers of the *H1* and Ry_{sto} genes were detected in the genotype of clone-sibling 99-10-1, *Sen1* and Ry_{sto} in the genotype of three species hybrids, *Sen1, H1* and *Gpa2* in 167-1-2008, *Sen1* and Ry_{adg} in all six two-species hybrids resulting from crossing *S. okadae* × *S. chacoense*.

3. The number of potato samples (*Solanum* L.) with identified DNA markers of resistance *R*-genes in different phenotypic classes

Marker (gene)	Phenotype		χ^2_Φ	r_A
	resistant	susceptible		
Resistance to *Synchytrium endobioticum*				
NL 25 (*Sen1*) (+)	13	7		
Nor found (–)	1	26		
Total	14	33	17.81*	0.62*
Resistance to *Globodera rostochiensis*				
TG 689 (*H1*) (+)	0	4		
57 R (*H1*) (+)	0	2		
57 R + N 195(*H1*) (+)	8	0		
TG 689+57 R + N 195 (+)	12	2		
Nor found (–)	4	27		
Total	24	35	18.53*	0.59*
Resistance to Potato Virus Y				
RYSC3 (Ry_{adg}) (+)	6	5		
YES3-3A (Ry_{sto}) (+)	3	0		
Nor found (–)	12	35		
Total	21	40	5.56	–

Note. "+" and "–" mean the presence and absence of a marker; χ^2_Φ is sample-based criterion χ^2, r_A is association coefficient. A dash means that the coefficient was not determined, since the marker-trait relationship was not established.

* The value is statistically significant at $\alpha = 0.1\%$.

Among the 47 genotypes evaluated for the resistance to *S. endobioticum* pathotype 1, 14 were resistant (Table 3). Among them, *S. tuberosum* prevailed (8 modern varieties and 3 Chilean indigenous varieties). Two clones which were derivatives of *S. alandiae* and one selected in the progeny resulting from the crossing of *S. okadae* × *S. chacoense*, were stable in two-year laboratory tests. Among the 59 genotypes evaluated for the resistance to *G. rostochiensis* pathotype Ro1, 24 were resistant (see Table 3). This group included two varieties (Meteor and Nayada) and 22 hybrid clones selected in the progeny resulting

from different combinations of two, three or more cultivated and wild *Solanum* species. Among them, 4 clones were derivatives of *S. alandiae*, 9 clones were two-species hybrids, derivatives of *S. okadae*, *S. chacoense* or *S. stoloniferum*, as well as previously created nematode resistance donors (clones 190-4, 90-7-7, 90-6-2) [21] and the progeny resulting from crossing 90-6-2 with varieties.

In studying the response of 61 potato genotypes to the PVY infection, we identified 21 samples resistant to PVY (see Table 3). The studied set contained only one PVY-resistant variety, the Meteor; the remaining breeding and indigenous samples were affected by the virus during artificial infection. Among 20 PVY-resistant hybrid clones, 9 were three-species hybrids the pedigree of which included *S. tuberosum*, *S. andigenum*, and *S. rybinii*. These are clones 90-6-2, 97-155-1, the donors of resistance to virus Y previously created at VIR [23] and the progeny resulting from their crossing with varieties. All two-species hybrids with *S. chacoense* were resistant to PVY: six clones were selected in progeny resulting from crossing with *S. okadae,* and two clones were derivatives of *S. tuberosum* × *S. chacoense*. Three complex multi-species hybrids were also resistant to the virus.

To evaluate the relationship between the presence of DNA markers and the resistance of potato varieties and hybrid clones to the nematode, potato wart and PVY, we used analyses of fourfold tables that presented the number of two phenotypic classes (resistant and susceptible samples) and groups with identified and not detected DNA markers of the corresponding resistance genes (see Table 3). The results of detecting different markers of the genes for resistance to the nematode and PVY took into account the total number (sum) of all detected markers. Assessment of the distribution of samples according to the phenotypic classes and groups using the χ^2 leads to the conclusion that there was a statistically proven relationship between the potato response to the nematode *G. rostochiensis* pathotype Ro1 and markers of the *H1* gene, as well as between the response of plants to the *S. endobioticum* pathotype 1 and marker of the *Sen1* gene. Sample χ^2 values significantly exceeded the critical ones (see Table 3), and the H_0-hypothesis was refuted at a high level of significance ($p < 0.001$). We also established a relationship between resistance to the nematode and potato wart and the detected markers of the corresponding R-genes, as evidenced by the significant association coefficients r_A (see Table 3). Among DNA markers of the *H1* gene used for molecular genetic screening, markers 57 R and N 195 had the strongest marker-trait relationship (Spearman's correlation coefficients $r_s = 0.72$ and $r_s = 0.79$).

Comparison of data on marker detection and assessment for resistance to the nematode *G. rostochiensis* Ro1 revealed false-positive results in Atzimba and Nevsky samples (marker 57 R detected), clones 160-1, 160-17, 159-31, 97-152-8 (marker TG 689) and clones 90-7-2, 39-1-2005 (all three markers of the *H1* gene). The probable reason for the presence of marker fragments amplified with primers used in potato samples affected by the nematode can be the complex structure of the *H1* locus which is characterized by the high-abundance of homologous fragments of other R-genes or genes encoding structural protein in the plant cell wall [28]. The fact that the markers of the *H1* gene were not detected in the nematode resistant clone 99-6-6 is consistent with the results of the molecular screening of the VNIIKH collection in which the markers of the *H1* gene were also not detected in four clones, including 99-6-6 [31]. Segregation in the progeny resulting from the self-pollination crossing of the clone 99-6-6 with the susceptible varieties Zagadka Pitera and Peterburgskii indicates the polygenic nature of the clone 99-6-6 resistance to the nematode [32] which explains the absence of DNA markers of the *H1* gene in PCR analysis.

In comparing data on the detection of the marker NL 25 of the *Sen1* gene and the assessment for resistance to *S. endobioticum,* false-positive results were obtained in one clone which is derivative of *S. alandiae*, four clones the pedigree of which included cultivated types of potatoes, and two multi-species hybrids. To explain the genetic nature of potato resistance to *S. endobioticum*, two models are proposed according to which the protective effect is manifested as the result of the expression of one dominant gene *Sen1* or the combined action of two dominant genes *Sen1* and *Sen1-4* localized respectively on the 11th and 4th chromosomes [30, 33]. It is possible that the differences in the efficiency of using the NL 25 marker of the *Sen1* gene for the screening of hybrid clones were associated with differences in the genetic control of the forms used as donors of resistance. False negative results of the samples resistant to *G. rostochiensis* Ro1 or to *S. endobioticum* could be obtained due to insufficient accuracy of phenotypic evaluation in laboratory tests or as a result of recombination at sites located between the marker fragment and the gene. It is also possible that other genes [7] provide resistance.

In this study, the χ^2 significance of the potato samples' distribution on the resistance to PVY and the presence of the markers of Ry_{adg} and Ry_{sto} genes was not proved due to the large number of resistant genotypes in which markers of known potato genes for PVY resistance were not detected (see Table 3). The RYSC3 marker of Ry_{adg} gene was detected in four samples susceptible to the PVY (the Chilean Frutilla form, the varieties Valeriy, Bintje, and clone 97-162-5). The recommendation to use the SCAR marker RYSC3 to identify samples with the Ry_{adg} gene was based on the results of studying 103 varieties and breeding clones, including the Bintje variety, in which the indicated marker was not initially detected [25]. Perhaps the differences in the PCR protocols led to false-positive results in the experiment. In the case of the YES3-3A marker of the Ry_{sto} gene, no false positive results were detected. This marker was detected in clone 97-155-1 resistant to virus Y and was not detected in clones 160-1 and 160-17 isolated in its progeny from self-pollination. In the experiment, we studied another donor of resistance to virus Y, the clone 90-6-2 and five clones isolated in the progeny of the 90-6-2 combination with variety Hertha or subsequent crosses. All the studied hybrid genotypes inherited the virus Y resistance, but markers recommended for identifying the *Ry* genes were not detected.

The RYSC 3 and YES 3A markers are DNA segments flanking *Ry* genes, the complete sequence of which is not yet known. Many researchers evaluated the diagnostic value of DNA markers of the Ry_{sto} and Ry_{adg} genes in PCR using a number of primers. In all experiments on screening varieties and breeding clones from different collections, as well as segregating populations, an incomplete concordance between the data of phenotypic evaluation and marker analysis was detected [34-36]. It is obvious that additional studies on the genetic nature of resistance to the virus Y in various potato forms are required, as well as the development of more advanced DNA markers of resistance genes.

For the first time in world practice, we carried out a multiplex PCR analysis of genetically diverse variety samples and selection clones of potatoes using DNA markers of eight R-genes. In the studied sample of 90 potato genotypes, the maximum number (five resistance genes) was detected in Meteor and Nur-Alem varieties. The results obtained are consistent with data from other researchers who, when studying varieties and breeding material of different origin, found no more than 5% of unique genotypes which were the potential sources of five to six resistance genes simultaneously [13, 17]. It is obvious that genotypes with a pyramid of potato genes for resistance to different pathogens are not widely spread, but their creation with purposeful breeding work seems to be an

achievable task. This is confirmed by the successful production of interspecific potato hybrids in which four to five *R*-genes for resistance to buckeye rot have been identified [37]. Molecular screening, of course, does not mean abandoning the phenotypic evaluation of breeding material, including in terms of resistance to diseases and pests. However, the use of multiplex PCR analysis to identify genotypes with several resistance genes of different specificity will accelerate the creation of potato varieties resistant to the complex of dangerous diseases and pests. The cost of multiplex PCR analysis for five markers of a single potato sample is 28 times less than the cost of assessing resistance to a single pest (cyst nematode or virus), and the economic benefit will be especially noticeable during mass analysis at the initial stages of breeding [9]. The apical meristem cultures and microclonal propagation are included in modern seed farming for healing seed potatoes from viruses. Similarly, multiplex PCR analysis should be integrated into potato breeding programs.

Thus, multiplex PCR analysis allows identification of genotypes with several (up to five) resistance genes of different specificity, including those providing resistance to the nematode *Globodera rostochiensis* Ro1, potato wart *Synchytrium endobioticum* (pathotype 1), and Potato Virus Y, among genetically diverse potato forms. A strong relationship exists between the 57 R and N 195 markers of the *H1* gene and the resistance to nematode *G. rostochiensis* Ro1 (association coefficient $r_A = 0.59$, Spearman's correlation coefficient $r_s = 0.72$-0.79) and between the NL 25 marker 5 of the *Sen1* gene and the resistance to potato wart ($r_A = 0.62$). To carry out marker-assisted selection (MAS) for Potato Virus Y resistance genes, it is necessary to study the genetic nature of the resistance of different potato forms and develop new effective DNA markers of resistance genes.

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USE OF SPELT WHEAT (*Triticum spelta* L.) IN BREEDING TRITICALE (*Triticosecale* Wittmack) FOR GRAIN QUALITY

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Abstract

Poor grain quality, mostly low proteins and gluten content, together with worse gluten quality, are the main obstacle to the widespread practical use of triticale. Hybridization of hexaploid triticale with certain *Triticum* species significantly expands the crop gene pool diversity and facilitates production of new forms with improved quality indicators. Spelt wheat is much suitable for crossing due to high protein content in grain (up to 25 %). The first Ukrainian study on hybridization between hexaploid triticale and spelt wheat was carried out in the Uman National University of Horticulture. The aim of the research was to improve triticale grain by intergeneric hybridization with spelt wheat and characterization of the hybrids for grain quality. The crosses resulted in a collection of more than 500 breeding samples of triticale which were grouped by plant height as medium, dwarf and short-stem forms. The best samples were analyzed for grain quality, i.e. content of proteins and gluten, the gluten quality, the 1000-seed weight and grain unite. Winter triticale Rarytet cultivar was the standard for medium height samples, and Alkid cultivar for low and short stem samples. Our research shows the improvement of triticale grain quality by intergeneric hybridization with spelt. Genotypes No. 455, 468 and 475 of medium height group, together with all dwarf and short-stem samples significantly exceeded the standards in grain protein and gluten content. The samples Nos. 455 (13.9 % protein, 30.2% gluten), 468 (13.0 % protein, 27.1 % gluten) and 473 (12.8 % protein, 28.0 % gluten) outstood for grain protein and gluten concentration. By the set of gluten quality indicators, the samples Nos. 455, 458, 451, 466, 488, 471 and 473 were assigned to group I, the rest of the samples belonged to group II. The samples Nos. 455 (56.0 g), 471 (55.3 g) and 473 (54.7 g) significantly surpass the standard in 1000-seed weight. No significant differences were found between the samples within each group for grain unit values. The highest values have Nos. 455 (700 g/l), 471 (690 g/l), 469 and 473 (685 g/l), 484 (682 g/l). As a result, two genotypes with high indicators of grain quality were selected, i.e. medium height sample No. 455 with protein content 13.9 %, group I gluten 30.2 %, 1000-seed weight 56.0 g, grain unit 700 g/l, and short-stem sample No. 471 with protein content 13.6 %, group I gluten 29.5 %, 1000-seed weight 55.3 g, and grain unit value 690 g/l).

Keywords: *Triticosecale* Wittmack, hexaploid triticale, *Triticum spelta* L., spelt wheat, hybridization, protein content, gluten content, 1000-seed weight, grain unite

Hexaploid triticale (*Triticosecale* Wittmack) is grown as a forage, food, and technical crop. Its planting acreage reaches 4 million ha [1, 2]. The priority directions of triticale selection are the increase in productivity, stability, and the improvement of grain quality indicators [2-5]. Low quality indicators of triticale grain, in particular, low content of protein and gluten in the grain, as well as low quality of gluten, are the main obstacles to the widespread introduction of the crop into production [6, 7]. Therefore, the efforts of many breeders are aimed at improving the quality of grain crops [8-10]. The insufficiently high plasticity of

cultivars and selective forms of triticale is associated with a limited genetic diversity of the parental material; it is required to expand the gene pool and increase the effectiveness of its selection with various methods, in particular, hybridization of hexaploid triticale with the genus *Triticum* L. species [11, 12]. As is noted by Hills et al. [13], it is an efficient way to increase the genetic diversity of the crop significantly. It is advisable to use spelt wheat (*Triticum spelta* L.), a hexaploid species ($2n = 6\times = 42$) with a genomic composition, homologous to the soft wheat (A^uBD) for hybridization. Spelt wheat grain contains up to 25% protein [14, 15]. It has been shown [16, 17] that spelt wheat grain contains essential amino acids, which are not synthesized in soft wheat grain and cannot be obtained from foodstuff of animal origin.

The idea of combining the genetic material of hexaploid triticale with spelt wheat belongs to Parii [11, 18]. As a result of experiments on transfer of the spelt wheat genetic material during hybridization of hexaploid triticale with spelt, a genetic pool of the Uman National University of Horticulture was created which includes more than 500 samples. It consists of recombinant forms that differ in economically valuable and morpho-biological characteristics.

The present paper represents the analysis of the hexaploid triticale forms obtained after hybridization with spelt wheat (according to protein and gluten content, gluten quality, 1000 seed weight and grain unit) and proves that it is possible to improve the quality of triticale grain in this way.

The aim of the research was to improve triticale grain characteristics by intergeneric hybridization with spelt wheat and characterization of the hybrids for the main indicators of grain quality.

Techniques. The hybridization of two types of grain crops and stabilization of the received samples were carried out during 2006-2012 (Central forest-steppe of Ukraine, the experimental field of the Uman National University of Horticulture, the Cherkasy Region). In hybridization, hexaploid triticale (*Triticosecale* Wittmack) Rozovskaya 6, Rozovskaya 7, or Ladnaya cultivars were a female parent, and the winter spelt wheat (*Triticum spelta* L.) Zarya Ukrainy cultivar was a pollinator. Hybridization was carried out by castration of flowers of the female parent, followed by controlled pollination with the seed parent. Glutenin proteins were electrophoretically separated in PAAG according to the description [19].

Grain quality indicators of the obtained breeding samples were analyzed ($F_{5\text{-}10}$, 2012-2017). All phenological observations and tests were conducted in accordance with the State Methodology for Qualifying Examination of Plant Varieties with the Definition of the Suitability Indicators for Proliferation in Ukraine (Ukrainian Institute for Plant Variety Examination, Kiev, 2012, vol. 2). Samples of triticale were grouped according to plant height based on the classification by Shchipak [20]. The productivity of the samples was evaluated in 5-fold repetition on 10 m^2 plots placed systematically. Winter triticale Rarytet cultivar was a medium-height standard, and winter triticale Alkid cultivar was dwarf and short-stem standard.

Experimental data were analyzed statistically with Microsoft Excel 2010 software. The mean values (*M*) and standard errors of means (±SEM) were calculated. The least significant difference (LSD_{05}) and the coefficient of variation (*Cv*) were calculated according to Ehrmantrout [21].

Results. The obtained F_1 hybrids were characterized by uniformity in spike morphology and general habitus of plants. Plants have dominative characteristics of spelt wheat (a long loose spike, rough glume, awnlessness, and worsened threshability).

By hybridization of hexaploid triticale and spelt wheat, mainly sterile F_1

plants appeared. Only a few samples formed fertile pollen grains. The sterility of plants obtained as a result of remote hybridization is highlighted by many authors [22, 23]. Thus, the F_1 hybrids from hybridization of three-species triticale and soft wheat are sterile, as in the case of hybridization of triticale and spelt wheat. The reason is the fact that between the AB triticale and wheat genomes, normal bivalent conjugation takes place because these genomes are homologous. Chromosomes of triticale R and spelt wheat D genomes do not have pairs for conjugation in meiosis. The process of meiosis among them is accompanied by significant anomalies, chromosomes form univalents that do not conjugate with each other. This leads to the formation of aneuploid gametes resulting in aneuploid plants. The fertility of such plants is reduced sharply.

The studies by Pershina and Trubacheva [23] show that it is possible to increase the pollen fertility of sterile F_1 hybrids by re-hybridizations with one of the parents. We also used this technique in this work and back-crossed the F_1 hybrids with hexaploid triticale. The spelt wheat-associated traits were less noticeable among the back-crossed descendants. Plant variability on the phenotype varied in F_1BC_1 hybrids and went beyond that of the parents.

Self-pollination of F_1BC_1 hybrids for several generations was used to stabilize the samples. After each subsequent self-pollination, the percentage of stable and well-grained forms of triticale increased. After the fifth self-pollination, 1137 plants with spike grain content equal to the original form were identified, 316 of them were grained for more than 80%, 471 – for 71-80% and 350 – for 61-70%.

Electrophoretic analysis of grain gluten proteins in PAAG revealed samples with genetic material from spelt wheat that carry spelt-specific gliadin- and glutenin-coding loci in chromosomes of the first homeological group, the *Gli-B1*, *Gli-D1*, *Gli-B5*, *Gli-A3*, *Glu-A1*, *Glu-B1*, and *Glu-D1*.

The resulting triticale forms were divided into three groups by plant height based according to the Shchipak's classification [20]: medium-height (100-120 cm), dwarf (80-100 cm) and short-stem (60-80 cm). The best genotypes were selected in each group for further analysis of economically valuable indicators.

The main purpose of hybridization of hexaploid triticale with spelt wheat was the genetic improvement of triticale, the increase in grain protein and gluten content which would improve its baking and technological properties. Lukaszewski [7] and Ukalska et al. [24] showed that triticale grain contains 10-12% protein, 20-25% gluten and is significantly inferior to soft wheat grain. Other researchers came to the same conclusions [8, 10, 17]. At the same time, it is admitted that the crop improvement potential in terms of productivity and quality is quite high, and the possibilities of its application are diverse [25].

In this experiment, a weak variation in the protein content (Cv = 8.9%) and average in the gluten content (Cv = 11.4%) occurred in the medium-height group. Sample No. 455 exceeded the standard (Table) in the protein content significantly (by 2.2% at LSD_{05} = 0.4). The same sample had gluten content (30.2%) significantly higher (by 5.7% at LSD_{05} = 1.1) than that of the standard. Other analysed samples exceeded the standard by 0.5 to 9.8% (LSD_{05} = 1.1). Samples No. 468 (13.0%), No. 475 (12.5%), and No. 458 (12.3%) of this group had high grain protein content. They also exceeded the standard cultivar in the gluten content (0.7-1.8% at LSD_{05} = 1.1). Gluten quality similar to that in group I was in samples Nos. 455 and 458. Other medium-height forms as per the indicators of gluten quality belonged to group II, which is mainly due to the low gluten deformation index (GDI), the main indicator characterizing its quality. The 1000 seed weight had the largest variation range (Cv = 18.7%). Sample

No. 455 (56.0 g) (LSD_{05} = 2.1) exceeded the standard in this indicator significantly. It is necessary to note that sample No. 455 has the highest indicators of the protein and gluten content, 1000 seed weight, and test weight (see Table) in the experiment.

Quality indicators of grain among selection samples of *Triticosecale* Wittmack/*Triticum spelta* L. (*M*±SEM, Ukraine, the Cherkasy Region, 2012-2017)

Selection sample	Protein, %	Gluten			1000-grain weight, g	Test weight, g/l
		content, %	GDI	quality group		
Medium stem (100-120 cm)						
Raritet (St)	11.7±0.04	25.5±0.11	75	I	50.4±0.24	670±12
455	13.9±0.02	30.2±0.06	75	I	56.0±0.32	700±8
458	12.3±0.03	26.2±0.07	70	I	48.9±0.20	660±14
461	11.5±0.04	24.1±0.05	60	II	48.4±0.21	662±11
465	11.5±0.05	24.6±0.07	60	II	49.3±0.23	665±9
468	13.0±0.03	27.1±0.08	55	II	50.2±0.25	670±5
475	12.5±0.03	27.3±0.09	60	II	51.4±0.27	678±8
LSD_{05}	0.4	1.1			2.1	28
Min	11.5	24.1			48.4	665
Max	13.9	30.2			56.0	700
Cv, %	8.9	11.4			18.7	10
S_x, %	4.6	3.5			3.3	4.3
Dwarf stem (80-100 cm)						
Alkid (St)	10.0±0.04	21.4±0.07	45	II	50.2±0.25	680±11
451	12.0±0.03	26.0±0.07	65	I	45.3±0.17	650±14
467	12.2±0.04	26.8±0.09	50	II	49.6±0.21	665±9
484	12.4±0.04	26.9±0.04	60	II	50.3±0.24	682±11
486	11.7±0.03	25.8±0.05	70	I	47.7±0.18	657±15
488	12.6±0.06	27.7±0.12	65	I	47.8±0.17	660±12
LSD_{05}	0.4	1.1			2.0	28
Min	10.0	21.4			45.3	650
Max	12.6	27.7			50.3	682
Cv, %	8.5	11.8			10.2	10
S_x, %	4.1	3.7			3.0	4.0
Short stem (60-80 cm)						
Alkid (St)	10.0±0.04	21.4±0.07	45	II	50.2±0.25	680±8
469	11.4±0.05	25.8±0.10	50	II	51.3±0.27	685±6
470	12.6±0.03	26.4±0.08	70	I	49.7±0.22	660±12
471	13.6±0.02	29.5±0.05	70	I	55.3±0.30	690±7
473	12.8±0.03	28.0±0.06	65	I	54.7±0.29	685±6
468	11.6±0.05	26.1±0.09	60	II	47.2±0.18	655±11
LSD_{05}	0.5	1.2			1.9	27
Min	10.0	21.4			47.2	655
Max	13.6	29.5			54.7	690
Cv, %	10.0	15.7			17.2	10
S_x, %	4.2	3.8			3.5	3.8

Note. GDI — gluten deformation index, S_x — test error.

Dwarf and short stem triticale cultivars are not used in agricultural production. As highlighted by Barnett et al. [26], this is due to the presence of a negative correlation between plant height and productivity. However, according to Kurkiev [27], such correlations are not absolute and manifested under adverse environmental conditions. Therefore, triticale forms which have high quality and productivity indicators combined with dwarf or short stems are of particular interest.

In these studies, all dwarf and short-stem forms were significantly superior to the standard in terms of the protein content (by 1.4-3.6% at LSD_{05} = 0.4-0.5) and gluten content (by 4.4-8.1% at LSD_{05} = 1.1-1.2) (see Table). The variation was average in gluten content (*Cv* = 11.8-15.7%), and low in grain protein content (*Cv* = 8.5-10.0%). The Alkyd cultivar was distinguished by high yield, but had low quality indicators: the protein content in the grain did not exceed 10.0% with 21.4% gluten. In the dwarf group, the increased protein (12.6 and 12.4%) and gluten (27.7 and 26.9%) contents were registered in samples Nos. 488 and 484, respectively. Samples Nos. 451, 486, and 488 showed group I gluten quality indicators. Sample No. 484 exceeded the standard cultivar for

1000 seed weight by 0.1 g, while samples Nos. 451, 467, 486, and 488 were inferior to it in this indicator. According to test weight, samples Nos. 484 (682 g/l), 467 (665 g/l), and 488 (660 g/l) (see Table) stood out.

Among the short stem forms, sample No. 471 was the best in the protein and gluten content, with 13.6% grain protein and 29.5% gluten, which was one of the highest indicators during the experiment. Samples No. 470 (12.6% protein, 26.4% gluten) and No. 473 (12.8% protein, 28.0% gluten) were slightly inferior. These samples differed from the rest due to combination of high 1000 seed weight, test weight, and gluten quality (group I).

Thus, these data evidence the possibility to improve triticale grain quality by intergeneric hybridization involving spelt wheat in breeding. In this work we obtained two hybrids with high grain quality indicators, i.e. the medium-height sample No. 455 (13.9% grain protein, 30.2% gluten of group I, 56.0 g 1000 seed weight, 700 g/l test weight) and short-stem sample No. 471 (13.6% protein, 29.5%gluten of group I, 55.3 g 1000 seed weight, 690 g/l test weight).

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SELECTION OF WINTER RYE (*Secale cereale* L.) INBRED LINES FOR GENERAL AND SPECIFIC COMBINING ABILITY AND ITS RELATIONSHIP WITH VALUABLE TRAITS

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Abstract

Creation of F_1 heterosis hybrids instead of population varieties is an advanced breeding technology since XX century. The rye as a strictly cross-pollinated culture is rather perspective for heterotic breeding. The success depends on the use of rye homozygous lines with high combining ability. The topcrossing allows effective estimates of the best genotypes to be used in synthesis of high heterotic hybrids. This paper is the first characterization of the authors' winter rye unique high-homozygous inbred lines, which are based on three Russian gene pools, with high combining ability intended for breeding programs. The purpose of our researches was to estimate by topcrossing method the general and specific combining ability of winter rye inbred lines. Test crossings in 2016 involved five sterile lines with type Pampas cytoplasm (H-649, H-577, H-842, H-1058, and H-1185), and four male fertile lines (H-451, H-1011, H-1247, and H-1071) as testers. In total, 20 simple F_1 interlinear hybrids were selected. In 2017, the hybrids were studied in plot tests (8 m^2 plots, 3 replications, and 500 grains per 1 m^2). F_1 hybrid yields varied from 5.02 up to 7.90 t/ha vs. 6.50 t/ha yield of population variety Valdai. The highest yield had F_1 (H-649 × H-1011, i.e. 7.9 t/ha that is 21.5 % higher compared to the standard Valdai variety. As a whole, the frequency of F_1 hybrids with high competitive heterosis is 7 of 20 (or 35%). Variation of F_1 productivity is mainly due to different combining ability of their parents. Note, both general and specific combining abilities (GCA and SCA) contribute to a dispersion of productivity trait, thought GCA effects account to 91.1% while SCA only 7.7%. Thence, additive gene effects but not intralocus dominance are the basic genotypic components of productivity trait variance. The obtained GCA/SCA values specify on high enough genetic divergence of the inbred lines used. Sterile analogues of lines H-1185 and H-649, and also of paternal line-tester H-1011 possess high GCA effects. The dwarf lines H-577 and H-1071 show lower general combining ability. High SCA effects are characteristic of the sterile lines H-1058 and H-649, and also the testers H-1071 and H-451. It was revealed that GCA of the used rye lines positively correlates with plant height (r = 0.85±0.10), 1000-grain weight (r = 0.80±0.13) and grain weight per ear (r = 0.64±0.21). These data emphasize the importance of inbred lines' selection for high own performance and combining of high 1000-grain weight and a dwarfism.

Keywords: *Secale cereale* L., winter rye, cytoplasmic male sterility, CMS, tester, homozygous inbred line, F_1 hybrids, general combining ability, GCA, specific combining ability (SCA), yield, 1000-grain weight, grain weight per ear, dwarfism

The choice of parents for crossing is a fundamental problem in the breeding of winter rye F_1 hybrids based on cytoplasmic male sterility (CMS). A clear proof of the presence of cytoplasmic male sterility in the Argentine variety of rye Pampa was presented by N.N. Geiger and F.W. Schnell (Hohenheim University — Universität Hohenheim, Germany) [1], and the first experimental

F_1 hybrids gave the impressive results. In comparison with the parent lines, the average heterosis in grain yield was 39%, in the number of grains in the ear 58%, in the 1000-grain weight 37%, in the plant height 31%, in the number of ears per 1 m^2 10% [2]. This was an incentive for private investment in methodology, biotechnology, and practical hybrid rye breeding, with the result that its share in the structure of the world plantings of this crop exceeded 20% [3]. In recent years, German breeding companies have created a series of improved hybrids that combine high yields with other valuable features (short stem, resistance to lodging, brown rust, and ergot) with improved baking qualities of grain and greater suitability for the production of feed and biogas [4].

At average, the grain yield in F_1 hybrids of winter rye is 15-20% higher than in population varieties [5]. In Germany, from 1982 to 2005, the annual yield increase in population varieties was 30 kg/ha, in hybrid varieties 51 kg/ha (70% higher) [6]. At the same time, the rate of breeding improvement at the hybrid level is much higher than at the population level. In Germany for 26 years (1991-2016) the yield of rye hybrids in the state testing increased by 23.3%, while in the population varieties by 18.1% [4]. In Poland, in the state testing, the yield increase of such varieties compared to the population varied was from 9.8 to 14.5 c/ha, while F_1 hybrids were more resistant to lodging and less affected by brown rust and powdery mildew [7]. In Canada in the areas of traditional cultivation of this crop, the decrease in its areas which lasted for a long time now stopped due to the introduction of the European hybrid varieties [8]. The State Register of Selective Breeding Results of the Russian Federation included five rye hybrids of German selection, the Picasso, Magnifico, Palazzo, KWS PABO (the originator is KWS Lochow GmbH, Germany); Helltop (the originator is Monsanto Saaten GmbH, Germany), and two Ukrainian hybrids (Pervistok and Yuryevets, the originator is the Yuriev Plant Production Institute, Ukraine), but the area under them is small; domestic linear hybrids are absent in the register. In Russia, heterotic breeding of rye with the use of CMS is almost not conducted currently. The exception is provided by the works involving three gene pulls of rye (from Nemchinovka, Saratov, and Kirov) with the use of multiple inbreeding plants from self-fertile populations and saturating crossings to obtain sterile analogues of lines like Pampa [9] using CMS, and the subsequent hybrid breeding (Federal Research Center Nemchinovka).

Evaluation of the genetic properties of homozygous lines with high productivity in interline crossings is the most difficult and costly stage in heterotic breeding. It is proposed to start the determination of the general combining ability (GCA) in the first generation of inbreeding (I_1-I_3), as the probability of line segregation on this trait is very small in the subsequent generations, and it is advisable to exclude them from further testing and self-pollination [10]. It is extremely important because the frequency of lines with high GCA is very low. For example, from 364 hybrid combinations in different types of crossings (polycrossing, topcrossing, variety-to-line crossing, interline crossing), such lines were about 20% [11], and less than 8% when taking into account only economically significant heterosis [12]. The reason is that those inbred lines that are superior to other lines can be genetically related, which reduces the effect of heterosis. Maximum heterosis can be achieved only in crossing genetically different inbred lines [13].

The analysis of F_1 hybrids from diallel crosses gives an objective assessment of the combining ability of parent lines. It is possible to determine the GCA of parent forms with one variety-tester, and the scheme in which a set of breeding valuable inbred lines serves as the tester allows breeders to characterize SCA of parent forms without diallel crossing [14]. The study of five inbred lines

of corn as testers showed [15] that the best, in this case, will be the forms with increased yield and high effects of the GCA. Topcrossing as a method of early estimates is considered effective in assessing the combining ability of winter rye lines [16]. It is important to take into account that the choice of the optimal number of testers in determining the GCA is largely influenced by the number of environmental test points in testcrossings [17].

The GCA of inbred lines is used in the selection not only for yield but also for other characteristics. The comparison of 40 simple interline hybrids of winter rye artificially infected with *Fusarium culmorum* (W.G. Sm.) Sacc. showed that the GCA variance on resistance to this pathogen was 10 times higher than the SCA variance [18]. On this basis, it was concluded on the predominance of additive interaction of genes controlling stability, which determined the strategy of selection. Similar data were obtained for winter triticale [19]: a close relationship between the effects of parental GCA and their own productivity, which makes it possible to reliably determine the expected resistance to ear head blight in F_1, was also determined.

The forecast for hybrid power in interline hybrids is of great interest. A prediction is possible both on the basis of the GCA effects and data on the productivity of inbred lines per se [20]. The structurally simple features, by the average value of which in the parent lines it is possible to estimate the expected expression of these features in hybrids, are the most convenient. In relation to complex structural characteristics, such as productivity, the accuracy of such prediction is reduced by the effects of dominance [21].

In the present paper, the unique high-homozygous inbred lines of winter rye, which were obtained on the basis of three domestic gene pulls, were characterized for the first time, and the forms with high combining ability, promising for breeding for productivity and other main economically valuable features (winter hardiness, short stem, 1000-grain weight, etc.) were identified.

The aim of the research is to determine the combining ability of inbred lines of winter rye using test crosses according to the scheme of complete topcrossing and to study the correlation of the obtained effects of the GCA with the main breeding traits.

Techniques. Inbred lines were obtained by multiple inbreeding of plants from hybrid populations derived from crosses between the rye varieties Alpha, Valdai, Voskhod 1, Voskhod 2, Saratovskaya 5, Bezenchukskaya 87, etc. with various donors of self-fertility. The lines were selected for winter hardiness, short and strong stem, ear productivity, 1000-grain weight, resistance to fungal diseases, the falling number and viscosity of the aqueous extract of grain meal. To create the sterile analogs of inbred lines in winter (in greenhouse conditions), the pair backcrossings were conducted by placing the ears of sterile and fertile plants under the same parchment isolator. The source of sterile cytoplasm of the Pampa type was one of the highly sterile inbred lines. The preservation of plants sterility after each backcrossing was controlled visually in the greenhouse and in the field.

When creating the parent lines A and B in the formula of simple hybrid, different gene pulls, unrelated to the synthetics-pollinator C, were used. The parent lines for A were five male sterile lines (N-649, N-577, N-842 N-1058, and N-1185), selected according to the complex of economically valuable traits (winter hardiness, short stem, large-grainy, etc.). Test crosses were carried out in 2016 (experimental field of the Federal Research Centre Nemchinovka, Moscow Province) under topcrossing scheme with four male fertile lines (N-451, N-1011, N-1247, and N-1071) as a paternal form. The resulting 20 interline F_1 hybrids were assessed in 2017 in field tests arranged in 3-fold repetition, with 8.0 m^2 plots and

the seeding rate of 500 grains per 1 m^2. The yield was harvested at full ripeness (a Winter-Classic small combine, Wintersteiger AG, Austria).

The effects of the GCA and the SCA of maternal sterile lines and paternal fertile testers were measured using the mathematical evaluation model proposed by Wolf and Litun [22].

Results. The weather conditions during the growing season deviated from the norm significantly. The partial death of plants due to the ice crust had happened in winter. April was abnormally cold, the cold weather also dominated in May, snow and rain fell often, rainfall was record high (88.4 mm at the rate of 52.4 mm). Heavy rains in June had a negative impact on trans-pollination. In July, the weather was also mostly cold and humid: rainfall was 34.6% higher than the rate; 420.3 mm of precipitation fell from April to July total at the rate of 249.5 mm (168.5% of the rate). This led to the lodging of some hybrids, reduction of 1000-grain weight and, as a result, reduced productivity, technological, and baking quality of grain.

In total, more than 2000 homozygous lines were obtained, from which the best for a number of features (winter hardiness, short and durable stem, early ripeness, a well-grained ear, large grain, resistance to fungal diseases, the high falling number, and viscosity of the aqueous extract of grain meal) were selected. On the basis of long-term data from this group, short-stem and valuable in terms of a complex of other features inbred lines were selected, from which sterile analogs were obtained, which were used in the "breeding conveyor" for the synthesis of interline F_1 hybrids based on the CMS of the Pampa type.

1. Yield (t/ha) **of winter rye (*Secale cereale* L.) simple interline hybrids F_1** (field tests, Moscow Province, 2017)

Sterile line	Fertile line (tester)				Average X_i
	H-451	H-1011	H-1247	H-1071	
H-649	7.02*	7.90*	7,03*	6,02	7,00
H-577	5.09	5.98	5,74	5,78	5,65
H-842	5.88	6.50	6,31	6,15	6,21
H-1058	6.89	7.03*	6,01	5,53	6,36
H-1185	7.39*	7.52*	6,89	7,19*	7,24
Average X_j	6.45	6.99	6,39	6,13	6,49
LSD_{05}	0.52				

* The hybrid yield is significantly higher than the average yield in the experiment.

The results of the field test of 20 winter rye interline F_1 hybrids (Table 1) showed 6.49 t/ha average yield in the experiment (6.50 t/ha in the standard population variety Valdai). Seven F_1 hybrids exceeded ($P \geq 0.95$) this standard for competitive heterosis by 0.5-1.4 t/ha (8.0-21.5%). The F_1 hybrid derived from N-649 × N-1011 showed the highest yield (7.90 t/ha), F_1 hybrid of N-577 × N-451 was the lowest in yielding (5.09 t/ha). The maternal sterile line N-1185 was present in the pedigree of the most productive hybrids, and the N-1011 line was present among the paternal testers. The hybrids produced with the participation of the N-577 and N-842 lines were the most low-yielding. The lines N-1185, N-649 and the tester N-1011 mostly gave high-yielding hybrids. The typical feature of these lines was that each of them gave three high-yielding hybrids when crossing with others. It indicates their high combining value. At average, the frequency of F_1 hybrids with significantly increased competitive heterosis in yield was 35% (7 out of 20). The genotype of the maternal lines caused a stronger variation in the yield of hybrids than the genotype of the tester lines.

The variation of yield in the studied hybrids was mainly due to the unequal parental combining ability. According to the results of the variance analysis, three components that affect the variance of yield significantly were found in the

yield genotypic variance, i.e. the GCA of sterile lines, the GCA of testers, and the SCA of the combination "line × tester" (Table 2).

2. **Dispersion analysis of the combining ability of the obtained inbred lines of winter rye (*Secale cereale* L.)**

Source of dispersion	SS	df	ms^2	F_{actual}	F_{05}
GCA of sterile lines	6.49	4	1.62	54.0	2.6
GCA of tester lines	1.92	3	0.64	21.3	2.9
SCA	2.27	12	0.19	6.3	2.0
Residual	0.97	38	0.03		

N o t e. GCA is general combining ability, SCA is specific combining ability.

It is important to note the relatively large variance of sterile maternal lines GCA (ms^2 = 1.62), and tester lines GCA (ms^2 = 0.64) compared to the variance of the SCA (ms^2 = 0.19). By summing up the variances, it can be found that the share of the GCA effects is 91.1% of the whole yield variability of the studied hybrids, and the SCA is 7.7% only. Consequently, the main component of the genotypic variance for yield is the additive interaction of genes, but not the intralocus dominance. The large value of the variances ratio (GCA/SCA) indicates the sufficiently high genetic divergence of inbred lines taken for crossing [23].

3. **Effects of general and specific combining ability** (GCA and SCA) **in the obtained sterile inbred lines and fertile lines of rye (*Secale cereale* L.) testers** (field tests, Moscow Province, 2017)

Line	GCA effect (g_i)	SCA effect (S^2_{ij})				ΣS^2_{ij}	σ^2_{Si}
		H-451	H-1011	H-1247	H-1071		
Sterile lines:							
H-649	0.50*	0.05	0.42	0.15	–0.62	0.58	0.173*
H-577	–0.84*	–0.52	–0.16	0.19	0.49	0.57	0.017
H-842	–0.28	–0.29	–0.21	0.20	0.30	0.26	0.067
H-1058	–0.13	0.57	0.17	–0.26	–0.48	0.65	0.197*
H-1185	0.75*	0.19	–0.22	–0.27	0.30	0.25	0.063
H-451	–0.04						
H-1011	0.50*						
H-1247	–0.10						
H-1071	–0.36*						
S^2_{ii}		0.72	0.32	0.24	1.03		
σ^2_{Si}		0.158*	0.058	0.038	0.235*		
			σ^2_{Si} = 0.134				
			σ^2_{Si} = 0.122				
Error Edg_i (lines)	0.156						
Error Edg_i (testers)	0.136						

* The indicator value is statistically significant at $p \leq 0.05$.

In the experiments, the studied lines had both positive and negative estimates for GCA. The sterile lines N-1185 and N-649 were characterized by the high GCA effects on yield; the short-stem line N-577 had significantly lower GCA (Table 3). Among the fathers-testers, the best GCA was in the N-1011 line, the worst in the N-1071 line (see Table 3). The F_1 hybrids involving these lines varied in yield greatly.

The crossing scheme used in this experiment made it possible to compare the lines not only for the GCA but also for the SCA and to assess the contribution of the heterosis effect to the yield potential for each pair of parents. If the heterosis value in the resulting combination is much higher than can be expected from the GCA value of the line, then such a line has a high SCA. The SCA indicator allows us to determine the use of which lines will give F_1 hybrids with the highest yield. Among the studied lines, the N-649, N-1058, N-1071, and N-451 showed significantly high SCA.

It should be noted that in the selection of inbred lines, their productivity is an important landmark. Miedaner et al. [24], studying the relationship between

the manifestation of eight traits of productivity in inbred lines and testcrosses, concluded that the selection of lines per se at the phenotypic level is important at all stages of hybrid breeding. It is noted that genotypic correlations (r_g) become weaker as the structure of the trait becomes more complex. The authors found the highest correlations ($r_g > 0.7$) for plant height, 1000-grain weight, as well as for the falling number and starch content [24]. According to the data of this experiment (Table 4), the sterile lines N-649 and N-1185, as well as the fertile tester line N-1011, which showed the highest estimates for the GCA as compared to the low-combination line N-577, were distinguished by a relatively long stem (from 86 to 97 cm vs. 78 cm), a larger grain (1000-grain weight is 24.4-30.0 g vs. 21.5 g) and a productive ear (grain weight per ear is 0.79-1.15 g vs. 0.62 g).

4. Productivity traits and their correlation with the effects of general combining ability (GCA) **in the obtained sterile inbred lines and fertile tester lines of rye (*Secale cereale* L.)** (field tests, Moscow Province, 2017)

Line	GCA effect	Plant height, cm	Weight, g		Grain and flour		
			1000 grains	grains per ear	falling number, s	protein content, %	grist water extract viscosity, cP
H-649	0.50	86	24.4	0.79	112	14.9	8.3
H-577	–0.84	78	21.5	0.62	261	12.8	10.3
H-842	–0.28	82	22.9	0.93	228	14.4	5.5
H-1058	–0.13	84	26.3	1.08	126	13.0	3.8
H-1185	0.75	95	29.5	1.15	234	12.8	8.5
H-451	–0.04	81	22.8	0.73	265	14.3	3.6
H-1011	0.50	97	30.0	1.12	116	13.7	9.4
H-1247	–0.10	80	22.8	0.63	222	13.1	6.2
H-1071	–0.36	79	23.0	0.71	173	13.7	3.8
Correlation coefficient (r) of GCA effect with the trait:		0.85*	0.80*	0.64*	–0.42	0.20	0.23

* The indicator value is statistically significant at $p \leq 0.05$.

In the experiment, we revealed close relationship between the GCA and some important breeding characteristics. For example, the high-confidence ($p \leq 0.05$) positive correlation between the estimates of the GCA lines and the plant height ($r = 0.85 \pm 0.10$), 1000-grain weight ($r = 0.80 \pm 0.13$) and the grain weight per ear ($r = 0.64 \pm 0.21$) was identified. It is important to note that the presence of conjugation between the GCA and plant height is undesirable, since long-stem lines are not suitable for hybrid breeding. However, the selection improvement of lines on this basis is generally achievable, especially if intensive and large-scale selection for the compatibility of the traits of large grain size and short stem is used. These features are characterized by a high coefficient of heritability; therefore, the selection of them per se among the used lines will allow being more confident to predict the best interline hybrids. The possibility of effective forecasting the productivity of simple interline rye hybrids based on the particular features of parental forms is also noted by other researchers [25, 26]. According to the latest data [3], in Germany, as a result of intensive breeding, modern self-pollinated rye lines exceed the productivity of the first inbred forms by 5-8 times, which accordingly affected the productivity of commercial hybrids. Due to breeding improvements of the parent forms, modern hybrid rye reached the level of such leading crops as maize and wheat in terms of yield.

Thus, according to the results of the field test of 20 topcross F_1 hybrids, 7 promising combinations were identified, which demonstrated significantly high competitive heterosis in yield. The highest yield had the simple F_1 hybrid N-649 × N-1011 (7.90 t/ha, that is the 21.5% excess compared to the standard variety Valdai). The sterile lines N-1185, N-649 and the tester-pollinator N-1011 also showed a high general combining ability in yield; therefore, they can be effectively involved in hybrid breeding. The observed variances ratio of

GCA/SCA indicates a significant genetic divergence of the crossed inbred lines. The GCA effects positively correlate with plant height, 1000-grain weight, and the grain weight per ear. The correlation analysis allows us to conclude about the possibility of early prediction of the combining ability of inbred rye lines for the compatibility of large grain size and short stem.

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TAXONOMIC COMPOSITION AND ORGANIZATION OF THE MICROBIAL COMMUNITY OF SODDY-PODZOLIC SOILS AFTER APPLICATION OF STRAW OF CEREAL CROPS AND USING OF THE BARKON BIOPREPARATION

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Abstract

The modern concept of the reproduction of soil organic matter (SOM) requires the sequestration of the carbon of plant residues in the soil by the formation of stable organic compounds. In this regard. the role of microbial preparations, accelerating the decomposition of straw are important. Learning the taxonomic structure of the microbial community in these processes is of great importance and not well understood. Microbial communities of arable soddy-podzolic soils decomposing straw of grain crops were studied in field and laboratory experiments. Straw (rye, wheat and oat) were crushed and inoculated with the Barkon preparation (complex association of microorganisms developed at the FGBNU ARRIAM). The functioning of the microbial community was assessed by the number and activity of microorganisms, the agrochemical properties of the soil. The composition of bacterial community of soils was determined by high-performance sequencing of 16s rRNA gene libraries. The rate of decomposition of straw was controlled by the ratio C:N in it: rye straw < wheat < oat. Barkon increased rate of decomposition of straw by 18-42% compared to soil microflora by 3 months of composting. Biopreparation is more effective when straw is incorporation in the 0-5 cm layer than by 9-12 cm. The effect of the Barkon on the number, biomass of microorganisms, and their respiration was not noticeable as compared with the growth of these parameters when introducing straw. The absence of an increase in carbon dioxide emissions with an increase in the rate of straw decomposition when Barkon is introduced, indicates an intensification of the processes of carbon sequestration in soil. The treatment with a biological preparation promotes the formation of microbial destructive communities with the highest efficiency of straw conversion and its conversion into labile organic compounds, and then into soil humus substances. Therefore, the use of Barkon, compared to the uninoculated straw, increased the content of total carbons in the soil by 4.8 to 8.4%. All studied factors (soil, straw, biological preparation, depth and time of decomposition) influence on the composition of microbial communities leading decomposition, the most significant of which is the type of soil. This confirms the high response of the composition of the microbial community to various factors while maintaining the crustal component of the microbiome characteristic of this soil. In the more acid soddy-podzolic soils, at the same humus content, in the taxon *Acidobacteria*, group 1 and group 2 prevailed, while in the soils with a neutral pH, group 6 predominated. The indicator of straw

application for sod-podzolic soil in all experiments is the increase of *Actinobacteria* from the family *Micrococcaceae*, particularly in variants with straw inoculation with Barkon, since *Micrococcaceae* is one of the microbial components of this biopreparation. Detected the influence of adding straw and application of the Barkon on the taxonomic composition of the bacterial community and the configuration of the destructive biosystem of soil microorganisms tuned for humification of plant residues. The decomposition of straw in the soil, as compared to that which was not planted, showed some weakening of Barkon's effect on the formation of the humification trophic chain, as evidenced by the lack of growth of labile humic substances in the respective variants. Based on the extended taxonomic data on the composition of the soil microbial community, it was found that minor groups of microorganisms participate equally with major groups, forming network fractal structures.

Keywords: microbial community, straw, a microbiological preparations, Barkon, the index of fractal structures

The decomposition of plant residues and the formation of organic matter (humus) resistant to microorganisms in arable soils play an important role in the global carbon cycle in the biosphere. The use of straw as an organic fertilizer becomes essential in the world, especially in connection with the developed no-till technology [1-5]. For example, due to the sharp reduction in Russia of the resources required for traditional organic fertilizers, the need for them in order to reproduce the soil humus can be satisfied only by 17-20%. The positive effect of the introduction of straw on the nutrient regime, the physical state of the soil, the processes of humus formation, the number and activity of soil microorganisms [2, 3, 5-7] is known. However, straw is used insufficiently because of its long-term decomposition, which is accompanied by a deficit of mineral nitrogen in the soil, released phytotoxic compounds and accumulated phytopathogens. The solution can be the straw treatment with biopreparations to accelerate its decomposition and eliminate possible negative consequences, which is especially important for the no-till technology [8-10]. Only 10-20% of straw with no treatment with special microorganisms is converted into humus when introduced into the soil. However, the modern concept of the reproduction of soil organic matter (SOM) involves the fixation of organic carbon in soil (sequestration) with a decrease in their mineralization to CO_2 [1, 11], so it is important that biopreparations for straw biodegradation enhance the inclusion of crop residue carbon into soil organic matter [2, 6, 12].

Management of the soil biological component to increase the production of agricultural products becomes one of the urgent tasks under the conditions of unstable climate and increasing soil degradation, since the possibilities of chemicals and “green revolution” reduced [12]. The significance of biodiversity and the total activity of microorganisms for the soil functioning are generally well studied, but the effect of the presence and ratio of certain species and genera of microorganisms (taxonomic structure) on biological processes, as well as self-organization of the microbial community in a particular soil difference [13-16] have not been studied sufficiently.

Studying the patterns of formation of the microbial community structure and its relationship with the properties and functioning of the soil is one of the most important areas of modern science [12-14, 16, 17]. The task is complicated by the so-called redundancy of the microbial system, when one function (cellulose decomposition, nitrogen fixation, denitrification) is performed by many microorganisms. A decrease in the number or absence of some species of microorganisms may not affect the general nature of the process in the soil (a particular soil function), since their role is shifted to others [13]. For this reason, the composition of the soil microbial community is not determined when assessing the soil nutrient regime, calculating the planned yield, or modeling large-scale cycles under stable conditions [13, 14]. However, at present, when abrupt climate changes during the season (abnormal heat or cold, drought or excess rainfall),

together with the soil degradation, are becoming the norm, information on the composition and structure of the microbial community can reduce the uncertainty of predictions regarding the result of plant residues biodegradation and even the yield forecast [13, 14, 16].

The decomposition of plant residues in soil is determined by many factors (chemical composition, soil physical properties, and hydrothermal conditions); therefore, significant differences are observed in the process speed, the quality of final products, humification coefficients and other indicators [1, 18-20]. Currently, there are three main hypotheses about the effect of biological factors and chemical composition on the final result of plant residues biotransformation [18]. According to the chemical hypothesis, regardless of the initial composition of residues and microbial destruction community, the same compounds will be obtained at the final stage of transformation (for example, humus of the same type). According to another hypothesis, various humic substances can be obtained depending on the initial chemical composition of plant residues. According to the third hypothesis, compounds are formed depending on the composition of the microbial community responsible for decomposition.

One of the ways to control the composition of the microbial community that decomposes straw is the introduction of biopreparations. Introduced microorganisms often work only at the initial stage, and then their number decreases, and the microbiocenosis returns to its original state [21, 22]. However, there is an option when changes in the microbial community composition after introducing biopreparation are maintained until the end of decomposition. The latter is possible not only as a result of direct exposure to the number of introduced microorganisms but also due to the ability of biopreparation to change the connections between them while making trophic chains in a new way. In both cases, it is possible to obtain substrates that differ quantitatively and qualitatively at the output. This issue has not been studied sufficiently, although it is very important for assessing the trend of soil-biological processes and the importance of the repeated introduction of biopreparation.

The Barkon preparation (All-Russian Research Institute of Agricultural Microbiology) is an association of microorganisms that capable of destruction of lignocellulosic substrates and their subsequent transformation into humic substances [23]. The preparation has a stimulating effect on the microbiological processes of straw transformation via increasing the number of microorganisms, microbial biomass, the coefficient of straw humification, and eliminating phytotoxicity [6, 23-25]. When processing the straw (stubble) with Barkon without embedding it in the soil, microorganisms participating in plant residues transformation form a humified trophic chain, providing more effective incorporation of both introduced and native microorganisms into humus-forming processes.

The present study for the first time establishes that straw inoculation with Barkon contributes to the formation of destructive microbial biosystems with a set of the most effective microorganisms from the soil community and organized action on the straw residues decomposition. It is shown that soil properties, rather than biopreparation, the type or depth of straw embedment, have the greatest influence on the composition of communities of microbial destructors of straw.

The purpose of the paper was to assess the taxonomic composition of the soil microbial community which decomposes the straw of cereals, and the role of microorganisms of Barkon biopreparation in the change of the soil microbiome.

Techniques. The composition and functioning of the microbial community of arable sod-podzolic soils when introducing straw of cereals inoculated with the Barkon biopreparation [23] was studied in lab and field experiments. The straw chopped into 1-2 cm pieces was treated with the preparation according to

the developer's recommendations (1 ml of the preparation + 25 ml of water per 10 g of dry straw). The control was options without straw (absolute control) and with straw treated with water.

In lab test 1, soft wheat straw (3.5±0.2% ash, 0.7±0.03% N, C:N = 69) was inoculated with the Barkon biopreparation or soil suspension (10 g of soil in 90 ml of water) and composted outside the soil for 1 month. The composted straw was introduced into the soil collected from the arable horizon of sod-podzolic soil (Leningrad Province, settlement Belogorka; $S_{hum.}$ 1.27±0.02%, N_{total} 0.11±0.003%, pH_{straw} 4.92±0.03) at a rate of 3 g/kg soil and stirring evenly. The experiment was carried out in 250 ml glass vessels at a constant humidity of 60% FMC (field moisture capacity) and a temperature of 25±2 °C. The duration of composting with the soil was 3 months, with 2 vessels per each term of estimation and 3 vessels for the final estimation.

In lab test 2, plastic 1.5-liter pots were filled with cultivated sod-podzolic soil from the arable horizon (St. Petersburg—Pushkin, Detskoselsky State Farm; $S_{hum.}$ 4.02±0.06%, N_{total} 0.316±0.02%, pH_{straw} 5.6 ±0.01). Rye straw (3.4±0.04% ash, 0.25±0.02% N, C:N = 193) was treated with Barkon or water and, after 1 h, added to pots at a rate of 3 g/kg soil. Two variants for embedding straw were studied, surface (0-3 cm) and deep (9-12 cm). The experiment was arranged in 5 replications 5, the test lasted 62 days at a constant humidity of 60% FMC and 25±2 °C. The paper presents data for 5 variants out of 13.

In the field experiment 3, oat straw (4.9% ash, 1.40±0.01% N, C:N = 34) was treated with Barkon or water, mixed with soil ($S_{hum.}$ 1.96%, N_{total} 0.194%, pH_{straw} 5.62) at the rate of 3 g/kg, then placed in nylon bags and put in the soil to a depth of 0-5 and 10-15 cm (experimental field of the All-Russian Research Institute of Agricultural Microbiology, Pushkin). The experiment lasted for 1 month amd was arranged in 9 replications.

Analyses of straw and soil (a mixed sample) were performed in 3-5 replications using standard methods [26, 27]. The content of undecomposed straw in the soil was determined by flotation in 0.5 normal Na_2SO_4 [28], the amount of total carbon by wet ashing with potassium bichromate, labile water-soluble organic carbon by the method of Schulz [29]. Movable humus compounds were isolated from soil by 0.1 normal Na-pyrophosphate (pH 7.0 or 10.0). The carbon content in the extracts was evaluated at λ = 340 nm (Ultraspec spectrophotometer, LKB, Sweden) [30]. Soil respiration was measured with a Tsvet 110 gas chromatograph (OAO Tsvet, Russia; the katharometer was a detector, and the gas carrier was helium). Microbial biomass in the soil was determined by substrate-induced respiration [31] as total (fungi + bacteria) and fungal biomass (treatment with streptomycin and rifampicin, 16 mg of antibiotic per 1 g of soil). In experiment 1, only fungal biomass was determined; in experiment 3, only the number of fungi was determined. Nitrogen and carbon of the microbial biomass were estimated using the rehydration method [32]. The number of physiological groups of microorganisms (ammonifying, amylolytic, cellulose-decomposing, humus-decomposing, micromycetes) was counted on dense nutrient media by soil suspension-plating method [33].

The structure of the soil bacterial community was determined using high-throughput sequencing of the 16S rRNA gene libraries for individual periods: 2 months for laboratory experiments (without replications, from mixed samples according to variants), 3 and 17 days in a layer of 0-5 cm for a field experiment (repeated 3 times).

The taxonomic composition of the bacterial community was determined using high-throughput sequencing of the 16S rRNA gene libraries. For this,

DNA was isolated from soil using MoBio kits (Qiagen, Germany) [34], libraries were produced by PCR with universal primers F515 and R806 for 16S rRNA gene [35]. Sequencing was performed using a GS Junior instrument (Roche, USA); the results were processed in the QIIME program [36].

The biodiversity of microbial communities was assessed according to the Shannon diversity index and the Sørensen-Czekanowski coefficient of similarity. Processing with Statistica v6 software (StatSoft, Inc., USA) involved standard methods of multidimensional statistics, i.e. principal component analysis, dispersion, correlation, fractal analysis, and graph analysis. The necessary calculations were performed using dispersion, correlation and fractal analyses [37-40] using original computer programs. Tables and text show mean values (M) with confidence intervals at $p \leq 0.05$ significance level ($t_{0.05} \times$ SEM). For comparison of libraries (with small frequencies), the probability that the frequency of membership in a taxon will be the same for two libraries was estimated [41].

The probability of the observed difference (significance) in the assignment to taxon T was estimated by the formula:

$$p(y|x) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!\,y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}},$$

where N_1 and N_2 are the total number of sequences for libraries 1 and 2, x and y are the number of sequences assigned to T, for libraries 1 and 2, respectively. One of the fundamental assumptions for this formula is that x and y are relatively small compared to N_1 and N_2 (less than 5% of the total), and N_1 and N_2 are relatively large (more than 500).

Results. Since the relationship between the nitrogen content in plant residues and the rate of their biotransformation [44, 43] is well known, it is likely that significant differences in the loss of straw in different experiments are related to the nitrogen content in it. The effect of the microbial community composition on the rate of biodegradation is unlikely: a decrease in mass for 1 month for wheat straw (experiment 1) under constant hydrothermal conditions was 2.6-3.5%; for oat straw in field conditions (experiment 3) this was from 20.0 to 31.8% at similar values of the total carbon content in the soil. Rye straw decomposed most slowly (experiment 2): for 2 months its weight loss was comparable to that for 1 month for wheat.

For 1 month of composting soil with straw, the use of Barkon did not have a statistically significant effect on the rate of straw decomposition, regardless of its type, depth of embedment and soil fertility. Decomposition under the influence of the biopreparation was significantly accelerated only at the beginning of month 3 (experiments 1 and 2): the weight loss of straw was 18-42% higher compared to the native soil microflora. Experiment 2 identified the efficacy of Barkon in biodegradation in the upper soil layers: the decrease in straw in the 0-3 cm layer was 14.8% more, and in the 9-12 cm layer was 7.6% less than with the native microflora. Decomposition of straw in the field also proceeded somewhat faster in a layer of 0-5 cm (a straw decrease of 30.8-31.8%) than in the lower layer (20.0-30.0%). The reason for the lower efficiency of the deep embedding of straw inoculated with Barkon may be that the preparation contains aerobic microorganisms [23, 24]. When creating facultatively anaerobic conditions in the lower layers (in separate niches), including that resulting from the activity of microorganisms, the effect of Barkon is worse, and the change in the trophic relationships of the microbial community even worsens the situation regarding the variant without biopreparation.

In experiment 1 with preliminary composting of wheat straw, the use of Barkon for 30 days increased the formation of water-soluble humus-like com-

pounds compared to the variant with native microflora: the absorption index was 9.0±0.2 vs. 8.0±0.1, E_{465}/E_{665} coefficient (humus content) 2.7±0.0 vs. 1.9±0.4. It should be noted that both in control and under the action of Barkon, the ash content changed equally (from 3.5±0.2 to 5.3±0.6%), as well as the content of nitrogen (from 0.7±0.05 to 1.07±0.08%), phosphorus (from 0.06±0.0 to 0.16±0.03%), and the C:N ratio (from 69 up to 28). It is the influence of Barkon during the preliminary composting that can be associated with a 25.6% increase in the content of newly formed labile humic substances in experiment 1 as compared to their amount when using soil inoculum (Table 1). In the variant straw + Barkon, humus-like compounds were formed when decomposing (see increase in $C_{lab.}$) which are probably less accessible to microorganisms. This led to an increase in the total carbon content in the soil. On the contrary, in the variant straw + soil inoculum, microorganisms increased by 67.4% (Table 2) and after dying out, because of low C:N ratio (3.4-5.8), were less included into humus. In addition, in this variant, humus was even lower compared to the control, due to the fact that either the microbial community strenuously decomposed not only straw but also labile humic substances, or they were formed less. Since the biomass of microorganisms was characterized by low C:N, humic substances probably contained more nitrogen, which resulted in an increase in the content of total nitrogen in the soil.

1. Agrochemical parameters of sod-podzolic soil when introducing straw and using Barkon biopreparation ($M \pm t_{0.05} \times$ SEM)

Variant	C_{total}, %	N_{total}, %	Total N-mineral, mg/kg	C-CO_2, mg · kg^{-1} · day^{-1}	C-labile humus, mg/kg
	в конце опыта		среднее за опыт		
Lab experiment 1					
Control	1.24±0.02	0.106±0.003	48.7±3.0	8.6±1.3	699±25
Straw + soil inoculum	1.25±0.05	0.111±0.001	19.8±0.2	23.6±1.8	636±51
Straw + Barkon	1.48±0.06	0.106±0.005	25.7±1.0	25.8±0.9	737±55
Lab experiment 2					
Control	3.78±0.05	0.313±0.001	26.9±0.8	8.8±0.1	7250±120
Straw, 0-3 cm	3.83±0.04	0.310±0.001	22.4±0.8	17.7±1.6	8110±320
Straw, 9-12 cm	3.81±0.14	0.306±0.010	18.1±1.7	13.8±0.8	7620±280
Straw + Barkon, 0-3 cm	3.84±0.03	0.305±0.010	22.9±0.0	20.7±1.9	6790±10
Straw + Barkon, 9-12 cm	3.89±0.08	0.307±0.010	21.8±1.5	15.2±3.2	7040±500
Field experiment 3					
Control, 0-5 cm	1.85±0.05	0.178±0.001	15.3±0.1	8.4±1.8	1354±5
Control, 10-15 cm	1.88±0.01	0.178±0.001	24.6±1.0	6.6±1.0	1335±22
Straw, 0-5 cm	1.98±0.05	0.187±0.002	15.3±0.1	22.6±3.9	1403±7
Straw, 10-15 cm	1.89±0.09	0.167±0.002	17.8±1.0	27.4±3.7	1413±9
Straw + Barkon, 0-5 cm	2.13±0.08	0.179±0.001	19.5±0.1	30.0±2.3	1369±14
Straw + Barkon, 10-15 cm	1.98±0.08	0.182±0.002	24.6±2.0	25.3±2.0	1357±22

Note. For a description of the experiments, see the *Techniques* section. Labile humus — C content in 0.1 normal pyrophosphate extract; pH 7.0 for experiments 1 and 2, pH 10.0 for experiment 3.

When embedding straw into the soil immediately after treatment with Barkon (experiments 2 and 3), no accumulation of newly formed humus compounds occurred (see Table 1), although it is impossible to make an unequivocal conclusion that this was not related to the type of straw. Nevertheless, the use of Barkon increases the content of total carbon in the soil compared to the variants without biopreparation due to the enhancement of straw transformation processes, including humification. For example, the complex index of humification [44] when introducing straw (experiment 2) was 2.87 in the control and 3.21 in the variant with biopreparation. Barkon somewhat weakened the negative effects of the straw introduction on the content of mineral forms of nitrogen in soils with a low content of organic matter (experiments 1 and 3). This can be considered as an advantage of Barkon, since its use does not require the obligatory introduction of mineral nitrogen for the straw decomposition [2, 3], which is especially valuable since usually biopreparation is used after harvesting grain crops

when nitrogen is not needed for plants.

The introduction of straw is expected to increase the respiration of microorganisms in all experiments (see Table 1). The influence of Barkon on this indicator vs. non-inoculated straw was insignificant. There was a tendency to an increase in the release of carbon dioxide from the soil only in experiment 2. Breathing enhancements were not observed in experiments with Barkon conducted by other researchers [6].

In the case of soils with a low total carbon content (experiments 1 and 3), the introduction of straw increased the number of the main groups of microorganisms 2 times or more (Table 2), which is consistent with the data on the greater efficiency of fertilizers and preparations on poor soils [2, 12, 45]. When introducing the straw, a short development of r-strategists occurs [2, 46]: in experiment 3 on day 3, there was an increase in the number of ammonifying and amylolytic microorganisms, which stopped on day 17. Treatment of straw with the preparation did not lead to significant changes in the number of cellulolytic microorganisms, except for experiment 2. In experiment 3, the influence of the biopreparation was even weaker and not permanent. So, on day 17, when introducing Barkon, a slight decrease in the number of cellulolytic microorganisms was in the 0-5 cm layer.

Barkon influenced the succession of microorganisms. Thus, in experiment 1, the coefficient correlation between the number of humus-decomposing microorganisms and the content of residual straw in the variants with Barkon and the soil inoculum changed its trend (–0.87 vs. 0.71). A significant influence on the strength and trend of the relationship between the dynamics of physiological groups and the amount of straw remaining in the soil was observed for other microorganisms: the correlation coefficients for the variants with the Barkon and without were 0.20 and –0.78 (cellulose-decomposing), –0.38 and 0.36 (amylolytic), respectively.

In all experiments, no significant effect of the introduction of straw, preparation and the depth of introduction on the number and/or biomass of fungi was found (see Table 2). Perhaps this is due to the fact that the role of bacteria increases in arable soils in the process of plant residues decomposition [45]. In the experiments, a certain increase in the number of bacteria occurred during the study, while the number of fungi varied slightly or decreased: their share of total biomass ranged from 14 to 22%. When introducing the straw, not only the number, but also the biomass of soil microorganisms increased, and the effect of treatment with biopreparation was insignificant (see Table 2). Under the action of Barkon, microbial biomass rather decreased, but its activity increased, it was expressed in the magnitude of nitrogen flows through biomass (serves as a function of the number and activity of microorganisms and reflects microorganisms work in the soil). The smaller size of the nitrogen flows in experiment 2 was caused by weak decomposition of rye straw and low nitrogen content in it, since the decomposition of straw in cultivated highly humus soil requires more available nitrogen [43].

The relationship between the structure of the microbial community and the agronomic properties of the soil during decomposition of straw follows from two tree diagrams [39] obtained for experiment 1, of which the first was drawn up according to microbiological (abundance, biomass, activity) and agrochemical indicators, and the second according to the taxonomic composition of the soil microbial community. Tree diagrams had an identical cluster structure (data not shown). The obtained results showed that the treatment with Barkon biopreparation had a strong effect on the microbial community structure, since this option was not included in one cluster with straw decomposed by soil inoculum.

2. Counts, content and activity of microbial biomass in sod-podzolic soil when introducing the straw and using Barkon biopreparation ($M \pm t_{0.05} \times$ SEM)

Variant	Ammonifying, mln CFU/g soil	Amylolytic, mln CFU/g soil	Humus decomposing, mln CFU/g soil	Cellulose decomposing, thous. CFU/g soil	Micromycetes, thous. CFU/g soil	$C_{m.b.}$, mg/kg	$N_{m.b.}$, mg/kg	N flow, mg/kg
Lab experiment 1, 2 months)								
Control	3.4±0.2	3.1±0.1	1.4±0.1	0.95	–	344±4	55.3±12.2	54.0±4.1
Straw + soil inoculum	6.0±0.1	8.2±0.4	4.2±0.4	15	–	576±56	98.7±19.9	181.0±7.2
Straw + Barkon	6.8±0.2	7.8±0.8	4.4±0.4	15	–	344±52	102.0±9.3	25.0±3.4
Lab experiment 2, 2 months)								
Control	12.0±0.8	8.7±0.6	6.3±0.6	15.8±2.1	23.5±3.5	571±60	43.9±0	87.0±6.5
Straw, 0-3 см	16.4±0.3	14.0±1.4	6.8±1.0	65.8±8.1	26.5±3.5	697±52	53.0±4.2	112.0±4.6
Straw, 9-12 см	12.9±0.8	14.1±1.1	9.5±0.7	61.8±14.0	20.4±2.1	807±39	53.4±4.9	87.0±2.3
Straw + Barkon, 0-3 cm	14.3±0.5	13.4±1.5	8.6±1.0	127.2±16.1	30.4±3.7	626±34	45.9±2.7	129.0±2.1
Straw + Barkon, 9-12 cm	13.8±1.0	13.8±1.6	8.7±0.9	98.9±14.0	13.0±1.4	603±72	41.9±5.6	99.0±9.6
Field experiment 3, day 3/day 17)								
Control, 0-5 cm	18.0±5.9/19.6±7.9	9.2±2.8/9.2±4.5	4.3±0.2/5.5±1.3	8.0±2.8/10.4±2.3	13.6±4.2/18.8±4.2	562±1/410±12	28.8±0/35.2±0	253.0±2.3
Control, 10-15 cm	18.6±6.8/32.1±3.3	9.7±2.0/9.5±4.6	4.9±0.5/8.1±0.8	6.7±0.5/8.5±0.8	14.5±1.1/24.0±5.3	498±25/460±61	27.9±0/38.2±0	262.0±13.8
Straw, 0-5 cm	107.1±20.2/27.6±3.4	67.8±16.7/20.3±8.4	8.8±0.3/12.0±2.4	7.3±2.4/10.3±3.4	10.9±3.6/23.8±6.6	694±48/597±118	35.2±1.2/54.0±4.8	513.0±5.9
Straw, 10-15 cm	59.3±0.7/46.4±0	69.4±1.5/23.2±0	9.8±0.4/14.5±0.8	4.7±1.3/3.9±0.8	12.5±3.3/23.2±2.7	557±60/640±6	24.6±6.1/60.6±11	821.0±7.8
Straw + Barkon, 0-5 cm	70.6±2.1/46.8±12.1	54.1±3.5/21.1±5.6	11.7±0.5/12.2±1.7	11.3±0.8/8.2±1.3	16.5±3.2/27.6±2.3	525±61/694±14	27.2±0/54.0±4.8	758.0±4.9
Straw + Barkon, 10-15 cm	57.3±3.5/61.2±20.2	44.0±13.7/28.1±9.3	11.3±0.6/11.90	9.7±1.4/7.0±2.3	14.5±4.7/29.3±0	442±86/710±10	25.9±9.3/59.7±9.5	858.0±13.1

Note. For a description of the experiments, see the *Techniques* section. $C_{m.b.}$ — C of microbial biomass, $N_{m.b.}$ — N of microbial biomass. Dashes indicate that the indicator was not determined. The flow of nitrogen through the microbial biomass [32] was calculated for the entire time of the experiment: experiment 1 — 103 days, experiment — 92 days, experiment 3 — 31 days. Cellulose decomposing microorganisms in experiment 1 was determined by the method of limiting dilutions.

3. Distribution of bacteria in sod-podzolic soil according to major taxa (% of the total number of operating taxonomic units, OTU) **when introducing straw and using the Barkon biopreparation** ($M \pm t_{0.05} \times$ SEM)

Variant	Major taxa (different rang)										I_F
	Archaea	*Acidobacteria*	*Actinobacteria*	*Bacteroidetes*	*Chloroflexi*	*Firmicutes*	*Gemmatimonadetes*	*Planctomycetes*	*Proteobacteria*	прочие	
Lab experiment 1											
Control	0.5	18.5	17.0	0.7	0.03	3.5	2.0	1.3	27.7	28.8	0.44±0.03
Straw + Barkon	1.3	23.7	12.6	1.8	0.10	1.8	1.8	1.6	32.8	22.5	0.75±0.03
Straw + soil inoculum	0.3	16.9	9.5	3.5	0.03	5.2	1.1	1.3	41.7	20.5	0.52±0.03
Lab experiment 2											
Control	1.0	4.1	24.5	0.3	3.5	3.9	3.6	1.2	55.5	2.4	0.60±0.03
Straw, 0-3 cm	1.4	5.4	26	0.5	3.7	3.1	3.8	1.9	51.1	3.1	0.58±0.03
Straw, 9-12 cm	1.1	3.6	32.4	0.8	4.2	4.6	3.6	2.6	44.2	2.9	0.62±0.04
Straw + Barkon, 0-3 cm	1.2	3.5	24.7	7.6	3.5	2.5	4.2	1.8	46.9	4.1	0.68±0.04
Straw + Barkon, 9-12 cm	1.1	4	28.9	0.7	4.5	3.5	4.8	1.0	49.0	2.5	0.58±0.04
Field experiment 3											
Control, 3 days	1.9	5.8	28.6	2.0	5.2	4.6	5.3	2.0	39.3	5.3	0.78±0.03
Straw, 3 days	1.7	5.5	30.7	2.6	5.5	4.7	4.2	2.3	37.6	5.2	0.88±0.03
Straw + Barkon, 3 days	2.4	5.3	34.9	3.8	4.9	5.1	4.1	2.2	32.8	4.5	0.79±0.03
Control, 17 days	2.7	6.8	28.7	2.9	6.2	6.3	5.8	2.5	32.5	5.6	0.83±0.03
Straw`, 17 days	2.2	6.9	25.6	3.8	5.4	6.2	4.7	2.4	35.9	6.9	0.80±0.03
Straw + Barkon, 17 days	2.9	6.0	29.9	3.1	5.6	5.6	4.8	2.5	34.2	5.4	0.83±0.03

Note. For a description of the experiments, see the *Techniques* section. Archaea phyla, due to their relatively small number, are represented by the entire domain. I_F — the index of fractal structures, calculated using full taxonomy data (at a genera level).

The bacterial community members in the studied soils were basically representatives of the phyla *Actinobacteria* and *Proteobacteria*; a significant number of microorganisms belonged to *Acidobacteria*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes* (Table 3).

Barkon preparation contained a significant number of microbial species (Fig. 1), which did not coincide with the soil microbiome. For example, Barkon containes one order less typical soil inhabitants, the *Acidobacteria*. At the same time, the share of *Bacilli*, *Sphingobacteria*, and *Gemmatimonadetes* is higher in the biopreparation compared to the soil. The Barkon biopreparation is intended for the decomposition of cellulosic waste; therefore, there are many actinomycetes in its bacterial community (*Cellulomonas*, *Corynebacterium*, *Micrococcaceae*) which decompose cellulose and other hard-to-reach organic compounds, and the differences with the soil were at least one order.

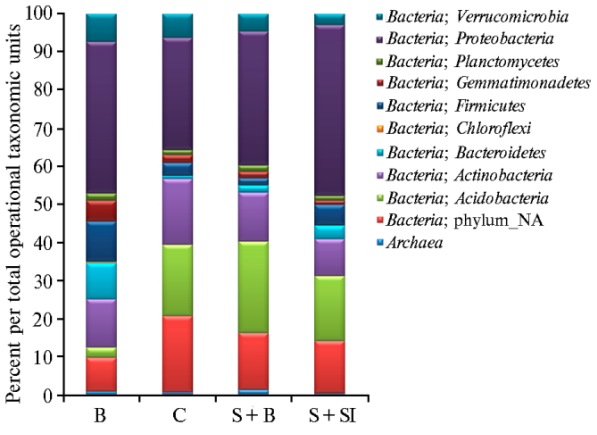

Fig. 1. The composition of the bacterial microbial community of the Barkon biopreparation and sod-podzolic soil when introducing straw and using the Barkon in experiment 1: B — Barkon, C — control (soil), S + B — straw + Barkon, S + SI — straw + soil inoculum.

When analyzing bacterial community of sod-podzolic soils, it was found that microbiomes in three experiments differed significantly between themselves (see Table 3). All three soils formed separate clusters, that is, the soil factor had the greatest influence on the structure of the microbial community (see Table 3). This conclusion differs from the statement that the main factors in the formation of the microbial community decomposing the straw are climatic conditions, not the type of soil [47]. However, the introduction of straw, the use of biopreparation, the period of analysis and the depth of embedding also had a significant impact on the microbial community composition. For example, when using Barkon as compared to soil inoculum (experiment 1), the number of *Firmicutes* (difference significance is 2.58×10^{-12}) [41], *Bacteroidetes* (6.34×10^{-5}), *Proteobacteria* (1.26×10^{-12}) decreased and the proportion of *Acidobacteria* (8.07×10^{-11}), *Actinobacteria* (1.2×10^{-4}) and archaea (7.8×10^{-4}) increased.

When analyzing the data obtained, particular attention was paid to several of the most important taxa, since a significant part of the microorganisms did not directly participate in the decomposition of straw. It should be noted that each soil was characterized by its own, not always the same set of families in each taxon. Thus, a sensitive indicator of soil pH may be not only the representation of the *Acidobacteria* but also its composition [48]. The experiments established that with a similar content of $C_{org.}$ in more acidic soils, *Acidobacteria* of the 1st and 2nd groups prevailed in this taxon (60 and 18%, respectively), in soils with pH close to neutral the 6th group prevailed (30%, for the experiment 1 1-3%), and the 1st and 2nd groups were not identified. *Acidobacteria* of the 6th group responded positively to the introduction of straw on acidic soil (an increase from 1 to 3%).

Actinomycetes play a significant role in the mineralization of hardly decomposable substrates, including straw [2, 17, 49]. The indicating group for the

introduction of straw for sod-podzolic soil in all experiments was the *Micrococcaceae* family. Its representatives are part of the Barkon biopreparation. The significant increase in the proportion of *Micrococcaceae* from all *Actinobacteria* in the variants with straw (Fig. 2) observed in experiment 3 on day 3 remained on day 17, although it was lower, by 10, 17 and 26%, respectively, for control, straw and straw + Barkon. The increased proportion of bacteria of this family was also observed in experiment 1 on day 61 (see Fig. 2).

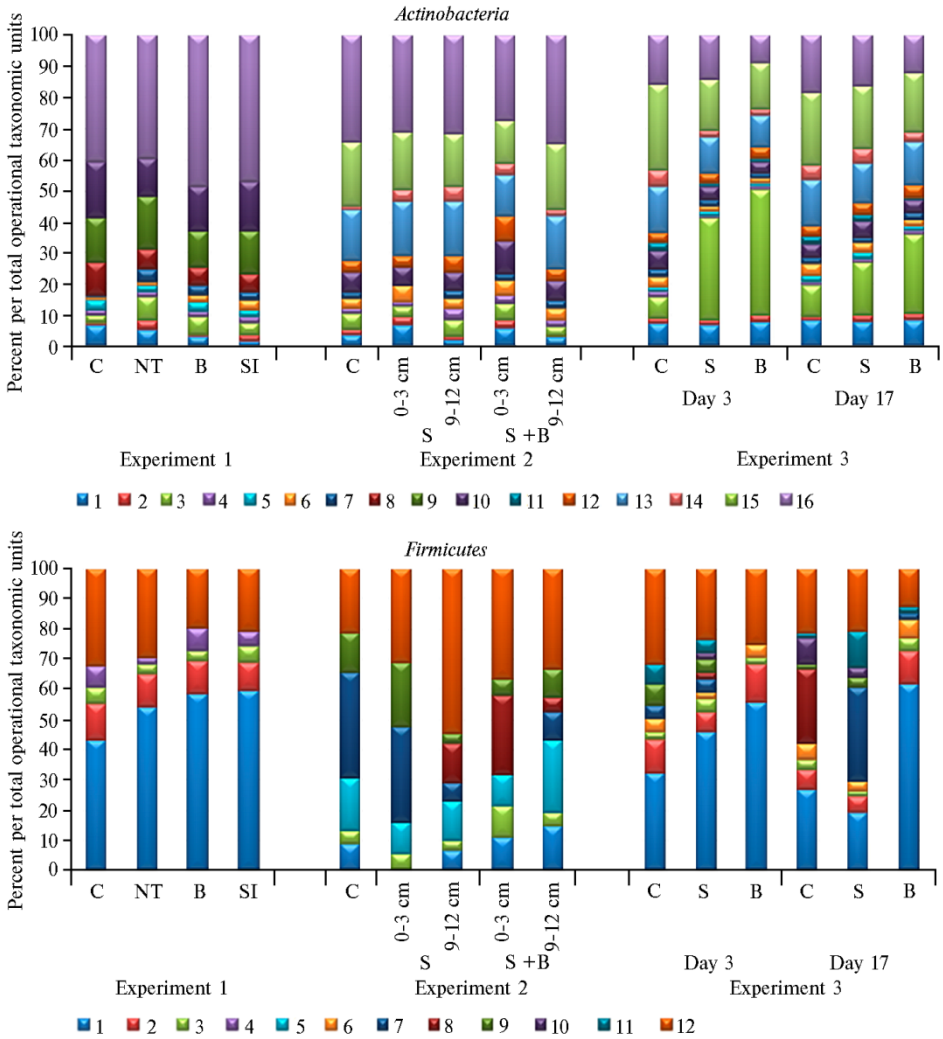

Fig. 2. Composition of individual phyla at the family level in the experiments (share of the total number of operating taxonomic units — OTU) **when introducing straw into the sod-podzolic soil and using the Barkon biopreparation:** C — control, WT — without treatment, B — Barkon, S — straw, SI — soil inoculum. Семейства *Actinobacteria* families: 1 — *Intrasporangiaceae*, 2 — *Microbacteriaceae*, 3 — *Micrococcaceae*, 4 — *Micromonosporaceae*, 5 — *Mycobacteriaceae*, 6 — *Pseudonocardiaceae*, 7 — *Streptomycetaceae*, 8 — *Thermomonosporaceae*, 9 — *Conexibacteraceae*, 10 — *Solirubrobacteraceae*, 11 — *Geodermatophilaceae*, 12 — *Nocardioidaceae*, 13 — *Gaiellaceae*, 14 — *Patulibacteraceae*, 15 — *Solirubrobacterales*, 16 — прочие. Семейства *Firmicutes*: 1 — *Bacillaceae*, 2 — *Paenibacillaceae*, 3 — *Clostridiaceae*, 4 — *Planococcaceae*, 5 — *Carnobacteriaceae*, 6 — *Alicyclobacillaceae*, 7 — *Staphylococcaceae*, 8 — *Lactobacillaceae*, 9 — *Streptococcaceae*, 10 — *Veillonellaceae*, 11 — [*Tissierellaceae*], 12 — others. For a description of the experiments, see the *Techniques* section.

Spore-forming microorganisms from phyla *Firmicutes* may also be involved in the decomposition of fresh organic matter. The biopreparation had a significant effect on its composition, reducing the number of genera (see Fig. 2).

The greatest influence was observed in the first days after the introducing the straw and the biopreparation (experiment 3), but it remained for later periods: in experiment 1, the number of genera in the control and in the variant with straw with soil inoculum was 17, in the variant with Barkon it was 10. In experiment 2 with soil rich in organic matter, the effect of Barkon occurred only when the preparation was embedded in the lower layer: the number of genera in this phylum for straw and straw with Barkon was 15 and 11, respectively.

The ratio of gram-positive and gram-negative bacteria characterizes the oligotrophic nature of soil processes. It is known that gram-negative bacteria require a richer substrate (fresh organic matter). The decomposition of straw increases their number, especially *Alphaproteobacteria* and *Gammaproteobacteria* [46, 48]. In our experiments, except the experiment 1, there was no increase in gram-negative bacteria, depending on the introduction of straw. Even in experiment 3, when introducing readily degradable oat straw, no increase in the proportion of gram-negative bacteria was found. The reason may be either in differences with the methods used by other researchers (the fatty acid method) or in a high proportion of uncultivated microorganisms.

To assess the influence of the studied factors on the diversity of microbial communities, we used the Shannon index. In experiment 2, when introducing the straw, it was increasing compared to the control as expected. Barkon somewhat reduced the diversity of the microbial community of the soil during the decomposition of straw in the lower layer (control — 5.04; straw in the layer 0-3 and 9-12 cm — 5.47 and 5.57, respectively; Barkon — 6.22 and 5.17). In experiments 1 and 3, no significa t differences according to the Shannon index were between the variants. The Sørensen-Czekanowski coefficient of similarity showed that the taxonomic composition of the microbial community of the biopreparation (for experiment 1) differed significantly from all soil variants (0.54-0.59 vs. 0.72-0.75). For experiment 2, the composition of the microbial community decomposing straw in the upper layer had the greatest similarity to the control (0.82), whereas Barkon treatment and decomposition in the lower layer reduced the similarity coefficient to 0.67-0.70. Consequently, when treating with biopreparation, the greatest differences in the composition of microbial communities according to both coefficients were when the straw is incorporated in the lower layer.

The principal component analysis revealed a significant differences between the soil and the biopreparation in experiment 1 (Fig. 3, A). Introduction of Barkon led to significant changes in the structure of the microbial community decomposing straw. Differences between replications were comparable to the differences between the variants (see Fig. 3, B), although at a low level (no more than 9%), especially for the version with straw on day 17. This is probably due to the presence of the detritosphere around the pieces of decomposing straw [46, 50, 51]. Nevertheless, the influence of Barkon on day 3 was quite clear. For experiment 2, a significant difference in microbial communities in different layers is shown (see Fig. 3, B).

The fractal analysis of molecular genetic data presented in the form of a fractal portrait [23, 37, 38] was limited to counting the number of primary fractal groups (PFGs). When searching for PFGs in the portrait, the arrangement of fractal triplets is sequentially analyzed, which include points that differ in the whole number parts of the frequency logarithms and are located on the same line. The absolutely accurate location of three points on one straight line is an exceptional situation. Therefore, all the found PFGs are characterized not only by the taxonomic parameters of the OTU groups, but also by the error (h) of the location of three points on one straight line.

One can assume that the microbial community in which a larger number of PFGs are present has greater consistency and efficiency in the joint transformational activity of microorganisms.

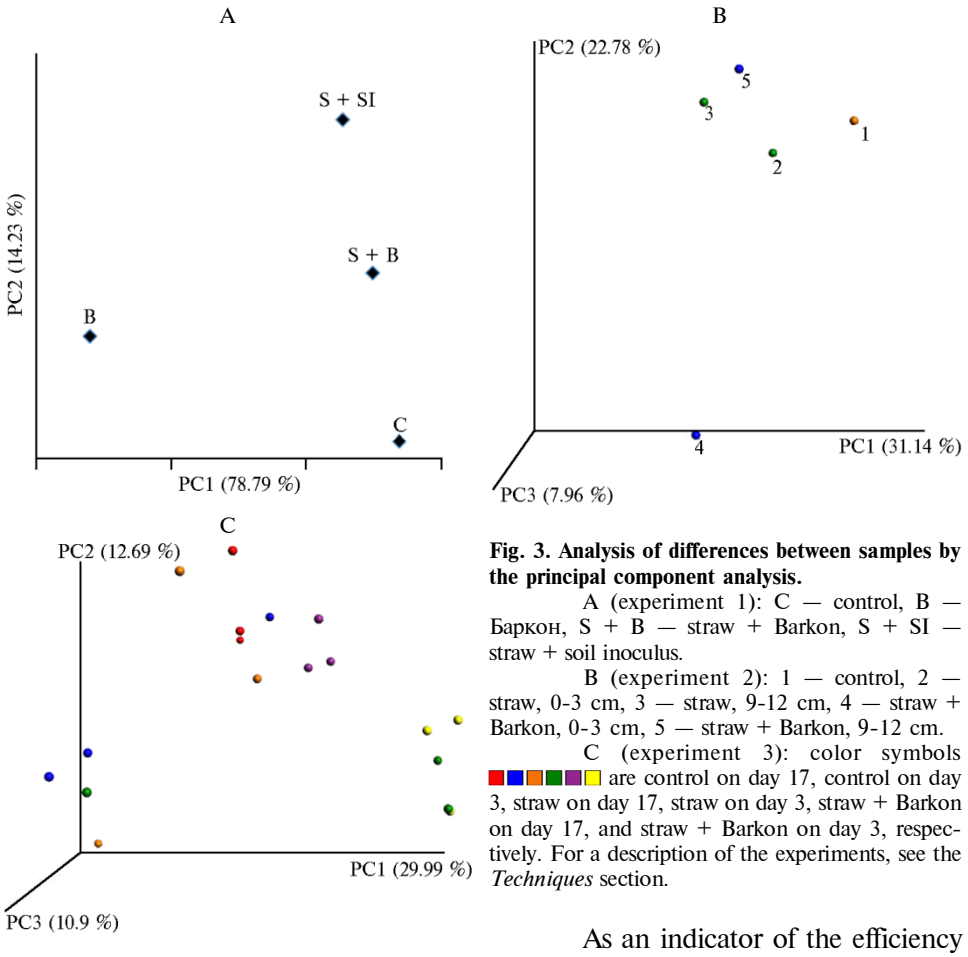

Fig. 3. Analysis of differences between samples by the principal component analysis.

A (experiment 1): C — control, B — Баркон, S + B — straw + Barkon, S + SI — straw + soil inoculus.

B (experiment 2): 1 — control, 2 — straw, 0-3 cm, 3 — straw, 9-12 cm, 4 — straw + Barkon, 0-3 cm, 5 — straw + Barkon, 9-12 cm.

C (experiment 3): color symbols ■■■■■■■ are control on day 17, control on day 3, straw on day 17, straw on day 3, straw + Barkon on day 17, and straw + Barkon on day 3, respectively. For a description of the experiments, see the *Techniques* section.

As an indicator of the efficiency of joint transformation activity of the microorganism community, it is proposed to calculate the index of fractal structures of the microbial community using the following formula:

$$I_F = N_{\text{ПФГ}}(h)/N_M ,$$

where $N_{\text{PFG}}(h)$ is the number of different OTU groups (operational taxonomic unit) in the primary fractal groups identified in the fractal portrait of the microbial community for $ln(h)$ <= –5...–3; N_M is the total number of OTU groups combining individual OTU with similar frequencies.

For successful utilization of straw, microorganisms form biosystems with the fractal organization of network structures in which the transformational biochemical roles of microorganisms are distributed and the order of joint transformational actions is coordinated. In the case of unsuccessful assembly of a destructive biosystem (for example, in the absence of necessary microorganisms), the efficiency of transformations of plant substrates in the soil is low, which leads to a decrease in the index of fractal structures of the microbial community. For example, on day 17 the index of fractal structures in experiment 3 was lower for non-inoculated straw (I_F = 0.80) than for straw and Barkon (I_F = 0.83) (Table 3). This means that microorganisms of the biopreparation are embedded in the soil

destructive biosystems in the absence of the necessary microorganisms in it.

For the formation of destructive biosystems, microorganisms need some time to tune in to work together, distribute transformational roles and establish the order of transformational actions. Therefore, the soil microbial community (with straw and Barkon) was not fully tuned ($I_F = 0.79$) in experiment 3 on day 3, but tuned in on day 17 ($I_F = 0.83$). If comparing the variants of experiment 2, which differ in the depth of introducing the straw inoculated by Barkon, then the efficiency of the destructive microbial biosystem was greater when straw was introduced into the upper (see Table 3; $I_F = 0.68$) than in the lower soil layers ($I_F = 0.58$). A likely explanation is the fact that the use of Barkon allows for the best results in terms of the formation of destructive microbial biosystems if the biosystems are characterized by the predominance of aerobic microorganisms, which can receive enough energy for the biochemical transformations of plant substrates by oxidizing organic substances.

In experiment 1, the indexes of fractal structures (see Table 3) in the control and at the straw inoculation with soil extraction were lower ($I_F = 0.44$ and 0.52) than when using straw inoculated with Barkon ($I_F = 0.75$). That is, during production of this biopreparation, microorganisms formed network structures that are ready for joint transformational biochemical activity.

Thus, treatment with the Barkon biopreparation promotes the formation of microbial destructive communities with the highest efficiency of straw transformation into labile organic compounds, and then into soil humus substances. At the decomposition of straw in the soil, as compared to unembedded straw, there is some weakening of the effect of Barkon on the formation of the humification trophic chain, as evidenced by the lack of growth of labile humic substances in the respective variants. The greatest influence on the composition of microbial communities, leading to the decomposition of straw, is exerted by the type of soil, the least — by its chemical, physical, biochemical characteristics. The interrelationship of the soil-microbiological parameters (the number and biomass of microorganisms, respiration) with the taxonomic composition of the microbiocenosis confirms the high response of the composition of the microbial community to various effects while maintaining the core component of the microbiome characteristic of a particular soil. According to the microbial profiles of soil samples, it was for the first time shown that under straw inoculation with the Barkon biopreparation the proportion of the *Micrococcaceae* family increases (*Micrococcaceae* representatives are among the constituent microbial components of this biopreparation). It is established that the minor groups of microorganisms participate equally with the major groups in the formation of network fractal structures. It is shown that the microbiological preparation increases the index of fractal structures of the microbial community, especially in the upper parts of the arable layer, that is, it creates conditions for the effective utilization of straw in the soil, increasing the rate of straw processing into humus compounds.

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DEGRADATIVE ACTIVITY AND PRODUCTION OF THE EXTRACELLULAR PEROXIDASES BY MICROMYCETES WITH DIFFERENT ECOLOGICAL STRATEGY

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Abstract

Environmental pollution by natural and man-made pollutants remains a serious problem. Agricultural areas are contaminated by major and hazardous pollutants such as oil, which comes from local oil-producing and oil-refining facilities, and polycyclic aromatic hydrocarbons (PAHs), which result from natural fires and from human activity associated with the use of flammable organic raw materials. This presents the hazard of accumulation of toxic substances in food and fodder plants. Natural ecosystems have powerful detoxifying potential, which is ensured by the degradative activity of microorganisms, including ascomycetes — one of the largest groups in the fungal kingdom. Here we examined the degradation of oil and PAHs by micromycetes with different ecological strategies and detected ligninolytic enzymes implicated in the oxidation of the pollutants. We used four ascomycete strains with different taxonomic affiliations and ecological strategies. These were *Fusarium oxysporum* IBPPM543, *Lecanicillium aphanocladii* IBPPM542, *Cladosporium herbarum* MUT3238, and *Geotrichum candidum* MUT4803. The fungi were grown in liquid media with different compositions that received additions of the pollutants used: oil, PAHs, and anthraquinone-type dyes. After 14 days of fungal growth, the elimination of the pollutants and the content of their main degradation products were examined by GC. Ligninolytic enzyme activity was estimated spectrophotometrically by the oxidation rate of the corresponding test substrates. All treatments in the experiments and analyses had no less than three replications, and each experiment was repeated no less than three times. Data were processed with Microsoft Excel 2003 software. All fungi oxidized oil; the utilization was from 46 to 82 % of the initial concentration of 5 g/l within 14 days. *C. herbarum* MUT 3238 metabolized all PAHs included in the study (anthracene, phenanthrene, and fluorene) almost completely (initial concentration, 0.05 g/l). *L. aphanocladii* IBPPM 542 degraded anthracene, phenanthrene, and fluorene by 40, 63, and 81 %, respectively. *F. oxysporum* IBPPM 543 utilized phenanthrene and fluorene only by 20 and 40 %, respectively. PAH degradation by *G. candidum* MUT4803 was not greater than 18 %. Anthracene was not degraded by *F. oxysporum* IBPPM 543 and *G. candidum* MUT4803. The degradation of the pollutants was accompanied by the production of extracellular peroxidases by all fungi except *G. candidum*. The activities of these peroxidases were largely stimulated by Mn^{2+}; this property makes them similar to the Mn-peroxidases of basidiomycetes. This is the first report on the production of extracellular peroxidases by *C. herbarum* and *L. aphanocladii*. Neither of the fungi produced lignin peroxidase or laccase. Identification of the PAH oxidation products allowed us to suggest a pathway for PAH degradation by the tested fungi with an extracellular Mn-peroxidase. The degradation proceeds through the formation of quinones and carboxylic acids (phthalic and 2,2′-diphenic), which indicates that the PAHs are utilized almost completely and that no toxic metabolites accumulate. The obtained results indicate that two widely distributed ascomycete species, *C. herbarum* and *F. oxysporum*, and a strain of the lesser-known and

poorly studied species *L. aphanocladii*, have degradative potential toward oil and PAHs, which presupposes their involvement in the self-cleaning of the environment from these pollutants. The detection of ligninolytic enzymes (Mn-peroxidases) and of the corresponding products of PAH degradation speaks in favor of an ecologically appropriate pathway for the utilization of PAHs, which reduces the negative consequences associated with the possible formation of toxic metabolites. In the *G. candidum* strain, the oxidation of oil and PAHs is possibly due to the activity of other enzymes, for example cytochrome P450 monooxygenase, because no ligninolytic enzymes have been found. In addition, it is highly possible that this strain has a "dye peroxidase", which requires a narrow range of substrates and catalyzes the degradation of anthraquinone dyes, as was also shown by us. The ability of all fungal strains to degrade pollutants makes them promising candidates for practical use in bioremediation and other biotechnologies.

Keywords: ascomycetes, *Fusarium oxysporum*, *Lecanicillium aphanocladii*, *Cladosporium herbarum*, biodegradation, polycyclic aromatic hydrocarbons, oil, ligninolytic enzymes, peroxidases

Environmental pollution by natural and man-made pollutants remains a serious problem. Agricultural areas are contaminated by major and hazardous pollutants such as oil and polycyclic aromatic hydrocarbons (PAHs): oil due to local oil-producing and oil-refining facilities [1], PAHs as a result of natural fires and human activity associated with the use of flammable organic raw materials [2]. This presents the hazard of the accumulation of toxic substances in fodder and food plants. Natural ecosystems have powerful detoxifying potential, which is ensured by the degradative activity of microorganisms, including ascomycetes, one of the largest groups in the fungal kingdom.

All species of the *Fusarium* genus are characterized by high metabolic activity and adaptive plasticity [3]. They are primarily known as harmful to agriculture, causing diseases and toxicoses in plants and animals. The *Fusarium oxysporum* species often serves as a model for studies of plant—pathogen interaction [4]. However, most of its representatives have a saprotrophic lifestyle in the soil, with using complex carbohydrates and lignocellulose as sources of nutrition. The strains that form a mutually beneficial symbiosis with plants and even protect them from diseases are known [5, 6]. The participation of *Fusarium* in the processes of pollutants degradation, including PAHs and oil [7, 8], is shown.

The fungi of *Cladosporium* genus make a significant contribution to the degradation of plant residues but can cause plant diseases, damage of hay and grain in storage, by releasing toxins hazardous for animals and humans. Not enough information is known about pollutants degradation with these fungi: *C. resinae* is described as creosote or kerosene fungus, corroding pumps; the ability to metabolize anthracene is shown in *C. herbarum* [9], fluoranthene is described for *C. sphaerospermum* [10].

Representatives of the *Geotrichum* and *Lecanicillium* genera inhabit other types of biotopes. *Geotrichum candidum* causes a variety of rots on organic loadings, including fruits, and is considered the opportunistic fungus, the causative agent of opportunistic mycoses. At the same time, strains of this species are used in the production of elite cheeses and a number of fermented milk products [11]. Data on the degradation properties of *Geotrichum* mainly concern the ability of this fungus to discolor synthetic dyes, including those containing condensed aromatic rings [12, 13], transform 2,4,6-trinitrotoluene [14] and detergents [15]. The *Lecanicillium aphanocladii* species (known as *Verticillium lecanii* until 2001) is described as entomopathogenic [16, 17] and parasitic upon other fungi [18]. Its degrading properties with regard to pollutants have not been studied virtually. The information about the representatives of the other species of this genus is known: *L. saksenae* is a pesticides destructor [19], *V. lecanii* is the destructor of 2,4-dichlorophenol, 2,4-dichlorophenoxyacetic acid [20], and anthracene [9].

The destruction of natural substances and xenobiotics by fungi is carried out with the help of extracellular and intracellular enzymes. The extracellular lig-

ninolytic enzymes, the laccases and peroxidases, are produced by many basidiomycetes and ascomycetes in the process of lignocellulose degradation and are often considered as key enzymes of pollutant degradation [21]. The reports about the production of similar enzymes in *L. aphanocladii* and *C. herbarum* were not found.

The intracellular enzymes involved in pollutant degradations are primarily represented by cytochrome P450-dependent monooxygenases (cytochrome P450-monooxygenases), which are present in the cells of fungi regardless of their ability to produce ligninolytic extracellular enzymes. It is assumed that the pathway of primary oxidation of PAHs by one or another enzyme depends on a number of conditions; in the case of the hydroxylation of the aromatic ring and a number of subsequent transformations catalyzed by cytochrome P450-monooxygenase, such powerful carcinogens as epoxides and transdihydrodiols can be generated. At the same time, oxidation of these substances mediated by peroxidase or laccase occurs with the formation of quinones, which are further metabolized by the fungus up to compounds that are less toxic than the original PAHs. Therefore, oxidation of PAHs by ligninolytic enzymes may be a more logical strategy for detoxifying the polluted environment [22].

Within this framework, it is interesting to evaluate not only the destructive activity of some ascomycetes, i.e. the constant and mass inhabitants of plant communities, characterizing their participation in the self-purification of the natural environment, but also to determine the presence of ligninolytic enzymes, lowering the environmental risks associated with the possible formation of toxic metabolites.

In this paper, the ability of a number of previously unexplored strains of ascomycetes of different generic assignment to destroy oil and PAHs actively, by producing extracellular peroxidases, was shown for the first time.

The work objective was to study the degradation of oil and polycyclic aromatic hydrocarbons by micromycetes with different environmental strategies, as well as to identify the ligninolytic enzymes involved in the oxidation of these pollutants.

Techniques. Four ascomycetes strains used were *Fusarium oxysporum* IBPPM543 (isolated from old creosoted wood tie; IBPPM, the Collection of rhizospheric microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms RAS), *Lecanicillium aphanocladii* IBPPM542 (isolated from sporocarp of basidiomycete *Lentinus* sp.), *Cladosporium herbarum* MUT3238 and *Geotrichum candidum* MUT4803 (obtained from the Mycotheca Universitatis Taurinensis, Turin, Italy).

Fungi were cultured in flasks in a rich medium for basidiomycetes containing (in g/l) NH_4NO_3 0.724, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, KCl 0.5, yeast extract 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot 7H_2O$ 0.0028, $CaCl_2 \cdot 2H_2O$ 0.033, glucose 10.0, peptone 10.0; pH 6.0 [23]. Due to the optical opacity of this medium, the Kirk [24] medium in the authors' modification was used to observe the discoloration of dyes containing (in g/l) KH_2PO_4 2.0, $MgSO_4$ 0.348, $CaCl_2 \cdot 2H_2O$ 0.143, NH_4NO_3 1.02; (in ml/l) microelement-containing solution 10, thiamine 0.5. The microelement-containing solution included (in g/l) nitrilotriacetate 1.5, $MgSO_4 \cdot 7H_2O$ 3.0, $MnSO_4 \cdot H_2O$ 0.5, NaCl 1.0, $FeSO_4 \cdot 7H_2O$ 0.1, $CoSO_4$ 0.1, $CaCl_2$ 0.082, $ZnSO_4$ 0.1, $CuSO_4 \cdot 5H_2O$ 0.01, $AlK(SO_4)_2$ 0.01, H_3BO_4 0.01, $NaMoO_4$ 0.01; 25 mM phosphate buffer for pH 6.0; maltose at a final concentration of 1% was the source of carbon and energy.

The degradation activity of fungi was evaluated with PAHs (anthracene, phenanthrene, and fluorene), anthraquinone synthetic dyes (Acid Blue 62 and Reactive Blue 4) and crude oil (alkane 47.4%, naphthenes 22.3%, low-molecular aromatic substances 4.4%, high-molecular aromatic substances 5.4%, resins

3.9%, asphaltenes 16.6%). PAHs and oil were introduced into the culture medium in the form of chloroform solution, anthraquinone dyes in the form of aqueous solution. The final concentration for PAHs and anthraquinone dyes was 0.05 g/l, for oil 5.0 g/l. The media were inoculated with 2-days fungi inoculate and cultured at 26 °C and aeration (120 rpm), after 2 days pollutants were introduced into the flasks, in the control variants 100 μl of solvent. After 14 days, a decrease in the amount of pollutants, the content of the main metabolic products and the activity of ligninolytic enzymes were estimated.

PAHs and their degradation products were extracted from the culture liquid by chloroform (three times by 5 ml), the extracts were combined, evaporated to dryness and analyzed by the gas-liquid chromatography (GLC) method on the GC-2010 chromatograph (Shimadzu Deutschland GmbH, Germany) with the flame photometric detector. The substances were separated on the HP5 column (Agilent Technologies Inc., USA), with carrier gas helium. The column temperature of 200 °C was maintained for 3 min and then increased up to 270 °C at the rate of 15 °C/min; this temperature was maintained for another 2 min. Prior to GLC, 2-carboxybenzaldehyde, 2,2′-diphenic and phthalic acid were methylated with CH_3COCl. Anthracene (retention time 4.15 min), 9,10-anthraquinone (5.39 min), phenanthrene (4.08 min), phenanthrene-9,10-quinone (6.86 min), fluorene (5.57 min), 9-fluorenone (4.33 min), 2-carboxybenzaldehyde (7.31 min), 2.2′-diphenic acid (6.7 min), phthalic acid (8.99 min) were used as markers for identification of PAHs and products of their oxidation.

The loss of dyes was tested spectrophotometrically, by taking 2 ml aliquots from the flasks at certain time intervals, followed by measurement of absorption at $\lambda = 590$ nm [25]. The residual oil from the culture medium was extracted with chloroform (three times by 5 ml); the extracts were combined and evaporated to dryness. The total oil content in the samples was determined by adsorption chromatography with gravimetric termination [26].

Enzyme activity was evaluated spectrophotometrically (Evolution 60, Thermo Scientific, USA): laccase by the oxidation rate of diammonium salt 2,2g-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at $\lambda = 436$ nm [27]; Mn-peroxidase by the oxidation rate of 2,6-dimethoxyphenol in the presence of H_2O_2 and Mn^{2+} at $\lambda = 468$ nm [28]; lignin-peroxidase by the formation of the oxidation product of veratryl alcohol at $\lambda = 310$ nm [29]. Peroxidase activity was calculated as the difference between the rate of substrates oxidation in the presence of H_2O_2 and without it. The amount of enzyme that catalyzed the conversion of 1 μmol substrate per minute was taken as a unit of activity (U/ml).

Repetition in all variants in experiments and analyses was not less than 3-fold; each experiment was repeated at least 3 times. The obtained results were statistically processed in Microsoft Excel 2003. The figures show mean values (*M*) and standard deviations (±SD).

Results. The studied fungi had oil-oxidizing activity. In all variants, there was an intensive growth of biomass, the mycelium grew in the form of pellets of different sizes; after 14 days, the oil was completely emulsified. *C. herbarum* MUT 3238 and *F. oxysporum* IBPPM 543 destroyed oil by 82%, *L. aphanocladii* IBPPM 542 by 60%, *G. candidum* MUT4803 by 46%. The literature presents quite a lot of data on the participation of micromycetes in oil degradation [30-33]; however, no publications that would report on the oil-oxidizing properties of *L. aphanocladii* were found.

Concerning PAHs, the activity of fungi differed more brightly. For *C. herbarum*, the decrease in the initial amount of anthracene, phenanthrene, and fluorene from the culture medium for 14 days was almost complete.

L. aphanocladii degraded anthracene, phenanthrene, and fluorene by 40, 63, and 81% respectively. *F. oxysporum* oxidized phenanthrene and fluorene by only 20 and 40%, *G. candidum* destroyed no more than 18% of these PAHs. Anthracene was not degraded by the last two fungi (Fig. 1).

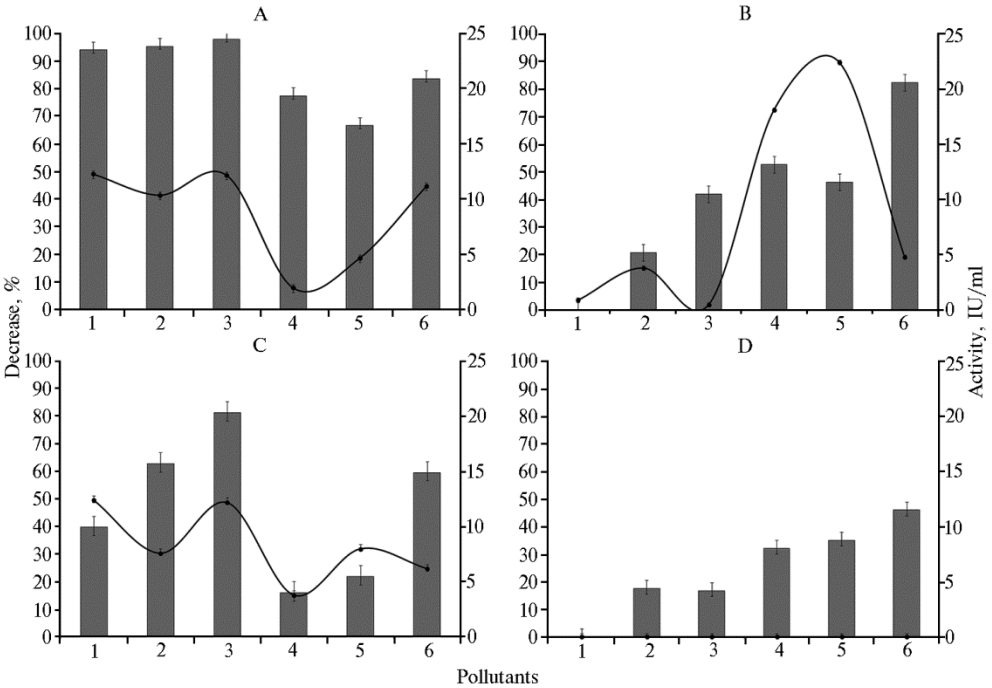

Fig. 1. Destructive activity (histogram) **for anthracene** (1)**, phenanthrene** (2)**, fluorene** (3)**, Reactive Blue 4** (4)**, Acid Blue 62** (5)**, oil** (6) **and extracellular peroxidase production** (graph) (day 14; $n = 3$, standard deviations).

A key role in the fungal degradation of various pollutants is attributed to extracellular oxidative enzymes, primarily ligninolytic [21]. In this regard, the studied strains were tested for the activity of laccase, lignin- and Mn-peroxidase. For primary screening of fungi for the production of these enzymes, their ability to discolor anthraquinone dyes is often used. As tests have shown, all four ascomycetes had this ability, which served as an indirect confirmation of the presence of such enzymes in them. However, in the conditions of this experiment, the activity of lignin-peroxidases and laccases in fungi was not detected. For three strains (except *G. candidum*), the presence of pollutants was accompanied by the production of extracellular peroxidases (see Fig. 1), which in control options (without pollutant) were absent. It was found that their activity was largely (up to 40%) stimulated by Mn^{2+} ions, which makes these enzymes similar to Mn-dependent peroxidases of basidiomycetes [34].

According to the literature, many micromycetes have ligninolytic enzymes. The representatives of the *Fusarium* genus produce Mn-dependent peroxidase, lignin-peroxidase and laccase involved in stress and degradation reactions of lignocellulose [35]. The role of laccases in the pathogenesis of fungi was confirmed [36]. The participation of the *Fusarium* enzymes in the degradation of PAHs was described in various options. For example, *F. solani* laccase is involved in the degradation of anthracene and benz(a)anthracene in mangroves polluted with PAHs, while lignin- and Mn-peroxidase were not detected [37]. When using *F. oxysporum* for the transformation of aromatic components in the dry waste of the olive mill, the activity of Mn-peroxidase and Mn-independent

peroxidase was detected, and the activity of laccase was not detected [38]. The members of the species *G. candidum* have three types of peroxidases, the participation of which in the process of degradation is widely discussed. These are lignin- and Mn-peroxidases [39, 40], as well as discoloring (dye-peroxidase) peroxidase, which is assumed to have a narrow substrate spectrum and serves as a key enzyme in the degradation of dyes, including those containing condensed aromatic rings [12, 13]. In this case, the revealed ability of the *G. candidum* strain to discolor anthraquinone dyes at very low oxidative activity against PAHs allows suggesting the presence of extracellular discoloration (dye)-peroxidase. In the publications of other authors, the information about the production of lignolytic enzymes by *L. aphanocladii* and *C. herbarum* was not found.

Certainly, in processes occurred in fungi, including the degradation of pollutants, the other enzyme systems, primarily oxygenases, which are known to be intracellular enzymes, are involved, and their participation is connected with the preliminary transportation of the substance into the cell. PAHs have low solubility, which probably determined the weak degradation of phenanthrene and fluorene (18%) by *G. candidum.*

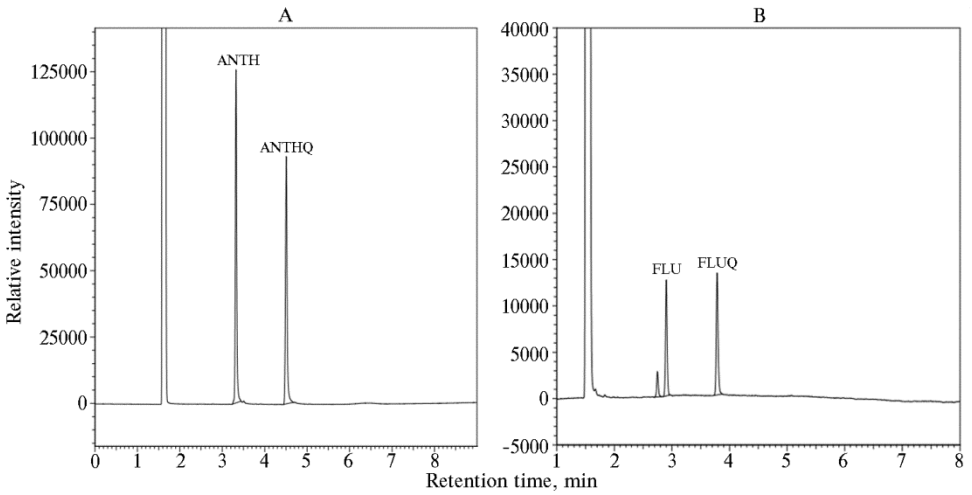

Fig. 2. Chromatograms of cultural liquid extracts of *Cladosporium herbarum* MUT 3238 after the degradation of anthracene (A) **and fluorene** (B)**:** the main peaks are ANTH — anthracene, ANTHQ — 9,10-anthraquinone, FLU — fluorene, FLUQ — 9-fluorenon (GC-2010 chromatograph, Shimadzu Deutschland GmbH, Germany; the flame photometric detector, column HP5, Agilent Technologies Inc., USA).

In the study of products of tricyclic PAHs anthracene and fluorene oxidation by the *C. herbarum* fungus with the GLC, the metabolite of anthracene degradation by *C. herbarum*, the 9,10-anthraquinone (Fig. 2), was identified, which after 14 days was almost completely destroyed with the formation of 2,2′-diphenic and phthalic acids. The metabolite of fluorene degradation by this fungus, the 9-fluorenol, was detected in trace amounts, which may be the result of rapid utilization of the initial PAH. As one of the final products of fluorene degradation, phthalic acid was found, which is known to be included in the main metabolism of fungi [41].

The formation and subsequent utilization of 9-fluorenol, 9-fluorenone and 2-carboxybenzaldehyde were observed in the degradation of fluorene by the *F. oxysporum*. Degradation of PAH by *C. herbarum* and *F. oxysporum* occurred with the formation and subsequent destruction of quinones, without the accumulation of toxic metabolites. It is necessary to note that the identified metabolites were identical to those found in basidiomycetes [42, 43]; this fact allows suggest-

ing the presence of similar metabolic pathways of PAH degradation for ascomycetes as well.

In the study of the PAH metabolism by *L. aphanocladii* and *G. candidum*, such unambiguous results were not obtained. In phenanthrene degradation by *L. aphanocladii*, trace amounts of phenanthrene-9,10-quinone were detected, which disappeared with an increase in the time of fungus culturing. The detection of this quinone and production of Mn-peroxidase by the strain allows us to suggest that *L. aphanocladii* has the pathway of PAH destruction which is similar to *C. herbarum* and *F. oxysporum*. Quinone metabolites during the degradation of PAHs by *G. candidum* were not identified.

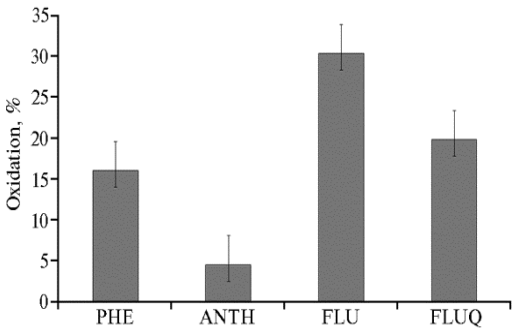

Fig. 3. Oxidation of polycyclic aromatic hydrocarbons by rough enzyme preparation of *Fusarium oxysporum* IBPPM 543 peroxidase: PHE — phenanthrene, ANTH — anthracene, FLU — fluorene, FLUQ — 9-fluorenone (day 2; $n = 3$, standard deviations are given).

As mentioned above, three of the four studied fungi produced peroxidase in response to the presence of pollutants in the culture medium. It is known that ligninolytic peroxidases are involved in the degradation of PAHs by oxidizing them to the corresponding quinones [21]. To clarify the role of the detected Mn-peroxidases of ascomycetes in PAH degradation, the direct oxidation reactions of three-cyclic PAH by an enzyme from *F. oxysporum* were investigated. A crude preparation of this enzyme was obtained, for which the fungal mycelium was cultured to a maximum of peroxidase production (20 U/ml); the culture medium was separated from the mycelium by filtration, concentrated 50-fold with ultrafiltration (Amicon PM-10 filter, Merck KGaA, Germany) and used as an enzyme source. It was found that this peroxidase oxidized both native PAH fluorene and phenanthrene, as well as 9-fluorenone, the oxidation product of fluorene (Fig. 3). Therefore, it is an extracellular fungal peroxidase that oxidized PAHs and also, at least, a fraction of polynuclear aromatic compounds of oil. It is important to note that in this experiment, fungi with different environmental strategies showed similar properties. All strains had high oil-oxidizing activity. Potentially entomopathogenic *L. aphanocladii* in terms of its destructive properties and production of Mn-peroxidase was similar to saprotrophs *C. herbarum* and *F. oxysporum*. The opportunistic *G. candidum* which did not show significant destructive activity against PAHs was significantly different from them, which may be the consequence of the absence of extracellular peroxidases similar to ligninolytic, although in the literature this species is referred to as a destructor of 2,4,6-trinitrotoluene [14]. *G. candidum* oxidizes oil and PAHs likely due to the activity of other enzymes, such as cytochrome P450-monooxygenase [44].

The obtained results give grounds to continue the research, using both classical biochemical methods of isolation, purification, and comprehensive study of enzymes and methods of pollutants degradation, and modern molecular biological approaches, making it possible to establish the presence and expression of the corresponding genes.

Thus, the representatives of two widely distributed in nature species of ascomycetes, *Cladosporium herbarum and Fusarium oxysporum*, as well as a strain of the less known and little studied species *Lecanicillium aphanocladii* have a high

destructive potential for oil and polycyclic aromatic hydrocarbons (PAHs), which implies the participation of these micromycetes in the processes of self-purification of natural ecosystems from pollutants. Identification of ligninolytic enzymes and related products of PAHs degradation indicates in favor of an environmentally appropriate way of PAHs utilization (with the formation of quinones), which reduces the negative consequences associated with the possible formation of toxic metabolites. The strain *Geotrichum candidum* has no ligninolytic enzymes and oxidation of oil and PAHs can be performed by other enzymes, such as cytochrome P450-monooxygenase. In addition, it is likely that this strain has a so-called discoloring peroxidase, which has a narrow spectrum of substrate specificity and catalyzes the discoloration of anthraquinone dyes. The ability of the studied strains to destroy pollutants makes them promising for practical use in bioremediation and other biotechnological processes.

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POLYMER GELS TO MANAGE WATER AVAILABILITY FOR WHEAT (*Triticum aestivum* L.) UNDER VARIOUS ENVIRONMENT CONDITIONS

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Abstract

During the last years, due to climate changes and reducing water availability for crops, special attention is paid to moisture-swelling polymers. In this paper we compared the influence of Russian hydrogel Ritin-10 (LLC RITEK—ENPTs, Russia) and polymer Aquasorb (SNF s.a.s., France) on spring and winter wheats in Russia and Kazakhstan. The effect of Ritin-10 hydrogel on spring wheat Esther variety water supply was studied in a field experiment (Russia, 2011) with the hydrogel dosage of 400 kg/ha and its combination with nitrogen fertilizers (N_{60}, N_{90}, and N_{120}). Analysis of soil moisture during different periods of vegetation showed that Ritin-10 significantly ($p < 0.05$) increases soil moisture as compared to the control. The moisture content in use of nitrogen fertilizers combined with the hydrogel varied from 19.33 to 31.60%, and in use of nitrogen fertilizers without hydrogel from 13.14 to 17.40%. In the control, the soil moisture during the vegetation period was from 11.36 to 17.10%. Reserves of productive moisture under Aquasorb application on winter wheat Glassy variety 24 crops at tillering (Kazakhstan, 2015-2016) were 10.30-19.00% higher compared to the control. When using N_{45}, the reserves of productive moisture were 23.90-31.00%. The use of Ritin-10 hydrogel on wheat crops leads to a significant ($p < 0.001$) increase in grain yield. The grain yield of spring wheat under a combined effect of Ritin-10 hydrogel and nitrogen fertilizers varied from 33.23 to 35.7 c/ha. In our tests, the combination N_{120} + Ritin-10 provided the highest grain yield which exceeded control by 10 c/ha. Aquasorb without fertilizers and with N_{45} yields grain harvest of 27.0-35.7 c/ha for winter wheat variety Glassy 24. This study showed that Ritin-10, like superabsorbent polymer Aquasorb, can effectively manage water availability and water supply of crops.

Keywords: *Triticum aestivum* L.), spring wheat, winter wheat, water-absorbing polymers, soil moisture, root system, water availability, yield

The use of moisture-swelling polymers is considered one of the innovative non-traditional approaches in modern agricultural technologies. Strongly-swelling (moisture-swelling) polymeric hydrogels represent hydrophilic polymer material of acrylate nature. They repeatedly increase in volume at swelling, possess a high water-absorbing ability; at the same time, they are stable at repeated cycles of drying and swelling [1]. When applied to the soil root layer, gel particles are located in the inter-aggregate space and swell when moisture enters, providing an increase in humidity compared to the indicators in untreated soil. As a result, the water balance is optimized and moisture conditions favorable for the growth and development of plants are created. The main part of the water in the hydrogel has a potential of $4.2 > pF > 2.0$ (values characterizing biologically available moisture) and is used by plants effectively. When drying, the absorbent takes its original crystalline form and is ready for a new cycle with subsequent humidification. The cyclical nature of absorption and the delivery of moisture for several years are inherent in the hydrogels of polyacrylamide type, so their use is the most appropriate when carrying out agricultural activities [2, 3].

When using hydrogels, water and fertilizers (in the form of a soil solution) are stored in the zone of the root system of plants and feed them. Many researchers note [4-6] that hydrogels increase the amount of available moisture in the root zone, implying longer intervals between watering. Moisture capacity depends on the soil texture, type of hydrogel, size of particles (powder or granules), and pH of the soil solution.

The studies of domestic scientists [7, 8] showed that hydrogels are one of the most powerful synthetic means of controlling the hydrophysical properties and water regime of soils. Doses of domestic polymer gels 0.10-0.25% by weight of the soil reduce its density by 1.2-1.5 times, which creates additional porosity and increases the total moisture content up to 30-40% against 23-25%. In foreign publications, it was reported [9-11] that the treatment of soil substrates with synthetic hydrophilic gels at doses of preparations from 0.1-0.3 to 0.5% of the soil weight contributed to better germination of seeds, stimulated the growth of crops and increased their yield by 30-40%. It was found that [12] Aquasorb (SNF s.a.s., France) absorbs 50% of water for 20 min and 100% of water for 120 min. Adsorption volumes vary from 30 to 500 liters per 1 kg of dry polymer. Such water absorption capacity can be effectively maintained for 4-5 years, and the water exchange between the soil and the polymer is reversible.

Hydrogels are used as additional additives in the cultivation of plants in regions where water resources are a limiting factor. The use of a superabsorbing polymer eliminates the effects of drought and contributes to the development of drought resistance in plants [13]. The use of hydrogels reduces the number of watering significantly, especially for soils with a rough structure [14, 15].

Ritin-10 is a cross-linked copolymer of polyacrylamide, synthesized by external exposure to ionizing radiation (technology of scientific and technical landfill of PAO LUKOIL—OOO RITEK-ENPTS, Russia) and is mainly used in the oil industry to improve the oil recovery of formations. The chemical composition of the hydrogel includes C 11%, N 4.7%, O_2 16.4%, Cl 1.01%, K 27.05% and Na 36.98%. It also finds application in crop production: 1 g of hydrogel holds about 300 ml of water, with the moisture availability for plants of 95% and productivity in the soil up to 5 years [16]. Agricultural tests of Ritin-10 were started in 2008 [17]. The results of field experiments on the cultivation of winter wheat under semi-fallow conditions showed the effectiveness of this hydrogel in the Stavropol Territory [18].

In the present paper, the domestic hydrogel Ritin-10 (OOO RITEK-ENPTS, Russia) obtained from the waste of oil industry was compared with the known water-absorbing polymer Aquasorb (SNF s.a.s., France) in field trials in Russia and Kazakhstan.

The work objective was to estimate the effectiveness of the hydrogel of Russian production (Ritin-10) in water supply of wheat plants depending on nitrogen nutrition and to compare this with superabsorbent Aquasorb when used on soils of different types.

Techniques. The effect of the hydrogel Ritin-10 (OOO RITEK—ENPTS, Russia) for the water supply of spring wheat (*Triticum aestivum* L.) Ester variety (the predecessor was the potato variety Skarb) was studied in the technological cycle seeding—commercial products in a field experiment (Agrophysical Institute, field station of the Menkovskii branch, the Leningrad Province, 2011) on sod-podzolic light loamy soils with different types of nitrogen nutrition. Agrochemical examination of the experimental site was performed according to the method of field experiment [19]. Soil samples were taken with a soil auger to a depth of arable layer; the acidity (pH of salt extract) was determined potentiometrically according to GOST 26483-85, the content of ammonium nitrogen

$N\text{-}NH_4$ according to GOST 26489-85, nitrate nitrogen according to GOST 26951-86, mobile forms of phosphorus and potassium by Kirsanov (GOST 26207-91), humidity according to GOST 28268-89. The hydrogel was introduced into the root layer (5-7 cm) in the pre-sowing period at a dose of 400 kg/ha. The size of the working plots is 80 m^2 (8×10 m), repetition is 2-fold. Combinations of hydrogel (400 kg/ha) with nitrogen fertilizers at doses of N_{60}, N_{90} or N_{120} were tested; the controls were the option without hydrogel and nitrogen fertilizers and the option with hydrogel (400 kg/ha) without nitrogen fertilizers. During the vegetation period, biometric and phenological observations were carried out according to the phases of plant development and soil samples were taken to determine the humidity.

The influence of Aquasorb absorbent (SNF s.a.s., France) on the water supply of winter wheat Steklovidnaya 24 variety was investigated in stationary field experiments (Kazakh Research Institute of Agriculture and Plant Growing, 2015-2016) on light-brown, light loamy soil. Two doses of the absorbent (20 and 40 kg/ha) and their combination with nitrogen fertilizer (N_{45}) were tested; the control was the option without the absorbent and nitrogen fertilizer.

Field and laboratory observations (analyses) and accountings were carried out by the method of field experiment [19].

Statistical processing was performed with Statistics 5.0 software (StatSoft, Inc., USA). The values of the mean (*M*) and standard deviations (±SD) were calculated. The significance of mean differences was assessed by two-factor analysis of variance (ANOVA), the differences were considered statistically significant at $p \leq 0.05$.

Results. Sod-weak- and sod-medium-podzolic soils are widespread in the Menkovskii branch. Among these soils, easy-medium-loamy soil and sandy loam on the moraine dominate. The granulometric composition of sod-podzolic light-loamy soil includes: physical clay 27.96%, large dust 22.4%, ooze 6.11% (K.G. Moiseev. The database of the soil cover of the Menkovskii branch of BSI Agrophysical Institute of RAA, the structure of the soil cover, geomorphological structure, physical, and geochemical properties of soils, 2013). The results of the agrochemical survey of the experimental site showed that the total nitrogen content is 0.37% with the high availability of phosphorus and potassium, i.e. P_2O_5 724.7 mg/kg, K_2O 280.9 mg/kg (according to Kirsanov). According to the pH_{KCl} 5.8, the soil refers to slightly acidic or close to neutral.

Fig. 1. The development of the root system in spring wheat (*Triticum aestivum* L.) Ester variety plants at tillering stage without the use of hydrogel (on the left) **and with the pre-sowing application of hydrogel Ritin-10 (Russia)** (on the right) (field trials, field station of the Menkovskii branch of the Agrophysical Institute, the Leningrad Province, 2011).

In the conditions of the Leningrad region, the introduction of hydrogel during the pre-sowing period had a positive effect on the development of the root system of spring wheat at tillering; a large part of the roots (Fig. 1, on the right) was in the area of hydrogel application. The effect of hydrogel was the greatest from tillering (in the North-Western zone of Russia, the end of May to the beginning of June is considered a dry period) to flowering and especially at the boot stage. These are the most critical stages of the development of spring wheat, when the crop is formed, and the lack of moisture in these periods affects the crop yield greatly [20].

The presence of hydrogel in the root layer affected the formation of biomass significantly. During the growing season, the increased plant growth with good standing density occurred. Phenological and biometric observations showed that plant biomass (compared to that in the control) significantly ($p < 0.001$) increased in vegetation stages, especially in variants where hydrogel was used together with nitrogen fertilizers at doses of N_{90} and N_{120} (Fig. 2).

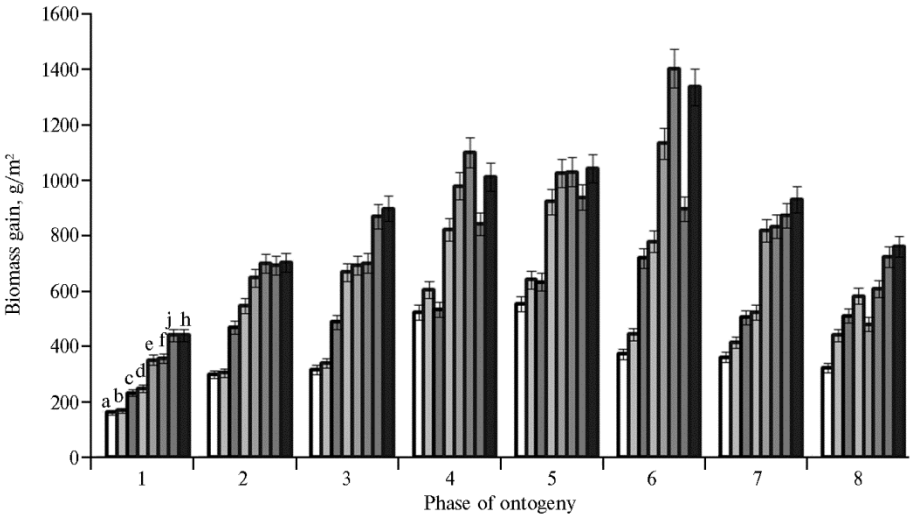

Fig. 2. Dynamics of biomass accumulation in spring wheat (*Triticum aestivum* L.) Ester variety plants under the influence of hydrogel Ritin-10 (Russia, 400 kg/ha) and nitrogen fertilizers: a — control 1, b — control 2 (control 1 + gel); c — N_{60}, d — N_{60} + gel; e — N_{90}; f — N_{90} + gel; g — N_{120}, h — N_{120} + gel; 1 — tillering; 2 — booting; 3 — earing; 4 — flowering; 5 — flowering-filling; 6 — milky ripeness; 7 — wax ripeness; 8 — full ripeness. The mean (*M*) and standard deviations (±SD) are given (field experiment, the field trials of the Menkovskii branch of the Agrophysical Institute, the Leningrad Province, 2011).

As is known [21-23], if water is available in the required quantities, a significant proportion of the energy released on the surface of actively developing plants is spent for transpiration. The intensity of transpiration is influenced by a large number of factors (temperature of the transpiration surface, soil moisture, relative humidity, coefficients of diffusion and air turbulent flow). The main part of the absorbed water, passing through the plant, transports minerals and evaporates through the stomata of the leaves into the atmosphere. With the lack of moisture, a decrease in transpiration due to the closure of stomata takes place, which leads to plant wilting. Hydrogel [23-25] is most effective in stressful conditions for plant growth and development (high temperature, lack of moisture in the soil). The results of soil moisture analysis in different periods of vegetation indicate that hydrogel ($p < 0.05$) increased this parameter compared to the control significantly. The moisture content in the options when nitrogen fertilizers were used in combination with hydrogel varied from 19.33 to 31.60%, in the presence of nitrogen fertilizers without hydrogel from 13.14 to 17.40%. In the

control group without hydrogel, soil moisture during the growing season was from 11.36 to 17.10%. Consequently, the moisture retained by the hydrogel was sufficient for use in transpiration, which had a positive effect on the growth, development, and productivity of plants. It is especially important to have a minimum reserve of soil moisture in the early stages of development of wheat plants. The introduction of the hydrogel contributes to improving conditions for seed germination and plant nutrition during formation of the climatically secured crop [25, 26].

Aquasorb is a superabsorbent based on anionic polyacrylamide, a water-insoluble cross-linked copolymer of acrylamide and potassium acrylate. The absorbent is hardly affected by biodegradation, does not hydrolyze, and does not bioaccumulate. Specific gravity is 1.10 g/cm^3, pH = 8.10, 1 g holds about 400 ml of water; adsorption in deionized water is 400 g/g, in soil 150 g/g, moisture retention at pF 1 is 980 ml/l with 95% water return (near the drying point) and cation exchange capacity of 4.6 mEq/g. Productivity in soil is up to 5 years [26]. Aquasorb superabsorbent is widely used in crop production, forestry, and horticulture (for transportation and planting of seedlings), floriculture, vegetable growing (in greenhouses), melon production, animal husbandry (as a litter for animals), landscape design (Alpine slides, lawns).

In stationary experiments in the conditions of Kazakh Research Institute of Agriculture and Plant Growing, the density of light-brown light loamy soil was 1.16 and 1.33 g/cm^3. The mechanical composition of the soil refers to light loam, with the content of physical clay 39-42%, large dust 45-50%, ooze 12-17%. The content of carbonates in the upper layers is 2.7-3.6%, in the carbonate horizon 6.5%. The amount of absorbed bases does not exceed 12 mg-eq. per 100 g of soil. The calcium accounts for 80-90%, magnesium for 10-20%, the amount of absorbed sodium is negligible. The provision of soil with easily hydrolyzed nitrogen is medium, mobile phosphorus is low, exchangeable potassium is medium (according to Kirsanov, GOST 26207-91), light-brown soil in the upper horizon contains 0.12-0.14% gross nitrogen, 2.02% humus (GOST 26213-91), with no salinization with water-soluble salts (the amount of salts in the upper layer does not exceed 0.12%) [27]. The predecessor of winter wheat was complete fallow. The reclaiming with the blade cultivator KRN-2-150 (Humanimal, Russia) was carried out before sowing at a depth of 20-22 cm.

Studies carried out in Kazakhstan have shown that the use of Aquasorb in winter wheat crops effectively increases moisture reserves, especially when grown without irrigation [28]. It was found that during the tillering of winter wheat, productive moisture reserves in the soil layer depth of 1 m when applying Aquasorb at doses of 20 and 40 kg/ha were higher by 13-19 mm (or 10.3-19.0%) compared to the control group (without Aquasorb). When using nitrogen fertilizers N_{45} with hydrogel, the reserves of productive moisture were 30-39 mm (or 23.9-31.0% higher than in the control). The reserves of productive moisture in the cultivation of winter wheat were the largest when applying Aquasorb (40 kg/ha) with a nitrogen fertilizer N_{45} and the smallest with nitrogen fertilizer N_{45} only, with moisture reserves only 6 mm (4.8%) higher than in the control.

When using Aquasorb without fertilizers and in combination with fertilizer, the yield of winter wheat ranged from 27.00 to 35.70 c/ha (Fig. 3, A). Aquasorb significantly ($p < 0.05$) increased grain yield, especially in combination with nitrogen fertilizers. The highest yield without the use of nitrogen fertilizers was obtained at 40 kg/ha of Aquasorb (32.20 c/ha, that is, by 5.20 c/ha higher than in the control), and the combination of Aquasorb 40 kg/ha + N_{45} gave an increase of 8.70 c/ha compared to the control. Treatment with hydrogel Ritin-10 led to a significant ($p < 0.001$) increase in the yield of spring wheat (see Fig. 3, B). This fig-

ure in the options with hydrogel in combination with nitrogen fertilizers ranged from 33.23 to 35.70 c/ha. The highest yield was obtained with N_{120} + Ritin-10 (grain yield was 10 kg/ha more than in the control without fertilizers and without gel). The increase in yield was 20.6% for N_{60} + Ritin-10, 33.0% for N_{90} + Ritin-10, and 38.9% for N_{120} + Ritin-10 as compared to the control.

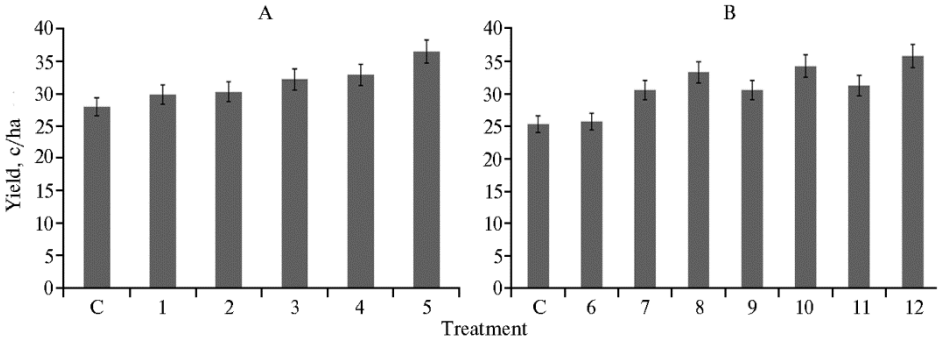

Fig. 3. The yield of wheat (*Triticum aestivum* L.) under application of hydrogels and nitrogen fertilizers: A — winter wheat (the Steklovidnaya 24 variety, Aquasorb, Kazakhstan), B — spring wheat (the Ester variety Ritin-10, Russia); C — control (without additives), 1 — N_{45}, 2 — Aquasorb 20 kg/ha, 3 — N_{45} + Aquasorb 20 kg/ha, 4 — Aquasorb 40 kg/ha, 5 v N_{45} + Aquasorb 40 kg/ha; 6 — Ritin-10 400 kg/ha, 7 — N_{60}, 8 — N_{60} + Ritin-10 400 kg/ha, 9 — N_{90}, 10 — N_{90} + Ritin-10 400 kg/ha, 11 — N_{120}, 12 — N_{120} + Ritin-10 400 kg/ha. The mean (*M*) and standard deviations (±SD) are given. Field experiments, field stations of Kazakh Research Institute of Agriculture and Plant Growing (2015-2016) and the Menkovskii branch of the Agrophysical Institute (Leningrad Province, 2011).

Thus, assessment of the effect of hydrogel Ritin-10 on the water availability for wheat crops have shown that pre-sowing introduction of Ritin-10 improves the development of the root system during plant tillering stage. It was revealed that the hydrogel accumulates a sufficient amount of soil moisture for normal growth and development of wheat plants during dry periods of the growing season, thereby increasing crop yield. In terms of the effect on the water availability of plants, hydrogel Ritin-10 shows high efficiency in comparison to the superabsorbent Aquasorb.

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EXPERIENCE WITH THE USE OF MATHEMATICAL STATISTICS METHODS FOR ASSESSMENT OF AGRICULTURAL PLANTS STATUS

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Abstract

Solving problems related to the assessment of the status of agricultural plants during the growing season, allows us to effectively use fertilizers, obtain favorable yields, improve the quality characteristics of plants, as well as the ecological condition of the field. To solve such problems of precision farming, the use of various methods of mathematical statistics is becoming an increasingly promising direction. The aim of our work was to assess the state of agricultural plants using an approach based on the combined use of kriging and binary regression methods, as well as the determination of nitrogen planting using the NDVI (Normalized Difference Vegetation Index) index. The studies were carried out at the site of an experimental agricultural field located on the territory of the branch of the Agrophysical Institute (Menkovo, Leningrad region) in 2015. With the help of aerial photographs taken from the automatized unmanned aerial vehicle complex Geoscan-401 (Geoscan Group of Companies, Russia), a set of NDVI (Normalized Difference Vegetation Index) vegetation index values was obtained at arbitrary points of the plot. A number of ground-based measurements were also conducted on the studied area of the field. The proposed approach to assessing the state of agricultural plants consisted in the joint use of two methods of mathematical statistics: ordinary kriging and logistic regression. A preliminary variogram analysis was carried out, and a variogram model was constructed. After this, the kriging method was used to calculate a series of predicted values of the parameter being studied. At the next stage, the threshold value of the parameter for the study area was established, and also a dummy variable was entered, taking the value 1 if the parameter value exceeded the threshold, and 0 otherwise. Then a logit model was built, in which one of the factors was a series of estimates of the parameter of interest, obtained using the ordinary kriging method. The input data for building logit models were as follows: $N(x_i)$ is the NDVI value at the location x_i, $i = \overline{1.78}$; variable $T = 1$, if $N(x_i) \geq 0.46$, otherwise $T = 0$; the variables X and Y are the coordinates of the observations, are considered as explanatory variables; $N_{pred}(x_i)$ is parameter values, predicted using the kriging method at the observed points. All calculations were performed using the R programming language. As a result of the experiment, three logit models were built with the dependent variable T: in the first model, the explanatory variables X and Y; in the second model — X, Y and N_{pred}; in the third model N_{pred}. Testing showed that when adding the N_{pred} variable, the logit model works better (2 times less than the erroneous determination of the level of the parameter under study). The results obtained suggest that adding in the binary regression factors a set of values predicted by the kriging method can significantly improve the accuracy of calculations.

Keywords: plant status, Normalized Difference Vegetation Index, NDVI, kriging, binary regression, language R

Evaluating state of crops during the growing season (availability of nutrients, watering parameters, weeds, diseases, etc.) is necessary for using fertilizers efficiently and producing a great and high-quality yield of [1-3]. In recent years, statistical testing and remote sensing data processing are becoming increasingly more effective ways to address these challenges [4-6].

One of the new approaches in agrophysics is based on binary regression

methods. Thus, Bure [7] describes the application of binary regression to yield forecasting. Norwegian and Dutch scientists have proposed methods for predicting the spatial distribution of soil types by means of multinomial logistic regression using digital terrain analysis [8, 9]. More sophisticated and advanced areas of precision agriculture include geostatistics, which helps map the soil content of nutrients (nitrogen, phosphorus, potassium, etc.) [10, 11], and soil electrical conductivity, pH, density, and humidity estimates [12-14] used to optimize land management. In the geostatistical approach, the soil is treated as a set of spatially continuous variables, changes wherein are described in terms of spatial dependency [15, 16]. It is only economics that combines geostatistics and binary regression methods [17]; no such methodology has yet been described in detail in relation to precision agriculture.

This paper is the first to predict the spatial distribution of the Normalized Difference Vegetation Index (NDVI) in an experimental field using Gaussian process regression (kriging) in combination with logistic regression as a subtype of binary regression. Test results show that the proposed approach allows a sufficiently accurate evaluation of the test-site parameter of interest.

The goal was to characterize the condition of crops by combining kriging and binary regression, as well as to find the availability of nitrogen to crops in terms of NDVI.

Techniques. Studies were carried out in 2015 (a test field of the Institute of Agrophysics in Menkovo, Leningrad Province). Aerial photographs taken by a Geoskan-401 (Geoskan, Russia) unmanned aircraft were used to obtain the Normalized Difference Vegetation Index (NDVI) values at arbitrary points, 78 in total.

The condition of agricultural plants was evaluated by a combination of two methods of mathematical statistics: ordinary kriging and logistic regression. A logit model was used as an approach that enabled simple parametric evaluation.

The spatial distribution of the parameter of interest was predicted by ordinary kriging for a set of measurements [18, 19]:

$$\hat{Z}(x_0) = \sum_{i=1}^{n} \lambda_i Z(x_i), \quad \sum_{i=1}^{n} \lambda_i = 1, \tag{1}$$

where n is the number of observations, $Z(x_i)$ is the value of the observed parameter at the location x_i, λ_i is the unknown weight for the parameter, $\hat{Z}(x_0)$ is the parameter value predicted for the location x_0.

The unknown weight was found by variogram analysis and constructing a theoretical variogram model $\gamma(h)$ based on the obtained experimental curve $\gamma(\hat{h})$.

To run a logistic regression, the value d (the threshold) was recorded for the test site and a dummy variable was inserted:

$$y(x) = \begin{cases} 1, Z(x) \geq d, \\ 0, Z(x) < d. \end{cases}$$

The values of $y(x_i)$ were known for the observed points, as the point-specific values of the parameter of interest, as well as its level in relation to the threshold, were known. At the preceding stage, a set of kriging-predicted values of the parameter was produced. This gave a set of inputs for logistic regression that would reflect that correlation between the threshold exceedance probability and the explanatory variables [20, 21]:

$$P(y(x_i) = 1 \mid \varphi_i) = p_i = \frac{1}{1 + \exp(-\varphi_i^T \beta)}, \tag{2}$$

where φ_i are the factors that explain the dummy variable $y(x_i)$.

The set of kriging-predicted values was used as one of the factors in the

logit model. The adequacy of the build logit model [2] was tested by the classical statistical tests i.e. the Walt test, W, and the likelihood ratio test, LR [22].

For each point of the test site, the research team computed the probability $P(y(x) = 1)$, which, when tending to 1 indicates that the parameter of interest exceeds the threshold d, while when tending to 0, it indicates that the parameter is below the threshold.

Calculations were run in R software (https://www.r-project.org), which is a popular solution used in precision agriculture [23].

Results. Fig. 1 presents an aerial photograph of the test field, as well as a data distribution map (circle parameters are proportional to the original values) made in R-statistics. The result is a set of NDVI values, which are known to correlate with the point-specific in-plant nitrogen content [24, 25].

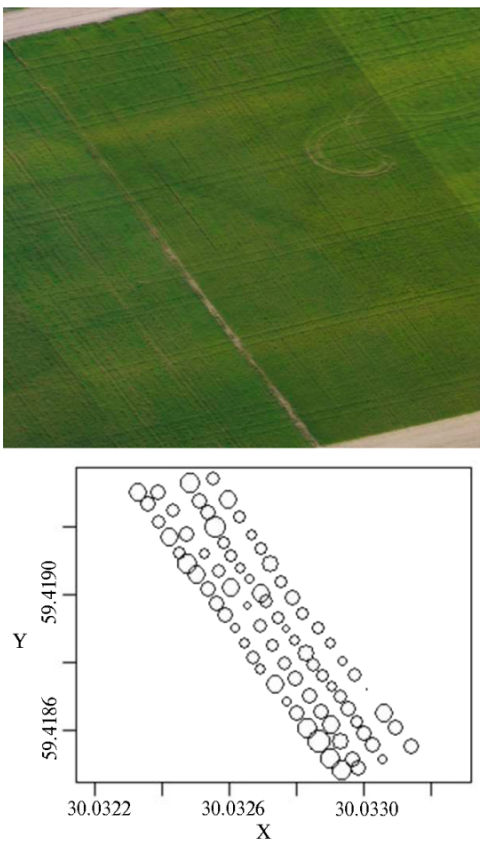

Fig. 1. Aerial view of the test site and observation distribution map (map location)**:** X and Y are the observation coordinates. The diameters of the circles are proportional to the value of the analyzed indicator, NDVI, as measured in Menkovo, Leningrad Province, 2015.

The approach proposed herein is to use a set of kriging-predicted values as one of the model factors. Accordingly, the first stage (predicting the spatial distribution of the parameter of interest) was to check whether the geostatistical conditions of stationarity and multinormality are held [26]. The detected outliers were cut at 2.5% bilateral quantiles. Besides, the research team would evaluate the linear correlation of the parameter with the coordinates. No spatial trend was identified. Verification by the Kolmogorov-Smirnov test did not allow rejecting the hypothesis of normal distribution (the attained significance was 89.75%).

The next stage was to run variogram analysis and to build a variogram model using the vgm function. Fig. 2 shows an experimental variogram of four directions (0, 90, 135, and 270°) for the configured variogram model. It was used for ordinary kriging (1): from a set of input observations, point-specific values were removed one-by-one, each time predicting the removed value by kriging using the krige function. As a result, the glm function produced three logit models. The value d = 0.46 was set as a threshold. The significance of the built models was evaluated by the LR test. The inputs for building logit models were as follows: $N(x_i)$ was the NDVI value at x_i, $i = \overline{1.78}$; the variable $T = 1$ if $N(x_i) \geq 0.46$, else $T = 0$; the variables X and Y were the coordinates of observations used as the explanatory variables; $N_{pred}(x_i)$ were the kriging-predicted point-specific values of the parameter.

The estimated coefficients of the second logit model were largely insignificant. The value α was assumed to equal 0.05, see Table 1. Statistical testing proved that the equation of this logit model is generally not significant, whereas

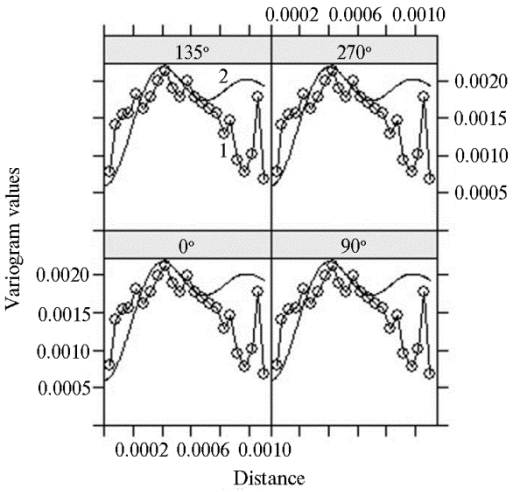

Fig. 2. Experimental variogram of the $\hat{\gamma}(h)$ spatial NDVI distribution on the test site (1), **with the theoretical model superimposed** (2) **in four directions (0, 90, 135, 270)** (Menkovo, Leningrad Province, 2015).

the equations of Models 1 and 2 are; testing those showed the third model was better.

The next stage was to test the adequacy of the three obtained models; to that end, points were removed from the input data one by one, the logit models were built again and revaluated to find how accurately each model would predict the probability of exceeding the threshold in the removed point. Testing showed that the first model made errors for 26 points (33.3%), the second one was wrong in 12 points (15.38%), see Table 2.

Similar experiments on simulated data show that the second complete model is better as long as it is statistically significant.

1. Results of constructing the logit models of spatial NDVI distribution; the models use different explanatory variables based on the aerial photography data (Menkovo, Leningrad Province, 2015).

Logit model 1 (the dependent variable is T, the explaining variables are X and Y)	
$P(T=1)=\frac{1}{1+e^{-479071+5717X+5173Y}}$	
Coefficient χ^2	5.53
Significance of	
coefficient 1 (constant term)	0.0263
coefficient 2 at X	0.0458
coefficient 3 at Y	0.0236
Logit Model 2 (the dependent variable is T, the explanatory variables are X, Y, and N_{pred})	
$P(T=1)=\frac{1}{1+e^{-326585,69+3587,44X+3683,28Y-26,65N_{pred}}}$	
Coefficient χ^2	11.049
Significance of	
coefficient 1 (constant term)	0.2424
coefficient 2 at X	0.3488
coefficient 3 at Y	0.2052
coefficient 4 at N_{pred}	0.0238
Logit Model 3 (the dependent variable is T, the explanatory variable is N_{pred})	
$P(T=1)=\frac{1}{1+e^{14,299-32,068N_{pred}}}$	
Coefficient χ^2	9.207
Significance of	
coefficient 1 (constant term)	0.00416
coefficient 2 at переменной N_{pred}	0.00439

Note. The observation coordinates X and Y, as well as the set of the kriging-predicted values $N_{pred}(x_i)$, were used as the explanatory variables for logistic regression.

Similar results were obtained by Fernandes et al. [17] who studied a credit scoring logit model using a spatial variable as an explanatory one. They compared two models, one that contained a spatial variable and one that did not. The results showed that the author-proposed method had better performance than conventional methods. In this paper, we studied an approach for predicting the spatial distribution of the parameter of interest, which is based on the combined use of kriging and binary regression; the complete model (where the logit

model incorporates a set of kriging-predicted parameter values) was better than the alternatives. Notably, it was only in the 2000s that binary regression found application in precision agriculture in Russia. Some reports [7, 20] give detail upon the opportunities to use logit and probit models in plant growing; however, those do not take into account the spatial variable.

2. Sample of the NDVI logit model testing results as obtained on the basis of aerial photography data (Menkovo, Leningrad Region, 2015)

Coordinate point No.	*X*	*Y*	*N*	*T*	N_{pred}	P	
						Model 1	Model 3
1	30.032934	59.418484	0.527	1	0.4894404	6.187555e-11	0.7519646
2	30.032902	59.418514	0.517	1	0.5037567	4.848053e-12	0.8326184
3	30.032835	59.418605	0.527	1	0.4917005	0.9999989	0.7661754
4	30.032695	59.418778	0.407	0	0.4396790	0.9999876	0.3652577
5	30.032673	59.418811	0.455	0	0.4261240	4.863455e-12	0.2654931
6	30.032588	59.418940	0.461	1	0.4387105	6.46559e-13	0.3366613
7	30.032477	59.419087	0.517	1	0.4614526	0.8979254	0.5382949
8	30.032327	59.419302	0.496	1	0.4600064	2.212942e-11	0.5256631
9	30.032472	59.419176	0.468	1	0.4688632	2.652915e-10	0.6007014
10	30.032528	59.419119	0.411	0	0.4656420	1.943197e-06	0.5943595

N o t e. *X* and *Y* are the observation coordinates, *N* are the values of input observations, *T* is the dependent variable, $N_{pred}(x_i)$ are the kriging-predicted parameter values. For models 1 and 3, the probabilities of threshold exceedance, predicted for the removed observation points, are presented.

Thus, the approach proposed herein is to use a set of the parameter-of-interest values predicted by ordinary kriging as one of the binary regression factors in the logit model. In general, combining kriging and binary regression to evaluate the plant condition seems to be promising and relevant. However, the experiments sometimes produced statistically insignificant models, this is why it is recommendable to use more examples to evaluate the proposed approach.

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PSEUDOMONADS ASSOCIATED WITH SOIL LUMBRICIDES AS PROMISING AGENTS IN ROOT ROT BIOCONTROL FOR SPRING GRAIN CROPS

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Abstract

Currently, crop yields can be increased by high farming standards which include environmentally friendly use of chemical fertilizers and pesticides, as well as their replacement by bioformulations having similar activity. That is why both search for new promising species, strains and isolates of bacterial antagonists for their potential use as biocontrol agents, and study of antifungal activity mechanisms, particularly the relationship between the activity in model tests and in agrocenoses, are relevant. The aim of this study was to estimate bacterial isolates from redworm coprolites as potential bioactive agents to control phytopathogenic fungi causing root rot of crops. The experiments were conducted in 2013-2015. In the preliminary laboratory screening for fungistatic and growth-promoting activity we selected two strains, *Pseudomonas* sp. GS4 and *Pseudomonas* sp. PhS1, and assessed their ability to decrease the growth rate of fungal colonies in Petri dish test on nutrient agar medium and to reduce seed infestation of soft wheat (*Triticum aestivum* L., Irgin cultivar) in sterile paper roll test. Seeds soaked in distilled water served as control. As a standard, we used seed treatment with a chemical fungicide Dividend® Star («Syngenta AG», Switzerland) (30 g/l difenoconazole, 6.3 g/l cyproconazole) at recommended rates. In field tests, we recorded root rots in soft wheat Irgin cultivar plants and in barley (*Hordeum vulgare* L.) Acha cultivar plants during tillering and beginning of blooming. The laboratory tests showed a statistically significant ($p < 0.05$) 1.5-2.5-fold decrease in the growth rate of phytopathogenic fungi *Fusarium oxysporum*, *Bipolaris sorokiniana* and *Alternaria* spp. as compared to control. In all experiments with bacterization, there was a 53-76 % decrease ($p < 0.05$) in total seed infestation by pathogens as compared to non-bacterized plants. The effect of the bacteria in planta was assessed in small model systems. The obtained data show a statistically significant ($p < 0.05$) reduction in the root rot disease incidence in bacterization with *Pseudomonas* sp. GS4 (by 33-37 %) and *Pseudomonas* sp. PhS1 (by 57-60 %). Root rot disease severity decreases 2.1-2.4-fold and 3.3-3.5-fold, respectively. In 2015, we revealed a tendency towards a 19-70 % increase in the total number of rhizosphere microorganisms at the beginning of plant blooming depending on the crop and type of bacterization. The number of phosphate-mobilizing bacteria in the rhizosphere under bacterization was, on average, 5.5-7.2-fold higher in wheat and 2.1-3.2-fold higher in barley than that without bacterization. Our results of root rot field study in the 2013-2015 showed the efficacy of both monocultures and complex bacterization which provided a decrease in wheat and barley root rot disease severity by 6.5-57.6 % and 18.6-50.0 %, respectively, depending on the bacterial culture and the weather conditions. The maximum biological efficacy of the isolates is noted at the beginning of blooming.

Keywords: biocontrol, rhizobacteria, *Pseudomonas*, *Eisenia fetida,* antifungal activity, phytopathogen, *Bipolaris*, *Alternaria*

Antifungal activity is a relatively common bacterial feature, which gives an environmental advantage in the environments that can support the growth of

mixed bacterial and fungal flora. There are a number of mechanisms, by which one organism suppresses the growth of its competitor: competition for the limited nutrient supply, production of siderophores [1-3], antibiotics, enzymes, and sundry compounds [4, 5]. Bacterial activity in vitro usually has a positive correlation with their ability to inhibit phytopathogen growth as well as with their stimulating activity in vivo [1, 6, 7]. However, the fungistatic and plant-growth stimulating effects of bacteria, albeit shown in the laboratory, are not always confirmed by in vivo experiments [8-10].

Despite the fact that bioformulations based on antagonistic bacteria are widely used in agriculture and have positive effects on plant growth and development, early studies noted the instability of results [11, 12]. A more detailed study showed [13-15] that the effectiveness of bioformulations depends on various factors: microorganism strain culturing parameters, preparative form, storage methods and time, soil properties, agro-climatic conditions, the host plant, the strain's capability of a strong symbiosis with this or that cultivated crop, as well as the microbial community status at the sowing of seeds treated with the bioformulation.

Several researchers have successfully protected plants by combining different bacterial strains [16, 17]. It was shown that the most effective way to control disease progression is to use a combination of strains and bi-component biofungicides [18, 19]. Pairing microbial strains sometimes allows for a reduction in the bioformulation concentration while improving its quality [7, 14]. In most such papers, Pseudomonas bacteria were one of the bioformulation components. This is due to their high antifungal activity and multifaceted effects on plant growth and development [1, 4, 11]. The second component is any bacterium widespread in the plant rhizosphere and capable of stimulating the growth and development of plants and of the primary component.

Active use of bioformulations in state-of-the-art agricultural technology requires searching for efficient strains that could be used for biological control of phytopathogens under various agrocenotic conditions. An in-depth study of the connection between the manifestations of bacterial bio-activity in the lab and in the field will contribute to the development of such bioformulations.

This paper is the first to find that *Pseudomonas* strains isolated from coprolites help reduce the infestation of soft wheat with infectious seed pathogens in a laboratory setting, which correlates with their ability to suppress the development of fungi (root rot pathogens) in an agrocenosis.

The purpose hereof is to evaluate the feasibility of using bacteria isolated from earthworm coprolites as the basis for bioformulations intended to control fungal phytopathogens (grain-crop root rot pathogens).

Techniques. Laboratory and field tests were carried out in 2013-2015. Bacterial cultures of *Pseudomonas* sp. GS4 and *Pseudomonas* sp. PhS1 were isolated from coprolites of earthworms (*Eisenia fetida*) after one-month culturing on a 1:2 peat and dung substrate, inoculated directly onto a growth medium based on fish hydrolyzate (FH broth) (State Research Center for Applied Microbiology and Biotechnology, Russia). Strains were selected after preliminary screening for fungistatic and growth-stimulating activity in laboratory tests [9].

Liquid enrichment cultures were produced by growth in flasks with FH broth (250 flasks, each containing 100 ml of the medium) using an ES-20/60 shaker (Biosan, Latvia) at 180 rpm and 28 ± 0.5 °C until the number of bacteria reached 1×10^9-9×10^9 cells/ml. FH broth contained pancreatic hydrolyzate of fish meal (8 g/l), enzymatic peptone (8 g/l), and sodium chloride (4 g/l). The bacterial population was controlled by counting in a Goryaev chamber (×400 magnification).

The test objects used to assess the antagonistic activity of bacteria were phytopathogenic fungi *Fusarium oxysporum*, *Bipolaris sorokiniana*, and *Alternaria* spp. (provided by the Department of Biological Plant Protection, Novosibirsk State Agrarian University). Those were cultured in 2% potato-glucose agar (0.23 l potato extract, 20.0 g glucose, and 0.77 l tap water). To determine the antifungal activity, bacterial suspensions were streaked on solid agar media edge-to-edge in Petri dishes, one suspension per sample. The dishes were incubated for two days. A cylindrical agar block, 2 mm in diameter, containing 6-day fungus mycelium, was placed in the center of the unoccupied medium surface. The dishes were then placed in a thermostat at 24±1.0 °C, and every 24 hours the radius of the fungal colony growth towards the streak was measured (the control sample was a fungal colony not exposed to bacteria). The fungistatic effect was evaluated by reduction of fungal colony growth on a dense growth medium in the bacteria-streaked samples as compared to the controls [20].

In laboratory experiments, a soft wheat (*Triticum aestivum* L.) cultivar Irgin seeds were soaked for 20 min in the bacterial suspension of test-strain monocultures (1×10^7-5×10^7 cells/ml). In case of co-inoculation, the amount of each strain-specific suspension was halved to 5×10^6-25×10^6 cells/ml. The controls were seeds soaked in tap water; for reference, the seeds were treated with Dividend® Star chemical fungicide (Syngenta AG, Switzerland) in the recommended dosage. This chemical is allowed for use in the Russian Federation and contains the following active ingredients: difenoconazole, 30 g/l; cyproconazole, 6.3 g/l. The fungistatic effect of the bacteria was evaluated by the reduction of seed infestation with the seed infection pathogens as detected by phytopathological assay using rolls of sterile filter paper [10, 20]. The effects of bacteria in *in planta* experiments were studied in small model ecosystems, each consisting of three parts: substrate (sterile coarse river sand), host plant (wheat), and bacteria. Bacteria were added to the system in a dose of 1×10^6 cells/seed. Control and experimental pots were placed in a GC-300TLH climatic chamber (Jeio Tech, Korea) for 10 days at 10 klx lighting, 16-hours light period, and 22 to 24 °C. After that, the signs of root rot in seedlings were scored [20].

Field trials (Siberian Agriculture and Peat Research Institute, Luchanovo, Tomsk Region) were carried using Irgin wheat cultivar and barley (*Hordeum vulgare* L.) Acha cultivar. The soil was gray podzolic mid-loamy, pH 5.0, humus content 4.87%, 24.9 mEq of absorbed bases per 100 g of absolutely dry matter (adm), $N\text{-}NH_4$ = 2.66, $N\text{-}NO_3$ = 8.48, P_2O_5 = 236.5, and K_2O = 99.2 mg/kg adm. The experiments were arranged according to Dospekhov's methodology [23], with 3-fold repetition and systematic placement of 40 m^2 plots (32 m^2 evaluated area per plot). The dose of nitrogen fertilizer (urea) was 45 kg/ha by nitrogen (N_{45}). Before sowing, seeds were bacterized with enrichment cultures (working titer of 1×10^7-5×10^7 cells/ml) at a rate of 100 ml/10 kg of grains. The seeding rate was 6.5 million seeds/ha for wheat, 5.5 million seeds/ha for barley.

To assess the fungistatic activity of the tested bacteria at tillering phase and at the flowering onset, at least 100 plants were randomly collected for each repetition/sample. The numbers of healthy, affected, or dead plants, and the damage score were used to calculate the disease prevalence and progression indices [10, 22].

The amount of microorganisms in the rhizosphere was determined by plating soil suspension serial dilutions on elective growth media. Total bacterial count was determined on FH agar, the phosphate-mobilizing bacteria were counted on Muromtsev agar (0.2 g/l K_2SO_4, 0.2 g/l $MgSO_4\cdot7H_2O$, 10 g/l glucose, 1.0 g/l asparagine, 3.3 g/l $CaCl_2$, 3.8 g/l Na_3PO_4, and 15 g/l agar-agar), and micromycetes were counted on Czapek medium (1.0 g/l K_2HPO_4, 2.0 g/l

$NaNO_3$, 0.5 g/l KCl, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 20 g/l glucose, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and 15 g/l agar-agar, HCl to adjust to pH 5.5).

The obtained data were processed with STATISTICA 6.0 software (StatSoft, Inc., United States). The growth rate of the fungal colony was calculated by linear regression method. The results of the phytopathological tests, as well as the prevalence of diseases during field vegetation are shown given Fisher's test for probabilities below 25% and above 75%. Statistical significance was evaluated by comparing sampling fractions given Student's *t*-test at 95% significance level for probabilities ranging from 25% to 75%; for any other probability, Fisher's test was applied. Root rot indices and microbial counts are presented as arithmetic means (M) with the confidence interval ($\pm\sigma$) taking into account Student's *t*-test for a 95% significance level, and compared by Student's *t*-test, with a significant threshold of $p < 0.05$.

Results. Laboratory experiments are an integral part of developing bioformulations to protect plants; such experiments help launch the production of new formulations faster. Promising microbial introducents and the introducent-based bioformulations should be further tested over several growing seasons that featured different weather.

Using standard streaking, we examined the effect of the bacterial isolates from coprolites on growth and development of some phytopathogenic fungi. Bacterial cultures isolated from earthworm coprolites are classified as *Pseudomonas* [23] by their morphological, physiological, and biochemical properties. The bacteria are short, single, motile, asporogenous, gram-negative bacilli that grew well on organic media. They liquefied gelatin, hydrolyzed starch, possessed catalase activity, reduced nitrates to nitrites, and produced acid on glucose- and sucrose-containing media. Besides, they are aerobic, and one of the selected strains, *Pseudomonas* sp. PhS1, can produce yellow water-soluble pigment and mobilize sparingly soluble phosphates.

It was found out that in the presence of bacteria, the growth rate of all the fungi under analysis is statistically significantly ($p < 0.05$) lower than that of the controls (a 1.5- to 2.5-fold reduction, see Table 1). Since the reduction in the growth rate of fungal colonies did not require direct contact of the fungal hyphae and bacterial steaks while the micromycetes stopped growing without reaching it, it is safe to say that the antifungal effect was associated with the soluble and media-diffusible bacterial metabolites.

1. Effects of *Pseudomonas* strains isolated from earthworms coprolites on the radial growth rate (mm/h) **of pure phytopathogenic fungi cultures** ($M\pm\sigma$, lab test)

Strain	*Fusarium oxysporum*	*Bipolaris sorokiniana*	*Alternaria* spp.
Control (water)	0.24±0.014	0.22±0.021	0.30±0.019
Pseudomonas sp. GS4	0.15±0.012*	0.09±0.006*	0.15±0.046*
Pseudomonas sp. PhS1	0.11±0.010*	0.07±0.005*	0.11±0.011*

* Difference from the controls statistically significant at $p < 0.05$.

According to literature data, the inhibition of fungal growth by the *Pseudomonas* bacteria is first of all related to various bacterial antibiotics, including phenazine-1-carbolic acid, derivatives of phloroglucinol, pyrrolnitrin, and other compounds [1, 5, 13]. Aside from antibiotics, siderophores (iron-transporting yellow-green pigments) produced by such bacteria are also important for inhibition. These bind ferric ions and form stable complexes with such iron, which deprives fungi of a necessary nutrient, inhibiting their development [4, 5, 15]. On MPA, *Pseudomonas* sp. PhS1 produced yellow medium-diffusing pigment. Apparently, in this case siderophores play an important role in inhibiting growth of the phytopathogenic fungi. *Pseudomonas* sp. GS4 had no pigmentation; apparently, their antifungal activity is related to the production of other antibiotics.

2. Reduction in seed colonization due to bacterization and chemical treatment as a percent of the untreated seed colonization ($M \pm \sigma$, soft wheat *Triticum aestivum* L., Irgin cultivar, lab test)

Variables	Total infection prevalence	Helminthosporium disease	Alternaria spot	Bacteriosis
Pseudomonas sp. GS4	54.0±2.0*	35.5±3.4	61.3±22.4	64.5±21.0
Pseudomonas sp. PhS1	76.2±2.1*	100.0±1.0*	29.0±7.8	100.0±1.0*
Pseudomonas sp. (GS4 + PhS1)	64.5±1.9*	100.0±1.0*	58.0±23.0	35.5±9.0
Dividend® Star, SC	74.1±1.7*	69.7±7.2*	25.4±7.9	100.0±1.0*

* Difference from the controls statistically significant at $p < 0.05$.

A statistically significant ($p < 0.05$) reduction in the overall seed infections was observed in all the bacterized samples, see Table 2. Treatment of wheat seed with a *Pseudomonas* sp. PhS1 monoculture was the most effective, resulted in the greatest reduction in infection and was comparable to the effect of a chemical protectant.

Vegetation modeling experiments *in planta* also demonstrated high biological activity of the analyzed bacteria. The prevalence of root rot was lower ($p < 0.05$) in samples bacterized with *Pseudomonas* sp. GS4 (33% to 37%) as well as with *Pseudomonas* sp. PhS1 (57% to 60%); the root rot progression index was 2.1 to 2.4 or 3.3 to 3.5 times lower than in the controls, respectively.

The weather conditions of the growing season 2013 were quite favorable for the growth and development of the crops used in the field tests. At the beginning and end of the growing season, temperatures were low while precipitation was frequent and intense; mid-July and early August were hot while precipitation was scarce. The early 2014 season was rather unfavorable, with low temperatures in May and early June accompanied by intense and frequent precipitation. The rest of the season, like 2013 season, was quite favorable for plant growth and development. In 2015, the sum of effective temperatures was accumulated from April to September; precipitation was above average in May, July, August, and September, while June was dry.

Microbiological analysis of the wheat and barley rhizosphere identified an upward trend in the microbial counts in the test samples. A marked reduction in the total number of lower fungi combined with a sharp increase in *Trichoderma* counts indicated a decrease in abundance of potentially hazardous microorganisms in the microbial rhizosphere community of the bacterized crops, while the proportion of biocontrol agents grew. The abundance of phosphate-mobilizing bacteria testified to highly competitive and successful colonization of the applied *Pseudomonas* sp. PhS1 culture in the plant roots. Thus, the counts of this microbial group in the rhizosphere of the test plants were 5.5 to 7.2 times higher for wheat and 2.1 to 3.2 times higher for barley as compared to the controls.

3. Microbial counts (million CFU/g of soil adm) **in the rhizosphere of soft wheat (*Triticum aestivum* L.) Irgin plants and barley (*Hordeum vulgare* L.) Acha plants after seed inoculation with bacteria isolated from earthworm coprolites** ($M \pm \sigma$, Luchanovo, Tomsk Province, 2015)

Variables	Total counts	Phosphate-mobilizing bacteria	Lower fungi	
			total number	*Trichoderma*
Wheat				
Control	102.0±26.5	2.1±0.2	0.71±0.01	0.01±0.002
Pseudomonas sp. PhS1	121.0±38.4	15.1±1.6*	0.24±0.03*	0.01±0.005
Pseudomonas sp. (PhS1 + GS4)	174.0±20.7*	11.6±2.9*	0.75±0.02	0.05±0.008*
Barley				
Control	60.0±2.1	5.0±0.2	1.42±0.27	0
Pseudomonas sp. PhS1	59.0±1.9	10.7±0.9*	0.58±0.01*	0.07±0.007*
Pseudomonas sp. (PhS1 + GS4)	94.0±1.5*	16.0±1.5*	0.67±0.07*	0.05±0.002*

* Difference from the controls statistically significant at $p < 0.05$.

Literature review [12] as well as the results of phytosanitary survey carried out annually by the Rosselhoztsentr, Tomsk Province, indicate that Helminthosporium disease and Fusarium root rot caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker and *Fusarium oxysporum* Schleht remain the most harmful crop diseases in the region [22, 24, 25]. The disease damages durum wheat, barley, soft spring wheat, and winter rye the most. Diseases reduce the wheat yield by 19% to 20%, the barley yield by 25% to 30%, or even more [22, 24, 25].

In 2013, there was a slight difference in the effectiveness of different bacterization options applicably to wheat and barley, see Table 4. Thus, wheat yielded the best results when treated with a mixture of bacterial cultures, with a statistically significant ($p < 0.05$) 34% reduction in root rot at the phase of earing and early flowering. Barley displayed the greatest reduction in root rot affection when bacterized with *Pseudomonas* sp. GS4 monoculture: 32% to 41%, and 39% to 44% during tillering and flowering, respectively.

4. Key indicators of root rot progression in wheat (*Triticum aestivum* L.) Irgin cultivar and barley (*Hordeum vulgare* L.) Acha cultivar after seed inoculation with bacteria isolated from earthworm coprolites ($M \pm \sigma$, Luchanovo, Tomsk Region)

Crop	Variables	Tillering		Flowering onset	
		incidence, %	severity, %	incidence, %	severity, %
		2013			
Wheat	Control	49.2±3.9	17.0±2.3	51.7±3.6	19.9±3.8
	Pseudomonas sp. GS4	56.4±2.7	16.4±1.0	44.4±2.6*	18.6±3.5
	Pseudomonas sp. (PhS1 + GS4)	56.7±5.1	18.0±1.2	36.7±5.7*	13.2±2.6*
Barley	Контроль	82.7±2.4	39.5±2.6	44.4±2.9	14.7±2.6
	Pseudomonas sp. GS4	53.3±7.6*	23.2±2.8*	24.4±8.1*	8.3±2.5*
	Pseudomonas sp. (PhS1 + GS4)	70.7±3.7*	27.3±3.6*	41.0±7.8	13.3±2.5
		2014			
Wheat	Control	66.3±14.2	26.7±4.8	60.6±8.9	20.4±3.7
	Pseudomonas sp. PhS1	74.4±6.4	27.3±3.6	52.2±12.6	17.2±8.5
	Pseudomonas sp. (PhS1 + GS4)	72.5±12.3	25.9±3.3	54.4±10.2	20.8±7.4
Barley	Control	84.0±6.7	41.3±4.0	86.7±12.9	43.6±10.6
	Pseudomonas sp. PhS1	84.0±6.7	37.5±7.6	73.3±10.1	35.5±7.6
	Pseudomonas sp. (PhS1 + GS4)	80.8±5.1	36.9±4.1	71.1±12.6	30.5±10.7
		2015			
Wheat	Control	55.6±17.8	31.2±3.8	61.1±17.5	21.7±6.6
	Pseudomonas sp. PhS1	55.6±17.8	27.5±4.0	50.0±17.9	15.8±4.2
	Pseudomonas sp. (PhS1 + GS4)	50.0±17.9	26.7±7.6	26.6±15.8*	9.2±3.7*
Barley	Control	55.6±17.8	21.7±5.5	66.7±16.9	28.3±3.7
	Pseudomonas sp. PhS1	57.8±17.7	22.5±6.1	46.7±17.8	14.2±5.0*
	Pseudomonas sp. (PhS1 + GS4)	51.7±17.9	22.5±7.8	57.8±17.7	20.8±3.8*

* Difference from the controls statistically significant at $p < 0.05$.

The root rot progression indicators were much higher in the growing season of 2014 as compared to 2013, see Table 4. Greater prevalence and progression of root rot was associated with the unfavorable climate conditions, as cold weather and frequent precipitation do not benefit crops in any way. Furthermore, efficient root colonization by inoculated bacteria requires a minimum temperature of 12 °C, while the optimum temperature range is 14 to 16 °C [13]. Lack of heat in May, with a monthly average temperature of 8.1 °C (2.5 °C below normal), which apparently prevented bacteria from successful colonization of the plant rhizosphere while inhibiting their ability to compete with the natural inhabitants and to fully manifest their defensive and stimulating properties. As a result, the 2014 growing-season field experiment did not reveal any statistically significant inoculation-attributable suppression of root rot in crops. Over the entire season, there was only a slight downward trend in the infestation of wheat and barley, with no clear correlation with the bacterization type or inoculation method. The favorable weather conditions of 2015 did have a positive effect on the phytosanitary situation: the prevalence of root rot was lower in both the controls and in the experimental samples as compared to the 2014 and 2014

values. A significant ($p < 0.05$) reduction in the root rot progression index was noted in wheat and barley at the flowering onset (55% to 59% and 25% to 28%, respectively) in the samples bacterized with a mixture of cultures, whereas in barley, significant reduction was also observed in the *Pseudomonas* sp. PhS1 monoculture-treated samples (by 48% to 51%).

Experimental root rot data prove the efficiency of complex bacterization and the feasibility of developing microbial bioformulations based on the complementary associations of microbial cultures that combine various functions, from providing plants with readily available nutrients to stimulating the growth and protection against phytopathogens [18, 19]. Similar data were obtained in the field experiments in 2011 [26]. Bacterial isolates helped reduce the prevalence of root rot in soft wheat and barley crops by 18% to 63%. Bacterization with *Pseudomonas* sp. GS4 was comparable to Dividend Star in terms of reducing the progression of root rot in wheat (at tillering and earing phase) as well as in barley (tillering phase). Bacterization with *Pseudomonas* sp. GS4 increased the wheat and barley yield by 19.6%, and the grain protein and gluten content by 0.9% to 1.3 % as compared to the Dividend Star-treated samples.

Therefore, *Pseudomonas* strains isolated from earthworm coprolites reduce the overall infestation of wheat seeds with seed infections in lab tests. The lowest infestation level is observed in samples treated with *Pseudomonas* sp. PhS1 (only a quarter of the control indicators). The laboratory-identified antifungal activity of bacteria correlates with their ability to suppress the development of root rot pathogen fungi in an agrocenosis. Over the three growing seasons, inoculating wheat and barley seeds with experimental strains helped reduce the root rot progression index; however, the observed fluctuations in the efficiency (5% to 58% depending on the bacterial culture and on the weather) necessitate further research to improve the stability of bacterial preparations and optimize their application.

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COMPATIBILITY OF ENTOMOPHAGES WITH BIOLOGICAL AND BIORATIONAL PESTICIDES

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Abstract

Severe adverse effects of chemical pesticides have driven demand for ecologically friendly technologies of plant growing with alternative pest control tactics. Traditional insecticides cause massive death of predatory ground beetles, bedbugs, coccinellids, lizards, flies and tachinid flies, parasitic trichogrammatids, ichneumonids, braconids and other useful species. Harmonized biological and chemical controls are becoming more relevant, which should include the use of beneficial entomofauna. This necessitates more data on sensitivity of entomophages to biologicals, biorational pesticides (i.e. natural substances and their synthetic analogues) and other selective chemistries. In this work, for the first time, we determined laboratory and field toxicity of several Russian and foreign conventional biologicals and chemicals for beneficial entomofauna of corn, potato and apple-tree agrocenoses. The originality of this study lies in its focus on searching commonly used biopesticides which can be integrated with entomophages in organic farming technology. The obtained data indicate that biorational insecticides Fitoverm® EC (emulsion concentrate) (Pharmbiomed, Russia, 1.3 l/ha), Vertimek® EC (Syngenta AG, Switzerland, 1.0 l/ha) and Atabron® SC (suspension concentrate) (ISK Biosciences, Belgium, 0.75 l/ha) are highly effective against harmful lepidopterans and aphids on corn, soy and pea crops without toxic effect on the massively used entomophagous *Habrobracon hebetor* Say and *Aphidius matricaria* Hal. Our findings also indicate effectiveness of combination of predatory bugs podisus (*Podisus maculiventris* Say) and perillus (*Perillus bioculatus* Fabr.) with biologicals against Colorado beetle on solanaceous crops. In using Bitoxybacillin® P (powder) (Sibbiopharm, Russia, 4 kg/ha) and Fitoverm® EC (1.3 l/ha), the survival rates of *P. masculentris* imagoes were 88% and 82%, respectively, with 64% for older larvae. When using the same pesticides, the survival rates of *P. bioculatus* imagoes were 97% and 91%, respectively, with 58% and 52% for fourth- to fifth-instar larvae. Fitoverm® at 1 l/ha rate recommended against aphids does not affect the viability of the aphidophages *Cycloneda sangvinea* Mul. and *Harmonia axyridis* Pallas on maize, vegetable pea and apple, and allows for survival of 85% adult beetles *C. sangvinea* and of 88% Asian ladybeetles *H. axyridis*. These data can be used in protocols for co-application of biologicals, biorational preparations and entomophages in organic and ecological farming to effectively control pests of maize (cotton moth, corn stalk moth, corn and cereal aphids), potatoes (Colorado potato beetle, potato aphids), peas (leguminous aphis), and apple trees (apple moth, Apple green aphid).

Keywords: biological preparations, entomopathogenic, insect sensitivity to pesticides, *Habrobracon hebetor* Say, *Aphidius matricaria* Hal., *Perillus bioculatus* Fabr., *Podisus maculiventris* Say, *Cycloneda sangvinea* Mul., *Harmonia axyridis* Pallas

Over the last 16 years, organic farming has been on the rise worldwide, with the total area use quadrupling to about 1% of all the farming land; more than 2 million organic producers have been certified, of which 75% are based in emerging economies [1-3]. The desire to advance organic production is overdue

in more than 170 countries, with even more joining the team as such products are in increasing demand [4-7].

Organic farming uses special formulations for pest control based on entomopathogenes, as well as predatory and parasitic laboratory-reproduced insects [8-11]. An early introduction of artificially grown entomophages helps improve the efficiency of biological pest control [12-14]. However, entomophages are often unable to restrict the pest population in the field to an economically imperceptible level. This can be caused by the asynchronous phenology of phytophages and entomophages, or by the very low post-winter population of the latter, which stimulates uncontrolled reproduction of the pests; however, it is the conventional chemical treatment that is believed to kill the beneficial entomofauna while not affecting the phytophagous populations which are resistant to many insecticides [15-18]. This makes it imperative to use biologicals and biorational products that will not affect the beneficial arthropods and additionally introduced entomophages and acariphages [19]. The former group comprises biologicals to combat pests, plant pathogens, and weeds; these are derived from living microorganisms or their metabolic products. The latter group comprises chemical compounds or natural substances that are low- or non-toxic to warm-blooded organisms (pheromones, essential and vegetable oils, growth regulators, etc.). The formulations of both groups feature quicker and eco-safer degradation compared to other preparations; besides, they are not accumulated in the food chain.

In green farming, pesticide load must be reduced by 50-75% (i.e. brought to the levels allowed for integrated protection); it is preferable to use biorational formulations (insect growth and development regulators, pesticides not affecting the beneficial entomofauna, etc.). Organic farming uses only biologicals and biorational products; conventional chemical insecticides are completely banned [14, 20, 21].

When choosing the protective agents, their toxicity for the beneficial organisms must be evaluated [22, 23]. The death of beneficial arthropods is most pronounced in perennial plantations (orchards, vineyards), as these cenoses contain numerous species of phytophages and their respective entomophage complexes that are important for controlling the population of the former. Using insecticides has been found to cause mass death of predatory ground beetles (*Carabidae*), *Pentatomoidea*, *Nabidae*, and *Anthocoridae* bugs, coccinellids (*Coccinellidae*), lacewings (*Chrysopidae*), flower flies (*Syrphidae*) and tachinids (*Tachinidae*), trichogrammatids (*Trichogrammatidae*), ichneumonids (*Ichneumonidae*), braconids (*Braconidae*), and sundry beneficial species. Annual crops also contain a significant number of entomophagous species (up to 200 in winter wheat, and up to 300 in peas). A one-hectare potato field contains 2,000 to 3,400 flower flies, more than 720 predatory spiders, and 2,400 to 2,800 ground beetles; conventional insecticides kill nearly all of them [24]. Treatment of wheat with insecticides targeted against corn bug (*Eurygaster integriceps* Put.) will negatively affect *Carabidae*, *Coccinellidae*, and *Scelionidae* entomophages. Fungicides and herbicides usually have a far lesser effect on entomophages than insecticides. It is preferable to use such biological and biorational protective agents that are safe for entomophages and acariphages [25]. Researchers have identified that biorational formulations based on thiamethoxam, chlorantraniliprole, tefluthrin, difenoconazole, fludioxonil, thiacloprid, imidacloprid, or their combinations (low-toxic, low- or moderately-hazardous) differ in the toxicity to the beneficial entomofauna [26-28]. Thus, Herold® WSC (water-suspension concentrate), a biorational diflubenzuron (240 g/l) preparation (ZAO Firma August, Russia), Proclaim® WSG (water-soluble granules) a biorational emamectin benzoate (50 g/kg) product (Syngenta AG, Switzerland), and Lepidocide® P (powder), a biological preparation based

on the spore-crystal complex *Bacillus thuringiensis* var. *kurstaki*, biological activity (BA) = 3,000 EA/mg (PO Sibbiofarm, Russia) do not inhibit the natural orchard entomophages: lacewings, *Nabidae* predatory bugs, and *Coccinellidae* beetles [29].

This paper is the first to demonstrate the compatibility of entomophages targeting Colorado potato beetles *Perillus bioculatus* Fabr. and spined soldier bugs *Podisus maculiventris* Say, the ectoparasites of cotton bollworm and sundry Lepidoptera species *Habrobracon hebetor* Say, aphid parasite *Aphidius colemani* Vier, predatory coccinellids *Cycloneda sangvinea* Mul. and *Harmonia axyridis* Pallas, with various biological and biorational insecticides.

The study objective is to determine the sensitivity of entomophages to biologicals and biorational agents while developing a system for the biological protection of crops against pests.

Techniques. The studied preparations were biologicals Bitoxybacyllin® P, Lepidocide® SC (suspension concentrate, PO Sibbiofarm, Russia), Helicovex® SC (Andermatt Biocontrol AG, Switzerland), the biorational products Vertimec® EC (emulsion concentrate) and Actara® WDG (water-dispersible granules) (Syngenta AG, Switzerland), Phytoverm® EC (Farmbiomed, Russia), Insegar® WDG and Atabron® SC (suspension concentrate, ISK Biosciences, Belgium), and the chemical insecticides Decis® Expert EC (Bayer AG, Germany), and Coragen® SC (DuPont, United States).

The compatibility of the biologicals Bitoxybacyllin® P (application rate of 4 kg/ha against lepidoptera, 3 kg/ha against aphids and Colorado potato beetle), Lepidocide® SC (2 l/ha against codling moth and aphids), Helicovex® SC (200 l/ha against Lepidoptera) and the biorational products Vertimec® EC (1 l/ha against Lepidoptera), Phytoverm® SC (1.3 l/ha against aphids and 0.2 l/ha against Lepidoptera), Coragen® SC (0.1 l/ha against Lepidoptera), Insegar® WDG (0.6 kg/ha against Lepidoptera), Atabron® SC (0.7 l/ha against Lepidoptera) with entomophages was compared in a laboratory tests using *Habrobracon hebetor* Say cocoons and cereal aphids infested with *Aphidius colemani* Vier., as well as in the field 40-m^2 potato plots in 4 repetitions (for the Colorado potato beetle entomophages *Perillus bioculatus* Fabr. and *Podisus maculiventris* Say), in a 4-ha apple orchard containing 10 model trees, in 50-m^2 maize and pea plots in 4 repetitions (for the ladybirds *Cycloneda sangvinea* Mul. and *Harmonia axyridis* Pallas).

For the laboratory culturing of the *Habrobracon*, the hosts were the larvae of middle-aged wax moth (*Galleria mellonela* L.). The larvae were placed in 0.5 l glass jars and infested with the parasite. The jars were closed with a calico cloth and a cotton swab soaked in 20% sugar solution to feed the entomophage and then placed in a thermostat (28 to 30 °C). Cocoons formed 7 or 8 days after the infestation; those were treated thrice with agents recommended for protecting maize against Lepidopteran pests. We used Bitoxybacyllin® P (BA = 1,500 EA/mg, with a titer of 20 billion/g, application rate of 4 kg/ha), Lepidocide® SC (BA = 2,000 EA/mg, with titer of 10 billion/g, application rate of 2 l/ha); Vertimec® EC (ai = 18 g/l, application rate of 1 l/ha), Helicovex® SC (7.5×10^{12} NPV/l, application rate of 200 ml/ha), Insegar® WDG (application rate of 0.6 kg/ha), Atabron® SC (application rate of 0.75 l/ha), Coragen® SC (application rate of 0.1 l/g), Decis® Expert EC (application rate of 0.1 l/ha). The controls were treated with distilled water.

Aphidius reproduction involved cereal aphids (*Schizaphis graminum* Ron.) cultured on wheat seedlings. On post-inoculation day 3 or day 4, *Aphidius* (*A. colemani*) were placed on the plants, the resultant mummies were treated with Phytoverm® EC (application rate of 1.3 l/ha), Lepidocide® SC (ap-

plication rate 2 l/ha); Bitoxybacyllin® P (application rate 3 kg/ha), Actara® WDG (250 г/kg, application rate of 0.2 kg/ha).

The toxicity of the protective agent for predatory bugs and coccinellids in the field was determined for the products recommended for use against Colorado potato beetle and aphids: Phytoverm® EC (2 g/l, application rate of 0.2 and 1.3 l/ha, respectively) and Bitoxybacyllin® P (BA = 1,500 EA/mg, application rate of 3 kg/ha). Experimental plots with entomophages, Colorado potato beetle and aphids were treated with a stock solution of products using a Pulverex hydraulic knapsack sprayer, Switzerland. The population of predatory bugs and coccinellids was counted before and after spraying.

Statistical processing followed the standard procedure [30]. The tables present the mean (*M*) and standard deviation (±SD). Statistics were run in Statistica 12.6 software (StatSoft, Inc., United States). Statistical significance was evaluated by Duncan's test at P = 95%.

Results. Table 1 presents the main characteristics of the compared biological and chemical products.

1. Sensitivity of ectoparasite *Habrobracon hebetor* Say to biological and chemical insecticides in a lab tests (*M*±SD)

Insecticides, ai	Dosage, l/ha, kg/ha	Cocoons before treatment, pcs.	Imagoes				
			day-specific number			total	% of initial number
			day 3	day 5	day 7		
Biological insecticides							
Lepidocide® SC (*Bacillus thuringiensis* var.*kurstaki*)	2.0	69.2	10.2±2.1	37.6±1.6	7.4±1.8	55.2^{ab}	79.8
Bitoxybacyllin® P (*Bacillus thuringiensis* var. *thuringiensis*)	4.0	46.6	6.8±1.5	19.3±3.4	3.1±2.3	29.2^{a}	62.7
Helicovex® SC (nuclear polyhedrosis virus of cotton bollworm)	200	83.4	22.5±3.7	39.6±2.1	21.3±3.4	83.4^{ab}	100
Biorational chemical insecticides							
Vertimec® EC (abamectin)	1.0	64.0	9.6±2.4	32.5±1.8	4.5±1.1	46.6^{bc}	72.8
Insegar® WDG (fenoxycarb)	0.6	80.3	14.7±1.5	51.9±3.3	12.4±1.1	79.0^{c}	98.4
Atabron® SC (chlor fluazuron)	0.75	76.2	24.8±3.2	44.3±1.3	9.9±1.6	76.2^{c}	100
Chemicall insecticides							
Decis® Expert EC (deltametrin)	0.1	87.5	0	0	0	0^{a}	0
Coragen® SC (chlorantraniliprole)	0.1	91.0	22.5±2.2	54.0±1.9	14.5±3.1	91.0^{ab}	100
Control		93.0	20.9±1.6	56.2±4.2	13.9±2.3	93.0^{c}	100

Note. The samples marked with the same letter index have no statistically significant difference by Duncan's test at P = 95% within the same column.

In the laboratory test (Table 1), application rates were as recommended for protecting maize, soybean, and sunflower against cotton bollworm and sundry Lepidopteran pests. The survival rates of the *Habrobracon* ectoparasite exposed to the biorational insecticide Vertimec® EC, biologicals based on *Bacillus thuringiensis* (Bitoxybacyllin® P) and Lepidocide® SC was 72.8, 62.7, and 79.8%, respectively. Coragen® SC data are interesting, as the *Habrobracon* imago release rate was 100% in the test. The biological Helicovex® SC based on nuclear polyhedrosis virus of the cotton bollworm was non-toxic for *H. hebetor* (100% post-treatment imago release indicates the complete entomophage compatibility with this product when used for the bioprotection of maize, soybeans, and tomatoes against cotton bollworm). Decis® Expert had the strongest suppressive effect, as the ectoparasite death rate was 100%.

American researchers found that insecticides based on cyantraniliprole, chlorantraniliprole, spinetoram, which are deemed eco-friendlier than carbamate-based products, had negative effects on the development of *Chrysoperla carnea* Stephens (*Neuroptera Chrysopidae*) and *Trioxys pallidus* Haliday (Hymenoptera: *Braconidae*) [31].

Evaluation of the sensitivity of the aphid parasite *A. colemani* to the biologicals and chemicals recommended for protecting winter wheat, fruits, and vegetables against aphids shows good insect viability when treated with Phy-

toverm® EC or Bitoxybacyllin® P, see Table 2. When using Actara® WDG, only 12 *Aphidius* species were released from 180 mummies. Russian researchers also found the biologicals Bitoxybacyllin® P and Lepidocide® SC to be non-toxic for such aphidophages as lacewings, coccinellids, and mirid bugs [29].

2. Sensitivity of *Aphidius colemani* Vier. to biological and chemical insecticides in a laboratory test (*M*±SD)

Insecticides	Dosage, l/ha, kg/ha	Mummies before treatment, pcs.	Parasites flied				
			day-specific number			total	% of initial number
			3-и сут	5-е сут	7-е сут		
Biological insecticides							
Lepidocide® SC	2.0	178.0±2.6	28.0±2.4	21.0±3.4	1.9±1.3	68^{bc}	38.2
Bitoxybacyllin® P	3.0	186.0±3.4	39.0±4.7	79.0±2.3	55.0±1.8	173^{ab}	93.0
Biorational chemical insecticides							
Phytoverm® EC	1.3	198.0±4.5	48.0±2.3	96.0±3.5	17.0±2.3	161b	81.3
Chemical insecticides							
Actara® WDG	0.2	180.0±3.8	4.0±1.6	8.0±1.7	0.0±0.0	12^{c}	6.6
Control		194.0±4.4	65.0±3.5	105.0±4.5	17.0±2.5	187^{a}	96.3

Note. The samples marked with the same letter index have no statistically significant difference by Duncan's test at P = 95% within the same column.

3. Sensitivity of *Asopinae* bugs to biological and chemical insecticides (*M*±SD, VNIIBZR test plot, Krasnodar, 2015)

Dosage, l/ha, kg/ha	Insect development	Insect survival rate on day 7, %	
		Podisus maculiventris Say	*Perillus bioculatus* Fabr.
Phytoverm® EC, 2 g/l			
0.2	Imago	81.8±0.10^{cd*}	90.9±0.10^{b}
	Older larvae (III-IV)	63.6±0.10^{c}	51.5±0.12^{a}
	Younger larvae (I-II)	24.2±0.06^{a}	0.0±0.00^{a}
Bitoxybacyllin® P, biological activity 1,500 EA/mg			
3.0	Imago	87.9±0.12^{bd}	97.0±0.06^{b}
	Older larvae (III-IV)	63.6±0.10^{c}	57.6±0.16^{ac}
	Younger larvae (I-II)	27.3±0.10^{a}	3.0±0.65^{a}
Actara® WDG, 250 g/kg			
0.2	Imago	0±0.0^{a}	0±0.0^{a}
	Older larvae (III-IV)	0±0.0^{a}	0±0.0^{a}
	Younger larvae (I-II)	0±0.0^{a}	0±0.0^{a}
Control			
0	Imago	97.0±0.06^{b}	93.9±0.06^{b}
	Older larvae (III-IV)	93.9±0.12^{b}	87.9±0.12^{bc}
	Younger larvae (I-II)	97.0±0.06^{b}	97.0±0.06^{b}

Примечание. Между вариантами, обозначенными одинаковыми буквенными индексами, при сравнении в пределах столбца нет статистически значимых различий по критерию Дункана при уровне вероятности P = 95 %.

Field trials with potato plants estimated the sensitivity of the predatory bugs *P. maculiventris* and *P. bioculatus* to Phytoverm® EC and Bitoxybacyllin® P, see Table 3. The products were tested at the application rates recommended for use against Colorado potato beetle. Actara® WDG was the chemical reference. Experiments carried out on the potato-field plots showed that Phytoverm® EC dosed at 0.4 l/ha was toxic for the predatory bugs' larvae; thus, day 7 survival rate of *P. maculiventris* I-II larvae was reduced drastically while that of *P. bioculatus* was 0%. Imagoes of predatory bugs were less sensitive to the product: the *Podisus* survival rate reached 81.8%, the *Perillus* survival rate was 90.9%. Bitoxybacyllin® P did not have toxic effects on the imagoes: *Podisus* had a survival rate of 87.9%, *Perillus* had a survival rate of 97.0%, see Table 3.

In an apple orchard, as well as in maize and peas, the biorational product Phytoverm® EC dosed at 1.3 l/ha did not affect the viability of *C. sangvinea* and *H. axyridis*. Imago survival rate was 85.0% for *Cycloneda* and 87.7% for *Harmonia*. Older larvae of both species were resistant to the product; younger *C. sangvinea* larvae had been wiped out by day 7. Egg treatment caused hatching and 100% death of larvae, while the survival rate of the control sample was

100%. Vertimec® EC was non-toxic for aphidophages. For reference, Actara® WDG was used, which killed 100% of insects. Phytoverm® EC was toxic for the younger larvae of predatory bugs and coccinellids, while Bitoxybacyllin® P and Ver-timec® EC kept the stock of entomophages alive. Similar results were obtained when testing the biological pesticide АкКөbelek™ (based on *Bacillus thuringiensis* var. *kurstaki*) against alfalfa, soybean, and maize pests in South East Kazakhstan; this product was not toxic for braconids, coccinellids, or *Nabidae* and *Miridae* predatory bugs [32].

Thus, as this research has shown, that Helicovex® SC, Bitoxybacyllin® P, Lepidocide® SC, and biorational pesticides such as Vertimec® EC or Phytoverm EC combined with entomophages *Habrobracon*, *Aphidius*, and ladybirds can protect maize against cotton bollworm, corn worm, and corn leaf aphids, as well as apple trees and peas against aphids. Coragen® SC, Insegar® WDG, and Atabron® SC are recommendable for ecologic farming, as they do not have negative effects on agrocenosis and can be combined with entomophages or used on their own. The biorational products Phytoverm® EC and Vertimec® EC recommended for protecting potatoes against Colorado potato beetle are compatible with predatory bugs and coccinellids. The results of these studies are usable for combining biologicals, bioactive products, and entomophages for effective pest control in organic and ecological farming.

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BIOLOGICAL CHARACTERIZATION, PHENOTYPIC AND GENOTYPIC STRUCTURE OF PREDATORY STINKBUG *Perillus bioculatus* Fabr. (*Heteroptera*, *Pentatomidae*) POPULATION IN KRASNODAR REGION

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Abstract

Perillus bioculatus Fabr. (1775) is a predatory bug in the family *Pentatomidae*, a prospective North American entomophage of the Colorado potato beetle. Earlier, Colorado potato beetle eggs were deemed the most preferable for the predator feeding and the main obstacle for perillus acclimatization on new territories. However, in monitoring perillus populations that have acclimatized in several areas of Krasnodar region for the last decade, we revealed the predators' feeding on ragweed leaf beetle (*Zygogramma suturalis* Fabr.) larvae and ragweed cutworm *Tarachidia candefacta* Hübn. (*Noctuidae*: *Lepidoptera*) caterpillars and also on all growth stages of Colorado potato beetle and other insects. In Krasnodar Krai perillus can give annually three generations and is present in three phenoforms, with red-black, orange-black and white-black scutellum. The abundance of *P. bioculatus* is influenced by feeding conditions and by other entomophages, e.g. egg-eaters of genus *Trissolcus* (*Hymenoptera*: *Scelionidae*) and tachina flies (*Diptera*: *Tachinidae*). Analysis of scutellum color inheritance in seven different crosses, including reciprocal ones, between red, orange (or yellow) and white parents showed that the F_1 and F_2 progeny from all combinations, including white individuals crossed with each other, have only red and orange shield (1:1). PCR analysis of *P. bioculatus* bugs in F_2 from crossing insects of different phenoforms revealed that the bugs with the same phenoform in F_2 are genetically different from each other for the frequency of certain RAPD markers. These data indicate non-Mendelian inheritance of scutellum coloration trait in perillus that additionally attract attention to the point. RAPD (random amplification of polymorphic DNA)- and ISSR(inter simple sequence repeat)-PCR analysis detected intra- and inter-population variation of the predatory bugs molecular genetic structure from different zones of the Krasnodar Krai. PCR analysis with almost all used specific primers revealed statistically significant differences ($p = 0.05$) in molecular genetic structure and DNA polymorphism for the populations studied. Geographic populations from the village of Staro-Nizhesteblievskaya and the city of Krasnodar (Krasnodar Krai) were genetically close while there genetic identities with the Moldavian village (Crimean region, Krasnodar Krai) population were low. This comports with their geographical location and indicates geographic variability of *P. bioculatus* populations' genetic structure. The researches executed by us confirm plasticity of this species and prospects for its further acclimatization within Colorado potato beetle area.

Keywords: predatory bugs, *Perillus bioculatus* Fabr., adventive species, acclimatization, PCR analysis, molecular genetic similarity, Colorado beetle, entomophages, biocontrol

Nowadays, Colorado beetle (Leptinotarsa decemlineata Say) has acclimatized in Europe and Asia from the southern regions of Denmark to Spain and Portugal, from the North-West of the European territory of Russia to Siberia and

the Far East [1-4]. The growth of the Colorado beetle population in the absence of permanent pesticide treatments can be limited by adverse weather conditions, lack of feed, or by entomophages and entomopathogens. However, until recently within the European areal of this pest, specialized entomophages that could reduce its number were absent. Despite the successful use of natural enemies against many species of ticks and harmful insects, such techniques against the Colorado beetle are either absent or limited to small areas, although its effective natural enemies of Colorado beetle are well known [5].

Systematically applied insecticide treatments ensure the preservation of crop yields [6], but lead to very large and in many cases unnecessary costs due to the lack of accounting for the useful activities of natural enemies of pests [7-10].

The opportunity to use the biological agents against the Colorado beetle attracted the attention of scientists after the pest spread widely in the United States, Canada, and then in Europe [11]. The subject of these studies was pathogenic microorganisms as the basis of microbiological preparations, local entomofauna (to identify effective natural enemies of *L. decemlineata*); the works on the introduction of entomophages of the Colorado beetle from North America were carried out [5, 12, 13].

The investigations on the acclimatization of the North American predatory bug *Perillus bioculatus* Fabr. have begun in France in the 1930s and resumed in the 1950s and 1960s actively [14]. In Europe, the representatives of the subfamily *Asopinae*, the *Perillus bioculatus*, *Podisus maculiventris* Say, *Oplomus nigripennis* var. pulcher Dull., as well as parasitic flies of the genus *Doryphorophaga*, were introduced [15-17]. Since 1966, *Perillus bioculatus* was brought to Belgium, France, Germany, Italy, the USSR, Slovakia, Yugoslavia, and Ukraine for the purpose of natural regulation of the *L. decemlineata* population [18, 19] which became insensitive to the used insecticides quickly [20-23] and showed cross-resistance [24-27]. An international working group of entomologists from Germany, Belgium, Czechoslovakia, Hungary, Bulgaria, and Poland was created for the acclimatization of the entomophage to carry out breeding, production and introduction of *Perillus bioculatus* in agrocenoses [14].

In the USSR, works on the introduction of this predatory bug (as the supposed most promising bioagent for the control of the Colorado beetle) were carried out in 1960-1970, but the attempts of acclimatization and/or seasonal colonization failed. In the last 25-30 years, the research of *Perillus bioculatus* in Russia has not been reported. In 2008, a naturally acclimatized population of *Perillus bioculatus* was found in Krasnodar [28, 29]. To identify the causes of its naturalization and adaptation, determine the areal and assess the prospects for practical application against Colorado beetle, monitoring of the biological characteristics of the predator and its morphogenetic structure in the Krasnodar population were carried out [30, 31]. In recent years, geographical populations of *P. bioculatus* Fabr. have also been identified in the Ust-Labinsky and Abinsky Districts of the Krasnodar Territory. Earlier, *Perillus bioculatus* was found in several areas of Kuban, Adygea, Rostov Region, Ukraine, Moldova, Turkey, and other countries, which gives the reason to assume its large-scale acclimatization in the Old World countries [32].

This paper summarizes the data on the biology and phylogeny of predatory double-eyed soldier bug *Perillus bioculatus* naturally acclimatized in the Krasnodar Territory. The geographical variability of the genetic structure and diversity of the Krasnodar population of this species new for Europe have been revealed for the first time with a description of the biotic factors affecting the dynamics of its population.

The work objective was to study the biological characteristics and phe-

netic structure of *Perillus bioculatus* populations by the color of the scutellum, including the analysis of the inheritance of this trait with the assessment of the genetic similarity of *P. bioculatus* populations.

Techniques. P. bioculatus from different geographical populations (Krasnodar Territory, Rostov Region, and the Republic of Adygea) collected in 2014-2015 were studied. Observations were carried out under stationary crop rotation (All-Russian Research Institute of Plant Biological Protection — ARRIPBP) and in the farms of the Krasnodar Territory which use organic agriculture systems (2014-2016). Eggs, larvae, and adults of Colorado beetle per 1bush (potato varieties of Gollandka, Udacha, Lugovskoy), the number of ragweed leaf beetle *Zygogramma saturalis* Fabr. per 1 m^2 area (plants, soil surface and its layer of 0-12 cm) were counted, as well as larvae and imago of *Perillus bioculatus* [33].

In lab tests, *Perillus bioculatus* was grown on eggs and larvae of *L. decemlineata*, as well as on caterpillars of wax moth (*Galleria mellonella* L.) of the 2nd and 3rd instars. Optimal conditions for the development of *Perillus bioculatus* were 24-25 °C, 70-80% relative humidity, and 16 h photoperiod. The previously developed technique for storage of adult bugs (imagoes) was used [29]. The methods to overcome diapause were applied to continuously maintain the laboratory populations (V.Ya. Ismailov et al., RF patent No. 2 14122878/10, 2015).

During hybridological analysis (reciprocal crossing), one pair of mature bugs of different phenological forms in different combinations, i.e. orange♂ × red♀, red♂ × orange♀, red♂ × white♀, white♂ × red♀, red♂ × red♀, orange♂ × orange♀, white♂ × white♀, were placed in Petri dishes in 5 repetitions. Insects were fed with eggs and larvae of Colorado beetle; after feeding of the 2nd and 3rd generations of the predator, the ratio of phenological forms was counted.

Twenty individuals were selected from each population to study genetic polymorphism. DNA was isolated by a modified CTAB method (V.I. Kil. Method of assessing DNA polymorphism of insect populations by RAPD and ISSR-PCR — recommendations. Krasnodar, 2009) with corresponding reagents (DiaEm, Russia). Purity (according to OD_{260}/OD_{280}) and concentration of DNA preparation were determined spectrophotometrically (Hitachi Perkin Elmer 124, Hitachi, Ltd., Japan).

DNA polymorphism was evaluated by RAPD- (random amplification of polymorphic DNA) and ISSR (inter-simple sequence repeat)-PCR methods (V.I. Kil. Method of assessing DNA polymorphism of insect populations by RAPD and ISSR-PCR — recommendations. Krasnodar, 2009) (iCycler, Bio-Rad Laboratories, Inc., USA) with electrophoretic separation of amplicons (Sub Cell-GT, a power source Power Pac-Basic, Bio-Rad Laboratories, Inc., USA; reagents of OOO DiaEm, Russia; Dialat Ltd., Russia.) DNA-polymerase (5 IU/μl, Dialat Ltd., Russia), a molecular weight marker (M 100bp, OOO SibEnzim, Russia) and standard primers designed in the Operon Technology, Inc. (the USA) (OP) and the University of British Columbia (Canada) (UBC), i.e. UBC880 (ISSR) and OPA02, OPA07, OPA20, OPB01, OPE07, UBC450, UBC531 (RAPD) (synthesized at OOO SibEnzim, Russia), were used. Amplified fragments were separated in 1.8% agarose (AppliChem GmbH, Germany). Photo documentation was performed on the transilluminator ESC-20-M (Vilber Lourmat, France). The compared DNA samples were amplified in one PCR in 2 analytical repeats.

The frequency of DNA markers and its variability were estimated by χ^2-criterion, the reliability of differences between the sample averages by Student's *t*-criterion. Statistical differences were estimated at 5% significance level. Polymorphism indicators were calculated as a percentage of polymorphic fragments to the total number of DNA markers. The genetic diversity of populations

and genetic distances were estimated according to M. Nei and C. Schennon with POPGENE v1.31 software (https://sites.ualberta.ca/~fyeh/index.html) [34].

Results. The populations of *Perillus bioculatus* were studied both in the field and in the laboratory. The modes of preservation of adult bugs (up to 10-15 days at 4 °C) were optimized for this purpose [29]. The laboratory population of *P. bioculatus* was maintained continuously due to the developed method of overcoming diapause by means of 10-15-day preservation of insects at low temperatures (3-4 °C) followed by laboratory breeding under optimal conditions (V.Ya. Ismailov et al., RF patent No. 2 14122878/10, 2015).

Biological features and phenetic structure of *Perillus bioculatus* populations. Observations of the populations of *P. bioculatus* and its victims were carried out during regular annual and seasonal observations. In 2015, the overwintered beetles *L. decemlineata* appeared in the 3rd ten-day period of April, the first egg-laying was on May 1 to May 3. In the first ten-day period of May, the first individuals of *P. bioculatus* were noted on the experimental site on potato plants; in the second ten-day period of May, the number of the bug began to increase gradually. At the beginning of the growing season, *P. bioculatus* was represented by a red phenological form; in the third ten-day period of May, individuals of a white phenological form appeared, and only in the third ten-day period of August a small number of bugs with an orange colored scutellum appeared. In this population, 80.0% (603 insects) were individuals with red-black scutellum, 17.0% (128 insects) were orange-black and 3.0% (23 insects) were black-white (total sample size of 754 insects). In 2012, the shares of these phenological forms were 71.3% (445 insects), 17.9% (111 insects) and 10.8% (69 insects), respectively; in 2013 these were 74.9% (540 insects), 20.1% (144 insects) and 5.0% (37 insects), in 2014 — 70.0% (480 insects), 15% (103 insects) and 15% (103 insects). The proportion of *P. bioculatus* individuals with different colors of the scutellum was compared over the years by χ^2-criterion in a form applicable to estimate the similarity of two or more empirical distributions [35]. The result was a value of $\chi^2 = 11.7$, which is less than the standard for the 5% statistical significance ($x^2_{st.[df = 11]} = 19.7$). In other words, the ratio of individuals with different coloring does not depend on the year of the study (differences by year are statistically unreliable).

Now, in the natural conditions of the Krasnodar Territory and the Republic of Moldova [36, 37], red, white, and also orange individuals can be met. In the studies conducted by E.M. Shagov (the city of Mukachevo, Transcarpathian Region, 1968), insects with yellow (orange) and white color of the scutellum were described [38]. E.M. Shagov noted that the higher the temperature, the lighter the color of the scutellum in *P. bioculatus* [38]. The observations of *P. bioculatus* in the natural conditions of the Krasnodar Territory confirm this dependence. In early May 2014, *P. bioculatus* was represented by the red morph (mean ten-period temperature in this period was about 20 °C), by the middle of May, orange individuals were noted (about 23 °C) and only by the middle of July (about 24.5 °C) a white morph of insects was found. The data we obtained are consistent with the report on the cleavage of the enzyme that affects the formation of the *P. bioculatus* scutellum color at elevated temperatures [39]. However, such dependence was not observed during lab culture. In the climatic chamber under the same conditions (the chamber supports the "day-night" mode) insects also had different colors of the scutellum (mainly red, rarely orange), while the white color was extremely rare.

It was found that *P. bioculatus* develops in three generations in the Central zone of Krasnodar Territory and its number during the whole vegetation period is from 2 up to 15 insects per 1 m^2 of a potato field, which is enough to suppress the

population of Colorado beetle and to abolish chemical treatments.

The observations have shown that the *P. bioculatus* population acclimatized in the Krasnodar Territory, when compared to that introduced in 1960-1970, has expanded trophic connections with the Colorado beetle: initially, *P. bioculatus* ate only eggs of this phytophage [14, 40], while the naturalized population had all the openly living stages of *L. decemlineata* (eggs, larvae of all ages, and adults) as a fodder base. Such feeding behavior of the predator allowed overcoming the asynchronous development of *P. bioculatus* and *L. decemlineata* in the second and third ten-day periods of April and the first ten-day period of May, which increases the survival rate of the predator and its bioregulatory activity.

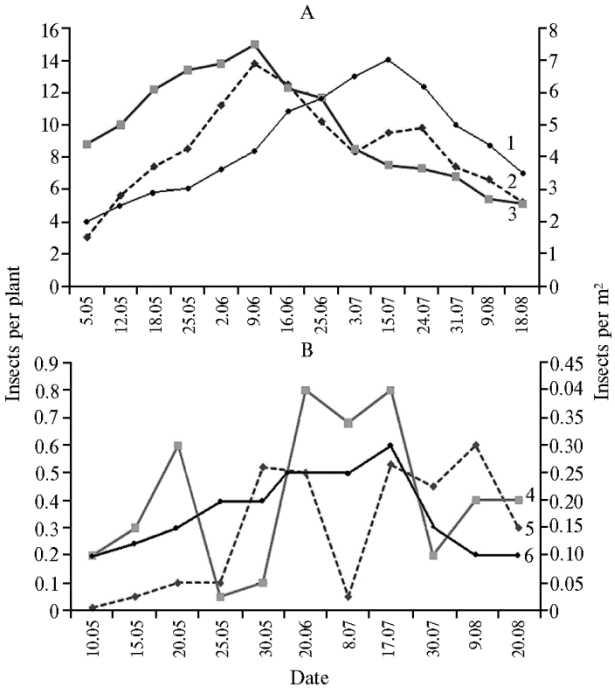

Fig. 1. Population dynamics of adults of ***Leptinotarsa decemlineata*** **Say,** ***Zygogramma saturalis*** **Fabr. and** ***Perillus bioculatus*** **Fabr. during the growing season in 2014** (A) **and 2015** (B)**:** 1 — *Z. suturalis*, 2 — *L. decemlineata*, 3 — *P. bioculatus*, 4 — *L. decemlineata*, 5 — *P. bioculatus*, 6 — *Z. suturalis* (the stationary crop rotation of ARRIPBP, Krasnodar).

In the village of Moldavanskoye (the Krymsky District, Krasnodar Territory, OOO Chistaya eda), the population of *P. bioculatus* was represented by the red and orange phenological forms in the ratio of 4:1. Annual planting of potatoes and other solanaceous crops (tomatoes and eggplants) in the farm, certified according to the organic standard, contributed to the development of the fodder base of the entomophage and created conditions for the development of its two summer generations. Chemical treatments in the fields of this enterprise were not carried out, and *P. bioculatus* during the entire vegetation period restrained the number of Colorado beetle on the nightshade crops at a level below the economic threshold of harmfulness.

To assess the impact of biotic factors on the dynamics of natural populations of *P. bioculatus*, we observed the predator at different stages of its development in natural stations. First of all, we studied the fodder base of *P. bioculatus* was studied, including estimates of both the predatory bug and its victims, the Colorado beetle *L. decemlineata* and ragweed leaf beetle *Z. suturalis* (Fig. 1). These observations revealed the determining role of a fodder base in dynamics of the predatory bug *P. bioculatus* population. For example, the decline of ragweed leaf beetle and the Colorado beetle populations in accounting stations (experimental fields of ARRIPBP) was the determining factor during a significant decrease in the predator population which reached in 2010-2012, 20-30 insets per 1 m^2 but was only 2-3 insects per 1 m^2 in 2014-2016. At the same time (in 2014-2016), in the South-Eastern regions of the Republic of Adygea, where the density of ragweed leaf beetles' and potato fleas' populations was high, the number of entomophages also remained high (up to 10-15 insects per 1 m^2).

Another equally important biotic factor is the activity of other entomophag-

es. In some years, the significant infection of the eggs of *P. bioculatus* with parasitic egg-eater *Trissolcus vassillievi* (Mayr) (*Hymenoptera*: *Scelionidae*) occurred, from 5 to 28%. *Phasiinae* flies (subfamily *Phasiinae*, family *Tachinidae*) infected 3-15%, and ectoparasitic mites up to 10% of the predatory imagoes.

P. bioculatus preferred the eggs of the Colorado potato beetle as food, but no less actively ate larvae of all instars and adult beetles. In natural conditions, in addition to the Colorado beetle, the predator fed larvae and imagoes of ragweed leaf beetle and caterpillars of olive-shaded bird-dropping moth *Tarachidia candefacta* Hübn.

Inheritance of scutellum color in *Perillus bioculatus*. In seven different combinations of crosses (including reciprocal) between red, orange (or yellow) and white parental forms in the offspring (both in F_1 and F_2), including the crossing of white individuals with white, only individuals with the red and orange scutellum were produced in a ratio of 1:1. Unlike natural, laboratory population did not contain white phenological forms. The lack of uniformity in F_1, the absence of white phenological forms and the equal ratio of red and orange morphotypes in F_2 indicate the non-Mendelian nature of the trait inheritance and its conditionality (along with the genetic component) with insect conditions (temperature, photoperiod duration, etc.).

Comparative analysis of geographical populations of *Perillus bioculatus* by DNA markers. In 2015, new geographical populations of *P. bioculatus* were discovered in the village of Varenikovskaya (Krasnodar Territory, the Krymsky District), in the village of Shuntuk (the Maikop station of the Vavilov All-Russian Research Institute of Plant Genetic Resources, the Republic of Adygea) and the Aksaysky District of the Rostov Province. By comparison of their survival depending on a type of feed, the population most suitable for lab culture was selected. The biological indicators of the population of *P. bioculatus* collected on potato plants in the village of Moldavanskoye were significantly different from that of Krasnodar.

As a result, the starting population of the village of Varenikovskaya and the city of Krasnodar was the most adapted to lab culture. When feeding with wax moth (*Galleria mellonella* L.), the number of eggs laid per female was to 70.4-81.6 pcs, whereas the population of the village Moldavanovskoye was unsuitable for the lab breeding of the predator. Winged bugs in this population when feeding its larvae with Colorado beetle were only 21.3%, and when converted to food with an additional victim, the wax moth, the survival of the Moldavanovskoye population of *P. bioculatus* fell sharply, which led to the loss of stock laboratory population, while the Krasnodar one continued to successfully develop and multiply.

The revealed features (along with differences in the phenotypic structure) lead to a conclusion about genotypic differences of *P. bioculatus* populations from the vicinity of Krasnodar and the Krymsky District.

1. DNA polymorphism and genetic structure of *Perillus bioculatus* Fabr. geographical populations (Krasnodar Territory, 2014))

Primer	DNA polymorphism, %		DNA fragment per individual, $M \pm SEM$		Number of detected DNA markers, n	Differences between samples in DNA marker frequency, χ^2
	SN	K	SN	K		
OPA02	100	100	7,9±1,4	8,2±0,7	26	25,6
OPA20	100	100	4,0±1,3	5,0±0,8	22	18,5
UBC880	100	100	4,0±0,7	2,0±0,2*	11	14,9

N o t e. SN means the village of Staro-Nizhesteblievskaya, K means the city of Krasnodar.
* Differences between samples are statistically significant ($t_{act.} \geq t_{05}$).

This was confirmed by the PCR-DNA analysis of *P. bioculatus* bugs, per-

formed with two (OPA02, OPA20) RAPD and one (UBC880) ISSR primers. As a result, no differences in DNA polymorphism between the Krasnodar and Staro-Nizhesteblievskaya (the Krymsky District, Krasnodar Territory) populations of insects (sample of 2014) were found (Table 1).

The high genetic similarity (0.99) was observed between the studied samples (Table 2). Intrapopulation genetic diversity in the studied geographical samples (Krasnodar and Staro-Nizhesteblievskaya) did not differ (see Table 2). The obtained data indicate a high migratory capacity of the species and that the samples under investigation probably belong to the same geographical population.

2. Genetic diversity of *Perillus bioculatus* Fabr. geographical populations in different years (Krasnodar Territory)

Indicator	2014		2015	
	SN	K	SN	M
Genetic diversity according to Shennon, I±SD	0.38±0.18	0.36±0.18	0,28±0,25	0,43±0,21*
Genetic identity according to Nei	0.99		0.86	

N o t e. SN means the village of Staro-Nizhesteblievskaya, K means the city of Krasnodar, M means the village of Moldavanskoye.

* Differences between samples are statistically significant ($t_{act.} \geq t_{05}$).

In 2015, the additional primers (OPA07, OPB01, OPE07, and UBC450) were used to obtain more detailed information on *P. bioculatus* polymorphism. The PCR analysis of the samples (from the village of Staro-Nizhesteblievskaya and the village of Moldavanskoye, 2015) revealed statistically significant differences in the genetic structure and DNA polymorphism with almost all used primers (Fig. 2, Table 3). The genetic diversity of these samples varied and their genetic similarity was relatively low (0.86) (see Table 2).

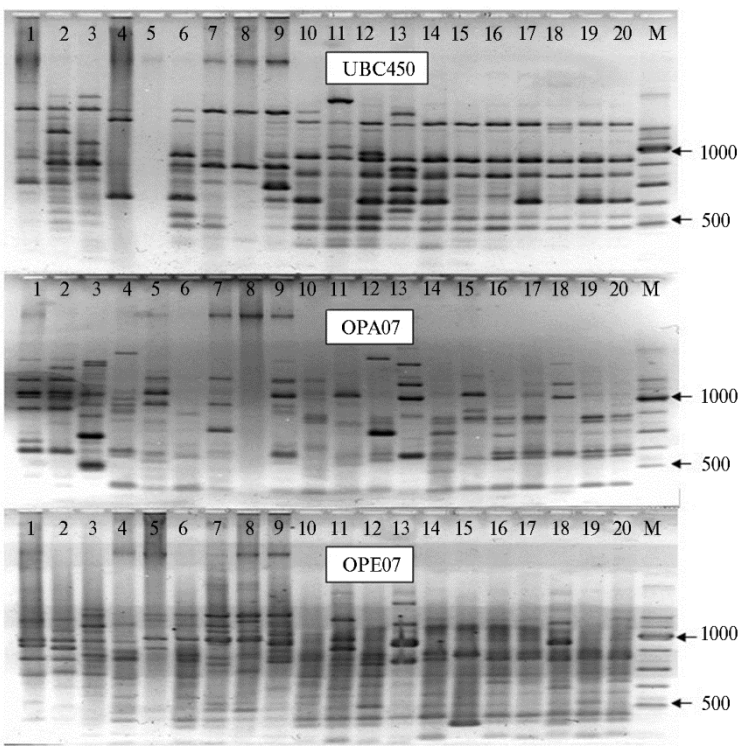

Fig. 2. RAPD-PCR analysis of *Perillus bioculatus* Fabr. from different geographical populations (Krasnodar Territory, 2015): 1-10 — the village Staro-Nizhesteblievskaya; 11-20 — the village Moldavanovskoye; UBC450, OPA07, OPE07 — the used primers; M — molecular weight marker (M 100bp, SibEnzim, Russia), 1.8% agarose.

3. DNA polymorphism and genetic structure of *Perillus bioculatus* Fabr. geographical populations (Krasnodar Territory, 2015 год)

Primer	DNA polymorphism, %		DNA fragment per individual, $M \pm SEM$		Number of detected DNA markers, n	Differences between samples in DNA marker frequency, χ^2
	SN	M	SN	M		
OPA02	100	100	6.2±1.4	5.8±1.0	25	25.1
OPA07	100	100	7.1±0.4	7.0±1.0	19	32.1*
OPA20	76.9	100	7.6±0.5	2.9±0.6*	10	30.5*
OPB01	75.0	100	5.5±0.5	5.0±0.7	12	23.4*
OPE07	93.3	100	10.1±0.7	9.8±1.0	21	50.8*
UBC450	100	100	5.1±1.2	6.3±0.7	11	27.8*

N o t e. SN means the village of Staro-Nizhestеblievskaya, M means the village of Moldavanskoye.
* Differences between samples are statistically significant ($t_{act.} \geq t_{05}$, $\chi^2_{act.} \geq \chi^2_{05}$).

The PCR analysis showed that the low viability of the starting populations of *P. bioculatus* from the village of Moldavanovskoye and its food preferences, which distinguish this population from the others, are due to genetic differences.

In general, it can be concluded that since 2008, when the first reports of acclimatization of the predatory bug *P. bioculatus* in the Krasnodar Territory [28, 32] had appeared, entomophage, due to the high migration capacity and the acquisition of additional trophic connections, managed to naturalize well in these new living conditions. Similar data were obtained on the rapid acclimatization of predatory bug throughout the entire territory of Moldova [36, 37], as well as in Turkey [39, 41, 42], Bulgaria [43], and Serbia [44]. The revealed significant influence of the fodder base and entomophages on the natural number of this bug allows us to suggest that native species of entomophages have adapted to the useful adventive species and are able to reduce its number. In some years, this can neutralize the useful activity of natural populations of the predator, thence, it is necessary to improve methods of artificial cultivation of *P. bioculatus* and create optimal conditions for its activation and reproduction in field conditions (the lack of chemical treatments and the presence of the fodder base).

Thus, in the Krasnodar Territory, acclimatized North American predatory bug *Perillus bioculatus* Fabr. has become an effective natural regulator of the Colorado beetle population. The study of the biological characteristics of *P. bioculatus* revealed a significant impact of a fodder base on the number of natural populations of the predator and indigenous species of parasitoids. The data obtained by crossing insects of different phenological forms of *P. bioculatus* indicate the non-Mendelian nature of the inheritance of the scutellum color and the different manifestations of this trait in the studied populations of the predator. The detailed genetic analysis of this trait and its variability depending on the environmental conditions is the fact of scientific interest and is also of practical importance for assessing the effectiveness of natural populations of entomophage. The results of the evaluation of predatory activity, the viability of the entomophage and the PCR analysis of differences in the genetic structure in the studied geographical populations are consistent.

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THE SPECTRAL LIGHT INFLUENCE ON YOUNG ORNAMENTAL PLANTS' RESISTANCE TO SHORT-TERM COLD STRESS

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Abstract

LED light sources allow for variations in the spectral composition which makes it possible to evaluate the effect of light with narrow spectral width on plants performance. The combination of such lighting with natural lighting can significantly change the direction and intensity of metabolic processes in plants to adapt to changing environmental conditions. In the present paper, we showed for the first time that additional illumination with narrow-spectrum light allows the seedlings of ornamental plants to successfully adapt to cold stress. The aim of our work was to estimate the effect of red light (600 nm) and blue light (400 nm) from LED panels on adaptation of tagetes (*Tagetis panula* L.) cultivar Karmen, snapdragon (*Antirrhinum majus nanum* L.) cultivar Flora shower white and petunias (*Petunia hybrida* L.) cultivar Mambo blue seedlings to spring frosts when transplanting to open ground. These plants are often used in landscaping settlements and are exposed to low positive and zero temperatures, especially in the first days after transplanting to open ground. The experiment was carried out in a greenhouse (18 °C, humidity 85%). During thirty days, one third of the plants were additionally red lightened (RL), and one third ones were blue lightened (BL) using Focus MC2 (JCC-12) LED panels (Russia) for 12 hours after the end of daylight hours. A third of the plants were grown under natural light only (C). After the end of the LED illumination period, a half of the plants in each variant were placed in the chamber with 2 °C for 24 hours. The second half plants were control without cooling. The effectiveness of lighting was assessed by the decorative state of plants, the change in the selective permeability of membranes, the content of salicylic and abscisic acids, the triggers of the cascade of protector reactions in the leaf tissues, and the survival of plants transplanted to the open ground. Our experiments showed that additional lightening with blue and red light contributes to seedling resistance to low positive temperatures. In RL- and BL-exposed plants, the leaf turgor was quickly restored, the habitus of plants was preserved, and budding began. In these variants, the cell membrane stabilization was noted, i.e. i.e. an increase in the outlet of potassium ions was two times lower than in the control. The changes of cell membranes was estimated by output of electrolytes from the 0.25 g leaves measured potentiometrically (ITAN, OOO SPE Tom'analit, Russia) with an ion-selective electrode (Elit-031, OOO NIKO ANALIT, Russia). Abscisic acid (ABA) and salicylic acid (SA), the important triggers and tuners of cascade responses to abiotic stress, were determined in a single sample, at the final stage, by isocratic HPLC method (Stayer system, ZAO Aquilon, Russia), with column PR-18 (250/4.6 mm) (Phenomenex, Inc., USA). ABA concentration in leaves of RL-exposed tagetes plants increased 4.4-fold and the SA 3.3-fold compared to the control, and in BL-exposed tagetes only SA content increased 1.4-fold ($p \le 0.05$). After cold exposure, the RL plants restored turgor, while in BL the restoration was slower and the leaf infiltration began. Similar changes were in control. In snapdragons and petunias, blue light also caused a 1.5-fild increase in the SA level in leaves, while red light led to insignificant changes. After cold stress, the RL- and SS-exposed plants of these species quickly recovered, their habitus was almost as in unaffected plants. The control plants recovered more slowly, and their decorativeness was worse. The survival rate of RL plants replanted to open ground was 100 % for tagetes and petunias, and 85-90% ($p \le 0.05$) for snapdragons. In BL-exposed plants, these indicators were 70% ($p \le 0.05$) for tagetes and 85-90 % ($p \le 0.05$) for petunias. The rooting of the control plants not exposed to RL or BL and subjected to the cold stress reached 60-70% ($p \le 0.05$). The physiological

and biochemical changes that we identified in the leaves of the seedlings of ornamental plants suggest that spectral light facilitates the modulation of plant metabolism and activates non-specific protective mechanisms aimed at preserving the ionic and redox cell homeostasis. An exposure of growing seedlings of ornamental plants to light with narrow spectral width, in addition to natural light, can be very effective to reduce the loss of re-planted plants from spring frosts.

Keywords: *Tagetis panula* L., tagetes *cv*. Karmen*, Antirrhinum majus nanum* L., snapdragon cv. Flora shower white, *Petunia hybrida* L., petunias cv. Mambo blue, narrow spectral light, cold stress, cell membranes, water soluble carbohydrates, salicylic acid, abscisic acid, ABA

Adaptation of plants to changes in the environmental temperature is often accompanied by stress. This is a complex multi-stage process, discovering the mechanisms of which is important for research and application.

Narrow-spectrum LED light combined with natural lighting may significantly alter the intensity and direction of plant metabolism [1, 2]. Using LED lamps and panels in new protected cultivation technologies becomes a trendy orangery technology [3]. However, the physiology and biochemistry of adaptation remain understudied. Light intensity, duration, and wavelength may trigger the expression of certain genes and the synthesis of a number of new substances affecting the generative organs of plants [4, 5]. One assumption is that the effects of narrow-spectrum light are related to the activation of COR genes that initiate the synthesis of cold shock proteins [6, 7]. This cascade of interrelated transformations involves multiple light-dependent responses, which causes significant readjustments in plant metabolism, as it alters the hormonal and carbohydrate status of cells and their membrane permeability, while also activating or inhibiting some enzymes [8-10]. Improved resistance of plants to hypothermia is associated with the suppression of oxidative stress due to binding the reactive oxygen species (ROS) and free radicals; this binding is enabled by antioxidant enzymes and the accumulation of low molecular weight organic antioxidants [11, 12]. When adapting to cold shocks, plants accumulate stress defensive agents, such as amino acids, soluble sugars, sugar alcohols, and sundry metabolites.

Some believe that COR genes are activated by exposure to red (λ = 660 nm) or blue (λ = 400 nm) light, i.e. the regulation of COR gene expression involves the phytochrome and cryptochrome light receptors [13, 14]. It is suggested that there exist primary elicitor signals of various nature; the suggestion is backed by the data on the stabilizing effects red and blue light has on gene expression regulation under stress. Low-temperature adaptation is modeled by a number of interrelated processes that enable plants to adjust their cellular and metabolic homeostasis [15]. Salicylic acid (SA) and abscisic acid (ABA) are important for the functioning of such cascade mechanisms. SA is one of the metabolites that initiate the expression of genes responsible for the synthesis of antioxidative enzymes, which helps control the ROC, preserve the integrity of cellular membranes, as well as the redox status of plant cells [16-18]. The accumulation of ABA in tissues triggers an ABA signaling cascade that culminates in the expression of COR genes, which in their turn determine the cold tolerance of the species [19].

This paper is the first to demonstrate that exposure to narrow-spectrum light enables the seedlings of ornamental plants to successfully adapt to cold shock.

The research objective is to find whether preliminary supplementary red or blue lighting will affect the resistance of seedlings to short-term low positive temperatures during spring frosts.

Techniques. The following plant species were selected: snapdragon (*Antirrhinum majus nanum* L.) short-stem cultivar Flora shower white; tagetes (*Tagetis panula* L.) short-stem cultivar Karmen; and petunia (*Petunia hybrida* L.) cultivar Mambo blue; all of those are popular ornamental plants used in open ground in

urban gardening. At the fifth-to-seventh leaf development stage, the plants were planted in pots with sand, 5 plants per pot, 15 pots per group. The plants were growing in a semi-controlled environment: the natural light was supplemented with red light (max $\lambda = 600$ nm), group 1, RL, or blue light (max $\lambda = 400$ nm), group 2, BL. The intensity for RL and BL was 2.58×10^{18} and 6.04×10^{18} photons/($m^2 \cdot s$), respectively. Extra light came from Focus MC2 (JCC-12) LED lamps (Russia). Supplementary lighting was on for 12 hours daily; the control group (group 3, or C) comprised plants exposed to natural light only. Seedlings were watered with distilled water daily, fed once a week with 150 ml of Knop's solution (0.25 mg potassium phosphate, 0.25 magnesium sulfate, 1 g calcium nitrate, and 0.125 g potassium chloride per 1 l of distilled water). After the supplementary lighting experiment was over, half of the plants from each group (RL, BL, and C) were placed for 24 hours in a chamber with a temperature of 2 °C; the remainders (the controls) were left non-cooled.

Samples for biochemical tests were taken on day 35 (when the experiment was over) and on day 37 (after 2 days of exposure to cold shock). To determine the functional status of cellular membranes, 0.3 g of leaves was placed in a bidistillate, kept for 24 hours in a thermostat at 26 °C; the conductivity of the eluate was measured, and the content of K^+ was quantitated by potentiometry using ion-selective electrodes (ITAN pH meter/ionometer, OOO NPP Tomanalit, Russia; Elit-031 ion-selective electrode, OOO NIKO Analit, Russia) according to earlier published guidelines [20].

The content of monosaccharides was found spectrophotometrically (Specol 1300, Analytik Jega AG, Germany) in terms of picric acid [21]. The concentration of salicylic acid (SA) and abscisic acid (ABA) was analyzed from a single sample: 2 g of fresh leaves were extracted by ethanol (80%); the extract was evaporated to an aqueous phase, which was divided into two parts of equal volume. To extract SA and ABA, the extract was purified using the laboratory-modified method [22]. At the final stage, the research team used isocratic high-performance liquid chromatography (a Stayer isocratic chromatograph, ZAO Aquilon, Russia) with an RP-18 (250/4.6 mm) column (Phenomenex, Inc., United States).

The data was processed statistically with Excel 2010 and Past v3.0 software [23]. The means of the analyzed values (M), standard errors of the mean ($\pm$SEM), and confidence intervals at 95% confidence level ($t_{0.05} \times$SEM) were calculated. The significance of differences between the groups was evaluated by non-parametric statistic (paired Shapiro-Wilcoxon test). The inter-group difference was deemed significant at $p \leq 0.05$.

Results. Diurnal temperature swings in spring and fall often damage or even kill plants. The temperature that was used to simulate cold shock (2 °C) is not lethal for the plants under analysis; however, it can significantly damage their leaves [24].

Supplementary red and blue lighting did not cause any significant habitus or morphology difference in tagetes between the groups, see Figure 1, A. Both experimental plants and controls had good leaf turgor; almost 30% began budding. In snapdragons and petunias, supplementary red lighting caused the aerial biomass of the plants to become 1.5 times and 2.4 times larger, while the root biomass became 1.6 times and 1.8 times larger, respectively, see Figure 1, B. Experimental plants did not differ from the controls in terms of flowering onset; however, the flowering performance was 19% to 23% better in the former. In May, when replanting the plants in open ground (at 10 to 12 °C in daytime and 2 to 5 °C in nighttime), the survival rate of RL- and BL-exposed plants varied from 100% for tagetes and petunias to 80% for snapdragons; the survival rate of

the controls was only about 60% to 70%.

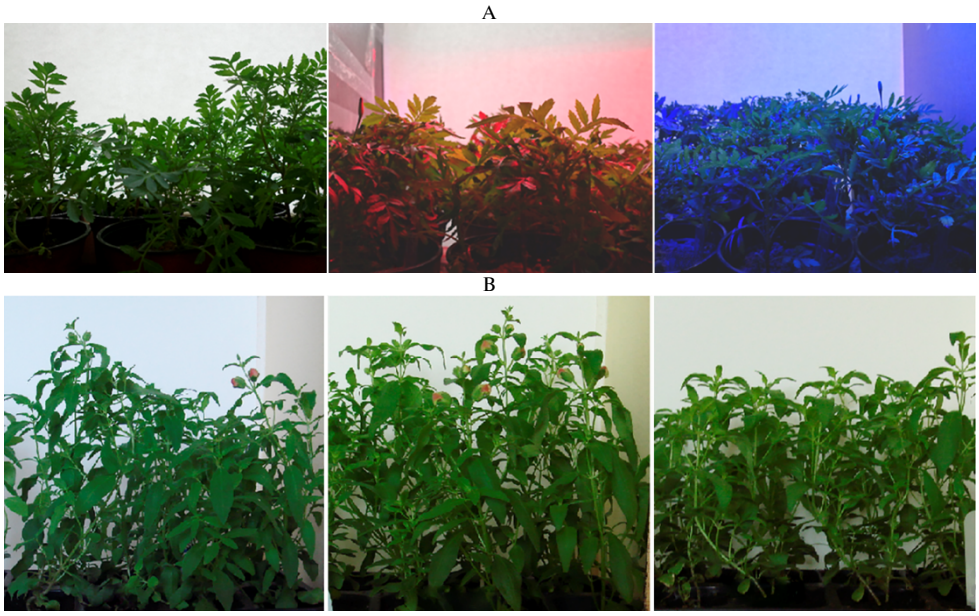

Fig. 1. Tagetes (*Tagetis panula* L., cultivar Karmen) (A) **and snapdragon (*Antirrhinum majus nanum* L., cultivar Flora shower white)** (B) **after 35 days of supplementary lighting for 12 hours a day:** left-to-right — controls (natural light only), red light (max λ = 600 nm) and blue light (max λ = 400 nm); Focus MC2 (JCC-12) LEDs (Russia).

Physiological parameters of cold-shocked ornamental plants as a function of supplementary narrow-spectrum lighting (pot experiment, 5-fold repetition)

Indicator	C (control)		RL				BL			
	1	2	1		2		1		2	
	abs. units	abs. units	abs. units	%	abs. units	%	abs. units	%	abs. units	%
Tagetes (*Tagetis panula* L.), cultivar Karmen										
A	41.0±1.8	45.3±2.1	27.4±1.3	67*	37.3±1.7	82*	40.6±1.5	99	39.6±1.7	88*
B	9.8±0.4	10.8±0.5	3.7±0.2	38*	6.8±0.3	63*	1.6±0.1	16*	1.2±0.1	11*
C	0.48±0.02	0.65±0.04	1.07±0.10	223*	0.46±0.09	71*	0.50±0.03	104	0.25±0.02	39*
Snapdragon (*Antirrhinum majus nanum* L.), cultivar Flora shower white										
A	58.7±2.4	82.4±4.1	63.1±3.5	108	74.6±3.9	91	85.2±4.1	143*	51.9±2.1	63*
B	10.0±0.5	16.3±0.6	7.1±0.3	71*	3.3±0.2	20*	8.5±0.4	85*	4.5±0.3	28*
C	2.33±0.15	1.19±0.13	2.57±0.16	110*	1.17±0.11	98	3.31±0.21	142*	1.32±0.12	111*
D	6.46±0.42	10.20±0.20	5.83±0.37	91	11.60±0.30	114*	6.50±0.44	101	12.30±0.30	121*
Petunia (*Petunia hybrida* L.), cultivar Mambo blue										
A	22.1±0.7	55.3±2.3	24.3±1.3	110	26.3±1.2	48*	32.4±1.5	147*	26.9±1.3	49*
B	9.2±0.4	14.0±0.5	5.3±0.2	58*	5.9±0.3	42*	4.7±0.3	51*	4.5±0.2	32*
C	1.23±0.12	1.39±0.13	1.52±0.16	124*	1.07±0.11	77*	1.49±0.13	121*	0.95±0.08	68*
D	3.05±0.19	3.24±0.16	2.88±0.14	95	3.53±0.21	109	2.93±0.16	96	4.43±0.24	137*

Note. A is the release of electrolytes (in terms of electrical conductivity), μS; B is the potassium ion concentration, μg/ml of solution; C is the salicylic acid content, μg/g of wet substance; D is the total free monosaccharides, μg/mg of dry substance; 1 is for day 35 (narrow-spectrum lighting experiment complete); 2 is for day 37 (after a 2-day cold shock). The control indicators were 100%. The confidence intervals $M \pm (t_{0.05} \times \text{SEM})$ did not exceed ±5% ($p \le 0.05$). Statistical processing used a nonparametric Shapiro-Wilcoxon test (pairwise comparison of each group against its respective control).

* Differences for pairwise comparisons (C1 и RL1, C1 и BL1; C2 и RL2, C2 и BL2) were deemed statistically significant at $p \le 0.05$.

Leaf cell membrane system status is an important indicator of plant stress tolerance. Preserving the selective permeability of plasmalemma to certain ions and water molecules helps sustain the cellular homeostasis [25]. To preserve it, a cell must accumulate sodium ions in its vacuole and maintain the physiological concentration of potassium ions and a high K^+/Na^+ ratio in the cytoplasm [26], i.e. the increase in the potassium ion release indicated negative changes in the cell internals. After the supplementary lighting experiment, tagetes leaf tissues

had the selective permeability of membranes decreased in the RL group, although the same indicator was within the control parameters for the BL group; on the other hand, exposure to RL increased the release of electrolytes in snapdragons and petunias insignificantly compared to the control and significantly compared to the BL group ($p \leq 0.05$), see Table. The release of potassium ions dropped significantly ($p \leq 0.05$) in both experimental groups compared to the controls, which preserved the high K^+/Na^+ ratio in the cytoplasm of the plants exposed to supplementary lighting.

Exposure to low positive temperatures deteriorated the membrane semipermeability in all plants, but it was the controls that displayed maximum deterioration. RL- and BL-exposed petunias had the best resistance to short cold shock: their leaf tissue electrolyte release was 51% to 52% lower than in the controls ($p \leq 0.05$). In tagetes, the reduction was 12% to 18%; in snapdragons, 9% to 37% ($p \leq 0.05$). Potassium ion release was significantly ($p \leq 0.05$) lower in all plants exposed to RL, even more so in the BL group. Notably, supplementary narrow-spectrum lighting accelerated the post-shock recovery of plants, especially in the RL group, whereas the controls recovered more slowly, and their decorativeness was worse, see Figure 2.

Fig. 2. Supplementary lightened tagetes (*Tagetis panula* L., cultivar Karmen) (A) **and snapdragons (*Antirrhinum majus nanum* L., cultivar Flora shower white)** (B) **after a cold shock (day 37):** left-to-right — controls (natural light only), red light (max λ = 600 nm) and blue light (max λ = 400 nm); Focus MC2 (JCC-12) LEDs (Russia).

Supplementary narrow-spectrum lighting did not affect the content of water-soluble carbohydrates in leaf tissue, see Table. Cold shock increased the monosaccharide content in snapdragons and petunias in both RL and BL groups as compared to the controls; the increase in water-soluble carbohydrates (21% for snapdragons and 37% for petunias) was most significant in the BL-exposed plants. It is known that monosaccharides are not merely a source of energy; they are also an important defensive agent that helps preserve cellular homeostasis under stress [27]. In this experiment, these agents might have contributed to a specific response that helped the plants survive hypothermic stress.

Salicylic acid is one of the signaling pathway triggers. Its role in triggering and regulating the adaptation mechanism is rather ambiguous [16]. Lack or excess of SA might amplify the stress effect, as the SA and ABA amounts correlate, while the SA level is associated with initiating the cascade of defensive responses [28] which is light-dependent and acts in conjunction with other defense mechanisms [29]. After the supplementary lighting experiment was over, the SA content was found to have risen in all plants as compared to the controls; the increase was the most significant in RL-exposed tagetes (223% higher than in the controls), see Table. Apparently, such exposure altered the hormonal balance of leaf tissue, causing plant-wide metabolism readjustments. On post-shock day 2, the SA content was lower in tagetes in both groups compared to the controls (29% to 61% lower) as well as to the pre-shock values (2.3 times lower in RL-exposed plants and 2.0 times lower in their BL counterparts). In snapdragons and petunias, exposure to RL and BL did not significantly alter the SA content compared to the controls. SA levels rose by 9% and 14% ($p \leq 0.05$) in RL-exposed plants, and by 21% and 37% ($p \leq 0.05$) in BL-exposed plants (supplementary lightened snapdragon and petunia, respectively) after cold shock.

Note that cold shock caused a quick loss of turgor in BL-exposed tagetes whose leaf edges were damaged. In this group, plants took a long time to recover and bloomed later; a third of plants did not survive, see Figure 2, A. Signs of damage were less pronounced in control plants; these survived cold better, but their decorativeness was worse (weak branching, small buds). RL-exposed plants had good leaf turgor, buds opened well. Perhaps, the RL-induced changes in the hormonal status of tagetes tissue helped trigger the defensive response cascade, which neutralized the negative effects of cold. BL-induced changes in the hormone ratio did not have any positive effects. Snapdragons (see Fig. 2, B) and petunias recovered quickly after shock in both RL and BL groups; their turgor, aerial biomass, and habitus were barely affected. In the control group, post-shock recovery of snapdragons and petunias was slow; the aerial organs were 15% lighter, and the underground organs were 10% lighter; the decorativeness of non-controls was worse than in case of supplementary lighting.

The initiation of cascade responses that form the response to abiotic stress also involves abscisic acid [19]. This hormone was only detected in tagetes tissue. After the supplementary lighting experiment was over, the ABA content rose in plants exposed to RL (to 0.191 ± 0.02 μg/g) and dropped slightly (to 0.037 ± 0.005 μg/g) in their BL counterparts as compared to the controls (0.043 ± 0.003 μg/g). The post-shock ABA content in leaf tissue was significantly greater in the BL group compared to the baseline (0.066 ± 0.008 μg/g), barely changed in the controls (0.048 ± 0.004 μg/g), and became 5 times lower (dropped to 0.038 ± 0.004 μg/g) in the RL group. This proves the hypothesis that the hormonal balance of tagetes leaves is affected by supplementary lighting, which therefore restructures plant-wide metabolism.

Perhaps the lights of different spectra triggered different defensive mechanisms activation pathways. Both SA and ABA can be such triggers, and their signaling pathways partially overlap. There emerges an information network that contains antagonistic and synergistic links [27]. In the case of BL, salicylic acid might have contributed to a faster transition of metabolic processes to an adaptive mode, whereas in RL-exposed plants, this mechanism might have been triggered after an ABA release, whereby salicylic acid was not involved in the expression of defense genes.

Therefore, the combination of physiological and biochemical changes in the leaf tissue of ornamental plants (habitus, morphology, biomass accumulation, electrolyte release, potassium ion content, salicylic and abscisic acid content,

total of free monosaccharides (glucose and fructose) gives rise to a suggestion that supplementary spectral lighting restructures the metabolic processes and the activation of nonspecific defense mechanisms that preserve the ionic and redox homeostasis of cells. This research is the first to show that supplementary narrow-spectrum lighting helps such plants successfully adapt to cold shocks. When re-planted in open ground (at 2 to 5 °C at night), their survival rate is 80% to 100% if exposed to supplementary lighting; cf. 60% to 70% in the controls. Therefore, supplementing natural lighting with narrow-spectrum light from LED panels while cultivating ornamental plants will help boost their survivability in a drastic change of habitat when used for landscaping.

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DYNAMIC REGULATION OF PHOTOSYNTHETIC PROCESSES UNDER VARIABLE SPECTRAL LED IRRADIATION OF PLANTS

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Abstract

In natural conditions of plant growth, along with changes in the intensity of light during different periods of time, there is a change in the spectral composition of the incident radiation. The ratio between the blue and red spectral regions changes from ≈ 0.50 for direct radiation of the sun, to ≈ 0.95 for diffuse solar radiation, depending on the height of the sun and the time of day. The work investigated the effect of light of different spectral composition on the functional characteristics of the photosynthetic apparatus of potato plants (*Solanum tuberosum* L.) of the Zhukovsky Early variety, grown by aeroponics in two vegetation chambers of a phytotron using LEDs sources with preferential irradiation of plants with blue light (LEDs BL, λ_{max} = 470 nm) or red light (LEDs RL, λ_{max} = 660 nm) of the spectral range of photosynthetically active radiation (PAR). With a total PAR intensity of 400±28 μmol · m^{-2} · s^{-1}, the proportion of blue light was 293.6 μmol · m^{-2} · s^{-1} (chamber 1), and of red light it was 262.0 μmol · m^{-2} · s^{-1} (chamber 2). As a result, the ratio BL/RL (when comparing the intensities of radiation in the two chambers of the phytotron) was about 0.7. The measurements were carried out on plants grown for a long time under irradiation in the PAR rang with the predominant blue (PAR + BL) or red light (PAR + RL). The dynamics of photosynthetic indexes were investigated 0, 1, 2, and 3 hours after the light regime changed from PAR + RL to PAR + BL or from PAR + BL to PAR + RL. When plants were irradiated with a larger share of the red light PAR spectrum (PAR + RL), a lower rate of photosynthesis was observed compared to plants grown with PAR + BL, both at 400 μmol · m^{-2} · s^{-1} and at saturating light intensity (1200 μmol · m^{-2} · s^{-1}). A change in the PAR + RL spectral mode for PAR + BL resulted in an increase in the rate of photosynthesis, a slight increase in the effective quantum yield and non-photochemical quenching. When the light mode changed from PAR + BL to PAR + RL, photosynthesis rate, electron transport speed, non-photochemical quenching decreased compared to plants irradiated with PAR + BL, but no change was observed in the maximum and effective quantum yield. The specific effects of blue and red light on the activity of light reactions in photosynthesis and the rate of photosynthesis in changing spectral composition after long-term plant exposure to environmental factors, which we detected for the first time in this work, make it possible to better understand the nature of plant adaptation in natural growth conditions. In plant growing with LED lighting, this allows directional use of LED emitters of different spectral composition, given the duration of predominantly blue or red irradiation.

Keywords: spectral regime, photosynthetic apparatus, rate of photosynthesis, electron transport, non-photochemical quenching, light-emitting diodes, *Solanum tuberosum* L., potato

Intensity and spectral composition of light are important for regulating activity of photosynthetic apparatus, and plant growth [1, 2]. In natural conditions of plant growth, along with changes in the intensity of light in different periods of time, there is a change in the spectral composition of the incident radiation. In this manner energy in blue (BL, λ = 400-500 nm) and red (RL, λ = 600-700 nm) spectral regions is distributed in 1:3 ratio in daytime [3]. Proportion of red and

far red component as compared to the blue one is increasing in morning and evening hours making BL/RL value as 0.74. According to some papers [4-6], the ratio between energy of the blue and red spectral regions is changing from ≈ 0.50 (direct radiation) to ≈ 0.95 (diffuse radiation), depending on the solar altitude.

Light regimes affecting photosynthetic activity are major elements of artificial lighting technologies and controlled plant growing in phytotrons, with LED-based sources being actively used therewith. White LEDs [7] together with red and red-blue ones [8, 9], as well as with various combinations of blue, red and other spectral regions [10-12] are widely spread. Depending on plant variety, the reaction thereof to RL and BL ratio may differ [2]. RL/BL ratio in ontogenesis to be changed is also worth noting [13].

Nowadays one of main approaches in studying ever-changing environmental plant adaptation is comparison of dynamic features of adaptive processes [14, 15], with major attention being paid to effect or aftereffect of various factors in time intervals [16, 17]. Such approach enables to assess response of photosynthetic apparatus over time similar to natural environmental change within several hours. It should be noted that mechanisms of plant light adaptation are complicated and to be further analyzed for appropriate usage of LED sources of various spectral composition in plant growing.

Prolong effect of monochromatic spectral irradiation sources influencing potato plants grown in vitro [18] has been analyzed by us earlier, and we have found out that no significant differences in activity of light reactions of photosynthesis are seen under low light. Changes in accumulation of dry substance by plants therewith were stipulated by stomatal conductance, as well as by light-independent reaction processes in photosynthesis, as efficiency of carboxylation reaction, rate of triose phosphate utilization.

The special effect of blue and red spectral regions under plant irradiation with all spectral range of photosynthetically active radiation (PAR) originally presented by us in this analysis is that change of red light to blue light is increasing photosynthesis rate, quantum efficiency and non-photochemical quenching, while under changing of blue to red light we observe converse effects.

The paper investigated the activity of photosynthetic apparatus of potato leaves exposed for a long time to various spectral compositions, as well as under changing of radiation spectrum with prevailing red or blue area.

Techniques. Potato plants (*Solanum tuberosum* L.) of Zhukovsky Early variety were grown by aeroponics in two environmentally-controlled phytotron chambers. The plants were cultivated by apical meristem method and tested for viruses and potato viroid by RT-PCR applying appropriate kits (OOO NPK Sintol, Russia). Each chamber contained 15 plants, with vegetation period thereof being 60 days, and 12 h photoperiod.

Radiation intensity in chamber 1 was increased by blue LEDs (BL LEDs, λ_{max} = 470 nm), with red LEDs being switched off, thereby under total intensity of 400±28 $\mu mol \cdot m^{-2} \cdot s^{-1}$ the proportion of BL was 293.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Radiation intensity in chamber 2 was increased by red light (RL LEDs, λ_{max} = 660 nm), with blue LEDs being switched off, thereby under total intensity of 400±28 $\mu mol \cdot m^{-2} \cdot s^{-1}$ the proportion of RL was 262.0 $\mu mol \cdot m^{-2} \cdot s^{-1}$. BL/RL value (if radiation intensity in two phytotron chambers is compared) is within 0.7-09. Other microclimatic conditions inside the chambers remained constant (CO_2 concentration is 395 ±12 $\mu mol\ CO_2 \cdot mol^{-1}$, air humidity is 60-80 %, temperature is 24 ±1 °C).

The analyzed values (0, 1, 2, 3 hours) were measured with plants in two variations: under stationary light mode with prevailing blue light (PAR+BL) or red one (PAR+RL) and after changing the light mode from PAR+RL to

PAR+BL or from PAR+BL to PAR+RL.

Rate of photosynthesis in leaves was registered in the morning (at 10 AM) under light intensity of 400 and 1200 μmol · m^{-2} · s^{-1}. Dependence between the process rate and light intensity was being found within PAR range of 0-1200 μmol · m^{-2} · s^{-1} at natural CO_2 concentration in the air of 395 ±12 μmol CO_2 · mol^{-1}. Values of variable fluorescence of chlorophyll *a* characterizing activity of photosystem II (PS II) were being assessed therewith [19].

Rate of photosynthesis was measured by portable gas analyzer LCPro+ (ADC BioScientific, Ltd, Great Britain), variable fluorescence — by fluorometer PAM-Junior (Heinz Walz GmbH, Germany). Spectral values inside phytotron chambers were controlled by spectrometers ASENSEtek, PG100N, UPRtek (Taiwan). Light curve was approached to by J.L. Prioul & P. Chartier model [20].

The experiments were made with 4-5-fold analytical and 3-fold biological replication. Calculations were made by one biological experiment data. Statistical data were processed by Statistica 10 application (StatSoft, Inc., USA). Arithmetic mean values (*M*) with standard error (±SEM) are given in the figures. Statistical significance of differences was found by Student *t*-test at P = 0.95.

Results. Plant growing under full PAR spectrum with prevailing either red or blue radiation (Fig. 1) resulted in morphological values changing. Under PAR+BL the plants were characterized by shortened internodes and differentiated root system, while under PAR+RL the plants were higher with less differentiated root system. Growth values observed are due to functional activity of photosynthetic apparatus.

Fig. 1. Spectral characteristics of phytotron chambers. PAR+BL (blue light, above): total photon flux is

408.6 μmol · m^{-2} · s^{-1}, blue and red spectral regions are 293.6 and 48.19 μmol · m^{-2} · s^{-1}, accordingly; PAR + RL (red light, below): total flux is 407.0, red and blue spectral regions are 262.0 and 35.97 μmol · m^{-2} · s^{-1}, accordingly. PAR — photosynthetically active radiation.

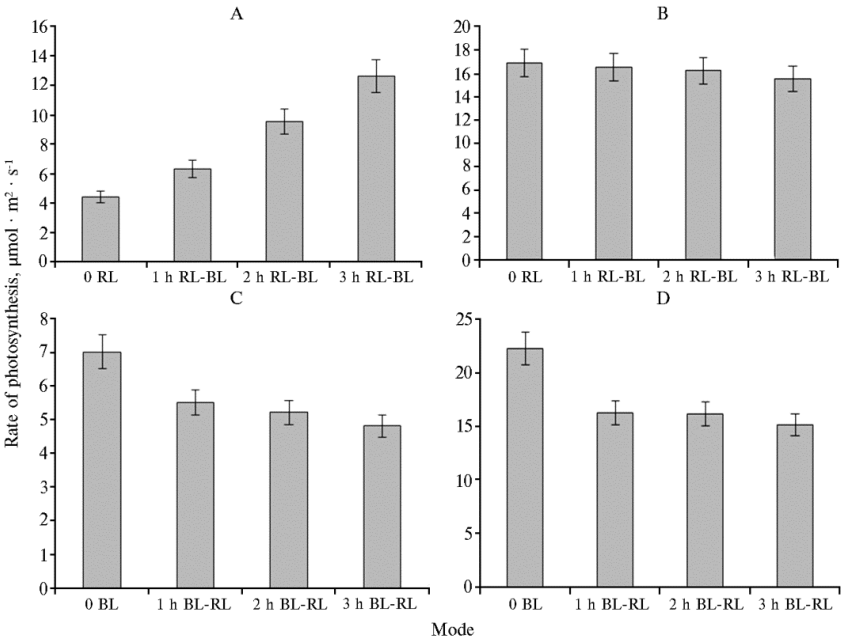

Fig. 2. Dynamics in CO_2-gas exchange in leaves of potato (*Solanum tuberosum* L.) Zhukovsky Early variety grown aeroponically at light intensity of 400 μmol · m^{-2} · s^{-1}, with prevailing red (RL, PAR+RL) **or blue** (BL, PAR+BL) **light after changing of prevailing light from one to another**: A, C — with no changing in light intensity (400 μmol · m^{-2} · s^{-1}), B, D —increase of light intensity to 1200 μmo l· m^{-2} · s^{-1}; PAR — photosynthetically active radiation.

Under PAR+RL as compared to PAR+BL the rate of photosynthesis was lower both at 400 μmol · m^{-2} · s^{-1} and 1200 μmol · m^{-2} · s^{-1} (Fig. 2). Mode changing from PAR+RL to PAR+BL resulted in increase in the photosynthesis rate at 400 μmol · m^{-2} · s^{-1} and in some increase at 1200 μmol · m^{-2} · s^{-1}. If light mode was changed from PAR+BL to PAR+RL at 400 μmol · m^{-2} · s^{-1} this value decreased ($p \leq 0.05$).

Light curves of plants photosynthesis at changing the spectral mode of irradiation were plotted together with analysis of prolonged exposure of PAR+RL and PAR+BL to photosynthetic apparatus activity. Change of mode from PAR+RL to PAR+BL resulted in increase in the rate of photosynthesis in the plateau of the light curve, increase of breathing rate and quantum yield, therewith saturation of light curve of photosynthesis being achieved at higher light intensity. On the contrary, when mode sequence was PAR+RL → PAR+BL, rate of photosynthesis and breathing and quantum yield were decreasing, while saturation of light curve took place at lower light intensity (Fig. 3).

Variations of photosynthesis rate observed are in particular due to different activity response in light stage thereof. According to analysis of variable fluorescence values the change of prevailing red light to blue one made no effect on the value of max quantum yield of photosystem II, but enabled to increase the rate of non-photochemical quenching and quantum efficiency ($p \leq 0.05$) (Fig. 4). The opposite reaction of photosynthetic apparatus (decrease in rate of electron transport and non-photochemical quenching) was noted at mode changing from PAR+BL to PAR+RL. Values of max and efficient quantum yields as compared to the ones in plants irradiated by blue LEDs remained the same (see Fig. 4).

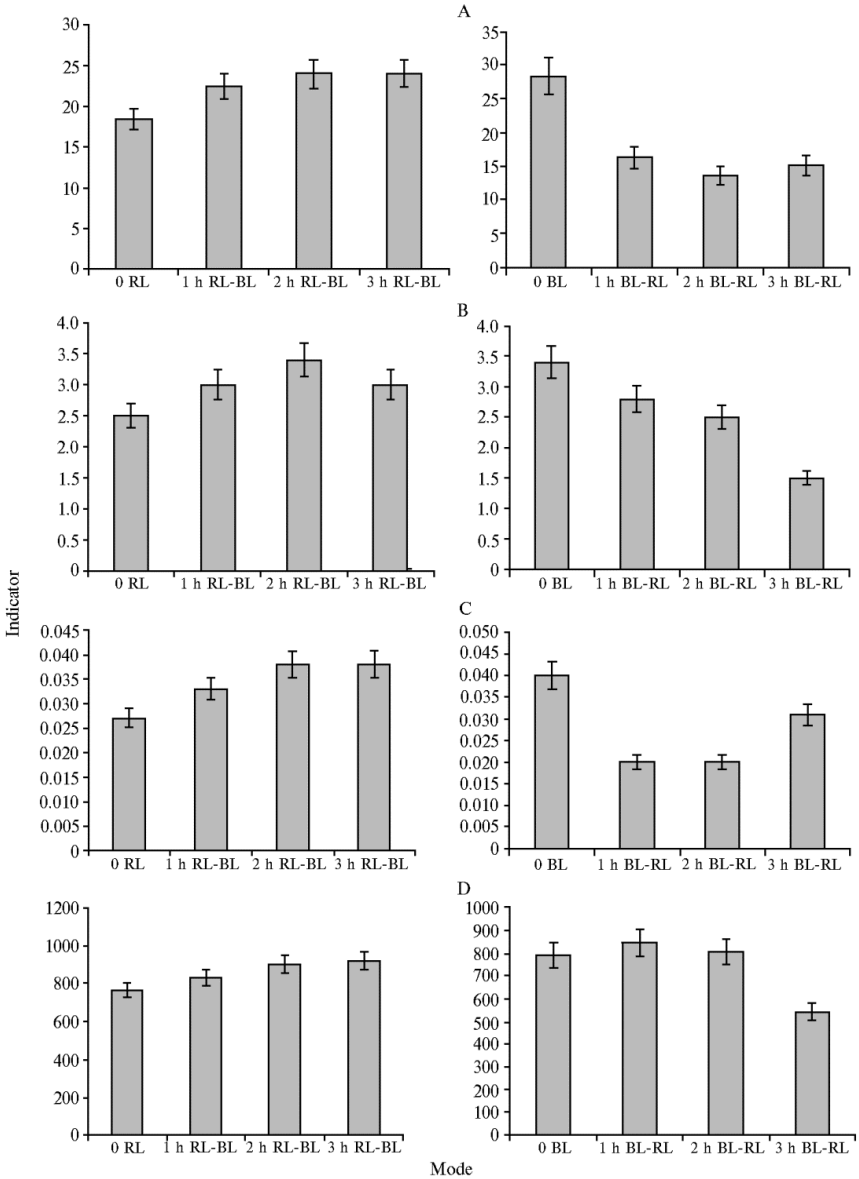

Fig. 3. Dynamics in CO_2 absorption rate, μmol · m^{-2} · s^{-1}, (A)**, breathing rate, μmol · m^{-2} · s^{-1},** (B)**, quantum photosynthetic efficiency, r.u.** (C) **and light intensity in the plateau of light curve, μmol · m^{-2} · s^{-1}** (D) **in potato (*Solanum tuberosum* L.) plants of Zhukovsky Early variety grown aeroponically at light intensity of 400 μmol · m^{-2} · s^{-1}, with prevailing red** (RL, PAR+RL) **or blue** (BL, PAR+BL) **light after changing of prevailing light from one to another** (RL-BL or BL-RL; PAR — photosynthetically active radiation).

PAR+RL and PAR+BL affecting the rate of photosynthesis observed in our experiments may be compared to data on photosynthetic apparatus decreased activity if plants are irradiated by monochromatic RL [21, 22]. Expansion of red or blue component in PAR spectrum noted by us resulted in no changes of max (see Fig. 3, A) and efficient (see Fig. 4, B) quantum yield of photosynthesis, electron transport rate (see Fig. 4, C), and rate of non-photochemical quenching (see Fig. 4, D). In this case the lower photosynthesis rate if irradiated by PAR+RL may be explained by lower activity of RuBisCO carboxylase enzyme, see paper by A.A. Tikhomirov et al. [2] and our study of monochromatic irradiation effect on potato plants [18].

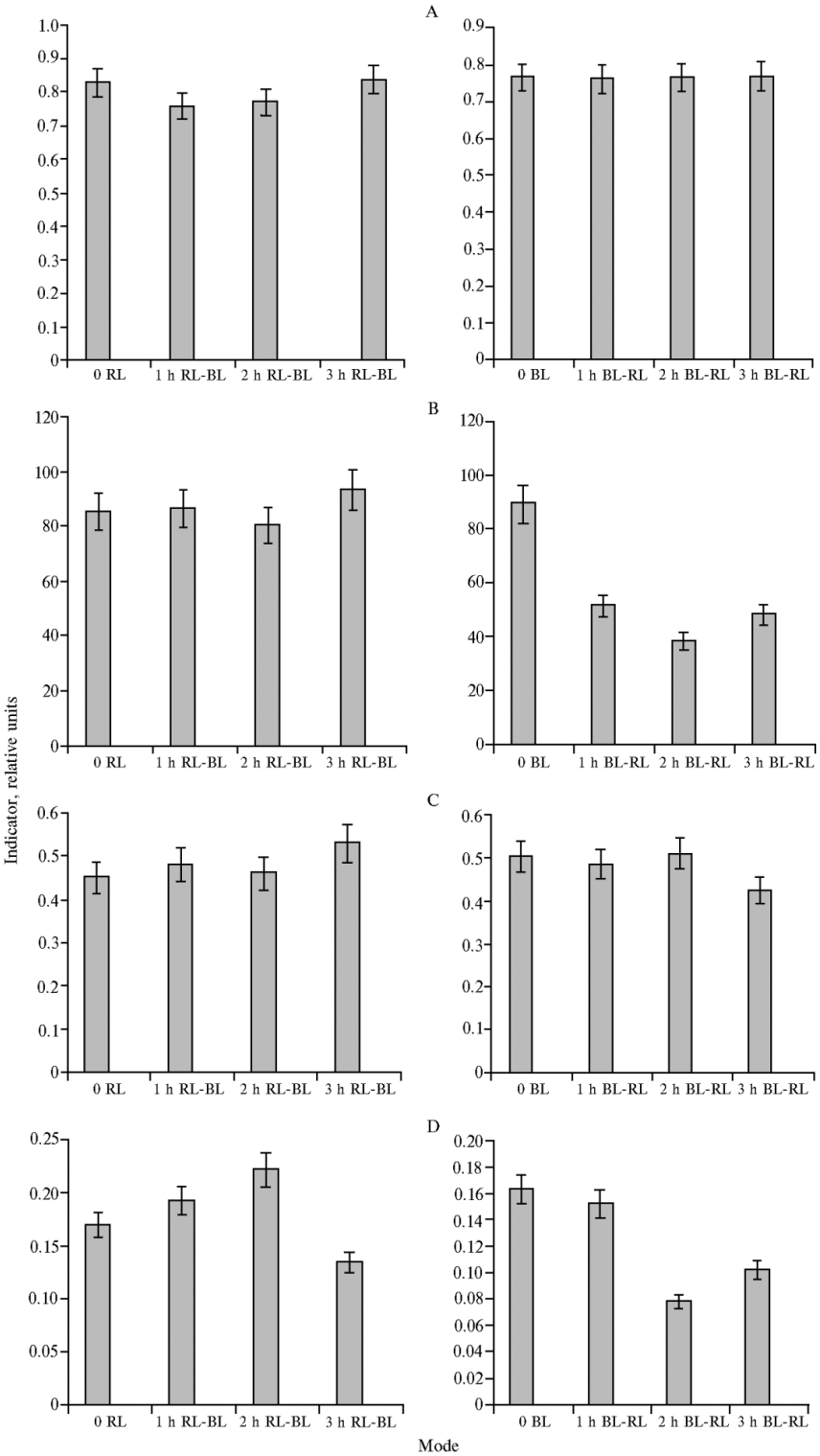

Fig. 4. Max quantum yield of photosystem II (A)**, quantum photosynthetic efficiency of photosystem II** (B)**, electron transport rate** (C) **and non-photochemical quenching rate** (D) **in potato (*Solanum tuberosum* L.) plants of Zhukovsky Early variety grown aeroponically at light intensity of 400 μmol · m^{-2} · s^{-1}, with prevailing red** (RL, PAR+RL) **or blue** (BL, PAR+BL) **light after changing of prevailing light from one to another** (RL-BL or BL-RL; PAR — photosynthetically active radiation).

Decrease in rate of non-photochemical fluorescence quenching (NPQ) in 3 hours after plants irradiation by PAR+BL may also indicate to increase of efficiency in using blue light in assimilation processes even at low value of BL/RL ratio. M. Košvancová-Zitovõ et al. [23] have noted high rate of photosynthesis induction at BL/RL ratio equal to 3:1. Decrease of blue (λ_{max} = 455 nm) or red (λ_{max} = 625 nm) light gave no differences in photosynthesis induction in leaves equally irradiated by BL and RL (1:1) and reducing BL in relation to RL (1:3). Significant changes in the rate of photosynthesis were found when changing the light mode of plant growing. Thus, 2-3-fold increase took place when changing PAR+RL to PAR+BL within 3 hours (see Fig. 2, A, C). Higher values of quantum yield of photosynthesis were obtained under the same conditions (see Fig. 3, C). At light saturation (1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) the rate of photosynthesis is actually remained unchanged (see Fig. 2, B, D) indicating high operational potential of photosynthetic apparatus produced by the plant irradiated mostly by red light in total flux. Therewith, one hour after changing of the light mode no significant activities of photosynthetic apparatus were seen.

Change of light mode from PAR+BL to PAR+RL resulted in decrease in the rate of photosynthesis at 400 and 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (see Fig. 2), as well as in decrease of dark respiration rate, the light intensity at saturation of the light curve, and quantum yield of photosynthesis (see Fig. 3). The observed changes in photosynthetic apparatus activity were due to reactions of photosynthesis light stage, i.e. delay in operation of electron-transport chain of chloroplasts (see Fig. 4, C). Decrease of electron transport rate and that of ATP synthesis [24] as a result thereof may be accompanied by RuBisCO decreased activity [25]. In this case reduction of inefficient energy loss (NPQ) may level the changing activity effect of reactions of photosynthesis primary stage.

Spectral distribution of light within irradiation sources affects the rate of photosynthesis both directly and indirectly, via imposition of effects from various parts of spectrum. A leaf is regulating the distribution balance of excitation energy between photosystems in response to relative spectral distribution of light [26]. As a result, irradiation of plants by either blue or red light with higher or lower intensity along the whole PAR area affected differently on the rate of reactions of light and dark photosynthesis stages.

Therefore, changing of red light to prevailing blue one is shown to result in increase of the rate of photosynthesis, effective quantum yield and non-photochemical quenching, while the effects are opposite when substituting blue light to red one. The found specific effects of blue and red light on the activity of light reactions in photosynthesis and the rate of photosynthesis in changing spectral composition after the plants are light-exposed for a long time, enable us to better understand the nature of plant adaptation in natural growth conditions. The obtained results may be used at programmed growing of plants in various indoor facilities.

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ANTHER-DERIVED CALLUS FORMATION IN BITTER MELON (*Momordica charantia* L.) AS INFLUENCED BY MICROSPORE DEVELOPMENT STAGE AND MEDIUM COMPOSITION

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Abstract

Anther culture is one of the powerful techniques to pure line production for the short time. This method can be applied to accelerate the breeding process of F_1 hybrids of bitter melon (*Momordica charantia* L., family *Cucurbitaceae*), which is an important commercial crop in tropical and subtropical countries of South America, Asia and Africa. Although, there are several factors affecting the success of this method, the effective protocol of bitter melon anther culture has not been developed at present. The results of this study showed that the microspore developmental stage has a significant ($p \leq 0.05$) effect on speed and rate of callus formation. It was revealed that the frequency and the rate of callus formation and the morphology of callus substantially depend on the composition and concentration of growth regulators in the nutrient medium. In addition, the dynamics of callus formation in bitter melon anther culture was first studied. The main objective of the work was to study the influence of the microspore developmental stage and the composition of nutrient medium on the ability of callus formation in bitter melon anther culture in vitro. Plants of the F_1 hybrid bitter melon Diago 26 were grown in field in Dailoc district, Quangnam province (Vietnam) in 2018 according to the standard technique for obtaining commercial fruits. The buds harvested at 5-7 am were stored in plastic bags in dark condition at 4 °C for 1 day. Before culturing of anther, flower buds were surfaced sterilized using 70 % ethanol and 5 % sodium hypochlorite (NaOCl). Anther removed from flower buds were inoculated in the induction medium in the horizontal laminar air flow; then incubated at 25±2 °C and a photoperiod of 16 h light/8 h dark for 4 weeks. Three variants of the Murashige-Skoog (MS) nutrient mediums were used, differing in composition and concentration of growth regulators: MK1 with the addition of 1.0 mg/l 2.4-D and 1.5 mg/l BAP; MK2 — 1.5 mg/l 2,4-D and 1.0 mg/l BAP; MK3 — 1.5 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l kinetin. The completely randomized design with statistical analysis using the software IBM SPSS Statistics Base 22 and Microsoft Excel 2013 were carried out. The cytological analysis result showed that each anther contained microspores with different development stages. Therefore, it is impossible to separate microspores at the unique developmental stage being suitable for anther culture; however, there is always a predominant stage. In this study, buds were divided into two main groups. The first group consisted of buds with the size of 4.0-5.0 mm with early and mid uninucleate microspores. The second group included buds with the size of 5.1-6.5 mm with late uninucleate and binucleate microspore. After inoculation, the beginning of calli formation was observed within the first week for anther of the buds with the size of 4.0-5.0 mm and within the second week for the buds with the size of 5.1-6.5 mm. The highest frequency of callus formation (93.75±2.55 %) was observed on MS medium supplemented with 1.0 mg/l of 2,4-D and 1.5 mg/l of BAP (MK1). Most calli were formed during the second and third week after cultivation. Also, there was the significant difference about the morphology of the calli obtained on three nutrient medium. Calli on medium MK1 were yellow, strongly dense and calli on MS medium with the addition of 1.5 mg/l of 2,4-D and 1,0 mg/l of BAP (MK2) being green, strongly dense. Green-yellow, dense calli were obtained on medium supplemented with 1.5 mg/l NAA and 1.0 mg/l BAP and 0.5 mg/l Kinetin (MK3). However, the effect of

the developmental stage of microspores on the morphology of calli was not revealed. Despite receiving a large number of calli, the formation of embryoids was not observed

Keywords: *Momordica charantia* L., bitter melon, callus, doubled haploid, anther culture, microspore stage

Bitter melon (*Momordica charantia* L.) is an economically important crop distributed widely in tropical and subtropical countries in South America, Asia and Africa [1, 2[. This crop has been cultivated throughout the year in Asia countries [3]. The area of bitter melon is about 340.000 ha, most of the production is concentrated in China and India [4]. The bitter melon fruit is used not only as a kind of vegetable, but also applied in medicine for treatment of various diseases including diabetes, gout, cancer [5, 6]. Chemical constituent analysis proved that *Momordica charantia* L. contains saponins (momordisin, momordin), which have shown promising biological activities including antibacterial, antifungal, antiviral, insecticidal effect [7].

Currently, about 80% of bitter melon seeds using in production are hybrids [8]. However, the range of bitter melon hybrids remains limited due to the complicated and prolonged breeding process. Nowadays the obtaining of F_1 hybrids is mainly depends on the pure lines production, while the process of pure lines production is very complicated [9]. By contrast for traditional selection methods, in with self-pollination for 5-7 generations has been carried on to obtain pure lines [10, 11], the technology of double haploids (DH) production can be applied to speed up the breeding process [12, 13]. DH plants have been obtained from more than 200 species. This technology is especially widely applied on cabbage and grain crops [14, 15]. One of the first and most common methods of DH production is anther culture. It is the singular method of DH plants receiving for some crops. The success of this technology depends on many factors, such as genotype, developmental stages of microspores, nutrient medium composition, addition of growth regulators, culture conditions, and the way of plant regeneration from callus [16]. Therefore, the anther culture method needs to be optimized for each crop, even for each genotype, especially in vegetable crops [17].

Researchers, cultivated the bitter melon anthers, received callus, but the plants generation from obtained callus could not be achieved [18-20]. Tang et al. [19] found that callus were formed from the anthers after flower buds pretreating whis low temperature of 4 °C for 48 hours and cultured on Murashige-Skoog medium (MS) with the addition of 3% sucrose, 0.5 mg/l 2.4-D (2.4-dichlorophenoxyacetic acid) and 2.0 mg/l BAP (6-benzylaminopurine). Supplementing vitamin C and $AgNO_3$ resulted in reducing of necrotic callus frequency [19].

There are few published studies that describe callus formation in the bitter melon anther culture, but there is still no clear opinion about the effect of a number of factors.

In the present research, we showed that the size of the flower buds, that is the stage of microspores development, affects significantly ($p \leq 0.05$) the ability of callus formation. We found out, that microspores of different developmental stages can be contained in the same anther. It was revealed that the calluses morphology, the frequency and the time of callus formation in bitter melon anther culture substantially depends on the composition and concentration of growth regulators in the nutrient medium. Besides, the dynamics of callus formation in the process of the bitter melon anthers cultivating was studied for the first time.

The aim of the present research was to study the effects of the microspore developmental stage and the nutrient medium composition on the callus formation in anther culture in vitro.

Techniques. The F_1 hybrid Diago 26 bitter melon was used in this re-

search, seeds producer company is Tropical Development and Investment Company Limited (Công ty trách nhiệm hữu hạn LLC), Vietnam. The plants were grown in 2018 on the farm in Dailoc district, Quangnam province, Vietnam, according to standard technique for commercial fruits obtaining. The flower buds were harvested in the morning at 5-7 am and stored in plastic bags at 4 °C for 1 day.

The developmental stage of microspores was monitored by microscopy using acetocarmine dye [21]. Microspores were isolated from the buds in a drop of glycerin by the clamp on a glass slide. Then they were transferred to a new glass slide containing a drop of 2% acetocarmine solution, covered by a cover glass and observed at magnification ×400 (Axio Imager.M2 microscope, Carl Zeiss, Germany). The developmental stage of microspores was determined by the number of nuclei and their location in the cell [22]. To assess the relationship between the size of the buds and the stages of microspores development, the buds were measured in sizes from 4.0 to 7.0 cm with a distance of 0.1 cm. 450 microspores from three anthers were observed in each group.

Before culturing of anther, flower buds were externally sterilized with 70% ethanol for 30 seconds and sterile distilled water for 15 seconds twice; then buds were immersed into 5% sodium hypochlorite (NaOCl) for 4 minutes and rinsed with sterile distilled water 5 times for 15 seconds. The anthers were taken from buds using tweezers (the filaments were tried to be removed) and placed on the induction medium in plastic Petri dishes of 6 cm in diameter, 8 anthers per dish, in the conditions of horizontal laminar air flow. Anther were incubated at 25±2 °C and a photoperiod of 16 h light/8 h dark for 4 weeks.

In this experiment, three variants of nutrient media based on the Murashige-Skoog (MS) nutrient medium were used. Nutrient media contained 3% sucrose and 0.7% agar (pH 5.8), variants differed in composition and concentration of growth regulators, i.e. 1.0 mg/l 2,4-D and 1.5 mg/l BAP for MK1, 1.5 mg/l 2,4-D and 1.0 mg/l BAP for MK2, 1.5 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l kinetin for MK3.

The effectiveness of callus formation was assessed by the ratio of the number of formed calluses and cultivated anthers, as well as by the color and density of callus. All the experiments were performed in 4 replications, the number of anthers in each replicate was 24.

The completely randomized design with statistical analysis using the software IBM SPSS Statistics Base 22 (IBM Corporation, USA) was carried out. The mean values of the studied parameters (M), standard errors of the mean (±SEM) and confidence interval were determined at 95% confidence level ($t_{0.05}$ × SEM). The significant difference between the variants was evaluated by Student's t-test. The significant test was set at $p \leq 0.05$. Graphs were built with Microsoft Excel 2013.

Results. The developmental stage of microspores is the key factor determining the success of anther culture technology [23]. Each stage corresponds to a definite range of buds size [24]. Therefore, the relationship between the buds size and the microspore developmental stages was investigated in order to select the suitable buds of the F_1 hybrid Diago 26 bitter melon for following culture.

As shown in figure 1, the single anther contains microspores at different developmental stages, from early uninucleate to binucleate. Therefore, it was impossible to select bitter melon buds with microspores at the definite developmental stage. The ratio of microspores at different developmental stages depended on the bud size. In buds of the same size this ratio was similar. We also observed that it was always possible to detect the predominant developmental stage (Fig. 2).

Cytological analysis of anthers found that the buds with the length of 4.0-4.5 mm contained microspores at four developmental stages, in which, the early uninucleate stage was predominant accounted for 60.19±2.32%, the percentages of microspores at mid uninucleate, late uninucleate and binucleate stages were 24.08±0.98%, 14.99±1.8%, 0.74±0.74%, respectively. For the 4.6-5.0 mm buds, there were also four stages of microspores development with the prevalence of the mid uninucleate stage stood at 71.98±0.42%. Therefore, it could not be separated the anthers with the unique development stage of microspores; however, it was possible to separate the anthers containing microspores at the predominant stage of optimal development. The anthers of the 5.1-6.0 mm long buds were found to contain microspores only at two stages (late uninucleate and binucleate), mostly at binucleate stage (82.64±0.59%). The buds with the length of 6.1-6.5 mm and 6.6-7.0 m had respectively 65.54±4.26%, 88.71±2.54% of binucleate microspores.

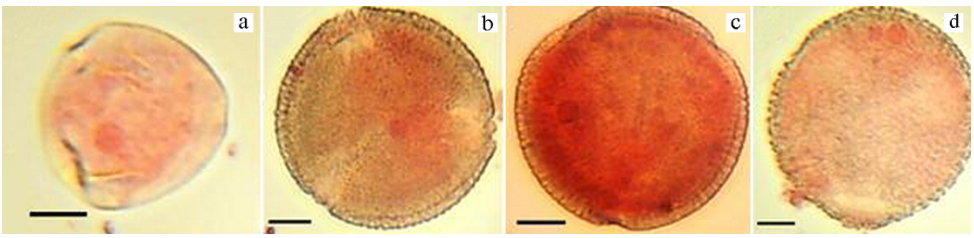

Fig. 1. The stages of microspores development of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26: a — early uninucleate, b — mid uninucleate, c — late uninucleate, d — uninucleate (Axio Imager.M2 microscope, Carl Zeiss, Germany; magnification ×400, scale bars = 10 μm).

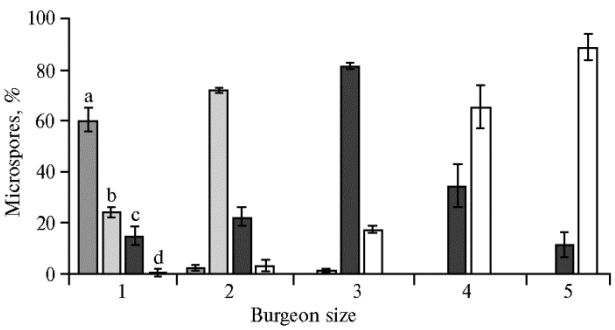

Fig. 2. The percentages of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 microspores at early uninucleate (a)**, mid uninucleate** (b)**, late uninucleate** (c) **and binucleate development stages in the buds of different sizes:** 1 — 4.0-4.5 mm, 2 — 4.6-5.0 mm, 3 — 5.1-6.0 mm, 5 — 6.6-7.0 mm.

The obtained in the present study results were different from previously published by other researches. As Tang et al. [18] reported, the buds size corresponds to a specific microspores developmental stage: 3.0-4.0 mm length bud contains early uninucleate microspores, 3.0-4.0 mm buds contain late uninucleate microspores, binucleate stage being observed at bigger buds (above 5.0 mm). This difference could be explained by a genotype specific relationship between the bud size and the developmental stages of anther. The same conclusions were also obtained for other crops [25]. Therefore, in order to achieve the best result of anther culture, it was necessary to determine the relationship between the bud size and the microspore development stages corresponding to each genotype.

To study effects of the developmental stages of microspores on the efficiency of callus formation, flower buds of two size ranges were cultivated in vitro: 1) 4.0-5.0 mm buds that contained early uninucleate and mid uninucleate microspores; 2) 5.1-6.5 mm buds with uninucleate and binucleate microspores. Two anther groups were cultured at three nutrient media, MK1, MK2 and MK3, with different concentrations and compositions of growth regulators. The result of the research revealed that the supplement of growth regulators and the

bud size had significant effects ($p \leq 0.05$) on the efficiency of callus formation in bitter melon anther culture; there was no effect of interaction of these factors. This finding is consistent with previous researches on the bitter melon as well as on other crops [18, 26, 27]. Using the buds containing early uninucleate and late uninucleate microspores induced the high frequency of callus formation. The average frequencies of callus formation were 79.17±4.57%, 68.23±4.00% when 4.0-5.0 mm of and 5.1-6.5 mm buds were used.

The results of the table show that callus formation was the most effective on MK1 medium with the 4.0-5.0 mm long buds. The frequency of callus formation was 93.75±2.55%. This is higher than the best result of Tang et al. [19] who reached 80.55% in culturing anthers on MS medium supplemented with 0.5 mg/l 2,4-D and 2.0 mg/l BAP. There was no significant difference between MK1 medium and MK3 medium. Culturing on MK2 medium resulted in the lowest frequency of callus formation (51.56±3.93%). Furthermore, there was no interaction effect between medium composition and bud size on the frequency of callus formation.

Effects of bud size and medium composition on callus formation in anther culture of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 (*M*±SEM)

Medium	Plant growth regulators, mg/l				Callus formation		Callus morphology
	2,4-D	BAP	K	NAA	percentage, %	starting time	
4.0-5.0 mm buds							
MK1	1.0	1.5	–	–	93.75±2.55[a]	week 1	Yellow, strongly dense
MK2	1.5	1.0	–	–	60.94±5.33[be]	week 1	Green, strongly dense
MK3	-	0.5	1.0	1.5	82.81±2.99[af]	week 1	Green-yellow, dense
5.1-6.5 mm buds							
MK1	1.0	1.5	–	–	73.44±2.99[cbf]	week 2	Yellow, strongly dense
MK2	1.5	1.0	–	–	51.56±3.93[de]	week 2	Green, strongly dense
MK3	–	0.5	1.0	1.5	79.69±2.99[af]	week 2	Green-yellow, dense

N o t e. For description of the media composition, see the *Techniques* section. 2,4-D is 2,4-dichlorophenoxyacetic acid, BAP is 6-benzylaminopurine, K is kinetin, NAA is 1-naphthaleneacetic acid. Different alphabets indicate statistically significant differences at $p \leq 0.05$.

Therefore, anthers of the F_1 hybrids Diago 26 bitter melon are able to form callus when containing microspores at various developmental stages. The early uninucleate and mid uninucleate stages are the most suitable, though late uninucleate and early binucleate stages are the best for *Brassicaceae* DH technology [28] and mid uninucleate and late uninucleate stages are the most suitable for cucumber [29, 30]. Tang et al. [18] proved that culturing of bitter melon anthers containing late uninucleate microspores resulted in higher efficiency of callus formation comparing to late uninucleate.

The date of start of callus formation varied and depended on the predominant stage of microspore development. For small size buds, calli formation could be observed within the first week of culture. Meanwhile, calli formation was found on the second week for larger buds. So, there sizes of calluses can be different on the same medium. It is very important to determine the time of callus formation for further sub-culturing or plant regeneration.

It was also found, that the rate of callus formation varies due to culture period. On MK1 medium, there was a difference between the buds of different sizes, and the largest number of callus was formed within the second and the third week (Fig. 3). On MK2 and MK3 medium, the highest callus formation frequency was within the second week. The callus formation was asynchronous. So, there were different sizes of calluses even at one Petri dish, in one bud passage.

There was also a significant difference in calli morphology depending on concentration and composition of growth regulators in nutrient medium after 4

weeks of culturing. Calluses on MK1 medium were yellow, strongly dense, calluses on MK2 medium were green, strongly dense and green-yellow, dense calluses were obtained on MK3 (Fig. 4). By contrast, effect of development stages of microspores on callus morphology was not observed.

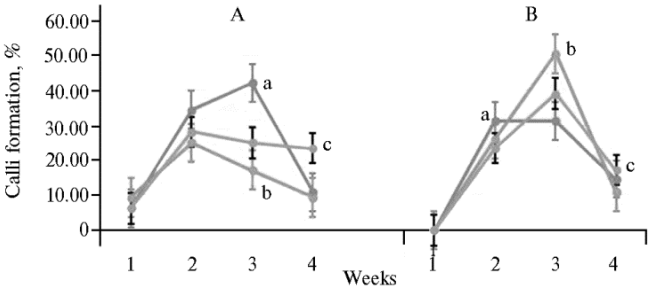

Fig. 3. The rate of callus formation in bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 derived from microspores of 4.0-5.0 mm (A) **and 5.1-6.5 mm** (B) **buds on MK1 medium** (a)**, MK2 medium** (b) **and MK3 medium** (c) **depending on culturing time** (for description of media composition, see the *Techniques* section).

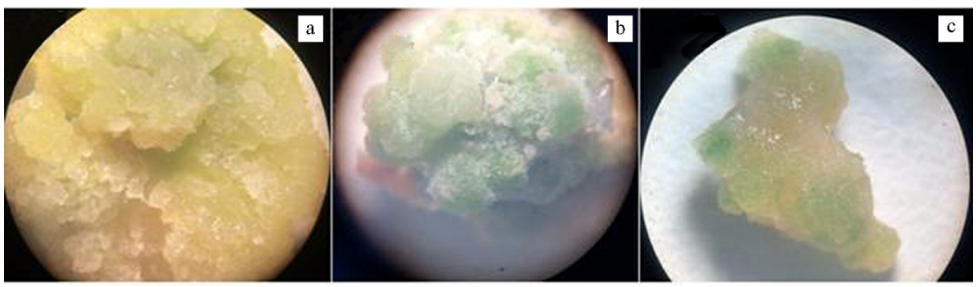

Fig. 4. Callus morphology of bitter melon (*Momordica charantia* L.) F_1 hybrid Diago 26 on different media after 4-week culturing: MK1 (a), MK2 (b), MK3 (c) (Axio Imager.M2 microscope, Carl Zeiss, Germany; magnification ×40; for description of media composition, see the *Techniques* section).

The time when callus formation began and growth rate of calli have not been mentioned in previous studies which have just focused on general calli formation time. However, the period of callus formation able to plant regeneration can vary depending on genotype and medium composition. For example, cucumber calli can form yellow embryoids after 6 weeks of culturing [12, 31], and this period can take 4 months for banana [32].

Although the high frequency of callus formation was obtained on all media, the formation of embryoids was not observed. Culturing for more than 4 weeks caused the calli to turn brown. This finding was consistent with the results of Tang et al. [20]. It is possible that the momordica callus cells accumulate secondary metabolites which prevent embryoid formation and organogenesis. By comparing concentration ratio of endogenous growth regulators in callus cells (gibberellic acid — GA3, zeatin, indole-3-acetic acid — IAA, abscisic acid — ABA), Tang et al. [20] found that stem tissues had higher concentration of Zeatin than callus formed from anthers. Besides, the regeneration occurred only in calli formed from stem tissues. In addition, callus able to plant regeneration had the lower ratios of IAA/zeatin and GA3/zeatin in [20].

Thus, the results of this study proved that the concentration of growth regulators significantly affects the rate, frequency of callus formation, and the callus morphology in anther culture of bitter melon (*Momordica charantia*) F_1 hybrid Diago 26. It has been established that one anther contains microspores at different developmental stages. The significant difference in the callusogenesis ability was revealed between the anthers containing early uninucleate, mid uninucleate microspores and those containing late uninucleate and binucleate microspores. For the highest frequency of callus formation (93.75%), anthers of flower buds of 4.0-5.0 mm length should be cultured on MS medium with 3%

sucrose and 0.7% agar supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 1.5 mg/l 6-benzylaminopurine.

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THE TRAITS DETERMINING PLANT LODGING AND ASSESSMENT OF LODGING RESISTANCE IN INTENSIVE AND EXTENSIVE RUSSIAN RICE (*Oryza sativa* L.) VARIETIES

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Abstract

Lodging is one of the main causes of rice crop loss due to adverse effects on photosynthesis and plant productivity. Plant bend hinders illumination and makes it difficult for the plastic substances to flow out of the stem and leaves to the panicle. This worsens grain filling, technological and sowing qualities. Lodging restricts potential productivity of rice varieties. The resistance of a variety to lodging depends on its genotype, the strength of the stem tissues, and the growing conditions. Insufficient stability of rice plant stems occurs when the crops are thickened or subjected to high nitrogen supply, deep water in the rice field, increasing dynamic loads due to sprouting, wind, rain, dew and diseases. The objective of this study was to determine morphophysiological traits causing resistance to lodging of rice (*Oryza sativa* L.) intensive and extensive varieties with a focus on th use of laboratory method for express estimates of lodging resistance. The studied Russian rice varieties were Rapan, Vizit, Gamma of intensive type and Sonata and Atlant of extensive type. Plants grew in concrete micro-check plots filled with soil from rice check plots in which rice irrigation mode was the same as in field conditions. The fertilizers, as ammonium sulphate, superphosphate and potassium chloride, were applied at $N_{24}P_{12}K_{12}$ and $N_{36}P_{18}K_{18}$ dosages. The study showed that during tillering to booting of intensive varieties Rapan, Vizit, Gamma, photosynthesis assimilates are more used for the formation of generative organs and less vegetative, resulting in high productivity of panicle, but less lodging resistance. Varieties of extensive types, Sonata and Atlant, during tillering—booting period use more for stem formation and less for panicle productivity elements formation. This leads to a decrease in panicle productivity and yield, while the resistance of sowings to lodging increases due to higher strength of the lower internodes. To quantitate lodging resistance of rice varieties, we measured mechanical resistance of lower part of stem, including the first and the second culm internode, to bend. This index averages 56-63 g for the intensive varieties, 66-80 g for extensive varieties 66-80 g, and correlates with lodging rate of tested genotypes under field conditions at $r = -0.99$ ($p \leq 0.050$). The increase in cellulose content per unit stem length and lower internodes resistance are the main traits for reducing the lodging of rice plants.

Keywords: *Oryza sativa* L., rice, intensive varieties, extensive varieties, panicle productivity, lodging resistance, cellulose, bending resistance, yield

Rice (*Oryza sativa* L.) is one of the most widely consumed staple foods for a large part of the world's human population. As of 2014-2015, the rice planting area in 116 countries was 160 mln hectares with yearly output about 740 mln tons, to be 781 mln tons by 2020 according to FAO forecasts (keeping the planted area) thereby exceeding 2-3 % demand for wheat [1, 2]. In 2018, the State Register for Selection Achievements to be applied was amended with 57 rice varieties, including 31 varieties being selections of All-Russian Rice Research Institute. Meeting the increasing demands in current climatic changes is

due to highly efficient technologies and varieties [3-5] with improved biotic and abiotic stress tolerance, quality and productivity [6, 7]. Potential rice yield has significantly increased after the "green revolution" through appearance of semi-dwarf varieties (1950s), three-way cross hybrids (1970s) and superhybrid rice (1996) [8, 9] with powerful heterosis [10] and improved donor-acceptor bonding [11], thereby enabling extra 12 % rise in the yield as compared with usual hybrids and inbred varieties of this crop. Besides, super-rice cultivation technology was improved [10].

Lodging is one of causes reducing productivity of the majority of cereals, sometimes resulting in productivity reducing almost twice especially with high-yielding rice variety [12-15]. Excessive nitrogen application, high density of planting, and global warming are demonstrated to result in lodging [16]. Heavy lodging is reducing transpiration, transport of nutrients and assimilants through xylem and phloem thereby reducing using thereof at grain-filling [17]. Besides, the lodged plant leaves may become favorable environment for diseases affecting the crop and grain quality because of high humidity [18].

Dwarfing genes were introduced into wheat and rice varieties to reduce plant height for better lodging resistance and crop yield [19-21]. However, low height may decrease photosynthetic activity and biomass of plant stands [22, 23]. Reducing the plant height by 6 cm is resulting in reduction of potential yield by 1 t/ha [24]. The analysis made has confirmed competitive connections between potential yield and lodging resistance. This problem in rice cultivation is partially solved by applying growth regulators and retardants [25, 26].

Strength of cereal stem is defined first of all by morphological characters and anatomic plant structure, with the latter being the result of growth and development at the cellular level and depends on environmental effects [27]. Shadowing of the corn results in reducing of the mechanical tissue thickness, amount of vascular bundles, xylem and phloem area [28], morphoanatomical changes are accompanied by high stem fragility [29]; longer and slender stem with lower density of tissue is formed in wheat at high density of crop thereby affecting lodging resistance [30]. Rice and wheat with high stability of stems and lodging resistance are characterized by larger outer diameter, stem wall and mechanical tissue thickness, large amount of big and small vascular bundles [31, 32]. However, A. Kelbert et al. [33] claims that thickness of extraxylary tissue of wheat varieties is not connected with resistance to lodging. Stem fracture strength, weight of the 2nd low internode, weight of grain in the main spike or plant are shown to be reducing at lodging of winter rye plants. Variability of these characteristics is largely due to degree of lodging than genotype. These characteristics are recommended to be used as selection criteria to evaluate resistance to lodging because of strong conjugation between elements of productivity, morpho-anatomical, dynamic stem features and degree of lodging thereof [34, 35].

Optimum nitrogen application is increasing rice yield [36, 37], but excessive application thereof may result in lodging [38, 39]. High nitrogen quantities reduce lodging resistance of rice and wheat by increasing a number of shoots, length of low internodes, plant height, reducing the dry weight per 1 cm of stem [40, 41]. The lodging is shown to be reduced by applying less nitrogen, i.e. risk of rice lodging proves to be low at average rate of nitrogen consumption [42-44].

To make selection of new high-yielding varieties considering anatomic and biochemical features of rice plants one should get such data from original parents and hybrid progeny thereof [13, 14]. Data on anatomic inherited features defining degree of lodging [45, 46] and variability thereof in hybrids of the first and next generations are also required. Additionally simple and informative

methods for analyzing both anatomic and biochemical features of rice plants are to be applied.

Morphophysiological traits causing greater lodging of Russian intensive and extensive varieties of rice depending on nitrogen status were originally demonstrated herein. The resistance is stipulated by low cellulose content in stems and, thereby weak mechanical bend strength thereof. Rice samples were assessed to lodging by loading causing stem bending closely connected with degree of lodging.

The objective of this paper was to analyze morphophysiological traits causing resistance to lodging of intensive and extensive varieties of rice (*Oryza sativa* L.).

Techniques. Micro-plot experiments were made in 2012-2015 [47]. Russian rice varieties Rapan, Vizit, Gamma (intensive type) and Sonata and Atlant (extensive type) were compared. The experiments were made in reinforced concrete tanks maintaining irrigation schedule typical to field conditions, with planting taking place on May, 5-7, and harvesting on September 1-5. The tank (3.6 m^2) was filled with meadow chernozem soil taken from rice irrigation system of All-Russian Rice Research Institute. Inorganic nutrition status are $N_{24}P_{12}K_{12}$ (optimum) and $N_{36}P_{18}K_{18}$ (high) (per 1 m^2). The plot area in the experiments was 1.2 m^2 with 3-fold replication. Density of plant crop is 300 per m^2.

Amount of shoots in certain plots was counted. During flowering period, the plants were taken to determine dry weight and weight of certain organs: leaves, stems and panicles. The crop area with lodged plants (as a percentage of total plants in the plot), with stem resistance to bend and content of cellulose in tissue thereof as well as crop yield, were considered [48] during full grain maturity stage. Stem strength was measured on day 28 of flowering start. Main shoots (by 10) were taken in 3-fold replication, with the low part (12 cm) including 1st and 2nd culm internodes cutting off, with no leaves on. Low part of the sample was support-fixed horizontally, the free end thereof being 30° bent using weight set and calculating weight of loading. Mean value thereof was calculated for assessing correlation with resistance to lodging.

The results were subjected to two-way ANOVA and regression analysis, arithmetical mean (M) and standard error of the mean (±SEM) were calculated with Doc Statpak program [49]. Statistical significance was found by Student's t-test, the results were considered significant at $p \leq 0.05$.

Results. Temperature conditions in the analyzing years were similar to long-time average annual values. The starting period with average temperature above 10 °C was May 7-18, the ending period was September 17-30, length being 122-146 days, sum of positive temperatures was 2,300-3,000 °C. Deviation from average monthly temperature in July did not exceed 2.0-3.5 °C.

Plant lodging of all analyzed varieties was seen under both optimum and high nutrient status, in Rapan and Gamma varieties being extremely high in the latter case. It should be noted that plant height has no statistically significant connection with the degree of lodging [50]. Varying resistance to lodging occurs as a result of unequal intensity of biosynthesis of gibberellins and auxins when applying average and high amount of nitrogen fertilizers.

Cellulose is the main component of cell walls and fiber vascular bundles responsible for the strength of skeletal tissues of the culm. In thick planting and at high nitrogen application the biosynthesis thereof is weakened thereby resulting in less cellulose in stems [51]. The amount thereof in the unit of stem length was decreasing under such conditions due to enhanced cell extension lengthwise against crosswise size thereof [52]. Changes in cellulose content in stems were

accompanied by plant lodging. Various resistance of rice varieties to lodging, first, was due to unequal cellulose content in stems to be defined by genotype and, second, unequal response to excessive nitrogenous nutrition (Table 1).

1. Content of cellulose in stems, resistance to bend and degree of lodging in rice (*Oryza sativa* L.) varieties at full maturity depending on inorganic nutrition dosage (*M*±SEM, 2012-2015)

Variety	Type	Cellulose, %	Cellulose per 1 cm stem length, mg/cm	Stem resistance to bend, g	Lodging, %
			$N_{24}P_{12}K_{12}$		
Rapan	I	31.27±0.58	4.45±0.05	60.00±0.71	43,30±2,35
Vizit	I	30.89±0.57	4.55±0.05	62.50±0.75	36,70±2,36
Gamma	I	29.70±0.55	4.79±0.05	60.00±0.73	40,00±2,04
Sonata	II	31.87±0.86	5.85±0.06	79.80±1.18	1,00±0,04
Atlant	II	32.40±0.87	5.01±0.06	73.80±0.90	13,30±0,95
r with degree of lodging		–0,70±0,25	–0.92±0.22	–0.99±0.04	
			$N_{36}P_{18}K_{18}$		
Rapan	I	28.41±0.54	4.19±0.04	56.20±0.88	53,30±2,39
Vizit	I	33.36±0.88	4.43±0.05	58.80±0.90	43,30±1,69
Gamma	I	29.40±0.55	4.40±0.05	57.50±0.95	50,00±2,04
Sonata	II	29.49±0.56	5.06±0.06	72.30±1.17	6,70±0,28
Atlant	II	29.01±0.53	4.50±0.06	66.40±0.91	23,30±1,67
r with degree of lodging		0,10±0,57*	–0.92±0.23	-0.99±0.03	
LSD_{05}		1,67	0.17	3.4	3.27

N o t e. I is intensive, and II is extensive variety; *r* — correlation coefficient.
* Unreliable value; other correlation coefficient values are statistically significant at $p \leq 0.05$.

Content of cellulose in stems of intensive Rapan, Vizit and Gamma varieties at $N_{24}P_{12}K_{12}$ dosage was much lower than in Sonata and Atlant varieties. And high concentration thereof at $N_{36}P_{18}K_{18}$ dosage was found in Vizit variety. Plant lodging took place at cellulose concentration in stem less than 4.8 mg for optimum nitrogen application, and less than 4.5 mg for high application. Close feedback was identified between amount of cellulose per 1 cm of stem length and rice plant lodging (*r* from –0.92 ±0.22 to –0.92 ±0.23): the less is the cellulose concentration per 1 cm of stem length, the higher is the lodging under other equal conditions.

Growing of varieties combining productivity and resistance to unfavorable conditions is a complicated problem, especially when it deals with yield enhancement closely connected with improvement of donor-acceptor relations combining spectrum of morphophysiological biometric traits being the basic models for both intensive and extensive rice varieties.

Mechanisms responsible for lodging resistance during plant maturation period deal with less number of morphophysiological traits. Importance of these mechanisms in the course of rice yield enhancement is great: incomplete realization of potential productivity in cultivated varieties is often seen due to insufficient density of crop and lodging during kernel filling [52].

Plant stems and panicles affecting coenosis resistance to lodging are formed during tillering-booting period. These processes continue in flowering period. Weight of stems and panicles of the analyzed varieties in this period was differed greatly due to various intensity of assimilant input (Table 2). The intensive varieties used less assimilants for stem formation and share thereof in shoot weight, while the extensive ones used more assimilants thereby increasing resistance to lodging thereof. This resulted in increase of carpophore productivity and crop in the intensive varieties but in less resistance to lodging.

Sonata and Atlant varieties were forming stems with resistance to bend but with less panicle productivity. We have found the connection of lodging with the weight of stem and panicle, as well as yield (see Table 2) thereby enabling to assess rice genotypes by these traits to find the promising forms.

2. Stem and panicle weight at flowering, degree of lodging and yield of rice (*Oryza sativa* L.) varieties depending on inorganic nutrition dosage (2012-2015)

Variety	Type	Lodging, %	Stem weight		Panicle weight		Yield, kg/m^2
			g	percent of shoot weight	g	percent of shoot weight	
				$N_{24}P_{12}K_{12}$			
Rapan	I	43.30±2.35	1.59±0.03	62.60±0.25	0.40±0.01	15,75±0,32	1,206±0,030
Vizit	I	36.70±2.36	1.49±0.03	63.14±0.30	0.33±0.01	13,98±0,33	1,078±0,020
Gamma	I	40.00±2.04	1.34±0.02	59.00±0.32	0.35±0.01	15,42±0,35	1,058±0,020
Sonata	II	1.00±0.04	1.64±0.04	67.77±0.33	0.32±0.01	13,22±0,31	0,994±0,020
Atlant	II	13.30±0.95	1.95±0.04	67.71±0.34	0.32±0.01	11,11±0,28	0,933±0,010
r with degree of lodging			–0,56±0,23	–0.88±0.27	0.71±0.20	0.71±0.19	0.700±0.190
				$N_{36}P_{18}K_{18}$			
Rapan	I	53.30±2.39	1.55±0.03	60.31±0.32	0.40±0.01	15,56±0,32	1,264±0,050
Vizit	I	43.30±1.69	1.67±0.04	66.80±0.33	0.31±0.01	12,40±0,29	1,207±0,020
Gamma	I	50.00±2.04	1.24±0.02	60.50±0.32	0.27±0.01	13,17±0,31	1,107±0,020
Sonata	II	6.70±0.28	1.69±0.04	67.06±0.35	0.32±0.01	12,70±0,32	1,098±0,020
Atlant	II	23.30±1.67	2.05±0.05	69.49±0.36	0.34±0.01	11,53±0,33	0,940±0,010
r с полегаемостью посевов			–0,55±0,23	–0.73±0.19	0.10±0.58*	0.53±0.23	0.600±0.220
LSD_{05}		3,27	0,08	1.72	0.02	0.52	0.05

N o t e. I is intensive, and II is extensive variety; *r* — correlation coefficient.

* Unreliable value; other correlation coefficient values are statistically significant at $p \le 0.05$.

Bending strength value was to 73.8-79.8 g in Atlant and Sonata extensive varieties, while 60.0-62.5 g in Rapan, Vizit and Gamma intensive varieties at optimum nitrogenous nutrition (see Table 1). The value for the first two varieties decreased to 66.4-72.3 g, while for the others to 56.2-58.5 g at high nitrogene nutrition. Therefore, variety differences in bending strength also remained the same at providing the plants with extra nitrogene nutrition. We found strong negative dependence between amount of load and degree of lodging (*r* from –0.99 ±0.04 to –0.99 ±0.03). It should be mentioned that the applied express assessment of resistance of low parts of stems to bend is quite simple and reliable revealing high efficiency for the last ten years as compared with labor-intensive analysis for cellulose concentration in rice stems.

Formation of varieties increased production is stipulated by efficient utilization of photosynthesis assimilants and plant reserve constituents for making grain yield [52, 53]. Higher macronutrients inflow to the panicle being formed is seen in high-yielding rice varieties thereby forming more spikelets and complete kernels defining higher yield of these genotypes, and resistance thereof to lodging is declining therewith [52]. Assimilants in intensive varieties are largely used for making vegetative organs, and for panicle productivity elements, to a lesser extent which results in better resistance of plants to lodging. In other words, assimilant distribution pattern along shoot organs is responsible for making morphological and physiological traits defining both amount of the yield and resistance to lodging.

We have noted the differences by resistance to lodging in various varieties analyzed due to unequal cellulose content in stems stipulated genotypically, depending therewith on varieties response to nitrogen supernutrition. Therefore, one of the approaches to control productivity and rice resistance to lodging (both on the genotype level and by agrotechnology optimization) may be based on cellulose content in stems [15, 51, 52]. The increase in cellulose content per stem length and lower internodes resistance are the main traits for reducing the lodging of rice plants. The analyzed role of nitrogene nutrition in rice resistance to lodging and relation thereof with productivity on the whole agree with other authors' reports [40, 42-44].

Therefore, susceptibility of rice plants to lodging is stipulated by cellulose accumulation in the stem that may be assessed by the mechanical resistance thereof to bend. Such resistance including the one dealing with the response to

nitrogene nutrition level is unequal in the analyzed varieties. The prevailing part of photosynthesis assimilants is used by intensive varieties for forming high-yielding panicle defining productivity of genotype and agrophytocenoses, but resistance to lodging thereof is declining therewith. Stems with more resistance to bend but with less panicle productivity are formed in extensive varieties. These features are to be considered in rice selection programs for productivity and resistance to lodging.

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PHYSIO-BIOCHEMICAL CRITERIA FOR APPLE TREE TOLERANCE TO SUMMER ABIOTIC STRESSES

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Abstract

In Russia's North Caucasus where drought is frequent the apple tree is one of the most important garden crops. Physiological and biochemical studies are necessary to assess adaptiveness of apple varieties to stressors during summer, in particular, to summer drought. The purpose of this work is to study physiological, biochemical and anatomical parameters of leaves to assess the water regime, photosynthetic activity of apple trees in summer conditions, and to identify the most drought tolerant varieties for cultivation in the North Caucasus region. Research was carried out in 2011-2013 in fruit-bearing plantations (Central'noe Farm, Krasnodar) on apple varieties of different eco-geographical origin and ploidy: Idared, Earle Mack, Dayton (United States), Ligol (Poland), Prikubanskoe, Rassvet, Fortuna, Soyuz, Rodnichok (Russia). Varieties Soyuz and Rodnichok are triploids, the rest ones are diploids. Monthly, fully formed leaves were collected from (from the middle part of annual shoots of three trees in 3 replicates for each variety, 10 leaves per replicate. Indicators of water regime (total, free and bound water contents) were analyzed gravimetrically. The total water was determined after drying samples at 105 °C to a constant weight. For anatomical examinations, leaf blade transverse sections (temporary preparations) were used. It was shown that the leaf tissue water content, as well as the ratio of the bound and free water depend on both the variety specificity and the meteorological conditions of the year. Leaf water content in Prikubanskoe, Fortuna, Soyuz, and Rodnichok trees during July and August decreased by an average of 1-4 % compared to June, and the bound-to-free water ratio was the highest. Also, direct correlation between the leaf area and water availability (r = +0.98), and negative correlation between the leaf area and air temperature (r = –0.99, ($p \le 0.05$) were characteristic of these varieties. Pair correlation coefficients between (a + b) chlorophylls and fruit yield (r = +0.87), and between water content of tissues and fruit bud initiation (r = +0.97) ($p \le 0.05$) indicate that water and temperature regimes influence the yield and fruit bud formation. In the varieties of Prikubanskoe, Fortuna, Soyuz, Rodnichok, the chlorophyll content was more constant during the summer, and the ratio of the sum of chlorophylls to carotenoids is the highest. A positive correlation was found between the carotenoids and the air temperature (r = +0.91) ($p \le 0.05$). Morpho-anatomical structure of the leaf has varietal characteristics and depends on temperature and water availability. In 2012, the varieties exhibited xeromorphic features of leaves to varying degrees, which determined the resistance to drought, and the highest palisade index (1.47-1.49) was characteristic of the varieties Prikubanskoe, Fortuna, Soyuz, Rodnichok. The obtained results are in line with the field data obtained in the gardens without irrigation. The varieties showed different responses to summer stress factors, i.e. high temperatures and drought. Idared, Earley Mack, Dayton, Ligol plants were "passive" with a reduced water content, high solids, and smaller leaves. The rest varieties maintained high water and pigments in leaves and showed sustainable growth. Thus, Russian apple varieties Prikubanskoe, Fortuna, Soyuz, Rodnichok possess greater ecological plasticity and adaptive reserves compared to the studied introduced foreign varieties. The revealed adaptive features make it possible to involve these varieties in breeding for drought resistance. The applied tests provide accurate assessments of apple drought resistance and can be

used in breeding.

Keywords: apple tree, adaptation, drought resistance, heat resistance, proline, frost resistance

The apple tree (*Malus domestica* Borkh.) is the important food crop occupying 60 to 95 % of crop areas in different zones of the North-Caucasus region of Russia. The stress factors of summer season, drought and high temperatures negatively affect the apple tree growth and development, with leaves and fruits falling down, and setting up of generative organs worsening thereby resulting in reduction in yields by 15-30 % [1-3]. Stress causes changing in plant metabolism, photosynthesis, water exchange affecting physiological, biochemical and anatomo-morphological values [4]. Analysis of the apple tree physiological and biochemical features in unstable climatic conditions is required for accelerating and enhancement of genetic-selection process efficiency and for revealing the highly adaptive varieties in various horticultural activities [5-8].

A leaf is the most plastic vegetative organ, responding to environmental changes [9-11]. Peculiarities of water regime, pigment complex, xeromorphous structure of the leaf are considered to be reliable criteria of the plants drought resistance [12, 13]. Genotypes with the largest potential of the drought resistance were taken from 40 apple varieties and forms by indicators of water content tissues, water deficiency, water-retaining capacity of leaves in central Russia. Column-like apple varieties as Kumir, Vasyugan, Stela were found to lose 17.7-19.3 % of water per green weight after being affected by thermal shock and wilting [14, 15]. In various soil and climatic zones chlorophylls and carotinoids content was used as drought resistance markers for fruit, nut and decorative crops [16, 20]. Chlorophyll content in hazel nuts was decreasing under temperature rising and water supply reducing, with carotinoids content increasing two times [20].

The anatomo-morphological indicators of the leaf may be used to identify the drought resistance [21, 22], however, correlations of the traits is to be analyzed in more detail. According to some data amount of mesophyll cells was decreasing, relationship between mesophyll tissues was retained and the cuticle was thickened under insufficient water delivery in various pear varieties in subtropical parts of Russia [23]. On the contrary, the other papers provide data that change in relation among the mesophyll tissues, and reduction of upper epidermis cells are due to peach drought resistance [24]. In the North Caucasus characterized by specific climatic conditions, mainly by acute fluctuations of both water and temperature regimes, peculiarities of the apple-tree drought resistance physiology are analyzed insufficiently.

Complex of physio-biochemical and anatomic indicators of the leaf blade is presented herein to find the resistance of the apple varieties of different eco-geographic origin to the summer period stressors in specific conditions of the North Caucasus. Peculiarities of these plant varieties to resist high temperatures and drought are indicated.

The objective of this paper is to analyze the peculiarities of water regime, photosynthetic activity of apple-tree in the summer time period, as well as to find the most drought-resistant varieties based on the leaf physio-biochemical and anatomic indicators, to be cultivated in the North-Caucasus region of the Russian Federation.

Techniques. The analysis took place in 2011-2013 in commercial plantation of Experimental Production Farm Tsentralnoe, Krasnodar. The apple varieties from various eco-geographical areas, Idared, Earley Mack, Dayton varieties (USA), Ligol (Poland), Prikubanskoe, Rassvet, Fortuna, Soyuz, Rodnichok (Russia), have been analyzed. Soyuz and Rodnichok varieties are triploid,

the other ones being diploid. Idared, Ligol, Prikubanskoe varieties were planted in 2010 using SK4 rootstock at 0.9 m×4.5 m planting scheme; Rassvet, Fortuna, Soyuz, Rodnichok varieties were planted in 2000 using M9 rootstock (2 m×5 m); Earley Mack and Dayton varieties were planted in 1998 with M9 (2 m×5 m) rootstock.

Every month fully preformed leaves were taken from 3 trees (middle part of one-year increment) of each variety in three-fold biological replication to be analyzed. Each replication consisted of 10 leaves. Content of both free and bound water was found by weighting [25]. The total water content in leaves was found by drying the weighed quantities in thermostat at 105 °C until the constant weigh thereof. The experiments were made in 3-fold analytical replication. Anatomic features of leaf blade were studied on temporary slides of transversal sections made with razor by hand applying elder-tree stem pith as an additional material. Sections without staining and fixing were microscoped in water drop with Olympus BX41 microscope (Olympus Corporation, Japan; magnification of ×400). Biometric values of the leaf blade were measured in microns by ocular micromere according to the specified procedure [26]. Pigment content was found spectrophotometrically in 85% acetone extract (spectrophotometer Unico 2800, United Products & Instruments, USA) at λ = 663, 644, 432 nm (red color-filter) [27].

Statistical analysis was made by B.A. Dospekhov [28]. All calculations were processed with Microsoft Excel 2010. Significance of differences between the analyzed values (LSD_{05}) was found with statistical reliability of 95%, arithmetic mean (*M*) and standard deviation (±SD) were calculated. Pair correlation coefficient (*r*) between physio-biochemical indicators was calculated with 95% statistical significance.

Results. The weather condition differed significantly by years. In 2011, the drought was noted in July, with maximum air temperature being 39.5 °C, and rainfall 3.1 mm. In 2012, the period from late July till the mid-August was abnormally hot and dry, maximum air temperature was 38.3 °C (above normal by 4.7 °C), the rainfall was 0.3-0.4 mm (2% of the norm). In 2013, maximum air temperature was 32 °C (above normal by 1.5-2.5 °C), the rainfall was 35 mm.

The adaptation to conditions of cultivation is of complex character and is based on plasticity of anatomic structures, change in physio-biochemical indicators, with limits thereof being determined by certain genotype. Extremely high temperatures and insufficient water supply negatively affect the water status of the apple-tree vegetative organs [29-31].

Water regime indicators for the apple-tree varieties are significant to assess drought resistance thereof. We have analyzed total water content of the leaf tissues taken from one-year shoots and fractional composition of water. Idared variety widely presented in the southern part of Russia was a control one. Water content in all varieties changed variously during summer period. In June, it ranged from 60.53 to 70.56% depending on variety features and environmental conditions (Table 1). In July and August, in the period of the highest stress factors (especially in dry year of 2012), water content in Idared, Earley Mack, Dayton and Ligol varieties was significantly reduced by 8-14%.

In other varieties the water content of leaf tissues decreased on the average by 1-4%. The largest reduction in water content of Idared, Earley Mack, Dayton, Ligol varieties in August was accompanied by enhancement of synthetic processes and accumulation of dry matter in the leaf tissues up to 48.71% (up to 47.69% in 2012) (Fig. 1). In other varieties dry matter content ranged from 29.44% in June to 34.87% in August.

1. Water content (%) **in leaves of various apple (*Malus domestica* Borkh.) varieties depending on month and year** (*M*±SD, Experimental Production Farm Tsentralnoe, Krasnodar)

Variety	2011			2012			2013		
	June	July	August	June	July	August	June	July	August
Idared (control)	69.12±5.71	59.26±5.65	58.63±5.92	68.45±5.42	57.84±5.89	58.88±5.28	69.21±6.05	58.24±6.24	57.25±6.27
Earley Mack	62.54±5.17	55.87±5.84	52.41±4.89	60.53±3.67	54.76±3.89	53.78±3.85	61.25±4.52	55.89±4.28	51.29±4.85
Dayton	65.28±6.58	54.26±6.27	52.84±6.28	64.28±6.84	53.26±6.58	52.31±6.78	66.58±7.12	55.43±7.18	52.43±7.58
Ligol	64.32±4.25	56.24±4.28	55.61±4.29	63.53±5.56	54.28±5.28	53.72±5.76	64.23±4.28	56.27±4.89	56.87±4.12
Prikubanskoe	70.56±0.94	69.27±1.03	68.71±0.49	68.57±0.58	67.53±0.82	67.58±0.46	68.75±0.28	69.12±0.85	69.58±0.45
Rassvet	67.81±1.15	65.28±1.28	65.28±1.86	66.21±0.58	65.24±0.46	65.41±0.83	68.23±1.53	65.13±1.59	66.57±1.28
Fortuna	69.31±1.25	68.71±1.48	66.41±1.46	69.87±1.49	67.24±1.27	66.23±1.53	68.72±0.48	68.42±0.27	67.24±0.46
Soyuz	70.12±0.86	69.25±0.78	68.42±0.49	69.21±0.58	68.27±0.58	68.21±0.27	69.82±0.78	68.52±0.58	69.27±0.46
Rodnichok	70.56±2.15	69.23±2.16	66.41±2.46	68.25±0.57	68.91±0.56	67.24±0.58	69.15±1.27	69.24±1.87	66.85±1.53
LSD_{05}	1.36	2.01	2.05	1.39	2.04	2.06	1.32	1.96	2.13

2. Pigments (mg/g dry matter) **in leaves of various apple (*Malus domestica* Borkh.) varieties in summers of 2011-2013** (*M*±SD, Experimental Production Farm Tsentralnoe, Krasnodar)

Variety	2011			2012			2013		
	(a + b)	c	(a + b)/c	(a + b)	c	(a + b)/c	(a + b)	c	(a + b)/c
Idared (control)	4.72±0.42	1.04±0.07	4.54±0.41	5.12±0.49	0.96±0.08	5.33±0.19	4.22±0.46	0.90±0.09	4.68±0.42
Earley Mack	4.22±0.13	1.24±0.01	3.40±0.24	4.42±0.18	1.24±0.02	3.56±1.33	4.16±0.28	1.42±0.07	2.92±0.34
Dayton	4.82±0.12	1.22±0.08	3.95±0.35	4.96±0.28	1.20±0.02	4.13±0.12	4.72±0.24	1.20±0.08	3.93±0.12
Ligol	4.70±0.34	1.28±0.23	3.67±0.28	5.02±0.35	1.22±0.51	4.11±0.17	4.42±0.28	1.36±0.02	3.25±0.43
Prikubanskoe	5.38±0.12	0.96±0.05	5.60±0.75	5.51±0.28	0.96±0.28	5.72±0.10	5.62±0.21	1.02±0.07	5.51±0.10
Rassvet	4.70±0.50	1.02±0.08	4.60±0.25	5.62±0.49	1.18±0.05	4.76±0.14	5.50±0.78	1.16±0.03	4.74±0.41
Fortuna	5.36±0.05	0.90±0.05	5.95±0.24	5.44±0.02	0.86±0.02	6.32±0.28	5.28±0.05	0.96±0.04	5.50±0.42
Soyuz	5.32±0.11	0.96±0.06	5.54±0.46	5.46±0.12	0.92±0.08	5.93±0.44	5.22±0.16	0.92±0.04	5.67±0.19
Rodnichok	5.58±0.04	0.91±0.09	6.13±0.57	5.66±0.05	0.84±0.04	6.73±0.19	5.62±0.02	0.96±0.03	5.85±0.42
LSD_{05}	0.34	0.22	0.41	0.25	0.24	0.51	0.27	0.28	0.45

N o t e. a + b — chlorophyll content, c — carotenoids.

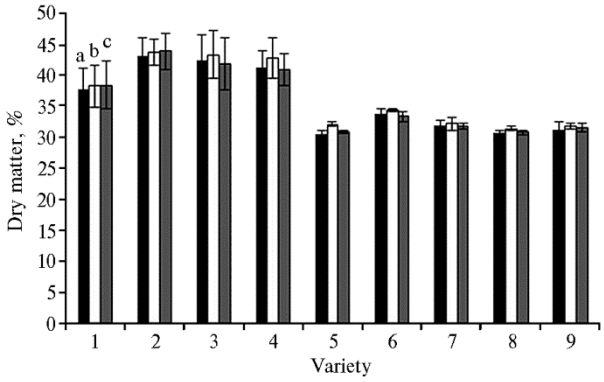

Fig. 1. Average content of dry matter in leaves of various apple-tree (*Malus domestica* Borkh.) varieties during summer of 2011 (a)**, 2012** (b) **and 2013** (c)**:** 1 — Idared, 2 — Earley Mack, 3 — Dayton, 4 — Ligol, 5 — Prikubanskoe, 6 — Rassvet, 7 — Fortuna, 8 — Soyuz, 9 — Rodnichok. LSD_{05}: a — 0.89, b — 0.68, c — 0.81 (Experimental Production Farm Tsentralnoe, Krasnodar).

The indicator of the plants resistance to the low water supply and drought is a bound to free water ratio ($K_{bound/free}$). The bound water provides water-retaining capacity of cells. The high coefficient of bound to free water quantitative ratio indicates the high drought resistance of the variety.

The highest values of $K_{bound/free}$ from 2.06 to 2.61 in 2011 and 2013 were found in Prikubanskoe, Fortuna, Soyuz, Rodnichok varieties (Fig. 2). In dry year of 2012, this value for all varieties was higher as compared to 2011 and 2013 varying within 1.53-2.99. In Earley Mack variety, the value $K_{bound/free}$ was the lowest ranging within 0.41-0.82 in the vegetation period, indicating low resistance to stress-factors of the summer time.

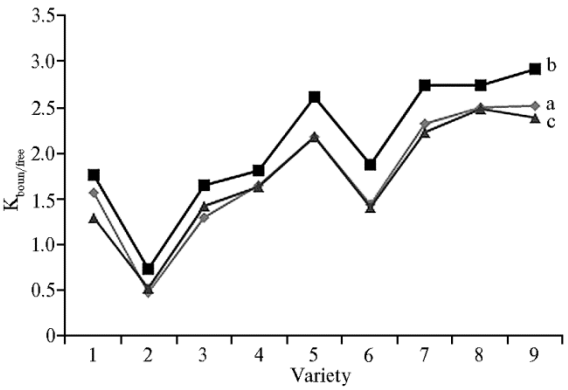

Fig. 2. Bound to free ratio of water ($K_{bound/free.}$) **in leaves of various apple-tree (*Malus domestica* Borkh.) varieties during summer of 2011** (a)**, 2012** (b) **and 2013** (c)**:** 1 — Idared, 2 — Earley Mack, 3 — Dayton, 4 — Ligol, 5 — Prikubanskoe, 6 — Rassvet, 7 — Fortuna, 8 — Soyuz, 9 — Rodnichok. LSD_{05}: a — 0.54, b — 0.58, c — 0.61 (Experimental Production Farm Tsentralnoe, Krasnodar).

Physiological condition of the plants is best characterized by increase of the leaf area. According to literature data, the leaf area was reducing in the periods of insufficient water supply with red currant and mango varieties (32, 33), and on the contrary remaining the same with almond (34). In our analysis, the linear parameters of the leaf within the vegetation period depended on the varieties and climatic conditions of the year. In this way the leaf area of Idared, Earley Mack, Dayton and Ligol varieties was decreasing in driest year of 2012 amounting to 68.29 cm^2 on the average (as compared to that of 72.52 cm^2 on the average in 2011) (Fig. 3).

Changes in the leaf size were insignificant in other varieties. The positive correlation was found between the leaf area and water supply ($r = +0.98$), and the negative one between the area and air temperature ($r = -0.99$) ($p \leq 0.05$). Both water and temperature regimes affect the crop productivity and fruit bud setting as well, specifying the yield for the next year and influencing the functional condition of assimilating apparatus, that was confirmed by the pair correlation coefficient between content of chlorophylls and crop productivity ($r = +0.87$), and between water content of the tissues and fruit bud setting ($r = +0.97$) ($p \leq 0.05$).

Drought and higher temperature affect the leaf pigment composition either

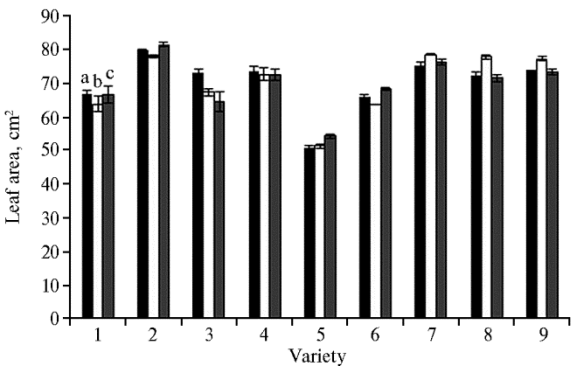

Fig. 3. Average leaf area of various apple-tree (*Malus domestica* Borkh.) varieties during summer of 2011 (a)**, 2012** (b) **and 2013** (c)**:** 1 — Idared, 2 — Earley Mack, 3 — Dayton, 4 — Ligol, 5 — Prikubanskoe, 6 — Rassvet, 7 — Fortuna, 8 — Soyuz, 9 — Rodnichok. NSR_{05}: a — 0.65, b — 0.51, c — 0.51 (Experimental Production Farm Tsentralnoe, Krasnodar).

[35, 36], that is also confirmed by our analysis. Correlation between content of chlorophylls (a + b) and carotenoids in the leaves of apple-tree varieties analyzed was changing variously during a summer period. Content of chlorophylls in Prikubanskoe, Fortuna, Soyuz, Rodnichok varieties was more stable during a summer period, but sharp accumulation of carotenoids was noted in July—August, that was confirmed by the positive correlation between the content of carotenoids and ambient temperature ($r = +0.91$) ($p \leq 0.05$). Carotenoids possessing the antioxidant properties play an important part in the plant defense reactions. The increased accumulation thereof under unfavorable conditions of the summer period is required for stimulating the adaptive response and reducing general stress. Quantitative correlation between the chlorophyll content and carotenoids indicating plant adequacy to unfavorable environmental conditions is considered to be the most informative value, with this value being the highest for all the varieties in 2012. The correlation between the chlorophyll content and carotenoids was higher in Prikubanskoe, Fortuna, Soyuz, Rodnichok varieties (5.72-6.73) than in the other ones. In 2011 and 2013, this value was 5.50-6.13 (Table 2).

Prikubanskoe, Fortuna, Soyuz, Rodnichok home-selected varieties proved to be highly resistant to dry weather conditions due to water regime and pigment complex.

Changes in physiological processes affect leaf anatomy and morphology. The apple tree leaf is dorsoventral, and mesophyll is differentiated into palisade and columnar tissues. The palisade tissue is composed of two cell layers. The stomata apparatus is of anomocytic type, the stomata are concentrated on abaxile side of leaf blades. The anatomo-morphological structure of the leaf had the variety features, and depended on temperature and water supply as well. Cells of leaf in triploid Soyuz and Rodnichok varieties were larger than in other varieties analyzed, and leaf thickness thereof was of maximum size.

The xeromorphous traits of leaves, indicating resistance to drought, namely an increase in thickness, in cuticle, palisade index, in stomata number per unit of leaf area, and reduction of stomata linear size, were found in dry year of 2012 in all varieties except Earley Mack.

The palisade index (relation between thickness of palisade and spongy layers) is the most informative estimate of drought resistance. In 2012, this index was growing in all varieties, with the largest value thereof being found in Prikubanskoe, Fortuna, Soyuz, and Rodnichok varieties (1.47-1.49) identified as the highly drought-resistant. Idared, Dayton, Ligol, Rassvet varieties with palisade index of 1.27-1.35 were marked as the drought-resistant, and Earley Mack variety with palisade index of 1.01 as a non-drought resistant (Table 3).

In some papers, the relationship between water supply and stomata number per unit of leaf area was established. Amount and size of pear stomata in

3. Leaf morphology indicators (μm) **in various apple (*Malus domestica* Borkh.) varieties during summers of 2011-2013** (*M*±SD, Experimental Production Farm Tsentralnoe, Krasnodar)

Variety	2011			2012			2013		
	TTLB	CE	PI	TTLB	CE	PI	TTLB	CE	PI
Idared (control)	176.2±2.65	10.0±0.12	1.27±0.01	180.3±2.34	10.2±0.12	1.30±0.01	175.4±2.34	10.0±0.12	1.29±0.01
Earley Mack	171.5±0.17	9.1±0.01	1.01±0.01	171.5±0.18	9.1±0.01	1.01±0.01	171.2±0.27	9.1±0.02	1.01±0.01
Dayton	191.3±3.75	10.1±0.23	1.28±0.01	198.5±3.45	10.5±0.23	1.29±0.01	192.8±3.57	10.1±0.21	1.28±0.01
Ligol	163.8±4.12	10.2±0.10	1.30±0.01	170.2±4.15	10.4±0.10	1.32±0.01	162.3±4.17	10.3±0.12	1.30±0.01
Prikubanskoe	199.2±3.45	11.2±0.21	1.47±0.01	205.3±3.48	11.5±0.19	1.49±0.01	199.5±3.27	11.1±0.21	1.46±0.01
Rassvet	169.4±3.12	10.3±0.05	1.32±0.02	175.4±3.27	10.3±0.05	1.35±0.02	170.7±3.17	10.2±0.03	1.31±0.02
Fortuna	178.5±2.81	11.1±0.10	1.46±0.01	181.4±2.47	11.3±0.11	1.47±0.02	175.6±2.42	11.2±0.13	1.45±0.01
Soyuz	213.9±15.23	11.2±0.12	1.45±0.01	243.6±15.21	11.4±0.15	1.48±0.01	221.3±14.27	11.1±0.17	1.46±0.01
Rodnichok	215.4±14.21	11.2±0.24	1.45±0.01	241.5±13.78	11.6±0.25	1.48±0.01	216.1±11.24	11.2±0.24	1.46±0.01
LSD_{05}	3.46	0.67	0.30	4.21	0.71	0.31	3.63	0.67	0.30

N o t e. TTLB — total thickness of leaf blade, CE — cuticle with upper epidermis, PI — palisade index.

4. Stomata apparatus (number of stomata and guard cell size) **of various apple (*Malus domestica* Borkh.) varieties during summers of 2011-2013** (*M*±SD, Experimental Production Farm Tsentralnoe, Krasnodar)

Variety	2011			2012			2013		
	number	width, μm	length, μm	number	width, μm	length, μm	number	width, μm	length, μm
Idared (control)	215.1±1.12	31.1±0.54	54.4±0.41	216.5±1.25	32.1±0.64	55.2±0.42	217.5±1.24	32.2±0.58	54.9±0.43
Earley Mack	189.4±0.15	34.0±0.04	56.2±0.52	189.7±0.14	34.1±0.07	55.6±0.51	189.5±0.27	34.1±0.02	55.2±0.49
Dayton	245.2±0.84	31.0±0.23	54.8±0.24	245.8±0.84	31.3±0.19	54.4±0.21	244.2±0.71	31.4±0.24	54.4±0.26
Ligol	231.6±0.46	32.1±0.21	54.2±0.10	232.4±0.42	31.7±0.18	54.3±0.12	231.60.43±	31.8±0.17	54.4±0.12
Prikubanskoe	270.4±3.87	30.1±0.27	53.7±0.25	275.3±3.57	30.2±0.22	53.6±0.25	267.4±3.84	30.5±0.16	53.2±0.27
Rassvet	236.7±1.02	31.7±0.17	54.3±0.31	237.4±1.01	31.4±0.17	54.7±0.34	235.4±1.04	31.4±0.18	54.1±0.31
Fortuna	265.3±2.34	31.0±0.27	53.2±0.42	270.6±2.54	31.5±0.42	53.8±0.42	267.3±2.47	30.6±0.42	54.0±0.42
Soyuz	281.4±5.13	30.1±0.24	53.2±0.58	289.7±5.24	30.6±0.24	53.2±0.56	280.4±5.27	30.4±0.22	54.2±0.54
Rodnichok	284.7±2.14	30.2±0.57	53.1±0.04	289.3±2.47	30.8±0.39	53.1±0.04	284.6±2.37	30.1±0.34	53.0±0.58
LSD_{05}	4.34	0.89	0.79	4.50	0.84	0.70	4.33	0.87	0.64

humid subtropical areas of Russia were changing depending on vegetation conditions: stomata density was decreasing together with increasing of size thereof in the years of intensive rainfall during active growth of shoots and leaves, with the reverse process taking place in dry years [23]. Peach varieties with high density of stomata analyzed by the Chinese scientists possessed higher drought resistance [24]. No such dependence is found in other papers. Water supply had no similar effects on the stomata density of young almond plants [34]. Increase in stomata number per unit of leaf area and reduction of the guard cells as compared to non-resistant varieties is typical of the drought-resistant varieties, according to our data (Table 4). Therewith the stomata number per 1 mm^2 of leaf surface in Prikubanskoe, Fortuna, Soyuz, Rodnichok varieties varied from 265.3 to 289.7, while in other varieties analyzed it ranged within 189.4-245.8. Maximum length and width of stomata (56.2 μm and 34.1 μm respectively) was in the non-drought resistant Earley Mack variety, while minimum size of stomata (53.2 μm and 30.1 μm) was in highly drought-resistant Soyuz variety. The obtained results agreed with those of field trials in non-irrigated gardens (data not given).

Therefore, by assessing physio-biochemical and anatomic parameters, we revealed the characteristic features of tolerance to summer stresses (high temperature and low water supply) among the apple varieties of different eco-geographical origin grown in the North Caucasus region of Russia. The introduced varieties Idared, Earley Mack, Dayton, and Ligol show passive drought resistance (water content reducing, high content of dry matter, reduced leaf area). Domestic varieties keep high water content, stability of both growing and synthetic processes, and high concentration of pigments. The domestically-derived apple varieties Prikubanskoe, Fortuna, Soyuz, Rodnichok possess better environmental plasticity and adaptation abilities than the introduced varieties of foreign selection. The adaptation features found in the apple varieties enable using thereof in breeding programs as the sources of drought resistance. The methods applied herein provide accurate estimates of apple tree tolerance to drought and may be used in selection process.

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THIONINS OF WHEAT *Triticum kiharae* Dorof. et Migush. ARE NOVEL POTENT INHIBITORS OF *Candida albicans* (C.P. Robin) Berkhout

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Abstract

Plants serve as a source of biologically active compounds, the most important of which are antimicrobial peptides (AMPs). AMPs represent an integral part of the defense arsenal of all living beings. Members of the thionin family found only in plants are effective inhibitors of plant pathogens, including bacteria and fungi, which opens up prospects for their practical application as biopesticides to protect plants from diseases. However, the effect of thionins on animal and human pathogens has not been sufficiently studied. Yeast-like fungi of the genus *Candida* are opportunistic pathogenic microorganisms that occur in 70 % of people without causing disease (M. Dadar et al., 2018). However, in immune-compromised individuals, they can cause a number of serious diseases, the frequency of which has increased significantly in the last two decades. Antimycotics traditionally used to treat *Candida* infections are not always effective and safe for humans. In this regard, the world is constantly searching for new natural antifungal agents. The aim of this work was to isolate thionins from the kernels of the highly pathogen-resistant wheat species *Triticum kiharae* Dorof. et Migush., determine their primary structure, and assay antifungal activity against *Candida albicans*. For the first time from the wheat *T. kiharae* using chromatography on chitin and reversed-phase high-performance liquid chromatography (HPLC), 2 thionins Tk-AMP-BP and Tk-AMP-AP1 were isolated, and their amino acid sequences were determined by automated Edman degradation. The primary structure of Tk-AMP-BP was confirmed by transcriptome high-throughput sequencing (NGS) of wheat seedlings. The study of antimicrobial activity of Tk-AMP-BP showed that it has potent fungicidal effect on *C. albicans* cells at very low concentrations (MIC = 0.78 μg/ml). The biological activity of the wheat thionin against *C. albicans* was higher than that of thionins from other plant species. The results obtained in this work allow us to consider the wheat thionin as a promising molecule for the development on its basis of next-generation drugs to treat *C. albicans* infections.

Keywords: plant immunity, antimicrobial peptides, *Triticum kiharae* Dorof. et Migush., wheat species, thionins, mycoses, *Candida albicans*

Although the plants have no adaptive immunity system as compared to superior vertebrates they possess congenital immunity enabling in finding pathogens and inhibit growth thereof in plant tissues [1]. Range of "chemical means" of protection including secondary metabolites and proteic substances like antimicrobial proteins (AMPr) and peptides (AMP) are used to inhibit plant pathogens [2-4]. Antimicrobial peptides are components of congenital immunity both in animals and plants. They are short (less than 100 amino-acid residues) positively charged amphiphilic polypeptides differed in primary and spatial structures and the so-called cysteine motif, i.e. arrangement of cysteine residues inside the peptide molecule [5-9]. There are several AMP families marked out under the struc-

tural similarity thereof: thionins, defensins, nonspecific lipid-transfer proteins, hevein- and knottin-like peptides, harpinins, as well as macrocyclic peptides (cyclotides) [5-7]. AMPs may be practically applied in agriculture as biopesticides to protect plants against diseases and in medicine when making new drug groups. As compared with usual antibiotics, the AMPs have some advantages: immediate impact, wide spectrum of antimicrobial activity, activity against antibiotic-resistant pathogen isolates, no persistent form of pathogens, if applied together with antibiotics they enhance the effect thereof, they possess activities useful for humans (e.g. inhibit sepsis).

Thionins are short (~ 5 kDa) cysteine-rich peptides first found in wheat flour [10]. Later they were found in a wide range of mono- and dicotyledonous plants. Over 100 thionin sequences from 15 plant species are known nowadays [11]. Thionins are divided into two main groups by number of cysteine residues, 6 and 8 ones, forming disulphide bonds [5]. There are 5 classes of thionins by structural features thereof [12]. For more than 60 years thionins are known to inhibit growth of pathogenic bacteria and fungi in vitro [13]. Inhibition of pathogenic bacteria growth was first demonstrated by R. Fernandez de Caleya et al. [14], and they made an assumption on the protection role of these proteins in plants. According to further analysis, thionins proved to inhibit the growth of both gram-positive and gram-negative bacteria and a number of phytopathogenic fungi and Oomycetes as well, with IC_{50} usually being within 1 to 15 *r*g/ml [11, 15, 16]. Therewith biological thionin activity with regard to pathogens and opportunistic pathogens in humans is poorly studied.

Candida albicans is a yeast-like fungus found in normal flora of 70% of humans (oral cavity, esophagus, intestinal tract, genital tracts and skin) provoking no diseases [17]. Immune compromised humans (HIV infected, oncology patients and after organ transplantation) may be seriously affected by this fungus, including blennosis and general lesion [18-20]. Infections induced by *Candida* spp. include full spectrum of such serious diseases as invasive candidiasis (*Candida* fungi in blood), chronic disseminated candidiasis, endocarditis, cerebral fever and entophthalmia [19].

Five classes of antimycotic agents are applied at invasive mycosis: azoles (imidazole and triazole derivatives), polyenes (polyene antibiotics), allylamines, echinocandins and fluoropyrimidines [21, 22]. However, treatment of infections with the above mentioned drugs is limited by spectrum of activity thereof, resistance of pathogens thereto, and fungistatic but not fungicidal activity thereof [23]. Besides, many antimycotic agents are toxic to mammalian cells. Therefore, natural antibiotics (AMP, like thionins) may be considered as the promising molecules against mycosis both in humans and animals.

Thionins of synthetic hexaploid wheat *Triticum kiharae* Dorof. et Migush were analyzed herein. They possess high resistance to phytopathogens due to large variety of AMPs found in seeds of this plant among which there are two thionins called Tk-AMP-BP and Tk-AMP-AP [24]. Short N-terminal amino acid sequences thereof were found by us earlier, and these AMPs were demonstrated to efficiently inhibit phytopathogenic fungi [24, our not published data] and exhibit antimutagenic activity protecting human cells against toxic effect of cadmium ions [25]. At the same time, full primary structure of these AMPs, and ability thereof to inhibit growth and development of pathogens in humans were not studied yet.

Primary structure of two thionins of *T. kiharae* wheat was first discovered herein, while the sequence of one of the, the Tk-AMP-BP, was confirmed by NGS (next-generation sequencing) method, and this thionin was demonstrated to have fungicidal effect on *C. albicans* cells at very low concentrations.

The aim of this paper was to isolate thionins from seeds of *T. kiharae* wheat, to find full amino acid sequences thereof, and analyze antifungal activity against *C. albicans*.

Techniques. Thionins were isolated from grinder-milled seeds of *T. kiharae* (10 g). The flour was being extracted by 50 ml of acid mixture (1 M HCl and 5% HCOOH, Khimmed, Russia) for 1 hour with constant stirring thereof. Protein-peptide fraction was pelleted (10,000 g, 15 min) from supernatant by five volumes of chilled acetone (high purity, Khimmed, Russia) during night at 4 °C. The residue was air-dried, dissolved in 5 ml of 50 mM ammonium-bicarbonate buffer (pH 7.8), and centrifuged (1,000 g, 10 min). The supernatant was mixed for 1 hour with 1 g of chitin (Sigma, USA) pre-washed with two volumes of 0.1% trifluoroacetic acid (TFA) (puriss. p.a., Fluka, Switzerland), two volumes of MQ water and balanced by three volumes of 50 mM NH_4HCO_3 (chemically pure, Reakhim, Russia). After immobilization of protein-peptide fraction on chitin, the latter was washed three times with 20 ml of 50 mM NH_4HCO_3 to remove not-bound components, then the chitin-bonded protein-peptide fraction was eluted with 30 ml of 0.1% trifluoroacetic acid (TFA) and separated by reverse phase highly performance liquid chromatography method (RP-HPLC) in Reprosil C_{18} column (4×250 mm, Dr. Maisch GmbH, Germany) in gradient of acetonitrile concentrations (UHPLC Supergradient, PanReac Quimica SLU, Spain): 10-50% solution B (80% acetonitrile in 0.1% TFA) in solution A (0.1% TFA) within 60 min (elution rate of 1 ml/min, detection at λ = 214 nm). The fractions were mass-spectrometricall analyzed (Bruker Daltonik GmbH, Germany).

To isolate individual components the thionin-containing fraction was re-chromatographed using Luna C_{18} column (4.6×150 mm, Phenomenex, Inc., USA) in gradient of acetonitrile concentrations (20-50 % solution B in solution A) for 30 min (elution rate of 0.75 ml/min, detection at λ = 214 nm).

Disulphide bonds were restored and alkylated as indicated in [24]. For this purpose 10 μg of the dried peptide was dissolved in 40 μl solution containing 6 M guanidine hydrochloride and 2 mM EDTA (BioUltra, Sigma-Aldrich, USA) in 0.5 M Tris-HCl buffer (pH 8.5). Then 2 μl of 1.4 M dithiothreitol water solution (BioUltra, Sigma-Aldrich, USA) was added. The obtained reaction mixture was vortexed and incubated for 4 hours at 40 °C, then 2 μl of 4-vinylpyridine (M_w ~ 60,000, Sigma-Aldrich, USA) was added. The mixture was incubated for 20 min at room temperature in the dark, then diluted by 100 rl of 0.1% TFA and injected onto the Luna C_{18} column (4.6×150 mm, Phenomenex, Inc., USA). The reaction products were separated in gradient of acetonitrile concentrations (0-50% in 0.1% of TFA) for 30 min.

RNA was isolated from wheat seedlings using Plant RNA Isolation Aid (Ambion, Inc., USA). Quality of RNA preparation was checked by Agilent 2100 Bioanalyzer (Agilent, USA). Ribosomal RNA fraction, unbound ribonucleotides and any residues of genomic DNA was removed from total RNA with the application of ready-to-use chemical sets (Illumina, Inc., USA) according to the manufacturer's protocol.

cDNA libraries were constructed as indicated in [26]. The cDNA libraries were sequenced (Genome Analyzer IIx, Illumina, Inc., USA). Transcriptomes were assembled by Trinity application (version 2.1.0) [27] with the digital normalization and maximum 50× coverage).

The elaborated algorithm described in [26] was applied for searching thionin precursor transcripts.

Mass-spectra were fixed by MALDI-TOF-MS (Ultraflex II TOF/TOF, Bruker Daltonik GmbH, Germany) either in linear mode or positive ion mode

using reflectron. 2.5-Dihydroxybenzoic acid (Ultra Pure, Sigma-Aldrich, USA) in concentration of 10 mg/ml in 50% (volume/volume) acetonitrile containing 0.1% TFA (volume/volume) was a matrix. Standard set of peptides and proteins in the molecular weight range of 700-66,000 Da (Sigma-Aldrich, USA) was applied for calibration.

For sequencing, reduced and alkylated thionins were vaporized in Savant SpeedVac Concentrator (Thermo Fisher Scientific, USA) till 50 μl volume. Amino acids were sequenced by Edman degradation (Procise 492 Sequencer, Applied Biosystems, Inc., USA) according to the manufacturer's protocol. Homologous sequences were found in the databases GenBank (https://www.ncbi.nlm.nih.gov/genbank/)/EMBL-Bank (http://www.ebi.ac.uk/embl/).

Antifungal activity was studied with 3-day culture of *C. albicans* grown on Sabouraud agar. Fungal cells were suspended in Sabouraud nutrient broth (1×10^6 cells/ml). Peptide sample was dissolved in nutrient broth to 200 μg/ml concentration and series of ten two-fold dilutions was prepared. A 50 μl aliquot of fungal suspension and 50 μl of peptide solution were added to wells of 96-well polystyrene plate for biological tests (BioCell, USA). Nutrient broth was a negative control, while 50 μl of fungal suspension and 50 μl of nutrient broth was the positive control. The plate was incubated for 72 hours at 28 °C. Then the control sample (without peptide) was diluted with Sabouraud medium (1:1000) and 100 μl was plated onto Sabouraud agar in Petri dishes. The dished were incubated for 48 hours at 28 °C. Fungus growth in control was taken as 100%. Test samples with various peptide concentrations were analyzed in the same way. Peptide minimum inhibiting concentration (MIC) fully (100 %) inhibiting the fungus growth was defined. The experiment was arranged in 3 replications.

Results. Wheat thionins were extracted in several steps: first, protein-peptide fraction was extracted by acid mixture [24], second, AMPs were immobilized on chitin [28], third, AMPs were separated by RP-HPLC method thereby obtaining thionin fraction; at the final step individual thionins were purified by re-chromatography. Chromatographic separation profile of wheat AMPs in the reversed phase column Reprosil C_{18} is presented in Figure 1.

According to mass-spectrometry analysis, the retention time for thionin mixture is 46.35 min. Components 4918 Da, 4801 Da and 4921 Da were found in this fraction, with the first two prevailing (see Fig.1). Re-chromatography of thionin-containing fraction in Luna C_{18} column (data not presented) resulted in purification of two peptides of 4918 Da and 4801 Da.

Amino acid sequences of the reduced and alkylated thionins were found by Edman automatic sequencing method (Fig. 2). Amino acid sequences of *T. aestivum* hexaploid wheat thionins and diploid species *T. urartu* and *T. monococcum* which are supposed donors of genome A of wheat polyploid species, as well as barley and rye thionins deposited in GenBank, are also indicated for comparison. Polypeptide chain length of both isolated purothionins is 45 amino acid residues. The *T. kiharae* 4918 Da thionin corresponded in weight and retention time to thionin Tk-AMP-BP characterized previously by us for N-terminal amino acid sequence [24]. Full amino acid sequencing of this peptide confirmed identity thereof to Tk-AMP-BP thionin. The second peptide with molecular weight of 4801 Da proved to be the new one and was not described previously. Identification of amino acid sequence thereof and comparison with the identified cereals thionins has indicated that it is also a thionin but referring to α-thionin family as opposed to Tk-AMP-BP. This thionin was marked by us as Tk-AMP-AP1. Both *T. kiharae* thionins just like other cereals thionins contain 8 cysteine residues (see Fig. 2). Alkylation of non-reduced peptides by vinylpyridine resulted in no changes in molecular weight thereof, and hence the molecules

thereof contain no free sulfhydryl groups, and all SH-groups form disulfide bonds typical to plant AMPs.

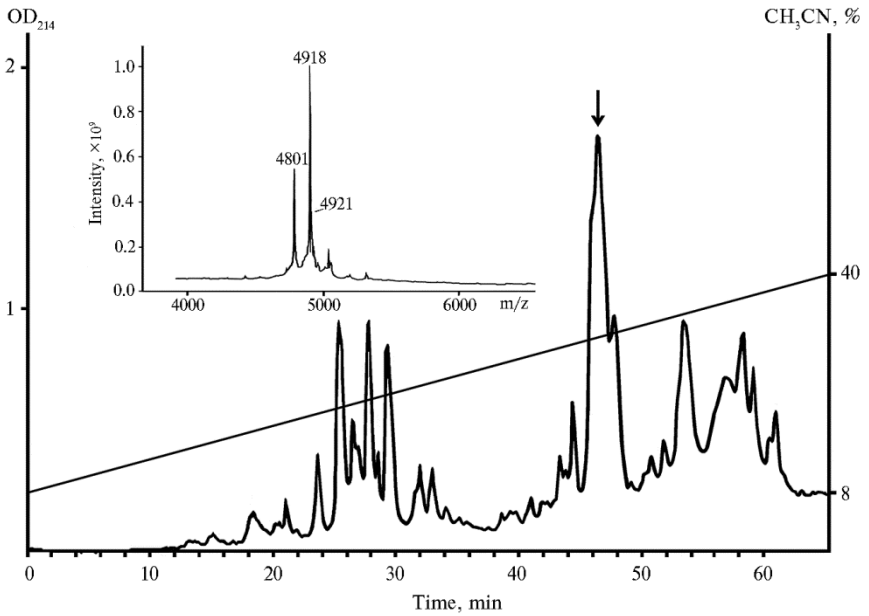

Fig. 1. Separation of antimicrobial peptides isolated from wheat (*Triticum kiharae*) seed by RP-HPLC method (highly performance liquid chromatography) in Reprosil C_{18} column (4×250 mm, Dr. Maisch GmbH, Germany) in gradient of acetonitrile concentrations. Thionin-containing fraction is marked by arrow. Mass-spectrum of this fraction is given in the insert (MALDI-TOF-MS, Bruker Daltonik GmbH, Germany). For description of chromatography conditions and mass-spectrometry, see *Techniques* section.

Species	Amino acid sequence	Peptide
Triticum kiharae	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	TK-AMP-BP
T. kiharae	KSCCRSTLGRNCYNLCRARGAQKLCAGVCRCKIASGLSCPKGFPK	TK-AMP-AP1
T. aestivum (CAA65312.1)	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. aestivum (CAA65313.1)	KSCCRSTLGRNCYNLCRARGAQKLCAGVCRCKISSGLSCPKGFPK	α_B-purothionin
T. urartu (ACL11920.1)	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. urartu (ACL11926.1)	KSCCKSTLGRDCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. monococcum (ACL11901.1)	KSCCKSILGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. monococcum (ACL11917.1)	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
Secale cereale (CAA65316.1)	KSCCKSTLGRNCYNLCRTRGAQKLCANFCRCKLISSTSCPKEFPK	purothionin
Hordeum vulgare (AAA32966.1)	KSCCRSTLGRNCYNLCRVRGAQKLCAGVCRCKLTSSGKCPTGFPK	α-hordothionin
H. vulgare (1206255A)	KSCCRSTLGRNCYNLCRVRGAQKLCANACRCKLTSGLKCPSSFPK	β-hordothionin

Fig. 2. Amino acid sequences of *Triticum kiharae* thionins and thionins of other cereals. Multiple alignments are made by CLUSTAL W2 program. Sequence numbers in GenBank are given in brackets. Cysteine residues are marked by white letters on the black background, divergent amino acid residues are marked by grey color.

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Tk-amp-bp   MGSKGLKGVMVCLLILGLVLEQVQVEGKSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK
Tk-amp-ap2  MGSKGLKGVMVCLLILGLVLEQVQVEGKSCCRTTLGRNCYNLCRSRGAQKLCSTVCRCKLTSGLSCPKGFPK

Tk-amp-bp   LVLESNSDEPDTMEYCNLGCRSSLCDYMVNAAADDEEMKLYVEQCGDACVNFCNADAGLTSLDA
Tk-amp-ap2  LALESNSDEPDTIEYCNLGCRSSVCDYMVNAAADDEEMKLYVENCGDACVNFCNGDAGLTSLDA
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Fig. 3. Amino acid sequences of thionin precursors of *T. kiharae* wheat. The signal peptide is underlined, the mature peptide is italicized. Cysteine residues in mature peptide are marked by white letters on the black background, divergent amino acid residues are marked by grey color.

Amino acid sequence of Tk-AMP-BP purothionins of *T. kiharae* wheat was confirmed under transcriptome analysis of seedlings by NGS method. Previously made algorithm was used for searching precursor transcripts in the dataset of sequencing [26]. As a result 15 transcripts encoding precursors of thionin-like peptides consisting of signal peptide, mature peptide and S-terminal prodomain were found in wheat. Sequence of the mature peptide in one of precursors fully

coincided with Tk-AMP-BP purothionin isolated from kernel (Fig. 3). In addition to this transcript another Tk-amp-ap2 transcript encoding close homolog of Tk-AMP-AP1 purothionin was found.

Comparison between sequences of the obtained purothionins and that of thionins of other cereals varieties has shown that Tk-AMP-BP sequence of *T. kiharae* coincides with β-purothionin sequence of *T. aestivum*, as well as with one of two identified β-purothionin sequences of *T. urartu* and *T. monococcum* (see Fig. 2). Hexaploid wheat *T. aestivum* is known to have three thionins marked as β, α_B and α_D purothionins. They are encoded by *pur A1*, *pur B1* and *pur D1* genes located on long arms of chromosome 1A, chromosome 1B and chromosome 1D, respectively [29]. As β-purothionin gene is bound to genome A, the similarity found between sequences of these thionins for genome A carriers (*T. kiharae* (AAGGDD), *T. aestivum* (AABBDD), *T. urartu* (A^uA^u) and *T. monococcum* (A^mA^m) is not surprising. Sequences of β-purothionins are also highly conserved: sequences of Tk-AMP-AP1 of *T. kiharae* and α-purothionins of *T. aestivum* differ by substitution in position 34 (Ser→Ala).

As Tk-AMP-BP thionin of *T. kiharae* was obtained in quantity enough for biological test, it was used for analysis of inhibiting activity against *C. albicans*. According to the study made this thionin under low concentration thereof (MIC = 0.78 μg/ml) is proved to fully inhibit growth of *C. albicans*, i.e. possessing high fungicidal activity against this pathogen. It should be mentioned that thionin activity of other plant species was lower. The thionin of *Arabidopsis* inhibited *C. albicans* growth by 80% at 2.5 μg/ml concentration only [18], while thionin-like protein of *Capsicum annuum* was less active (IC_{50} = 10 μg/ml) [30]. Mechanism underlying effects of wheat thionin on *C. albicans* cells is not known. Biological activity of thionin is considered to be due to direct interaction with cell-target membranes, and three models of such interaction were proposed [11]. Study of purothionin affection on *Rhizoctonia solani* demonstrated that cytolysis of this fungus was caused by steep increase of membrane permeability [31], which was likely due to pore formation [32]. The similar mechanism may be also supposed in *C. albicans* but this assumption is to be experimentally studied.

Thus, two purothionins, the Tk-AMP-BP and Tk-AMP-AP1, were isolated by us for the first time from wheat (*Triticum kiharae*) kernel, with full amino acid sequence thereof being found. Sequence of Tk-AMP-BP purothionin was confirmed under transcriptome analysis of seedlings by NGS method. Tk-AMP-BP purothionin is found to have high fungicidal impact on *Candida albicans* cells acting at very low concentrations (MIC = 0.78 μg/ml) and being superior to thionins of other plant species in activity. High fungitoxicity of wheat thionins against *C. albicans* offers great opportunities in applying this antimicrobial peptide for making new drug products thereunder against candidiases.

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ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL METABOLITES OF *Bacillus subtilis* STRAINS BZR 336G AND BZR 517 USING THE MODIFIED BIOAUTHOGRAPHY METHOD

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Abstract

The ability of *B. subtilis* to produce a large number of biologically active metabolites of diverse structure and properties can largely determine its fungicidal effect against particularly dangerous pathogenic fungi. In this regard, we have studied the fungicidal activity and properties of the antifungal metabolites of active strains *B. subtilis* BZR 336g and BZR 517. The approach we used, including purification by extraction with ethyl acetate, thin-layer chromatography (TLC), UV fluorescence, tests with detection reagents, spectroscopy and bioautography, showed the potential of the studied *B. subtilis* strains to accumulate the complex of active metabolites exhibiting antifungal effect from fungistatic to fungicidal. Bioautographic method was modified by use of the causative agents of harmful diseases, *F. oxysporum* var. *orthoceras* and *Alternaria* sp. as test organisms, which allowed us to assess in vitro the antifungal activity. Patterns of the antifungal compounds differed between the strains in TLC mobility and UV fluorescence. The impact on fungal growth also differed, from slight changes in mycelium growth to total suppression. In strain *B. subtilis* BZR 336g the most pronounced inhibition of *F. oksysporum* var. *orthoceras* BZRP1 growth was in bands with Rf 0.39 and 0.96. Significant *Alternaria* sp. BZRP8 growth inhibition was also observed in two bands, with Rf 0.42 and 0.96. *B. subtilis* BZR 517 synthesizes metabolites exhibiting fungitoxic properties in four bands with Rf 0.42, 0.84. 0.92 and 0.96 against *F. oxysporum* var. *orthoceras* BZRP1, and in two bands with Rf 0.42 and 0.96 against *Alternaria* sp. BZRP8. It is known that, in addition to the direct action, surfactins and fengicins of *B. subtilis* prevent the adhesion of competitive microbes on the plant. In out tests, spraying TLC plates with distilled water revealed bands with hydrophobic properties (non-wetting white zones), which can enhance the antagonistic effect of *B. subtilis.* Test with detection reagents visualized the presence of aromatic amines, phenols, phenolic steroids, methyl hydrazines, that is, compounds that probably play a certain role in the bioactivity manifestation. The weak staining of the metabolites with ninhydrin, as well as the absence of characteristic precipitation during acidification of the culture medium or adding ammonium sulfate, may indicate the presence of only minor amounts of amino acids and peptides. Spectrophotometrically we detected absorption peaks at 205-217 nm wavelengths, which indirectly evidences the presence of polyene structures. Our data allow us to hypothesize mechanisms underlying antifungal effects in the studied *B. subtilis* strains. These effects may be due to hydrolysis of phenolic compounds, since the detecting reagents indicate various phenolic derivatives, and, according to many researchers' opinion, active strains, when interacting with a phytopathogen, produce hydrolytic enzymes. As a result, the active groups of phenols can be released, which enhances the antifungal activity of the synthesized metabolites via effect on both fungal cell walls and other cell structures.

Keywords: *Bacillus subtilis*, microbiological preparations, antifungal metabolites, fungitoxicity, thin-layer chromatography, bioautography, detection reagents, phenolic compounds, polyene structures

Hay bacillus (*Bacillus subtilis*) produces various antimicrobial metabolites: lipopeptides, polypeptides, enzymes, non-peptide compounds [1-3], which

largely determines its fungicidal effect against particularly dangerous pathogenic fungi. The structure and mechanism of action of lipopeptide fungicides, which include active peptides from the families of iturins, surfactins, and fengycins [4, 5], are studied in most detail. It has been proven that the synthesis of lipopeptides of *B. subtilis* plays a key role in the suppression of pathogens in natural conditions, and the production of iturins and fengycins is determined by the presence of phytopathogens in the environment. High efficacy against filamentous fungi is associated with the ability of *B. subtilis* metabolites to influence membranes through interaction with ergosterol, with the formation of pores and subsequent release of monovalent cations from cells which are therefore lysed [5, 6]. For lipopeptides from different families, the specific pore formation mechanisms are different [7]. As a rule, bacteria strains with a high content of lipopeptide antibiotics have a higher antagonistic activity and a wide spectrum of action.

On the other hand, plant polysaccharides stimulate the formation of surfactin produced in the first hours of the interaction of bacilli with root tissues [8, 9]. The fungicidal activity is also associated with the presence of surfactants in bacilli, which are amphipathic molecules with polar and hydrophobic regions [10]. Surfactins are among the most effective biosurfactants (surfactants of biological origin). Having a structure similar to iturins and antagonistic properties, surfactin molecules, in contrast to iturins, contain amino acids with hydrophobic radicals and β-hydroxylated fatty acid [11]. In addition to direct action, surfactins and fengycins of *B. subtilis* prevent the adhesion of competitive microbes and can induce systemic resistance to pathogens and unfavorable abiotic factors in plants [12). Interestingly, lipopeptide antibiotics can be perceived by plant cells as a signal to initiate defense mechanisms, that is, to be elicitors [13]. Surfactins and fengycins, as elicitors of the host plant immune response, have been shown to significantly induce the defensive response of beans to the causative agent of gray mold *Botrytis cinerea* [14].

In this research, metabolites that suppress the development of harmful phytopathogenic fungi were first detected in the *Bacillus subtilis* BZR 336g and BZR 517 strains. Using the original analytical methods and approaches, we determined the properties and chemical structure of the identified compounds. The results obtained give reason to consider both of the studied strains as potential producers of biofungicides.

The aim of this research was to isolate, study the chemical structure and quantify the antifungal activity of metabolites released into the culture fluid by two promising strains of *B. subtilis*.

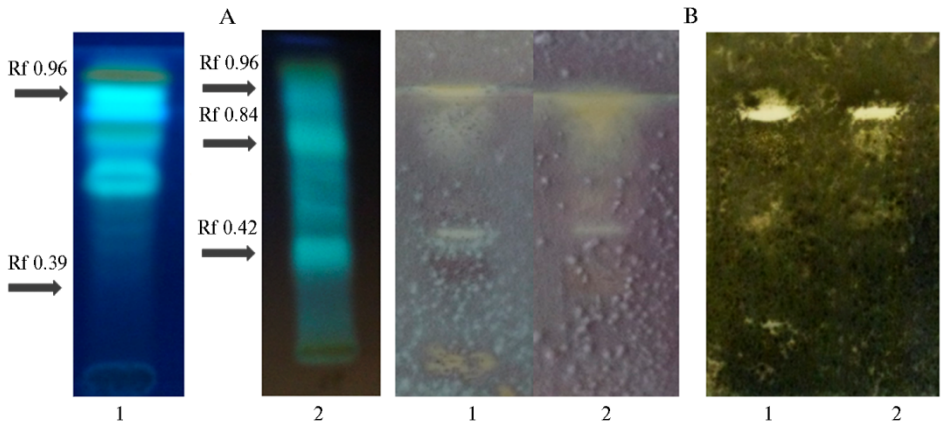
Techniques. We used *B. subtilis* BZR 336g and BZR 517, the producers of experimental biologicals [15-17], as well as cultures of phytopathogenic fungi *Fusarium oxysporum* var. *orthoceras* BZRP1 (a causative agent of root rot in grain and oil crops) and *Alternaria* sp. BZRP8 (a causative agent of Alternaria blight in fruit crops) from the collections of the All-Russian Research Institute of Biological Plant Protection (ARRIBPP).

The liquid culture of biofungicide based on bacterial strains was obtained by the method of periodic culture (New Brunswick Scientific thermostating systems, Excella E25, USA) for 48 h at 180 rpm. The composition of the nutrient medium and the culture conditions are commercial secrets of ARRIBPP (Order No. 42-p dated November 28, 2012). After the culturing was completed, the liquid culture of *B. subtilis* was centrifuged for 20 min at 12,000 g, the supernatant was extracted for 1 h with three volumes of ethyl acetate. The ethyl acetate extract was evaporated, the dry residue was dissolved in a minimum amount of ethyl acetate and analyzed by ascending thin-layer chromatography (TLC) on

20×20 cm plates with modified Kieselgel 60 silica gel (Merck, Germany), layer thickness 2 mm, solvent system ethyl acetate:ethanol:water (40:15:15). After viewing under UV light at λ = 366 nm and removing traces of solvents, the TLC-plates were soaked in the potato-glucose nutrient medium, then in a suspension of propagules of the test fungus and placed for 48-50 h in a moist chamber at 24 °C for *Alternaria* sp. BZRP8 and 28 °C for *F. oxysporum* var. *orthoceras* BZRP1. The localization of the active components was detected by the formation of zones of absence or inhibition of the fungus growth [18, 19].

For further study of the active components of the culture fluid, the required amount of ethyl acetate extract was separated by TLC as described above. The zones corresponding to the active antifungal compounds were scraped off and eluted with ethyl acetate. For qualitative analysis of eluates, specific detection reagents were used [19]. The absorption spectra of the active fractions of *B. subtilis* BZR 336g and BZR 517 metabolites were determined at λ = 200-400 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, USA).

Results. When viewing the TLC-plates under UV_{366} light, we found a significant set of produced metabolites which differed in chromatographic mobility and character of fluorescence (Fig., A) in both strains of *B. subtilis*. At the same time, the observed patterns of the strains differed.



The results of thin-layer chromatography (A, fluorescence in UV_{366} light) **and bioautograms** (B, on the left with the *Fusarium oxysporum* var. *orthoceras* BZRP1 test culture, on the right with *Alternaria* sp. BZRP8) **of ethyl acetate extracts isolated from culture liquids of *Bacillus subtilis* BZR 336g** (1) **and BZR 517** (2) **strains** (plates with modified Kieselgel 60 silica gel, Merck, Germany; after chromatographic separation of metabolites, silica gel was soaked in the potato-glucose nutrient medium, then in a suspension of propagules of the fungus and placed for 48-50 h in a moist chamber).

To identify the active fractions of metabolites by the method of bioautography, phytopathogenic fungi *F. oxysporum* var. *orthoceras* BZRP1 and *Alternaria* sp. BZRP8 were used as test objects to evaluate the antifungal effect against causative agents of root rot in grain crops and Alternaria blight in fruit crops. Both studied strains synthesized a complex of active compounds with fungistatic and fungicidal properties (see Table). Four most pronounced zones of complete growth inhibition of the fungus *F. oxysporum* var. *orthoceras* BZRP1 were found for BZR 517 strain, and only two for BZR 336g. The character of the fungus growth also differed from the usual due to the fungistatic effect of metabolite compounds (see Fig., B). The number of metabolite zones with fungitoxic effects significantly reduced on bioautograms with the *Alternaria* sp. BZRP8 fungus: only two most distinct zones of inhibition of the fungus were identified in both strains (see Table). However, in BZR 517, the manifestation of antifungal properties was more pronounced than in BZR 366g (see Fig., B).

Mobility, UV_{366}-fluorescence and antifungal properties of ethyl acetate extracts from culture fluid produced by *Bacillus subtilis* strains

Strain	Rf	Fluorescence	Antifungal activity	
			Fusarium oxysporum var. *Orthoceras* BZRP1	*Alternaria* sp. BZRP8
B. subtilis BZR 336g	0.32	Weak gray	–	–
	0.39	Bluish-gray	+++	–
	0.42	Bluish-gray	+	++
	0.51	Gray-blue	–	–
	0.57	Gray	–	–
	0.73	Bright whitish	+	–
	0.78	Bright greenish	+	–
	0.81	Dark blue	+	–
	0.84	Light greenish	-	–
	0.87	Dark green	+	–
	0.91	Bright whitish	+	–
	0.94	Bright whitish	+	+
	0.96	Intense dark green	+++	+++
B. subtilis BZR 517	0.32	Gray	–	–
	0.39	Bright light blue	+	–
	0.42	Gray	+++	++
	0.48	Light blue	+	–
	0.56	Light blue	+	–
	0.67	Gray	+	–
	0.75	Bright light blue	+	–
	0.84	Gray	++	–
	0.92	Light blue	+++	+
	0.96	Greenish yellow	+++	+++

Note. "+++" means fungicidal activity, "++" means average fungistatic activity, "+" means weak fungistatic activity; dashes indicate the absence of growth inhibition (separation of components by thin-layer chromatography with modified Kieselgel 60 silica gel, Merck, Germany).

Spraying the plate with distilled water allowed us to identify components with hydrophobic properties (manifested as white spots), one zone with Rf 0.84 in BZR 517 strain, two zones with Rf 0.75 and 0.84 in BZR 336g. According to many authors, the presence of surface-active metabolites can enhance the antagonistic properties of bacteria due to the influence on their ability to quickly spread over the leaf surface and in the rhizosphere [8].

With detecting reagents, we found functional groups that can contribute to the appearance of the biological activity of metabolites in the strains studied. When sprayed with a freshly prepared mixture of 0.1 M $FeCl_3$ and 0.1 M potassium ferricyanide (1:1), one zone of a BZR 517 metabolite (with Rf 0.92) and three zones of BZR 336g metabolites (with Rf 0.42, 0.57 and 0.73) were stained in a bright dark blue color, which indicates the presence of active compounds of aromatic amines, phenols and phenolic steroids. Navy blue staining of zones with a solution of Na_2CO_3 and Folin's reagent in BZR 517 and BZR 336g strains probably indicates the presence of methyl hydrazines and phenolcarboxylic acids. Using a 0.25% ninhydrin solution, the presence of free amino groups is detected in the same zone with Rf 0.73 for BZR 336g strain (a yellow-orange spot). Two zones with Rf 0.42 and 0.73 in BZR 336g stained in dark red with 0.5% "fast red B" salt solution may indicate the presence of phenols and hydroxybenzophenones [20].

According to the study, some assumptions can be made regarding the nature of the action of antifungal metabolites in the *B. subtilis* BZR 336g and BZR 517 strains. Since the detecting reagents indicate the presence of various phenolic derivatives, and the active strains, when interacting with the phytopathogen, produce hydrolytic enzymes [21, 22], then one of the mechanisms by which the phytopathogenic microorganisms are suppressed can be the hydrolysis of phenolic compounds. At the same time, the active groups of phenols are released, which contributes to the enhancement of the antifungal activity of the

synthesized metabolites. Since the attempt to precipitate the studied metabolites by acidification or ammonium sulfate was not successful, we assume that most of them have a non-peptide structure. There is evidence that some strains of *B. subtilis* form polyene antibiotics with conjugated double bonds that can inhibit the growth of phytopathogenic fungi [23]. A spectrophotometric study of the active fractions of metabolites in *B. subtilis* BZR 336g and BZR 517 showed that both strains produced compounds with an absorption maximum at λ = 205-217 nm, which serves as indirect evidence of the presence of short polyene chains in the structure of the studied substances. For long polyene chains, the absorption bands fall into the visible region, that is, the compound becomes colored (Rf 0.96) [24].

Many authors have shown that enhanced production of active metabolites contributes to an increase in the antagonistic activity of *B. subtilis* strains selected as promising biofungicidal agents. In cotton disease caused by *Rhizoctonia solani*, the antifungal effect of the *B. subtilis* NCD-2 strain on the pathogen was found to be mainly related to fengycin lipopeptides [25]. On melon leaves, lipopeptides from the families of iturins and fengycins [26] played a key role in the antagonism of four strains of *B. subtilis* against the pathogen *Podophaera fusca*. Fengycins synthesized by *B. subtilis* were of primary importance in the protection of leguminous plants from *Pythium ultimum* [27, 28]. The *B. subtilis* CMB32 strain synthesizes lipopeptides responsible for the antifungal activity against *Colletotrichum gloeosporioides* which causes anthracnose in plants [29]. In the strains we studied, the antifungal metabolites, unlike the known lipopeptides [30], appear to have a non-peptide nature.

Thus, *Bacillus subtilis* BZR 336g and BZR 517 strains synthesize a significant amount of fungitoxic metabolites. Thin-layer chromatography, bioautography, detection with developing reagents and optical spectroscopy methods allowed for data directly or indirectly characterizing the properties and structure of bioactive compounds produced by the studied strains. Their prospective value lies in the ability to synthesize a large set of antifungal metabolites, including, probably, non-peptide compounds. The results obtained confirm the high potential of *B. subtilis* BZR 336g and BZR 517 strains when used to develop means of protection against phytopathogenic fungi.

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THE FIRST MYCOTOXICOLOGICAL INVESTIGATION OF WHITE MUSTARD (*Sinapis alba* L.)

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Abstract

Crops widely represented on cultivated lands and often found in natural botanical formations have attracted increasing attention of researchers in recent years. This is due not only to their economic importance, but also to the high value of both experimental facilities that allow studying the features of the formation of the diversity of biocenotic connections and ecological equilibria. According to modern concepts, a complex of secondary substances in plants is increasingly seen as a joint product of their associations with microorganism communities, mainly microscopic fungi (**S.** Kusari et al., 2012). Recently, Russian researchers performed the first cycle of studies aimed at a comparative study of the content of mycotoxins in cereals and legumes in industrial crops and in the natural habitat (G.P. Kononenko et al., 2015; A.A. Burkin et al., 2017). In the present work, we obtained first information about the nature of the contamination of cruciferous plants with toxic metabolites of microscopic fungi, revealed for the first time differences in their localization in vegetative and generative organs, as well as changes accompanying the full development cycle. The aim of this work was to study the composition and content of mycotoxins in the white mustard (*Sinapis alba* L.), a cultivated plant of wide application, which also easily populates agricultural land and occurs in natural grass stands. For analysis, we used overground parts of plants and their organs (leaves, stems, flowers, pods) collected in the white mustard monoculture in 2017 during distinct phases of plant development. These phases were i) the beginning of the growing season after the completed formation of plant basic structure, ii) mass flowering, iii) the formation of green pods and iv) full ripening. The mycotoxins determined by the enzyme-linked immunosorbent assay were T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), aflatoxin B_1 (AB_1), sterigmatocystin (STE), roridin A (ROA), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR) and ergot alkaloids (EA). AOL, CPA, and EA were found in the mycotoxin complex of *Sinapis alba* organs during vegetation period, and all other metabolites were absent or detected sporadically. The very moderate accumulation of mycotoxins in this plant is a useful economic property, and previously no such slightly contaminated cultures were detected among examined cereals and legumes. During the vegetation of the mustard, the composition of mycotoxins and the quantitative ratios between them were generally stable, but the content of AOL and CPA decreased as the plant matured. Mass flowering was accompanied by the appearance in the plant of fusariotoxins DAS, DON, FUM, which were not detected in the next phase (pod formation). In experiments with individual organs of *Sinapis alba*, multiple and intense flower contamination with all analyzed mycotoxins, complete absence of fusariotoxins in green and ripe pods, as well as increased levels of AOL accumulation in leaves compared with stems are established for the first time. Possible causes of this phenomenon, the scientific and practical significance of new information on the degree of contamination, seasonal dynamics and accumulation of mycotoxins in this plant, as well as the prospects for further scientific research are discussed.

Keywords: white mustard, *Sinapis alba*, mycotoxins, T-2 toxin, diacetoxyscirpenol, deoxynivalenol, zearalenone, fumonisins, alternariol, aflatoxin B_1, sterigmatocystin, roridin A, cyclopiazonic acid, emodin, ochratoxin A, citrinin, mycophenolic acid, PR toxin, ergot alkaloids, ELISA

In the recent years, a strong focus has been given to the crops that are widely grown as well as widespread naturally. This is due to the crops' economic importance and involvement in ecological research of biocenotic relations and biological balance. Russian researchers have recently completed the first cycle of a comparative study of mycotoxins in cereal and legume grasses in commercial crops and natural habitat [1-3]. The complex of secondary substances in plants is increasingly seen as a joint product of their associations with microbial communities, mainly microscopic fungi [4].

The cultures of the *Cruciferae* (*Brassicaceae*) family represented in agrobiocenoses by a variety of genera and species have not yet been examined in this aspect. Among them, annual plants are the most attractive as, along with high yields and a short growing season, they differ in soil requirements, resistance to drought, lower temperatures, diseases and pests. These plants are used as the green manure due to their active role in the formation of coenoses — in improving soil quality and soil decontamination, in particular, neutralization of heavy metals [5].

White mustard (*Sinapis alba* L.) is an annual cultivated plant that easily populates agricultural land and natural grass stands, is found on waste grounds, along roads, in gardens and orchards. It is cultivated for seeds that are used to produce edible and technical oil; its young greens are used for food; the crops are used for grazing, grass meal, silage, and in early phases before the formation of pods the white mustard's grass stands are mown for livestock feed.

Recent years have been marked by major achievements in the selection of this culture, especially due to the development of a genomic approach in assessing the diversity of germplasm [6-9]. However, despite its versatile use, the data on the secondary metabolites of white mustard is very limited. For instance, acetylcholine was detected in the leaves and stems, and histamine in the seedlings of the white mustard [10]. Its seeds contain B vitamins, phytosterols, unsaturated fatty acids and sinalbin glucoside [11], as well as groups of nitrogen- and sulfur-containing compounds, the glucosinolates [12, 13]. A detailed analysis of the genetic apparatus disclosed the mechanisms of the biosynthesis of erucic acid, glucosinolates, and the phytochelatin, a fragment of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ peptides formed as a result of a reaction involving phytochelatin synthase (EC 2.3.2.15) [14, 15]. No information was found in the available literature about the toxic properties of micromycetes colonizing this culture, nor about the content of fungal metabolites in these plants or formed *in planta* together with endophytic fungi.

In our research, we were the first to study the nature of the contamination of cruciferous plants by toxic metabolites of microscopic fungi. We have revealed differences in their localization in vegetative and generative organs, as well as changes along the entire development cycle.

Our research focused on the composition and content of toxic metabolites of microscopic fungi in white mustard and their distribution in the plant's organs.

Techniques. The studied samples were mustard plants sown on May 28, 2017 on the trial ground of the Timiryazev Moscow Agrarian Academy. Samples were collected first on days 18 and 25 after sowing as soon as the basic structure of the plant was formed (stage I) and later on days 39 and 41 during the blooming (stage II), on days 53 and 58 during green pods (stage III), and on days 67 and 97 at maturity (stage IV). The above-the-ground parts of the plants were cut at a height of 3-5 cm from the ground. From day 39 after sowing, the plants were sorted into vegetative (stalks, leaves) and generative (flowers, pods) organs, which were later analyzed along the same indicators. The samples were dried in

a shaded ventilated room and then crushed in a laboratory mill. For extraction, a mixture of acetonitrile and water was used (84:16, 10 ml per 1 g of sample). Extracts after a 10-fold dilution with phosphate-saline buffer (pH 7.5) were used for indirect enzyme-linked immunosorbent assay.

The content of mycotoxins T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), aflatoxin B_1 (AB_1), sterigmatocystin (STE), roridin A (ROA), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR), and ergot alkaloids (EA) were determined using certified test systems [16]. The lower limit of measurement corresponded to the 85% antibody binding.

The data are presented as arithmetic mean values (M) and sample mean error (±SEM); statistical processing was done using Microsoft Office Excel 2013 and the Wilcoxon non-parametric total rank test with continuity correction by R version 3.4.3 [17, 18].

1. Mycotoxin incidence (n^+) and content in whole white mustard (*Sinapis alba* L.) plants (μg/kg) **after formation of the basic structure** (stage I), **during bloom** (stage II) **and at green pods** (stage III) (Moscow, 2017)

Mycotoxin	Plant development stage		
	I (n = 8)	II (n = 7)	III (n = 5)
T-2	–	–	–
DAS	–	3 (115±15)	–
DON	–	6 (81±2)	–
ZEN	–	–	–
FUM	–	1 (62)	–
EA	8 (7±1)	5 (3±0)	5 (5±1)
AOL	8 (37±2[a])	7 (43±2[a])	5 (20±1[b])
AB_1	–	1 (2)	–
STE	2 (16, 25)	–	1 (20)
ROA	–	–	–
CPA	8 (370±9[a])	7 (190±7[b])	5 (235±8[b])
EMO	–	1 (25)	–
OA	1 (6)	–	1 (5)
CIT	–	–	1 (40)
MPA	4 (22±1)	–	–
PR	–	–	–

Note. T 2 is T-2 toxin, DAS is diacetoxyscirpenol, DON is deoxynivalenol, ZEN is zearalenone, FUM is fumonisins, EA is ergot alkaloids, AOL is alternariol, AB_1 is aflatoxin B_1, STE is sterigmatocystin, ROA is roridin A, CPA is cyclopiazonic acid, EMO is emodin, OA is ochratoxin A, CIT is citrinin, MPA is mycophenolic acid, PR is PR toxin; n is the number of investigated samples; n^+ is the number of positive samples. Stage I is days 18 to 25 after sowing, stage II is days 39 to 41, stage III is days 53 to 58. Specific mycotoxin levels identified in positive samples or M±SEM are given in parentheses. Dashes indicate that no positive samples were found. Values on the same line with different superscript indices (a, b, c) differ significantly at $p < 0.05$.

Results. Three of 16 mycotoxins: CPA (hundreds of micrograms per 1 kg), AOL and EA (1-2 times less quantities) were regularly detected in young plants before blooming (stage I) (Table 1). MPA was detected only in half of the samples, and CTE and OA in just a few. Such non-uniform detection of substances may have been due to the fact that their concentrations were near the sensitivity threshold of the method or were outside the measurement range.

At stage II (blooming), along with CPA, AOL and EA, we also detected DAS, DON and FUM, and AB_1 and EMO among the mycotoxins with a basal level in single samples. Interestingly, STE, OA and MPA that are rarely detected at the beginning of the growing season were not found in the flowering plants. DAS was dominant among fusariotoxins, while the frequency of DON and FUM were a lot less. A similar relationship with the predominance of DAS was noted earlier in other objects, but its cause is still unclear. In plants with already formed pods (stage III), the same three mycotoxins were present, the CPA, AOL and EA, with the rare detection of CTE, OA and CIT. Statistical processing of the results showed a significant ($p < 0.05$) decrease in the content of AOL and CPA in the ground parts of the mustard during the growing season, but at different times (Table 1). The CPA concentration decreased during flowering and remained the same afterwards; the AOL concentration decreased at a later stage of the formation of pods. The same trend was earlier revealed with the CPA concentration in wild-growing cereal grasses by the end of their vegetation in August—September [1].

The green mass of the mustard is generally characterized by a moderate accumulation of mycotoxins, which is considered a useful economic trait. Among the significant diversity of the studied legume grasses, crops that would be so weakly contaminated have not yet been identified [19]. This can be considered as a manifestation of resistance to fungal diseases, which, along with high yields, soil tolerance, drought tolerance and a short vegetation period, serve as the criteria for assessing the viability of annual oil-bearing crops of wide application. Apparently, the accumulation of mycotoxins is the result of genetically-derived plant relationships with microscopic fungi, which determine the internal mycobiota and the specific profile of secondary metabolites.

The presence of three contaminants in all the examined samples probably indicates that they were caused by endophytic fungi. Given the low accumulation of AOL and EA, there were no highly active producers among those fungi or their habitat conditions did not contribute to intense metabolic reactions. Significant accumulation of CPA, which may be associated with infection with certain *Aspergillus* species [20], is not characteristic of the mustard but it was found in other plants, as often and in larger concentrations [19]. It cannot be ruled out that those metabolites were products of associated biosynthesis involving fungi and plants.

2. Mycotoxin incidence (n^+) and content in whole white mustard (*Sinapis alba* L.) plants (μg/kg) **at massive blooming** (day 39 and day 41, stage II) (Moscow, 2017)

Mycotoxin	Organs		
	stems (n = 4)	leaves (n = 4)	flowers (n = 4)
T-2	–	–	4 (2.8±0.3)
DAS	–	–	4 (365±8)
DON	–	–	4 (170±10)
ZEN	–	–	4 (28±2)
FUM	–	–	4 (100±3)
EA	3 (6±2)	3 (4±2)	4 (5.8±0.8)
AOL	4 (18±1[a])	4 (39±5[b])	4 (77±8[c])
AB_1	–	–	4 (3.3±0.3)
STE	–	2 (14. 14)	4 (26±3)
ROA	–	–	–
CPA	4 (40±8[a])	4 (250±29[b])	4 (455±61[c])
EMO	–	–	4 (45±7)
OA	–	–	2 (8.8)
CIT	–	–	4 (39±2)
MPA	–	–	4 (33±6)
PR	–	2 (100. 115)	4 (335±42)

N o t e. T 2 is T-2 toxin, DAS is diacetoxyscirpenol, DON is deoxynivalenol, ZEN is zearalenone, FUM is fumonisins, EA is ergot alkaloids, AOL is alternariol, AB_1 is aflatoxin B_1, STE is sterigmatocystin, ROA is roridin A, CPA is cyclopiazonic acid, EMO is emodin, OA is ochratoxin A, CIT is citrinin, MPA is mycophenolic acid, PR is PR toxin; n is the number of investigated samples; n^+ is the number of positive samples. Specific mycotoxin levels identified in positive samples or M±SEM are given in parentheses. Dashes indicate that no positive samples were found. Values on the same line with different superscripts (a, b, c) differ significantly at $p < 0.05$; for differences in the AOL and CPA concentrations in the plant's organs the p-value was 0.02857.

Most pathogens of cruciferous fungal diseases are not among the producers of the analyzed substances. Nevertheless, the detection of a relatively large group of background mycotoxins in said plants may be associated with the toxigenic species of the *Penicillium*, *Aspergillus* genera from the so-called secondary fungi associated with the pathogen complex. Among the few harmful diseases of the cruciferous which may cause the accumulation of mycotoxins, alternariosis should be noted along with fusariosis. According to Russian researchers, 9 pathogens of pheodictiosporic hyphomycetales were found, including 5 species of the genus *Alternaria* (including *A. tenuissima*), as well as the fungi belonging to the *A. infectoria* complex [21]. The participation of *Alternaria* toxigenic small spore species can lead to the AOL accumulation in the plant [22, 23), however, judging by the low concentration of this metabolite without evidence of obvious variation during the vegetation, its presence is either not associated with pathogenic species or the activity of the AOL producers is limited by the physiological characteristics of the culture.

To get an idea of the nature of the distribution of mycotoxins in the white mustard and the dynamics of their concentration during the plant's devel-

opment, we investigated the vegetative and generative organs in different periods (Tables 2, 3). In the flowering phase (see Table 2), the stems and leaves retained the signs characteristic of young plants of the 1st collection, except that half of the leaf samples contained PR. Concentrations of AOL and CPA in the leaves significantly ($p < 0.05$) exceeded the ones observed in the stems. The mustard flowers had a very peculiar profile of mycotoxins: they contained all substances except ROA, while DAS, CPA and PR were detected in significant quantities (hundreds of micrograms per 1 kg). It is important to note that we could measure the concentrations of MPA, STE, AB_1, EMO and PR, which were previously assigned to the group of background mycotoxins, i.e. they fell into the scope of definition. Among the newly discovered were CIT as well as the T-2 and ZEN fusariotoxins. Obviously, the expanded composition of mycotoxins in the green mass of mustard that was noted in the flowering phase was mainly due to the contribution of flowers with the partial participation of stems and leaves.

The appearance of fusariotoxins in the flowers may have been due to infection with *Fusarium* fungi along with the complex of toxigenic species. Along with trichothecenes T-2 and DAS characteristic of the fungi *F. sporotrichioides* and *F. langsethiae*, we identified DON and ZEN characteristic of *F. graminearum*, and FUM whose main producers are considered to be *F. verticillioides* and *F. proliferatum* [24]. The weak accumulation of T-2, DON, ZEN, and FUM, as well as the absence of fusariotoxins in the leaves and stems could be due to a short flowering period and a rapid fall of flowers. However, an unusually high accumulation of DAS in the mustard flowers (an average of 365 μg/kg), which was twice larger than the amount of T-2, has not been explained so far. Given the multiple contamination of flowering plants when grown for food, earlier mowing is advisable.

3. Mycotoxin incidence (n^+) and content in whole white mustard (*Sinapis alba* L.) plants (μg/kg) **at phases of green pods** (day 53 and day 58, stage III) **and after pod ripening** (day 67 and day 97, stage IV)

Mycotoxin	Stage III			Stage IV	
	stems (n = 7)	leaves (n = 3)	pods (n = 8)	stems (n = 18)	pods (n = 19)
T-2	–	–	–	–	–
DAS	–	–	–	–	–
DON	–	2 (83, 100)	–	–	–
ZEN	–	–	–	–	3 (28±2)
FUM	–	–	–	–	–
EA	6 (10±2)	2 (2, 4)	6 (3±1)	–	1 (2)
AOL	7 (25±5[a])	3 (68±16[b])	8 (30±4[a])	7 (69±24)	6 (40±5)
AB_1	2 (2, 2)	3 (3±1)	3 (2±0)	–	–
STE	2 (15, 19)	–	2 (15, 16)	1 (13)	5 (14±2)
ROA	–	–	–	–	–
CPA	7 (205±37[a])	3 (220±91[a])	8 (275±31[a])	–	18 (140±7[b])
EMO	–	3 (37±6)	3 (22±2)	2 (25, 28)	2 (21, 26)
OA	–	–	1 (6)	–	1 (5)
CIT	–	1 (40)	–	–	1 (16)
MPA	–	1 (26)	1 (26)	–	–
PR	–	–	–	–	–

Note. T 2 is T-2 toxin, DAS is diacetoxyscirpenol, DON is deoxynivalenol, ZEN is zearalenone, FUM is fumonisins, EA is ergot alkaloids, AOL is alternariol, AB_1 is aflatoxin B_1, STE is sterigmatocystin, ROA is roridin A, CPA is cyclopiazonic acid, EMO is emodin, OA is ochratoxin A, CIT is citrinin, MPA is mycophenolic acid, PR is PR toxin; n is the number of investigated samples; n^+ is the number of positive samples.. Specific mycotoxin levels identified in positive samples or M±SEM are given in parentheses. Dashes indicate that no positive samples were found. Values in the same line with different superscript indices (a, b) differ significantly at $p < 0.05$.

At the third stage, AB_1 and STE were detected in the stems, along with the previously found EA, AOL and CPA. The composition of the analyzed substances turned out to be wider in the leaves and, in addition to EA, AOL, CPA and AB_1, we detected EMO, CIT and MPA; from among the fusariotoxins, only DON was found in some samples (see Table 3). In green pods, the complex of

mycotoxins in the total number of detected substances coincided with their composition in the leaves, with a few differences in the minor components, i.e. STE and OA were detected instead of DON and CIT.

The AOL concentration in the pods did not differ from the stems, but it was lower than in the leaves, and the CPA concentration remained the same as in the leaves and stems. Rare or absent fusariotoxins in vegetative organs (stems, leaves) and pods indicate that there is something precluding their spread throughout the plant, which may be due to the short flowering period and the rapid fall of flowers. At this stage of development, the AOL concentration in the leaves was significantly higher ($p < 0.05$) than in the stems and pods, with no differences with respect to CPA.

The contamination of the plant generally decreased at stage IV, what followed from sporadic or absent mycotoxins in the stems and ripe pods. AOL was found in less than half of the samples, STE and EMO – only in rare cases. Some samples of the pods carried ZEN, OA and CIT in small quantities; CPA and EA, which were found in the green plants, were not detected in the stems. Nevertheless, we could measure the CPA concentration in all samples of the yellowed ripe pods, except one. Its concentration significantly decreased compared to the green pods ($p = 0.000219$) (see Table 3). It is possible that such a sharp decrease in the concentration of this metabolite is associated with the suppression of the functions of endophytic fungi or the completion of the life cycle of the plant.

Interestingly, the first experiments with the sunflower and leguminous plants (red clover, white clover, spring vetch, sweet clover, galega orientalis, lupinus polyphyllus), divided into leaves, stems and flowers, have also revealed regular differences that are yet to be assessed. The uneven distribution of mycotoxins in plants can be the result of migration of both the metabolites and toxigenic fungi, as well as the result of physiological and metabolic contacts within the fungal community and with the host organism. Interpretation of the obtained results from the point of view of the role of pathogenic and endophytic fungi in the symbiosis, with the differentiation between the groups of basic (constitutive) and induced mutualism, will be the next important step in understanding the mechanisms of interaction between higher plants and microscopic fungi.

The mature mustard pods were generally very weakly contaminated with mycotoxins. This fact is of great practical importance, since the mustard's seeds have been lately considered as a unique and promising source of physiologically active substances. For instance, it has been revealed that the mustard seeds contain a complex of compounds that inhibit the formation of tumors as well as have antibacterial and antioxidant effects [25]. It has been experimentally proven that the products of the hydrolysis of glucosinolates, 4-methylsulfanyl-3-butenyl isothiocyanate in particular, have chemoprotective properties and can suppress carcinogenesis processes [26]. Waste from seed squeezing or extracting is used in feeding animals, although it is recommended to use mustard cakes and residues with care because of the simple nitriles that are formed during the specific enzymatic hydrolysis of different glucosinolates [27]. It is equally important to study the contamination of this animal food with mycotoxins because the example of other oilseeds, the sunflower, has revealed significant differences in the composition of toxicants in the seeds vs. products of its processing and storage [28].

In our experiments, the white mustard seed was characterized by a tendency to decrease the AOL and CPA concentration as the plants mature. Considering that seasonal mycotoxin dynamics in leguminous grasses, on the con-

trary, revealed that their concentration remained the same or grew [19], it seems important to undertake mycotoxicological examination of *Sinapis alba* from mixed naturally formed communities.

In the future, such projects are advisable for other cruciferous plants that are considered promising for introduction in feed production and farming, such as oil radish (*Raphanus sativus* var. *oleifera* Metzg.), redberry (*Camelina* sp.), abyssian cramba (*Crambe abyssinica* Hochst.), winter rape (*Brassica napus* L. ssp. *oleifera* Metzg.), winter rape (*Brassica rapa* L. ssp. *oleifera automnalis*) and others, as well as the wild-growing members of this family.

Therefore, a complex of toxic metabolites of mycogenic origin in the white mustard under monoculture conditions is mainly represented by alternariol, cyclopiazonoic acid, and ergot alkaloids, and its composition remains stable at all phases of the plant development. For alternariol and cyclopiazonic acid, preferential localization was revealed in the leaves vs. stems, while the flowers have a multiple contamination, with an increased accumulation of metabolites and the presence of fusariotoxins. The concentration of cyclopiazonoic acid decreases in the plant during the blooming, while alternariol decreases during the green pods phase. This new data and the identified patterns are very important for continuing the fundamental research of the biological role of fungal metabolites in plants, as well as for finding appropriate measures to prevent intoxication due to the contamination of food, feed and the environment with mycotoxins.

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