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MICROBIOLOGICAL CONTROL IN PHYTOSANITARY OPTIMIZATION TECHNOLOGIES FOR AGROECOSYSTEMS: RESEARCH AND PRACTICE

(review)

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Abstract

Phytosanitary optimization of agroecosystems targeted to control harmful arthropods and plant pathogens should use a complex of multifunctional biologics based on microbial antagonists of pathogens, producers of bioactive substances and entomopathogens (V.D. Nadykta et al., 2010; Rohini et al.; 2016, M. Ghorbanpour et al., 2017). The most promising microbial strains for plant protection are those possessing not only a direct target effect but also the ability to increase plant disease resistance due to phytoregulatory activity (I.I. Novikova, 2016). The holistic concept of microbiological protection involves the development and use of biological products based on living cultures of entomopathogenic microorganisms and antagonistic microbes with preventive and prolonged action, as well as formulations based on metabolite complexes to quickly reduce the density of phytopathogen populations (I.I. Novikova et al., 2016). Developing multifunctional biological products for plant protection is based on technological strains with high biological activity that are safe for humans and warm-blooded animals. It has been shown that the role of entomopathogenic viruses, microsporidia, bacteria and fungi in the dynamics of the number of phytophagous insects is determined by the type of pathogenesis (obligate or facultative). In case of intracellular obligate parasitism of baculoviruses and microsporidia, mass epizootics were observed in unpaired silkworms (*Lymantria dispar* Linnaeus), leafworms (family *Tortricidae* Latreille), cabbage whitewash (*Pieris brassicae* Linnaeus), meadow and corn moths (*Loxostege sticticalis* Linnaeus, *Ostrinia nubilalis* Hübner), ginger pine (*Neodiprion sertifer* Geoffroy) and black bread (*Cephus pygmeus* Linnaeus) sawflies, Siberian silkworm (*Dendrolimus sibiricus* Tschetverikov), cotton (*Helicoverpa armigera* Hübner) and gray grain scoops (*Apamea anceps* Denis & Schiffermüller) (I.V. Issy, 1986; A. Vey et al., 1989; A.N. Frolov et al., 2008; V.A. Pavlyushin et al., 2013). The regulatory role of *Entomophthora* infection is most pronounced in various species of aphids and some species of locusts (G.R. Lednev et al., 2013). For facultative parasitism which is characteristic of entomopathogenic fungi of genera *Beauveria*, *Metarhizium*, *Lecanicillium*, etc. (E. Quessada-Moraga et al., 2004), as well as bacteria of *Bacillus thuringiensis* group (N.V. Kandybin, 1989) and genus *Xenorhabdus* members, the most important factor of virulence is toxigenicity against host insects (M. Faria et al., 2007). Hydrolytic enzymes (chitinases, lipases, proteases), toxins, and antiphagocytic defense are factors of entomopathogenic fungi virulence. Microbiological protection of plants from diseases is based on the use of highly competitive strains that synthesize complexes of hydrolases and biologically active compounds and efficiently colonize suitable ecological niches (I.V. Maksimov et al., 2015; I.I. Novikova, 2016; I.I. Novikova et al., 2016). A number of active compounds produced by rhizosphere microorganisms possess elicitor activity and trigger induced resistance (J.W. Kloepper et al., 2009; N. Ohkama-Ohtsu et al., 2010). The effectiveness of biologicals developed at the All-Russian Research Institute of Plant Protection against the main harmful diseases of crops reaches 60-90%, which provides a 20-25 % increase in productivity and improves the quality of crop production (I.I. Novikova, 2017). The plant microbiological protection concept relies on the search for promising producers of novel biologicals among wider range of microbial species and strains, on the design of new formulations optimal in specific environmental conditions, and on biological plant protection and integrated plant protection management which combines biological products for various purposes depending

on the specific complex of plant pathogens and the local phytosanitary situation in general (N.A. Belyakova et al., 2013).

Keywords: biologicals, bio-effectiveness, entomopathogenic microorganisms, antagonist microbes, harmful arthropods, plant pathogenic fungi, plant pathogenic bacteria, usable pesticides, bioactive complexes, elicitors

Phytosanitary optimization of agroecosystems via use of ecologically low-hazard products for plant protection can effectively improve crop production and quality to solve the pressing problems worldwide. The environmental effects, resource and energy efficiency of agro-technologies which provide optimized conditions for useful organisms and increase the stability of agrobiocenoses are the main indicators of the adaptability and efficiency of agriculture [1-3]. In the last decade, the strategy and tactics of phytosanitary optimization with environmentally low-hazard plant protection products and innovative technologies for their application have been significantly rethought. The general concept of plant protection as a scientific discipline is the construction of intensive and ecologically sustainable agroecosystems based on the optimization of trophic connections and other mechanisms of biocenotic regulation.

Here, we aimed to characterize the state and current trends in the development of microbiological plant protection in the Russian Federation and abroad based on fundamental and applied research data.

At present, anthropogenic impact results in a deep transformation of the structure and functional patterns of various types of agroecosystems [4, 5]. In agrobiocenoses, as monodominant systems, there is an increase in the population density and harmfulness of a number of plant pathogens, harmful arthropods and weeds with intensive formation of groups of dominant and superdominant species. Cases of their mass reproduction occur more and more frequently, zones of severity expand, and microevolution within populations is activated. Along with a general decrease in the diversity of biological communities, these factors worsen the phytosanitary state of crops, leading to a global problem.

The higher human impact inevitably leads to significant changes in the pedosphere which ensures transformation of photosynthetically assimilated atmospheric carbon during the carbon cycle in the biosphere. In particular, the main soil functions (matter and energy transformation, the role of a protective and buffer biogeocenotic barrier, sanitary function) become wane [6, 7]. Low amount of organic fertilizers, crop rotation neglect and monoculture practice speed up soil dehumification, depletion of soil microbiocenoses, a decrease in natural soil suppressivity, and the accumulation of soil infectious agents. These weaken the sustainability of agroecosystems self-regulation and cause phytosanitary destabilization.

Plants are the core of complex biogeocenotic consortia which comprise various groups of heterotrophs, including plant pathogens and pests, as well as their antagonists and hyperparasites. The biocenotic process which involves organisms of each trophic level determines stability and phytosanitary well-being of agroecosystems. Biocenotic principles of microbiological plant protection are the essence of a bioprotection approach, and biodiversity of multifunctional microbial entomopathogens, antagonists and hyperparasites is, in turn, a cornerstone of the microbe-based protection methods. Phytophagous insects significantly affect phytosanitary state of agroecosystems. Among 80 insect species that reduce crop yield and quality, there are super dominants in a state of ecological explosion with a significant spatial expansion and rising harmfulness, e.g. locust beetles (family *Acridoidea* MacLeay), pest bug (*Eurygaster integriceps* Puton), Colorado potato beetle (*Leptinotarsa decemlineata* Say), meadow moth (*Loxostege sticticalis* Linnaeus), cotton moth (*Helicoverpa armigera* Hübner), cereal ground

(*Zabrus tenebrioides* Goeze) etc. Plant protection against harmful insects requires more chemical treatments, which led to the appearance in Russia of about 40 resistant populations of phytophagous insects. Entomopathogenic microorganisms (viruses, microsporidia, bacteria, fungi, nematodes) play a significant role in the dynamics of insect populations, causing massive epizootics of insect hosts or a decrease in the number of pests (mostly during overwintering) by 15-30%. For example, entomophthora of the Italian locust (*Calliptamus italicus* Linnaeus), migratory locust (*Locusta migratoria* Linnaeus) and pea aphid (*Acyrthosiphon pisum* Harris) affects up to 100% of the population. The regularly occurring microsporidiosis of cabbage butterfly (*Pieris brassicae* Linnaeus) has practically led to its elimination in the northwestern region of the Russian Federation, and no harmfulness has yet been observed [8, 9]. However, low incidence of epizooties and the limited number of species of the obligate entomopathogens do not allow us to rely on their constant and stable sanitary effect while biologicals based on selected highly virulent and technologically advanced producer strains can provide effective protection against phytophagous insects in agrocenoses [10, 11].

Fundamental research (All-Russian Research Institute of Plant Protection — VIZR, Institute of Systematics and Ecology of Animals SB RAS, All-Russian Research Institute of Agricultural Microbiology, All-Russian Research Institute of Biological Plant Protection) made it possible to advance in understanding the mechanisms of pathogenesis when insects are infected by entomopathogenic viruses, microsporidia, and micromycetes. The investigations have established the role of hydrolytic enzymes (chitinases, lipases, proteases), toxins, and antiphagocytic defense factors in the virulence of entomopathogenic fungi (*Beauveria bassiana*, *Metharrizium anisopliae*, *Verticillium lecanii*) upon insect infestation [10, 11]. Virulence in fungi is of a polydeterminant nature, including the ability of spores to germinate on the cuticle of the host insect, the activity of the formation of enzymes that ensure the penetration of the pathogen through the cuticle, the rate of accumulation of fungal biomass in the body cavity, and the synthesis of toxins. Genetic improvement of strains in virulence is based on the specified set of traits.

The role of entomopathogens in the dynamics of phytophagous insect populations is determined by the host-parasite relationships. Ultimately, the type of pathogenesis (obligate or facultative) determines whether the abundance of insect pests is regulated, or the usual decrease in the population density of phytophages occurs. In case of intracellular obligate parasitism of baculoviruses and microsporidia, which are well adapted for survival in the external environment with subsequent constant persistence in natural populations of insects, there is a high incidence of epizootics in the gypsy moth (*Lymantria dispar* Linnaeus), leafworms (*Tortricidae* Latreille), cabbage white (*P. brassicae*), meadow moth (*Loxostege sticticalis* Linnaeus), and corn moth (*Ostrinia nubilalis* Hübner) [12-14]. Mass viral epizootics were noted in red pine and black-yellow sawflies, Siberian silkworms, cotton and gray grain moths [11].

Entomophthora fungi which are obligate pathogens of insects penetrate into the host's body through the cuticle and parasitize in organs and tissues without penetration into cells with pathogenesis duration up to 14 days. The regulating role of entomophtoras is mostly manifested against various aphids (pea aphid *A. pisum*) and some species of locusts: the Italian locust (*C. italicus*), Moroccan locust (*Dociostaurus maroccanus* Thunberg), and migratory locust (*L. migratoria*). A distinctive feature of the relationship between the host and the parasite in obligate parasitism is the absence of pronounced symptoms of toxicosis in insects, which indicates a low synthesis of toxins in upon viral, microsporidia, and entomophthora infections [12-14].

Under facultative parasitism, which is characteristic of entomopathogenic fungi from the genera *Beauveria*, *Metarhizium*, *Lecanicillium*, *Conidiobolus* and *Isaria*, *Bacillus thuringiensis* bacteria and the genus *Xenorhabdus* representatives, the most important factor of virulence is toxigenicity towards insect hosts. Most of the entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, *L. muscarium*, and *Conidiobolus obscurus*) enter the body of host insects through the cuticle or spiracles, with germ hyphae secreting proteases, lipases, and chitinases to accelerate degradation of the integument. In the body of diseased insects, hyphae bodies and the embryonic mycelium appear, and the produced toxins accelerate pathogenesis and death of the host. The toxins of these fungi are low molecular weight cyclic peptides [15], organic acids, glycoproteins, and other metabolites. The causative agents of muscardinosis produce bovericin, boverolid, warfarin and piridoverin. It was shown that bassiacridin the *B. bassiana* toxin, is highly active against larvae of the migratory locust (*L. migratoria*). Noted that the pathogenesis in larvae is accompanied by intense tissue melanization, and phagocytic defense is not effective, since when granulomas appear, fungal elements produce growing hyphae from cell aggregations [16].

The pathogenic effect of the gram-positive spore-forming soil bacterium *B. thuringiensis* (Bt), whose host insect range reaches hundreds of species, has been studied in detail. During oral infection, under the influence of proteases in the insect intestine, the lysis of the protein crystal and the activation of protoxin occur, which leads to the death of the host [17]. It is characteristic that in bacteriosis, the biotrophic phase is practically not observed, and the main accumulation of bacterial cells in the host's body occurs postmortem. Protein δ-endotoxins, which are encoded by *cry* and *cyt* genes located on the Bt plasmids, play the main role in the toxigenicity of the Bt group. More than 100 genes of Bt β-endotoxins are known [18]. Other entomotoxins, namely α-, β- and γ-exotoxins, are also involved in the Bt virulence [18].

Bt-based commercial biologics provide the bulk of microbiological plant protection against phytophagous insects. At present, a total of 171 mycoinsecticidal biologicals based on *B. bassiana*, *M. anisopliae*, *Isaria fumosorosea*, and *L. muscarium* strains have been developed, of which 75% are worldwide marketable [19, 20]. The formulations based on selected strains of entomopathogenic fungi of genus *Lecanicillium*, affecting sucking insects (greenhouse whitefly *Trialeurodes vaporariorum* Westwood), aphids (*Aphidoidea* Latreille), etc., are suggested (a totola of 20 biological products). Experimental batches of Verticillin M (FGBNU VIZR, Russia) based on a toxigenic strain of *Lecanicillium muscarium* were effective in protecting greenhouse crops against whiteflies (*Aleyrodidae* Westwood), aphids (*Aphidoidea* Latreille), thrips (*Thripuidae* Stevens), and tetranium ticks [20].

In modern plant protection technologies, special attention is paid to bioinsecticides based on microbial metabolites [21, 22]. Russian actinomycete-based preparations Fitoverm®, EC (LLC NBTs Farmbiomed, Russia) and Agrovertin (new name Akarin; CJSC Agrovetservice, Russia) contain avermectins, a natural macrolide compounds, insecticidal preparation SpinTor™ (Dow AgroSciences Vertriebsgesellschaft mbH, Austria) is based on spinosad which contains macrocyclic lactones of the spinosyn group in the active complex [23, 24]. Streptozonin, ossamycin, and deoxyossamycin are the actinomycetes-based preparations with spiroketal macrolides as an active ingredient, which are highly effective against insect and mite pests [25]. Among the widely known microbial insectoacaricides, it is worth noting a group of milbemycins [25], similar in properties to avermectins, and niccomycin, a specific inhibitor of chitin synthesis [26]. For a success of the antiresistant plant protection program, and given the characteristics of entomophages, the main biocontrol agents, the means for plant protec-

tion should include a wide range of biologicals with active ingredients of different nature, which are effective, environmentally friendly and entomophages compatible. Therefore, the search continues for new microbial strains that produce metabolites with a wide spectrum of insectoacaricidal action.

The search for insecticidal streptomycetes in soils of different climatic zones allows for a more targeted identification and selection of strains to develop more specialized biologicals [27]. Particularly, the soils from India, China, Egypt, Vietnam, Ukraine and Western Siberia have been surveyed. Our long-term studies have shown the maximum biodiversity of insectoacaricide producers in chernozem and sod-podzolic uncultivated soils of Western Siberia [28]. In the State Collection of Microorganisms Pathogenic for Plants and Their Pests (GCM VIZR, Russia) (WFCC WDCM No. 760, http://www.ckp-rf.ru/usu/200616/), there are over 1000 actinomycetes strains as potential producers of bioactive substances (BAS) of various chemical nature.

The VIZR model of microbial stepwise screening on a wide range of test insects and ticks has been used to develop a number of biological products not affecting warm-blooded animals and humans and compatible with entomophages. In the conditions of the Leningrad region, in Tajikistan, Belarus and Georgia, experimental batches of *Streptomyces aurantiacus* 0775-based biological Aleucid [28], with 9-dimethyl-piericidin as an active ingredient, showed high efficiency against harmful sucking arthropods, in particular in all life stages of the greenhouse whitefly (*T. vaporariorum*). Test batches of the Indocid based on *Streptomyces loidensis* P-56 strain from the India soils are active against various aphids (*Aphididae* Latreille), thrips (*Thripidae* Stevens), and common spider mite (*Tetranychus urticae* C.L. Koch). The insecticidal products of the strain are depsipeptides of the ostreogricin type. For test batches of Gerben, a *S. herbaricolor* S-100 strain-based biological developed at VIZR, the death rates of melon aphids (*Aphis gossypii* Glover), *peach aphids* (*Myzodes persicae* Sulz.), spotted greenhouse aphids (*Neomyzus circumflexus* Buckton), legume aphids (*Aphis fabae* Scopoli), pea aphids (*A. pisum*) aphids, and common spider mite (*T. urticae*) reached 60-100% [28, 29].

The beneficial microorganisms of the rhizo- and phyllosphere are in constant and dynamic associative relationships with plants [30]. Bacteria from the genera *Pseudomonas*, *Bacillus*, *Streptomyces*, and *Serratia* are of the greatest importance for the biocontrol of plant pathogenic species [31-33]. The mechanisms of microbial-plant interactions resulting in the suppression of plant pathogen populations are complex and diverse [34-36]. Plant protection involves the use of highly competitive microbial strains that can synthesize complexes of hydrolases and bioactive compounds and effectively colonize appropriate ecological niches [37-39]. Many compounds produced by rhizosphere microorganisms possess elicitor activity and can trigger mechanisms of induced resistance [40-42].

The synthesis of various antibiotics classes (peptides, macrolides, polyene compounds, aminoglycosides, etc.) is of paramount importance for bioactivity of microbial antagonists of plant pathogens [43]. Antibiotics can disrupt the synthesis of proteins and cell wall components, leading to membrane dysfunction, and inhibit oxidase activity. E.g., phenazine produced by *Pseudomonas* strains inhibits growth of *Fusarium oxysporum* and *Gaeumannomyces graminis*, affecting the redox potential in fungal cells, and 2,4-diacetylphloroglucinol inhibits germination of *Pythium* spp. zoospores due to membrane lysis [43]. Metabolites of *Bacillus* strains (proteins, peptide and polyene antibiotics, cyclic lipopeptides, phenolic compounds, and cyanide) are active against gram-negative and gram-positive bacteria, as well as various plant pathogenic fungi (*Fusarium*, *Alternaria*, *Drechlera*, *Colletotrichum*, *Verticillium*, *Phoma*, *Phomopsis*, *Sclerotinia*, *Puccinia*, etc.)

[43, 44]. Chitinases, glucanases, proteases, and lipases, which lyse cell walls of plant pathogenic micromycetes and cause degradation of their effector molecules, mainly peptides, are essential for the antagonistic mechanisms [45]. Micromycetes *Trichoderma* spp. synthesizing rich hydrolase complexes occupy a special position as producers of polyfunctional biofungicides. Together with high hyperparasitic, antagonistic activity and suppression of plant pathogenic soil micromycetes (due to produced antibiotics and enzymes), they increase plant disease resistance, have a phytoregulatory effect, improve nitrogen utilization via stimulation of *Azotobacter* and nodule bacteria. It should be noted that *Trichoderma* strains are involved in the decomposition of complex organic polymers, enriching the soil with nutrients available to plants [45].

The ability of beneficial microorganisms of the rhizo- and phyllosphere to synthesize metabolites with hormonal and signaling functions that affect plant growth and resistance is essential for ensuring a comprehensive protective effect. Among the discovered natural growth regulators are abscisic (ABA), jasmonic and salicylic acids, cytokinins, gibberellins, and auxins [46-48]. It has been shown that many bacterial strains of *Azospirillium*, *Pseudomonas*, *Bacillus*, etc. can synthesize auxins that activate plant root growth, which allows plants to accelerately pass through the phases sensitive to infection [49-51]. *Bacillus* strains are capable of producing gibberellins [52]. Members of *Bacillus*, *Rhizobium*, *Arthtrobacter*, *Azotobacter*, *Azospirilium*, and *Pseudomonas* genera produce cytokinins. Inoculation of plants with cytokinin-producing *B. subtilis* strains leads to a significant increase in the content of chlorophyll and cytokinins, as a result of which the root biomass and the aerial part increase [53]. Representatives of *Bacillus*, *Pseudomonas*, *Azospirillum*, *Brevibacterium*, and *Lysinibacillus* genera show the ability to produce ABA. In other words, beneficial microbiota can optimize the endogenous hormonal balance of plants [54-56].

Proper mineral nutrition is essential to increase plant disease resistance and provide diseases control. Many rhizosphere microorganisms can solubilize phosphates due to certain metabolites, e.g. organic acids and phosphatases [56-59]. The role of plants in plant-microbial interactions is also active [60, 61]. It should be especially noted that bioactive substances synthesized by microorganisms have elicitor properties and activate the mechanisms of systemic induced resistance in plants [62, 63].

Many bacterial determinants (MAMPs, microbe-associated molecular patterns), in particular antibiotics, siderophores, hormone metabolites, biosurfactants, lipopolysaccharides, flagellin, and volatile organic compounds, induce defense reactions in plants [59, 60].

The biosurfactants produced by *Pseudomonas* and *Bacillus* bacteria are cyclic lipopeptides of three families, iturins, surfactins, and fengicins. By decreasing the surface tension coefficient of water and forming gels, they increase the availability of hardly soluble hydrophobic compounds for roots [64, 65]. Microbial lipopeptides, due to biofilms formed on the surface of rhizoplane, protect plants against pathogen invasions and bacterial cells from unfavorable environmental factors. As biofilms can change the permeability or destroy the structure of the cytoplasmic membrane by binding to the lipid bilayer, fengicins and iturins possess antifungal activity, and surfactins exhibit antiviral, antimycoplasmic, and antibacterial properties [66, 67]. Fengicin and iturin form pores in the membrane, while surfactin dissolves it [68, 69]. Surfactin and fengicin stimulate synthesis of secondary metabolites with an increase in the activity of enzymes of lipoxygenase pathway that results in elicitor activity [70]. It has been shown that the *Pseudomonas* strains are capable of forming lipopeptides of four families, amphysines, syringomycins, tolaazines, and viscosines.

Massitolide A from the viscosine group produced by *P. fluorescens* SS101 exhibits direct antagonism towards *Phytophthora infestans* and induces disease resistance in tomato plants [71].

The main mechanism of action of siderophores is the competition for Fe^{3+} between beneficial rhizosphere microorganisms and plant pathogens. However, it has been also shown that siderophores can induce resistance in plants [72]. Pseudobacins produced by *Bacillus* sp. SLS18 can suppress *F. oxysporum* in iron-poor soils, by *P. putida* WCS 358 can inhibit *Ralstonia solanacearum* on eucalyptus plants, by *B. cinerea* on tomato plants, and by *Erwinia carotovora* on tobacco plants [73]. In contrast, strains that did not produce pseudobacin failed to induce plant resistance to systemic diseases. Pseudobacin of *P. fluorescens* WCS 374 induced systemic resistance against rice blast caused by ascomycete *Magnaporthe oryzae* due to activating synthesis of phenolic compounds and H_2O_2 in the epidermis and strengthening the plant cell wall in the infected zone [74].

Thus, to date, a diverse set of information has been accumulated on the physiological, biochemical and ecological characteristics of microorganisms inhabiting the rhizo- and phyllosphere, as well as the mechanisms of their antagonistic activity [75, 76].

Fundamental research determines strategy to select beneficial microorganisms, and to develop technologies for manufacturing formulations of biologicals that provide a protective effect, increase yields and improve product quality. The researchers of the All-Russian Research Institute of Plant Protection have proposed an original paradigm of crop protection given multifactorial nature and diversity of the target objects upon a long-term phytosanitary destabilization. The paradigm is based on the on the harmful species dynamics control, crop immunity, and selective and multifunctional biologicals, given the regularities of the parasitocenoses functioning and plant-microbial communities [77]. It is applicable for intensive crop production, greenhouses and organic farming conditions. This is a continuation of the research on microbiological plant protection, entomology, phytopathology and mycology conducted in the VIZR since the 1930s and initiated by the researchers of leading Leningrad and Moscow scientific schools (E.N. Pavlovsky, G. Ya. Bei-Bienko, A.V. Znamensky, I.V. Vasilev, N.F. Meyer, L.S. Zimin, A.A. Yachevsky, N.A. Naumov, K.M. Stepanov, M.S. Dunin, V.P. Pospelov). Long-term studies have substantiated the prospects of using antagonistic microbes and entomopathogenic species to control populations of plant pathogens and harmful arthropods in agroecosystems [78]. As a result, conceptual approaches to the creation and use of two types of multifunctional biological products have been developed. The first group of the preparations provides direct suppression of the reproduction of plant pathogens, while the second group increases plants resistance and improve plant physiological state. Biologicals derived from living microorganism have preventive and prolonged effect and are the strategic mainstay of microbiocontrol, while those based on active metabolite complexes can be used tactically to quickly suppress actively propagating species, for example, powdery mildew and rust fungi. The development of such multifunctional products involved the use of strains that are technologically advanced and safe for warm-blooded animals and humans, with high and complex activity, i.e. bactericidal, fungicidal, antiviral properties, plant growth regulation, virulence, and toxicity [78]. Promising strains should have high ecological plasticity, competitiveness, and the ability to synthesize a set of substances with high target effects. The adaptability should also be regarded, namely the ability of the strain to utilize available and cheap substrates, resistance to drying and concentration methods, prolonged targeted activity and

viability in different formulations.

Ecological plasticity and high adaptability allow strains to effectively restrain an increase in the density of plant pathogen populations for a long time. Golovlev [79] introduced the concept of "ecological tactics" of microorganisms, that is, ways of responding to changes in environmental conditions and types of behavior in the same environment, the number of which can be very large. A microorganism's strategy is environmental tactics combination. Ecologically plastic species of the genera *Streptomyces* and *Bacillus* which dominate in various soil ecosystems (chernozems, gray forest soils, salt marshes, etc.) play a significant role in ensuring the dynamic stability of soil microbiocenoses [79]. *Bacillus* strains in an optimal habitat show growth parameters characteristic of r-strategists. On the contrary, under unfavorable conditions they form endogenous spores, like L-strategists. In addition, in microbial saturated rhizo- and phylloplanes communities, bacilli exhibit the K strategy. Actinomycetes can also show a mixture of K and L strategies. In our opinion, it is bacilli and actinomycetes of some genera that are most promising for introduction into agroecosystems for a long-term control of plant pathogens.

Polyfunctionality of microbial producers is due to substances with various target bioactivities produced as a result of strict natural selection during evolution of soil-living microorganisms under the habitat saturation. The concept of "phenotype metastability" by Golovlev [80] implies phenotypic variability within the framework of a constant genotype, which can be considered as a way of adaptation to a changing environment and the result of a specific form of natural selection under these conditions. Phenotype metastability emerged evolutionarily as a way of species stabilization rather than the generation of diversity and further divergence [80]. The species capable of forming a wide range of secondary metabolites are the most competitive during adaptogenesis. For the development of multifunctional biological products, it is these species and strains that are of greatest interest.

Bacillus and *Streptomyces* strains are widely used in biotechnologies due to diversity of metabolic processes and low pathogenicity. Bacilli and streptomycetes are well suited for biotechnologies of manufacturing biologicals and to the greatest extent meet the requirements for strains to be introduced into agrobiocenosis for biocontrol of plant pathogens. *Bacillus* strains are one of the most diverse and widespread groups of microorganisms [33, 35, 37]. They synthesize a variety of bioactive substances, mainly of a protein nature, which are important in the induction of plant disease resistance [36, 38]. Actinomycetes are also valuable in industrial biotechnology as producers of antibiotics and bioactive substances. The efficiency of spore dispersal, resistance to drying and temporary lack of nutrients determines the wide distribution of actinomycetes in nature and their high technological effectiveness [81]. Strains of the genus *Streptomyces* is one of the most numerous groups among actinomycetes [82]. Peptide, macrolide, and polyene antibiotics produced by streptomycetes in addition to hydrolases are of great importance for soil suppressivity [83]. Although actinomycetes have long been widely used in medicine and veterinary medicine, they have little use in agriculture is limited. It should be noted that, among almost 14000 known active microbial secondary metabolites, about 9000 are produced by actinomycetes, 80% of which belong to the genus *Streptomyces* [81]. In this regard, it is obvious that the physiological and biochemical characteristics of actinomycetes determine the expediency of their use as producers of specific biologically active compounds for plant protection.

At present, biological products in the world pesticide market still make about 2%, but their use has been increasing by 20% per year in recent years,

while the production of chemical pesticides has been increasing annually by only 3%. The USA and the EU produce over 75% of the world's biopesticides. Annual sales of biocontrol products by the largest companies Valent Bioscience (USA), Certis (USA), Koppert Biological Systems (Netherlands), Pasteuria Bioscience (USA), Isagro (Italy), Terra Nostra Technology (Canada) overcome USD 100 million. In the world market, 90% of commercial biopesticides are *B. thuringiensis*-based, followed by, according to the degree of commercialization, entomopathogenic nematodes, micromycetes, and, finally, antagonist microbes. The largest pesticide manufacturer, Bayer AG (Germany), produces commercial biofungicides Sonata® (*Bacillus pumilus* QST 2808), Rhapsody® and three formulations of Serenade® (*Bacillus subtilis* QST 713) (https://www.agroxxi.ru). In Russia, biologicals Fitosporin (LLC Bashinkom), Baktofit (LLC PJSC Sibbiopharm), Rizoplan (LLC Biopesticides), etc., are widely used to protect plants against diseases.

In the State Collection of Microorganisms Pathogenic to Plants and Their Pests (SCM VIZR), the total number of accessions reaches 8120. Among them, over 200 strains of various taxonomic affiliations are biocontrol microbial agents that perspective for plant protection against pests and diseases. Particularly, these strains have been used to develop formulations of six multifunctional biological products, Gamair, Alirin-B, Vitaplan, Trichotsin, Sternifag, and Glyocladin (registered jointly by the FGBNU VIZR and ZAO ABT-group), which protect crops against diseases and increase yields as plant growth stimulators.

Alirin-B (*B. subtilis* B-10) is intended for plant protection against fungal diseases. The strain synthesizes polypeptide and polyene antibiotics, and the main active substance alirin B_1 is assigned to bacteriocins [84]. *B. subtilis* M-22-based Gamair is effective against mycoses and bacterioses; the Gamair A strain synthetizes the polypeptide which is close to bacillin and belongs to mediocidin subgroup 1A, as well as gamair B, C, and D strains — the hexaene antibiotics of different structure [85].

Lab samples and pilot batches of several new biopreparations based on the most active strains show high efficiency for different crops. A promising strain *Streptomyces felleus* S-8 synthesizes alirinomycin C, the original antibiotic which belongs to the subgroup of basic macrolides of the carbomycin-cirramycin type and is highly active against plant pathogenic micromycetes [86]. Strains *S. chrysomallus* P-21 and *S. globisporus* L-242 produce a variety of compounds with high fungicidal, antiviral activity and phytoregulatory action. The *S. chrysomallus* P-21 strain produces chrysomal A, the original polypeptide antibiotic classified as a threonine-type peptidolactone. Both strains also produce heptaene aromatic antibiotics of the polyester group. Globerin and chrysomal C are assigned to the subgroup of aromatic heptaene polyenes [87]. Polyene antibiotics bind to certain components of fungal surface and have a selective membranotropic effect. According to the classification, the original antibiotics chrysomal C and globerin are assigned to the subgroup levorin-partricin-trichomycin. It was found that chrysomal C is closest to levorin, while globerin is closest to partricin [88].

The use of multifunctional formulations based on several strains producing bioactive substance is an innovative approach which can significantly increase the effectiveness of biologicals and expand the range of their action. An example is *B. subtilis* BKM B-2604D- and *B. subtilis* BKM B-2605D-based biopreparation Vitaplan, SP. *B. subtilis* BKM B-2605D produces a polypeptide close to bacillin, as well as hexaenic antibiotics one of which is attributed to the mediocidin subgroup. *B. subtilis* BKM B-2604D synthesizes antibiotics of various structures (polypeptide antibiotic which belongs to bacteriocins and polyene antibiotic) [84, 85].

The analysis of experimental data shows the prospects for the use of microbial producers of antibiotics as the basis of multifunctional biologicals for protecting plants from pathogens and clarifies the role of secondary microbial metabolites of various chemical structures in the mechanism of complex activity of producer strains. Nevertheless, the selection of a stable, highly active strain is a necessary, but not sufficient condition for effective microbiological protection. The key problem is development of formulations which are suitable for manufacturing, fully correspond to the producer strain biological characteristics, and thus ensure long-term microbial cell viability and biological activity during storage and application [89].

Effectiveness of *Bacillus subtilis*-based biologicals for integrated and biological crop protection systems in the regions of the Russian Federation [90-92]

Biologials (developer)	Crop, variety of hybrid, location	Disease	Effectiveness, %	Increase in yield
Alirin-B, DP (VIZR)	Winter wheat variety Bezenchukskaya 380, SKhPK Grachevskii, Lipetsk Province	Root rot Septoria Brown rust Fusarium head blight	60-80 85-90 75-80 60-70	8-10%
	Sugar beet, hybrid XM-5455, OOO Zarech'e, Voronezh Province	Complex of diseases (cercosporosis, phomosis, bacteriosis)	60-65	93.3 c/ha
	Appli tree variety Idared, OPKh Tsentral'noe, Krsnodar	Scab, powdery mildew	96-99	10-20 c/ha
	Strawberry variety Zenga Zengana, CT Sad, Voronezh Province	Gray rot	87	28.8-38.6%
Gamair, DP (VIZR)	Sunflower variety Rodnik, SKhPK Grachevskii, Lipetsk Province	White rot, phomosis	80-98	22%
Vitaplan, DP (VIZR)	Spring wheat variety Pobeda, GNU NV NIISKh test field, Volgograd Province	Root rot Septoria Powdery mildew	61-67 55-59 52-62	12.8-14.1%
	Winter wheat variety Rufa, Experimental Farm of Kuban Agrarian University, Krasnodar Territory	Root rot Septoria Powdery mildew	64-77 61-71 66-73	8.3-16.6%
	Spring barley variety Mamlyuk, Luk'yanenko KNIISKh, Krasnodar Territory	Net blotch	48-62	23.1-26.2%
	Winter barley variety Dobrynya, Experimental and Training Farm of Kuban Agrarian University, Krasnodar Territory	Root rot Net blotch	64-80 63-64	21.5-26.7%
	Potato variety Svetlyachok 1, Moldova. PMR GU Republican toxicological laboratory	Late blight, Rhizoctoniae, Alternaria	60-80	42.0-45.0%
	Carrot variety Nantskaya, OOO Nadezhda-2, Volgograd Province	Alternaria	58-70	11.8-12.4%
	Watermelon variety Sakharnyi malysh, OOO Nadezhda-2, Volgograd Province	Root rot and wilt Anthracnose	69-73 72-77	14.5-17.4%
	Melon variety Lada, OOO Nadezhda-2, Volgograd Province	Peronosporosis Root rot and wilt	56-67 49-69	8.4-12.8%
	Apple tree varieties Sinap Orlovskii and Pamyat' voinu, OOO Maslovskie sady, Orel Province	Scab, moniliosis, powdery mildew	93-100	10-25 c/ha
	Grape variety Bianka, SKNIISiV, Karsnodar, OOO AF Yuzhnaua, Temryuk region (the Anapa-Taman' agro-eco zone)	Mildew Oidium	64-74 86-100	29-39% for yield, 13-19% for sugar content
	Bulb onion variety Khaltsedon, GNU NV NIISKh, Volgograd Province	Peronosporosis	62-77	8.9%
	White cabbage variety Stakhanovka 1513, AF Moskovskii, Moscow Province	Blackleg, slimy bacteriosis	58-68	15.7-34.2%
	Sugar beet variety L'govskaya 52, beetroot variety Bordo 237, OOO RusAgro-Druzhba, Belgorod Province	Root rots (Fusarium, Phoma, Pythium) Cercosporosis	58-82 64-75	10.5%

Long-term on-farm tests showed the effectiveness of the biologicals suggested by us against diseases of cereals, vegetables, fruits and berries, pota-

toes and sugar beets showed by three biologicals [90-92] (data are summarized in the table).

Thus, 40 biological products have been developed in Russia to protect plants. Together with foreign producers, microbiological protection of agricultural crops against pests, diseases and weeds is provided by 300 biological products, which, of course, is a significant resource for phytosanitary optimization and achieving environmental safety in crop production [93]. Biologicals are effectively integrated into zonal protection systems for grain, potatoes and vegetable crops in the Russian Federation. Technologies for bioprotection of vegetable and ornamental crops in greenhouses have also been created which effectively combine biological products and entomophages [94].

So, the stable protective effect of biologicals is based on i) constant monitoring of harmful and useful populations in the agrobiocenosis; ii) obligatory preventive treatments using products with both protective and phytoregulatory properties; iii) coincidence of the conditions optimal for entomophages' reproduction and high virulence of microbial producers; iv) the complex action which ensures effectiveness towards diseases, harmful arthropods and depression of rapidly reproducing species; v) compatibility of entomophages, biologicals and other elements of bioprotection system. The range of biological products should expand both by involving new genera, species and producer strains, and by improving the formulations for different environmental conditions. In biological and integrated crop protection, multifunctional targeted biological products adjusted to the composition of harmful species and the phytosanitary situation in general are the most prospective.

REFERENCES

1. Ghorbanpour M., Omidvari M., Abbaszadeh-Dahaji P., Omidvar R., Kariman K. Mechanisms underlying the protective effects of beneficial fungi against plant diseases. *Biological Control*, 2018, 117: 147-157 (doi: 10.1016/j.biocontrol.2017.11.006).
2. Nadykta V.D., Volkova G.V., Dolzhenko V.I. *Zashchita i karantin rastenii*, 2010, 11: 9-11 (in Russ.).
3. Rohini, Gowtham H.G., Hariprasad P., Singh S.B., Niranjana S.R. Biological control of Phomopsis leaf blight of brinjal (*Solanum melongena* L.) with combining phylloplane and rhizosphere colonizing beneficial bacteria. *Biological Control*, 2016, 101: 123-129 (doi: 10.1016/j.biocontrol.2016.05.007).
4. Pavlyushin V.A., Fasulati S.R., Vilkova N.A., Sukhoruchenko G.I., Nefedova L.I. *Antropogennaya transformatsiya agroekosistem i ee fitosanitarnye posledstviya* [Anthropogenic transformation of agroecosystems and its phytosanitary consequences]. St. Petersburg, 2008 (in Russ.).
5. Pavlyushin V.A., Vilkova N.A., Sukhoruchenko G.I., Nefedova L.I. *Vestnik zashchity rastenii*, 2016, 2(88): 5-15 (in Russ.).
6. Zvyagintsev D.G. *Pochva i mikroorganizmy* [Soil and microorganisms]. Moscow, 1987 (in Russ.).
7. Polyanskii A.M., Golovchenko A.V., Polyanskaya L.M., Zvyagintsev D.G. *Mikrobiologiya*, 2002, 71(5): 675-680 (in Russ.).
8. Pavlyushin V.A., Yakutkin V.I., Tavolzhanskii V.N. *Vestnik zashchity rastenii,* 2016, 1(87): 14-22 (in Russ.).
9. Pavlyushin V.A., Ivashchenko V.G. *Vestnik zashchity rastenii,* 2017, 3(93): 5-16 (in Russ.).
10. Borisov B.A., Serebrov V.V., Novikova I.I., Boikova I.V. V sbornike: *Patogeny nasekomykh: strukturnye i funktsional'nye aspekty* [In: Insect pathogens: structural and functional aspects]. Moscow, 2001: 352-427 (in Russ.).
11. Lednev G.R., Dolgikh V.V., Pavlyushin V.A. *Vestnik zashchity rastenii*, 2013, 3: 3-17 (in Russ.).
12. Issi I.V. V knige: *Mikrosporidii. Seriya: Protozoologiya* [Microsporidia. Series: Protozoology]. Leningrad, 1986, vol. 10: 6-135 (in Russ.).
13. Frolov A.N., Malysh Yu.M., Tokarev Yu.S. *Entomologicheskoe obozrenie*, 2008, 87(2): 291-302 (in Russ.).
14. Pavlyushin V.A., Issi I.V., Tokarev Yu.S. *Vestnik zashchity rastenii*, 2013, 2: 3-12 (in Russ.).
15. Vey A., Riba G. Toxines insecticides issues de champignons entomopathogenes. Etat actuel des conaissances dutilsation de leurs activites. *C. K. Acad. Agr.*, 1989, 75(6): 143-149.

16. Quessada-Moraga E., Vey A. Bassiacridin, a protein toxic for locusts secreted by the entomopathogenis fungus *Beauveria bassiana*. *Mycol. Res.*, 2004, 108: 441-452 (doi: 10.1017/S0953756204009724).
17. Kandybin N.V. *Bakterial'nye sredstva bor'by s gryzunami i vrednymi nasekomymi: teoriya i praktika* [Bacterial control of rodents and harmful insects: theory and practice]. Moscow, 1989 (in Russ.).
18. Augustyniak J., Dabert M., Wypijewski K. Transgenes in plants: protection against viruses and insects. *Acta Physiologiae Plantarum*, 1997, 19(4): 561-569 (doi: 10.1007/s11738-997-0054-1).
19. Faria M., Wraight S.P. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control*, 2007: 43(3): 237-256 (doi: 10.1016/j.biocontrol.2007.08.001).
20. Mitina G.V., Kozlova E.G., Pazyuk I.M. *Vestnik zashchity rastenii*, 2018, 2(96): 25-32 (in Russ.).
21. Sharma M., Dangi P., Choudhary M. Actinomycetes: source, identification, and their applications. *International Journal of Current Microbiology and Applied Sciences (IJCMAS)*, 2014, 3(2): 801-832.
22. Aggarwal N., Thind S.K., Sharma S. Role of secondary metabolites of Actinomycetes in crop protection. In: *Plant growth promoting actinobacteria: A new avenue for enhancing the productivity and soil fertility of grain legumes*. G. Subramaniam, S. Arumugam, V. Rajendran (eds.). Springer, Singapore, 2016: 99-121 (doi: 10.1007/978-981-10-0707-1_7).
23. Méndes W.A., Valle J., Ibarra J.E., Cisneros J., Penagos D.I., Williams T. Spinosad and nucleopolyhedrovirus mixtures for control of *Spodoptera frugiperda* (*Lepidoptera: Noctuidae*) in maize. *Biological Control*, 2002, 25(2): 195-206 (doi: 10.1016/S1049-9644(02)00058-0).
24. Kirst H.A. The spinosyn family of insecticides: realizing the potential of natural products research *J. Antibiot. (Tokio),* 2010, 63(3): 101-11 (doi: 10.1038/ja.2010.5).
25. Baker G.H., Dorgan R.J., Everett J.R., Hood J., Poulton M.E. A novel series of milbemicin antibiotics from *Streptomyces* strain E225. II. Isolation, characterisation, structure elucidation. *J. Antibiot. (Tokyo)*, 1990, 43(9): 1069-1076 (doi: 10.7164/antibiotics.43.1069).
26. Dzhafarov M.Kh., Vasilevich F.I., Mirzaev M.N. Production of avermectins: biotechnologies and organic synthesis (review). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(2): 199-215 (doi: 10.15389/agrobiology.2019.2.199eng).
27. Boikova I.V. *Biologicheskie osobennosti streptomitsetov — osnovy novykh insektitsidnykh biopreparatov. Avtoreferat kandidatskoi dissertatsii* [Biological features of streptomycetes — the basis of new insecticidal biological products. PhD Thesis]. St. Petersburg, 1998 (in Russ.).
28. Boikova I.V., Pavlyushin V.A. *Informatsionnyi byulleten' VPRS MOBB*, 2002, 33: 102-113 (in Russ.).
29. Boikova I.V., Kozlova E.G., Anisimova O.S., Kononenko A.V. *Zashchita i karantin rastenii*, 2007, 9: 40-41 (in Russ.).
30. Tikhonovich I.A., Provorov N.A. *Simbiozy rastenii i mikroorganizmov: molekulyarnaya genetika agroekosistem budushchego* [Plant-microbe symbioses: molecular genetics of future agroecosystems]. St. Petersburg, 2009 (in Russ.).
31. Doornbos R.F., van Loon L.C., Bakker P.A.H.M. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. a review. *Agronomy for Sustainable Development*, 2012, 32(1): 227-243 (doi: 10.1007/s13593-011-0028-y).
32. Pieterse C.M.J., Zamioudis C., Berendsen R.L., Weller D.M., van Wees S.C.M., Bakker P.A.H.M. Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology*, 2014, 52: 347-375 (doi: 10.1146/annurev-phyto-082712-102340).
33. Compant S., Duffy B., Nowak J., Clйment C., Barka E.A. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology*, 2005, 71(9): 4951-4959 (doi: 10.1128/AEM.71.9.4951-4959.2005).
34. Beneduzi A., Ambrosini A., Passaglia L.M.P. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genet. Mol. Biol.*, 2012, 35(4, suppl. 1): 1044-1051 (doi: 10.1590/S1415-47572012000600020).
35. Kumar P., Dubey R.C., Maheshwari D.K. *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiological Research*, 2012, 167(8): 493-499 (doi: 10.1016/j.micres.2012.05.002).
36. Ye Y.F., Li Q.Q., Fu G., Yuan G.Q., Miao J.H, Lin W. Identification of antifungal substance (Iturin A2) produced by *Bacillus subtilis* B47 and its effect on southern corn leaf blight. *J. Integr. Agric.*, 2012, 11(1): 90-99 (doi: 10.1016/S1671-2927(12)60786-X).
37. Dunlap C.A, Schisler D.A, Bowman M.J, Rooney A.P. Genomic analysis of *Bacillus subtilis* OH 131.1 and co-culturing with *Cryptococcus lavescens* for control of Fusarium head blight. *Plant Gene,* 2015, 2: 1-9 (doi: 10.1016/j.plgene.2015.03.002).
38. Novikova I.I. *Vestnik zashchity rastenii*, 2016, 89(3): 120-122 (in Russ.).
39. Novikova I.I., Popova E.V., Boikova I.V., Pavlyushin V.A., Tyuterev S.L. *Zashchita i karantin*

rastenii, 2016, 8: 35-43 (in Russ.).
40. Yang L., Quan X., Xue B., Goodwin P.H., Lu S., Wang J., Wei D., Wu C. Isolation and identification of *Bacillus subtilis* strain YB-05 and its antifungal substances showing antagonism against *Gaeumannomyces graminis* var. *tritici*. *Biological Control*, 2015, 85: 52-58 (doi: 10.1016/j.biocontrol.2014.12.010).
41. Maksimov I.V., Veselova S.V., Nuzhnaya T.V., Sarvarova E.R., Khairullin R.M. *Fiziologiya rastenii*, 2015, 62(6): 763-775 (doi: 10.7868/S0015330315060111) (in Russ.).
42. Tan S.Y., Jiang Y., Song S., Huang J.F., Ling N., Xu Y.C., Shen Q.R. Two *Bacillus amyloliquefaciens* strains isolated using the competitive tomato root enrichment method and their effects on suppressing *Ralstonia solanacearum* and promoting tomato plant growth. *Crop Protection*, 2013, 43: 134-140 (doi: 10.1016/j.cropro.2012.08.003).
43. Duffy B., Schouten A., Raaijmakers J.M. Pathogen self-defense: mechanisms to counteract microbial antagonism. *Annual Review of Phytopathology*, 2003, 41: 501-538 (doi: 10.1146/annurev.phyto.41.052002.095606).
44. Arinbasarova A.Yu., Baskunov B.P., Medentsev A.G. *Mikrobiologiya*, 2017, 86(2): 258-260 (doi: 10.7868/S0026365617020057) (in Russ.).
45. Benитez T., Rincyn A.M., Limyn M.C., Codyn A.C. Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology*, 2004, 7(4): 249-260.
46. Sivasakthi S., Kanchana D., Usharani G., Saranraj P. Production of plant growth promoting substance by *Pseudomonas fluorescens* and *Bacillus subtilis* isolates from paddy rhizosphere soil of Cuddalore district, Tamil Nadu, India. *International Journal of Microbiological Research*, 2013, 4(3): 227-233 (doi: 10.5829/idosi.ijmr.2013.4.3.75171).
47. Bakker P.A.H.M., Pieterse C.M.J., van Loon L.C. Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology*, 2007, 97(2): 239-243 (doi: 10.1094/PHYTO-97-2-0239).
48. Van Loon L.C. Plant responses to plant growth-promoting rhizobacteria. *European Journal of Plant Pathology*, 2007, 119: 243-254 (doi: 10.1007/s10658-007-9165-1).
49. Porcel R., Zamarreco Б.M., Garcна-Mina J.M., Aroca R. Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. *BMC Plant Biology*, 2014, 14: 36 (doi: 10.1186/1471-2229-14-36).
50. Kilian M., Steiner U., Krebs B., Junge H., Schmiedeknecht G., Hain R. FZB24® *Bacillus subtilis* — mode of action of microbial agent enhancing plant vitality. *Pflanzenschutz-Nachrichten Bayer*, 2000, 1/00(1): 72-93.
51. Dobbelaere S., Vanderleyden J., Okon Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences*, 2003, 22(2): 107-149 (doi: 10.1080/713610853).
52. Arkhipova T.N., Prinsen E., Veselov S.U., Martynenko E.V., Melentiev A.I., Kudoyarova G.R. Cytokinin producing bacteria enhance plant growth in drying soil. *Plant and Soil*, 2007, 292(1): 305-315 (doi: 10.1007/s11104-007-9233-5).
53. Belimov A.A., Dodd I.C., Safronova V.I., Dumova V.A., Shaposhnikov A.I., Ladatko A.G., Davies W.J. Abscisic acid metabolizing rhizobacteria decrease ABA concentrations in planta and alter plant growth. *Plant Physiology and Biochemistry*, 2014, 74: 84-91 (doi: 10.1016/j.plaphy.2013.10.032).
54. Cohen A.C., Travaglia C.N., Bottini R., Piccoli P.N. Paticipation of abscisic acid and gibberellins produced by endophytic *Azospirillum* in the alleviation of drought effects in maize. *Botany*, 2009, 87(5): 455-462 (doi: 10.1139/B09-023).
55. Kumar P., Dubey R.C., Maheshwari D.K. *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiological Research*, 2012, 167(8): 493-499 (doi: 10.1016/j.micres.2012.05.002).
56. de Werra P., Pйchy-Tarr M., Keel C., Maurhofer M. Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Applied and Environmental Microbiology,* 2009, 75: 4162-4174 (doi: 10.1128/AEM.00295-09).
57. *Final Screening Assessment of Bacillus megaterium strain ATCC 14581*. Environment and Climate Change Canada. Health Canada, February, 2018.
58. Kurdish I.K., Chuiko N.V., Bega Z.T. *Prikladnaya biokhimiya i mikrobiologiya*, 2010, 4(1): 58-63 (in Russ.).
59. Junior I.T., Schafer J.T., Corrка B.O., Funck G.D., Moura A.B. Expansion of the biocontrol spectrum of foliar diseases in rice with combinations of rhizobacteria. *Revista Cikncia Agronфmica*, 2017, 48(3): 513-522 (doi: 10.5935/1806-6690.20170060).
60. Ohkama-Ohtsu N., Wasaki J. Recent progress in plant nutrition research: cross-talk between nutrients, plant physiology and soil microorganisms. *Plant and Cell Physiology*, 2010, 51(8): 1255-1264 (doi: 10.1093/pcp/pcq095).
61. Kloepper J.W., Gutierrez-Estrada A., McInroy J.A. Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Canadian Journal of Microbiology*, 2009, 53(2): 159-167 (doi: 10.1139/w06-114).
62. Verhagen B.W.M., Trotel-Aziz P., Couderchet M., Höfte M., Aziz A. *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defense responses in grapevine. *Journal of Experimental Botany*, 2010, 61(1): 249-260 (doi:

10.1093/jxb/erp295).
63. Ongena M., Henry G., Thonart P. The role of cyclic lipopeptides in the biocontrol activity of *Bacillus subtilis*. In: *Recent developments in management of plant diseases (Plant pathology in the 21st century), vol. 1*. U. Gisi, I. Chet, M.L. Guillino (eds.). Springer, Dordrecht, 2010: 59-69 (doi: 10.1007/978-1-4020-8804-9_5).
64. Meena K.R., Kanwar S.S. Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. *BioMed Research International*, 2015, 2015: Article ID 473050 (doi: 10.1155/2015/473050).
65. Falardeau J., Wise C., Novitsky L., Avis T.J. Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis* lipopeptides on plant pathogens. *Journal of Chemical Ecology,* 2013, 39: 869-878 (doi: 10.1007/s10886-013-0319-7).
66. Cawoy H., Mariutto M., Henry G., Fisher C., Vasilyeva N., Thonart P., Dommes J., Ongena M. Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production. *Molecular Plant-Microbe Interactions*, 2014, 27(2): 87-100 (doi: 10.1094/MPMI-09-13-0262-R).
67. Henry G., Deleu M., Jourdan E., Thonart P., Ongena M. The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related responses. *Cellular Microbiology*, 2011, 13(11): 1824-1837 (doi: 10.1111/j.1462-5822.2011.01664.x).
68. Patel H., Tscheka C., Edwards K., Karlsson G., Heerkotz H. All-or-none membrane permeabilization by fengycin-type lipopeptides from *Bacillus subtilis* QST713. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 2011, 1808(8): 2000-2008 (doi: 10.1016/j.bbamem.2011.04.008).
69. Ongena M., Jourdan E., Adam A., Paquot M., Brans A., Joris B., Arpigny J.-L., Thonart P. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental Microbiology*, 2007, 9(4): 1084-1090 (doi: 10.3389/fmicb.2019.02327).
70. De Vleesschauwer D., Hцfte M. Rhizobacteria-induced systemic resistance. *Advances in Botanical Research*, 2009, 51: 223-281 (doi: 10.1016/S0065-2296(09)51006-3).
71. Chetverikov S.P., Suleimanova L.R., Loginov O.N. *Prikladnaya biokhimiya i mikrobiologiya*, 2009, 45(5): 565-570 (in Russ.).
72. Luo S., Xu T., Chen L., Chen J., Rao C., Xiao X., Wan Y., Zeng G., Long F., Liu C., Liu Y. Endophyte-assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth-promoting endophyte *Bacillus* sp. SLS18. *Applied Microbiology and Biotechnology*, 2012, 93(4):1745-1753 (doi: 10.1007/s00253-011-3483-0).
73. Bakker P.A.H.M., Pieterse C.M.J., van Loon L.C. Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology*, 2007, 97(2): 239-243 (doi: 10.1094/PHYTO-97-2-0239)
74. De Vleesschauwer D., Djavaheri M., Bakker P.A.H.M., Hцfte M. *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiology*, 2008, 148(4): 1996-2012 (doi: 10.1104/pp.108.127878).
75. Nikoo F.S., Sahebani N, Aminian H. Induction of systemic resistance and defense-related enzymes in tomato plants using *Pseudomonas fluorescens* CHAO and salicylic acid against root-knot nematode *Meloidogyne javanica*. *Journal of Plant Protection Research,* 2014, 54(4): 383-398 (doi: 10.2478/jppr-2014-0057).
76. Lachin Mokhtarnejad, Reza Ghaderi, De Vleesschauwer D., Djavaheri M., Bakker P.A.H.M., Hцfte M. *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiology*, 2008, 148(4): 1996-2012 (doi: 10.1104/pp.108.127878).
77. Pavlyushin V.A., Vilkova N.A., Sukhoruchenko G.I., Nefedova L.I. *Materialy 9-i Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Biologicheskaya zashchita rastenii — osnova stabilizatsii agroekosistem»* [Proc. 9 Int. Conf. «Biological plant protection — the basis for sustainable agroecosystems»]. Krasnodar, 2016: 504-508 (in Russ.).
78. Novikova I.I. V sbornike: *Biologicheskie sredstva zashchity rastenii, tekhnologii ikh izgotovleniya i primeneniya* [In: Biological plant protection products, technologies for their manufacture and use]. St. Petersburg—Pushkin, 2005: 303-332 (in Russ.).
79. Golovlev E.L. *Mikrobiologiya*, 2001, 70(4): 437-443 (in Russ.)
80. Golovlev E.L. *Mikrobiologiya*, 1998, 67(2): 149-155 (in Russ.)
81. *Biologiya otdel'nykh grupp aktinomitsetov* /Pod redaktsiei N.A. Krasil'nikova [Biology of individual groups of actinomycetes. N.A. Krasil'nikov (ed.)]. Moscow, 1965 (in Russ.)
82. Zenova G.M., Zvyagintsev D.G., Manucharova N.A., Stepanova O.A., Chernov I.Yu. *Pochvovedenie*, 2016, 10: 1214-1217 (doi: 10.7868/S0032180X16100166) (in Russ.).
83. Zenova G.M., Dubrova N.S., Gracheva T.A., Kuznetsova A.I., Stepanova O.A., Chernov I.Yu., Manucharova A.S. *Vestnik Moskovskogo universiteta*, 2016, 17(4): 43-46 (in Russ.)
84. Shenin Yu.D., Novikova I.I., Kruglikova L.F., Kal'ko G.V. *Antibiotiki i khimioterapiya*, 1995, 40(5): 3-7 (in Russ.).
85. Novikova I.I., Shenin Y.D. Isolation, identification, and antifungal activity of a Gamair complex formed by *Bacillus subtilis* M-22, a producer of a biopreparation for plant protection from mycoses and bacterioses. *Applied Biochemistry and Microbiology*, 2011, 47(9): 817-826 (doi:

10.1134/S0003683811090031).
86. Shenin Yu.D., Novikova I.I., Kaminskii G.V., Ivanova I.A. *Antibiotiki i khimioterapiya*, 2001, 46(2): 10-16 (in Russ.).
87. Novikova I.I., Shenin Yu.D., Tsyplenkov A.E., Fominykh T.S., Suika P.V., Boikova I.V. *Vestnik zashchity rastenii*, 2009, 2: 3-19 (in Russ.).
88. Shenin Yu.D., Novikova I.I., Suika P.V. *Biotekhnologiya*, 2010, 2: 41-53 (in Russ.).
89. Novikova I.I. *Zashchita i karantin rastenii*, 2017, 4: 3-6 (in Russ.).
90. *Osnovnye itogi raboty Rossiiskoi akademii sel'skokhozyaistvennykh nauk za 2013 god* [The main results of the work of the Russian Academy of Agricultural Sciences, 2013]. Moscow, 2014 (in Russ.).
91. *Otchet otdeleniya sel'skokhozyaistvennykh nauk RAN o vypolnenii fundamental'nykh i poiskovykh nauchnykh issledovanii v 2014 godu* [Report of the Department of Agricultural Sciences RAS on fundamental and exploratory research, 2014]. Moscow, 2015 (in Russ.).
92. *Otchet otdeleniya sel'skokhozyaistvennykh nauk RAN o vypolnenii fundamental'nykh i poiskovykh nauchnykh issledovanii v 2014-2016 gg* [Report of the Department of Agricultural Sciences RAS on fundamental and exploratory research, 2014-2016]. Moscow, 2017 (in Russ.).
93. Shternshis M.V. *Vestnik Tomskogo gosudarstvennogo universiteta. Biologiya*, 2012, 2(18): 92-100 (in Russ.).
94. Belyakova N.A., Pavlyushin V.A. *Mat. 3-go Vserossiiskogo s"ezda po zashchite rastenii* [Proc. 3rd All-Russian Congress on Plant Protection]. St. Petersburg, 2013, t. 2: 7-10 (in Russ.).

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VIRUSES OF CEREAL CROPS AND THEIR VECTORS IN THE SOUTH OF THE RUSSIAN FAR EAST

(review)

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Abstract

The review presents data on the current taxonomic status and ecology of 10 viruses infecting cereals (*Poaceae*) in the South of the Russian Far East. Barley stripe mosaic virus (BSMV) (*Virgaviridae*, *Hordeivirus*) is the most common virus affecting about a third of crops (with the exception of oat, which is slightly infected with BSMV). The maize chlorotic stripe disease occurring in Primorye is also etiologically linked with BSMV. Unlike the European part of Russia, Brome mosaic virus (BMV) (*Bromoviridae*, *Bromovirus*) is slightly represented among cultivated cereals in the South of the Russian Far East since there are no its numerous vectors from perennial plants-reservoirs. In the South of the Russian Far East Poa semilatent virus (PSLV) (*Virgaviridae*, *Hordeivirus*) was isolated from wheat (*Triticum* spp.), swamp (*Poa palustris*) and meadow (*P. pratensis*) bluegrass. Vectors for this virus have not yet been established. The epicenter of the Northern cereal mosaic virus (NCMV) (*Mononegavirales*: *Rhabdoviridae*, *Cytorhabdovirus*) strain diversity in the South of the Russian Far East is located in the Amur region whereas in the Primorsky and Khabarovsk territories this virus is much less common. Latently infected perennial wild grasses are a natural reservoir of NCMV that is effectively spread by small brown planthopper (*Laodelphax striatella*). In the body of planthopper NCMV is capable of replication as well as overwintering in larvae. Russian oat mosaic virus (ROMV) (*Bunyavirales*: *Phenuiviridae*, *Tenuivirus*) infects wide range of cereals and is known by several synonymous names. It is possible that at least some variants of this virus are a mixture with phytoplasma. In the Primorye territory this virus is mostly found together with NCMV and is also carried by small brown planthopper. The main vectors of Rice stripe virus (RSV) (*Phenuiviridae*, *Tenuivirus*) in the South of the Russian Far East are small brown and brown (*Nilaparvata lugens*) planthopper as well as rice beetle (*Oulema oryzae*). Rice beetle is a mechanical vector of Rice spotted mosaic virus (RSMV). Far Eastern aphid (*Hemiptera*: *Aphidoidea*) species are vectors of the Maize dwarf mosaic virus (MDMV) (*Potyviridae*, *Potyvirus*) and Barley yellow dwarf virus (BYDV) (*Luteoviridae*, *Luteovirus*). In the rice fields of Primorye 44 species of aphids were identified, among which green com (*Rhopalosiphum maidis*), bird cherry-oat (*Rh. padi*), English Grain (*Sitobion avenae*) and greenbug (*Schizaphis graminum*) aphids dominate. Large foci of diseases caused by Wheat streak mosaic virus (WSMV) (*Potyviridae*, *Tritimovirus*) were found in the far Eastern agrocenoses, which can be transmitted by herbivorous mites (*Trombidiformes*: *Eriophyoidea*). Spider mites (*Trombidiformes*: *Tetranychidae*) are presumably the carriers of BYDV, NCMV, MDMV, and WSMV. The basis of the presented review is the data of long-term (since 1962) regular monitoring of cereal crop viruses carried out by the Laboratory of Virology in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch RAS. The strains obtained during this monitoring are stored in the Russian Collection of East Asia viruses (the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch RAS).

Keywords: plant viruses, cereals, taxonomy, vector, aphid, planthopper, gall mites

The southern part of the Far East is the area of a moderately monsoon extratropical climate, with a flat-ridged relief and accumulative depressions. Soils are of heavy granulometric composition with low water permeability, but regular over-moisture, a small amount of organic matter and phosphorus are common here [1].

Grain crop cultivation has a long history in the region. In the 7th century, commercial grain production was crucial in emergence of Bohai, the ancient state on the territory of Manchuria, Primorye and the northern part of the Korean Peninsula [2]. Since the 10th century, the Jurchen Jin state appears here and the land use system characteristic of China begins to form [3]. Zoning of European grain crops in the Far East began in the 17th century during its active development by the Russian Empire. The expansion of the area of grain crop cultivation falls on the period of P.A. Stolypin's agrarian reform (1906-1913) with a massive re-settlement of peasants to the Far East [4]. Changes in land use patterns were inevitably accompanied by the intensification of intra- and interpopulation interactions, the penetration of new pathogens into the local flora, and the adaptation of natural focal viruses to new crops [5, 6].

The fundamentals of grain production in the Far East developed and put into practice in the USSR, to this day not only provides local needs for grain, but also allows the territory to enter the grain market of the Asia-Pacific region. Spiked cereals (*Poales* Small, 1903: *Poaceae* Barnhart, 1895) cultivated in the Russian Far East are wheat (*Triticum* L., 1753), barley (*Hordeum* L., 1753) and rye (*Secale cereale* L., 1753), and paniculate cereals are rice (*Oryza* L., 1753), millet (*Panicum* L., 1753), oats (*Avena* L., 1753), sorghum (gaoliang) (*Sorghum bicolor* Moench, 1794 subsp. *chinense*), corn (*Zea mays* L., 1753) and plague (*Setaria italica* P. Beauv., 1812 subsp. *italica*). The advanced development of the Far East is associated with an increase in the regional grain production, particularly due to control of viral infections of cereals.

This review uses the data of the multi-year (since 1962) regular monitoring of viruses in cereal crops carried out by the Laboratory of virology of the Federal Research Center of Biodiversity of Terrestrial Biota, Far East Branch RAS (until 2018, the Biology and Soil Institute, the Far Eastern Branch RAS). Isolated viral strains are deposited in the Russian collection of viruses in East Asia (FSC Biodiversity FEB RAS) [7-9].

From 1962 to 2019, the prevalence of virus-like diseases in plot crops and breeding nurseries of the Primorsky and Khabarovsk territories was significantly higher than in industrial crops: 0.2-1.9% vs. 0.01-0.03% for wheat, 0.3-2.5% vs. 0.02-0.8% for barley, 0.3-6.0% vs. 0.05-0.4% for oats, 0.7-14.0% vs. 0.06-0.7% for corn, and 0.02-0.1% for rye (no industrial rye crops in the south of the Far East). The differences are associated with more favorable conditions for infection spreading in plots due to small area, thinness of crops, easier access for insects, increased risk of mechanical spread of infections. The maximum infection extensiveness both in nurseries and in row crops is typical for the Amur Region, where the most common members of the *Virae* kingdom that infect cereals are Barley stripe mosaic virus (BSMV) (*Virgaviridae*, *Hordeivirus*), Brome mosaic virus (BMV) (*Bromoviridae*, *Bromovirus*), Russian oat mosaic virus (ROMV) (*Bunyavirales*: *Phenuiviridae*, *Tenuivirus*) and Northern cereal mosaic virus (NCMV,) (*Mononegavirales*: *Rhabdoviridae*, *Cytorhabdovirus*). In some agrocenoses, large foci of diseases caused by Wheat streak mosaic virus (WSMV) (*Potyviridae*, *Tritimovirus*) and Poa semilatent virus (PSLV) (*Virgaviridae*, *Hordeivirus*) are found. Mixed infections of up to three or four viruses are widespread [10-15].

BSMV is the most common cereal virus in the south of the Russian Far

East, with about a third of crops (especially a small area) affected. In plots, the rate of BSMV infection in wheat is 2-5 times less than in barley. Oats are slightly affected by BSMV, regardless of the variety. BSMV is easily spread through contacts and seeds [13, 15]. A wide variety of strains of this virus is noted in both wild and cultivated phytocenoses [16, 17]. It has been shown that BSMV is the etiological agent of maize chlorotic stripe disease widespread in the Far East; damage of plants by BSMV results in a decreased seed setting and ugly ears [18-20].

Reconstruction of BSMV molecular evolution using barley seeds from archaeological excavations shows that at least 2000 years ago, the predecessor of this virus emerged in North Africa and the Middle East [21]. Apparently, it initially circulated in the natural area of wild barley (*Hordeum vulgare* L., 1753) [22]. Smith et al. [21], basing on the data of phylogenetic position analysis of modern Chinese line BSMV [23-26] widespread in East Asia, assumed that medieval BSMV strains penetrated here in the 13th-15th centuries due to intensified traffic along the Great Silk Road under the control of Mongol Empire [27]. Note, however, that barley was known on the Korean Peninsula in the ancient Ko Choson state at the beginning of the 1st millennium [28, 29]. Possibly even more ancient BSMV genetic lines have survived in East Asia, and their detection is most likely in the south of the Russian Far East, which historically was somewhat isolated although influenced by China [2, 3].

The stripe mosaic, etiologically associated with BMV and widespread on cereals in the south of the European Russia [30, 31], is insignificant in the south of the Far East (no more than 5% of the total plot number) [32]. Since the virus is mainly transmitted mechanically, without a massive vector, the efficiency of BMV spreading from perennial reserve plants to cultivated cereals is low. In the Far East, such reserve plants are awnless rump (*Bromopsis inermi*s Leyss., 1761), creeping wheatgrass (*Elytrigia repens* Desvaux ex Nevski, 1933), Siberian graybeard (frost grass) (*Spodiopogon sibiricus* Trinius, 1820), Langsdorf's reed grass (*Calamagrostis langsdorfii* Trinius, 1824), cock's millet (*Echinochloa crus-galli* Beauvois, 1812), and timothy grass (*Phleum pratense* L., 1753). Wheat is most affected by this virus (up to 50% of diseased plants), barley and oats are affected insignificantly [32].

PSLV was first discovered in slender wheatgrass (*Elymus trachycaulus* Gould ex Shinners, 1833) and fowl bluegrass (*Poa palustris* L., 1759) in Canada [33]. Wheatgrass (*Elymus* L., 1753) and bluegrass (Poa L., 1753) are widespread in the extratropical zones of both hemispheres of the Earth. In the south of the Russian Far East, PSLV was isolated from wheat, fowl bluegrass, and Kentucky bluegrass (*P. pratensis* L., 1753). The virus was identified immunochemically with polyclonal antibodies (provided by the Department of Virology of the Lomonosov Moscow State University). Given possible serological cross-reactivity between various members of *Hordeivirus*, the independent taxonomic status of the Far Eastern PSLV strains cannot be ruled out. The PSLV vectors have not been identified, and its distribution occurs by contact [13, 14].

The NCMV strain diversity epicenter in the Far East is in the Amur Region while in Primorsky and Khabarovsk territories, this virus is much less common [11, 12]. NCMV is effectively spread by small brown planthopper (*Laodelphax striatellus* Fallen, 1826). Complex relationships between thee planthoppers and the plant communities determine how the virus spreads. NCMV replicates directly in the vector, making the infected insect a permanent (up to death) source of infection; in addition, due to high mobility of the vector species, each individual can infect a large number of plants [11, 34, 35]. The pathogen overwinters in infected larvae. A significant part of perennial grasses is resistant to NCMV. So, according to Mamaev [11], in a latent form and to an insignificant

extent, redtop (*Agrostis gigantea* Roth, 1788), creeping bentgrass (*A. stolonifera* L., 1753), meadow foxtail (*Alopecurus pratensis* L., 1753), American sloughgrass (*Beckmania syzigachne* Fernald, 1928), chee reedgrass (*Calamagrostis epigeios* Roth, 1788), and blue-joint reedgrass [*C. canadensis* (Michx.) P. Beauv. var. *langsdorffii* (Link) Inman]. The NCMV spreading largely depends on the characteristics of agrocenoses. For example, in the Amur Region, where cereals are grown on vast areas without natural barriers, the infection is restrained only by entomophages and agrotechnical methods that reduce the number of small brown planthopper [36]. In Primorsky and Khabarovsk Territories, the NCMV spreading is limited by natural barriers, as the fields are located among forests, their areas are significantly inferior to those in the Amur Region, and the planthoppers-vectors reproduce much less intensively. If in the Amur Region in August 1000-1500 planthoppers are caught for 100 strokes of the net on annual cereals and fodder oats, while in the Primorsky and Khabarovsk territories there are only 30-40 individuals [11]. The low NCMV prevalence in Primorye is apparently due to the less favorable pronounced monsoon climate conditions. In addition, in most parts of the Primorsky and Khabarovsky territories, winter forms of cereals are absent. This undermines the food supply of the coastal aphids in the early spring and late autumn periods.

As to ROMV, the history of its study and common synonyms should be taken into account. The virus was described by Sukhov [37] in 1940 as a cereal pupation (pseudo-roset) virus. Later Fedotina [38] found bacilli-like viral particles and phytoplasmas (*Acholeplasmatales*: *Acholeplasmataceae*) in diseased plants and assumed a mixed etiology of the disease. Further studies did not add the understanding of the pathogen taxonomic affiliation (some authors attributed it to tenuiviruses, others to phytorabdoviruses), and, moreover, further confused the situation due to using synonymous names, e.g. oat pupation (pseudo-roset) virus, barley pupation (pseudo-roset) virus, wheat pupation (pseudo-roset) virus, etc. At present, this virus, which infects a wide range of cereals, causing dwarf bushiness, is called ROMV [39]. However, a mixed infection of ROMV with phytoplasmas cannot be ruled out, for which a plant bushiness (multi stemmed plants) is characteristic. In the Primorsky Territory, ROMV mostly occurs together with NCMV and is also transmitted by small brown planthopper [12, 38, 40]. A distinctive feature of tenuiviruses is the synthesis of a large amount of the so-called soluble antigen, a low-molecular-weight protein that forms loop-shaped intracellular inclusions [41]. Therefore, the isolation by Sukhov [37] of a “soluble antigen” from plants “sick with bulging” (according to the author’s definitions) indicates that the pathogen he studied belongs to tenuiviruses, while different morphology of the particles indicates a concomitant infection. Since the symptom complex of cereal diseases does not allow an unambiguous identification of the pathogen (especially in mixed infections common for this group of plants), viral isolates can differ significantly.

Another tenuivirus, Rice streak virus (RSV) (*Phenuiviridae*, *Tenuivirus*), was discovered in Primorsky Krai [10]. In the lab tests, RSV was successfully transmitted by small brown planthopper. Among the vectors in the Primorsky Territory, brown planthoppers (*Nilaparvata lugens* Stal, 1854) are of great importance. In total, 47 species of planthoppers (*Hemiptera* L., 1758: *Cicadellidae* Latreille, 1802) were identified in the rice paddies of Primorye. It is known that tenuiviruses can reproduce in insect tissues and are transmitted transovarially [42, 43].

Rice leaf beetle (*Oulema oryzae* Kuwayama, 1931) which in the south of the Russian Far East for a long time was mistakenly associated with red-throated cereal leaf beetle (*Oulema melanopus* L., 1758), is apparently another RSV vector

in the region. In this case, the virus is transmitted by a non-persistent route. In addition, this leaf beetle is a vector of another and yet unidentified Rice spotted mosaic virus (RSMV) which causes panicle deformation (axial shortening and pubescence) [10].

Aphids (*Hemiptera*: *Aphidoidea* Latreille, 1802) have been pests of cultivated crops and effective vectors of plant viruses since the beginning of arable farming. The richest species diversity of cereals and various ecological condi-tions for their growth have been the prerequisites for the equally diverse aphid fauna of cereal plants [44, 45]. These insects have moved to grain crops from their wild relatives, the natural reservoirs of many plant viruses, and retained trophic links with them [46, 47]. However, there are few cereal viruses transmitted by aphids. Of these, the most well-known and harmful are Maize dwarf mosaic virus (MDMV) (*Potyviridae*, *Potyvirus*) and Barley yellow dwarf virus (BYDV) (*Luteoviridae*, *Luteovirus*), causing large losses in cereal crops in the south of the Russian Far East [48-51] and neighboring countries, i.e. China and Japan [52-55]. Fifteen to twenty species of the Far Eastern aphid fauna are vectors of these viruses, among which the most significant are bird cherry-oat aphid (*Rhopalosiphum padi* L., 1758), corn leaf aphid, or corn aphid (*Rh. maidis* Fitch, 1856), wheat aphid (Sitobion avenae Fabricius, 1775), and greenbug (*Schizaphis graminum* Rondani, 1852). Currently, we have found BYDV in oats and barley plants in all regions of the south of the Russian Far East [7-9].

Our surveys of the aphid fauna in rice fields with the identification of vectors and the pathways for their possible migration in the Primorsky Territory (Far Eastern Experimental Rice Station, Spassky District; Sivakovskoe Rice Farming, Khorolsky District) and in forbs near rice fields revealed 44 species. Of these, 35 species (80%) are occasional visitors to rice plants and rice field weeds, and the 10 species are direct inhabitants and crop pests, i.e. dogwood-grass aphid (*Anoecia corni* Fabricius, 1775), rose-grass aphid (*Metopolophium dirhodum* Walker, 1849), apple-grass aphid (*Rhopalosiphum insertum* Walker, 1849), green corn aphid (*Rh. maidis*), waterlily aphid (*Rh. nymphaeae* L., 1761), Bird cherry-oat aphid (*Rh. padi*), greenbug (*Schizaphis graminum*), grain aphid (*Sitobion avenae*), elm sack gall aphid (*Tetraneura ulmi* L., 1758), and mealy plum aphid (*Hyalopterus pruni* Geoffroy, 1762). In rice plants, four of these aphidids dominate, the *Rh. maidis*, *Rh. padi*, *S. avenae*, and *Sch. graminum*. As for *H. pruni*, the species is not the most abundant in rice crops, but, apparently, can colonize it. By chance, polyphagous aphids, which generally develop on other fodder plants, can occasionally appear on rice plants, e.g. green peach aphid (*Myzodes persicae* Sulzer, 1776), cabbage aphid (*Brevicoryne brassicae* L., 1758), melon aphid (*Aphis gossypii* Glover, 1877), cowpea aphid (*A. craccivora* C.L. Koch, 1854). All of these aphids are virophorous (transmitting RSMV), which aggravates their harmfulness [13, 56, 57].

Currently, in the Primorsky Territory, the rice crops lost in the 1980s and 1990s are being restored. However, on abandoned rice paddies, wild phytocenoses with their vectors of plant viruses have formed, and in each case, special approach is required to assess harmfulness and prevalence of infectious agents [58]. The constantly increasing cargo and passenger flow between the Russian Federation and the People's Republic of China creates additional risks of the penetration of Chinese rice viruses into the Russian Far East [59-61]. Therefore, ecological and virological monitoring must be more strict, including tracking diversity of the rice viruses that have not yet been detected in the south of the Russian Far East, i.e. the Rice dwarf virus (RDV) (*Reoviridae*, *Phytoreovirus*) (62), Rice gall dwarf virus (RGDV) (*Reoviridae*, *Phytoreovirus*) [63], Rice bunchy stunt virus (RBSV) (*Reoviridae*, *Phytoreovirus*) [64], Rice ragged stunt virus (RRSV) (*Reoviridae*, *Oryzavirus*)

Taxonomic description and ways of spreading viruses of cereal crops in the south of the Russian Far East

Taxonomic position			Name	Typical transmission ways
order	family	genus		
Incerti ordinis	*Bromoviridae*	*Bromovirus*	Brome mosaic virus (BMV)	By contacts
Incerti ordinis	*Luteoviridae*	*Luteovirus*	Barley yellow dwarf virus (BYDV)	By aphids *Rhopalosiphum padi*, *Rh. maidis*, *Schizaphis graminum*, *Sitobion avenae*
Bunyavirales	*Phenuiviridae*	*Tenuivirus*	Russian oat mosaic virus (ROMV)	By small brown planthopper (*Laodelphax striatella*)
			Rice stripe virus (RSV)	By small brown planthopper (*Laodelphax striatella*), brown planthopper (*Nilaparvata lugens*), and rice leaf beetle (*Oulema oryzae*)
Incerti ordinis	*Potyviridae*	*Potyvirus*	Maize dwarf mosaic virus (MDMV)	Via seeds, by contacts and aphids (*Myzodes persicae*, *Brevicoryne brassicae*, *Aphis gossypii*, *A. craccivora*)
		Tritimovirus	Wheat streak mosaic virus (WSMV)	By contacts and mites *Aceria tosichella*, *A. tulipae*, *A. tritici*
Mononegavirales	*Rhabdoviridae*	*Cytorhabdovirus*	Northern cereal mosaic virus (NCMV)	By small brown planthopper (*Laodelphax striatella*)
Incerti ordinis	*Virgaviridae*	*Hordeivirus*	Barley stripe mosaic virus (BSMV)	Via seeds, by contacts
			Poa semilatent virus (PSLV)	By contacts
Incerti ordinis	*Incertae familiae*	*Incerti genus*	Rice spotted mosaic virus (RSMV)	By rice leaf beetle (*Oulema oryzae*) and aphids *Myzodes persicae*, *Brevicoryne brassicae*, *Aphis gossypii*, *A. craccivora*

N o t e. Taxonomic groups are sorted in alphabetical order of family names, since not all viruses have order-level taxonomic status. Rice spotted mosaic virus (RSMV) that is not currently classified is the last in the list.

Oryzavirus) [65], Rice black streaked dwarf virus (RBSDV) (*Reoviridae*, *Fijivirus*) [66], Southern rice black streaked dwarf virus (SRBSDV) (*Reoviridae*, *Fijivirus*) [66], Rice grassy stunt virus (RGSV) (*Bunyavirales*: *Phenuiviridae*, *Tenuivirus*) [67], Rice yellow stunt virus (RYSV) (*Mononegavirales*: *Rhabdoviridae*, *Nucleorhabdovirus*) [68], Rice tungro bacilliform virus (RTBV) (*Caulimoviridae*, *Tungrovirus*), Rice tungro spherical virus (RTSV) (*Picornavirales*: *Secoviridae*, *Waikavirus*) [69].

Gall four-legged mites (due to reduction of the posterior pair of limbs at postembryonic stages) (*Trombidiformes* Reuter, 1909: *Eriophyoidea* Nalepa, 1898) [70] are vectors of WSMV. The mites themselves are microscopic in size (~ 0.1 mm), but the colonies they form are clearly distinguishable visually. The main vector for WSMV is wheat curl mite (*Aceria tosichella* Keifer, 1969) [71-73] (synonyms dry bulb mite *A. tulipae* Keifer, 1938 and wheat curl mite *A. tritici* Shevtchenko, 1970) [73].

Among vectors of cereal viruses, spider mites (*Trombidiformes*: *Tetranychidae* Donnadieu, 1875) should be noted [74]. Robertson and Carroll [75] described the transmission of Barley yellow streak mosaic virus (*Mononegavirales*: *Rhabdoviridae*, *Cytorhabdovirus*) by brown wheat mite (*Petrobia latens* Muller, 1776) in barley crops in Canada and the United States. Later Smidansky and Carroll [76] confirmed rapid reproduction of the virus in imagoes and preimagoes of *P. latens*, and also showed the transovarian transmission.

The field detection of spider mites is difficult due to their small size (~ 0.5 mm), whereas the symptoms they cause per se resemble a virus-induced pathology (chlorotic spots, yellowing of veins, twisting of the leaf blade). In Primorsky and Khabarovsk territories, strawberry spider mite (*Tetranychus turkestanicus* Ug. et Nik.) and European red spider mite (*Panonychus ulmi* Koch, 1836) are widespread, which, being polyphagous, often invaded cereal plants and can presumably be BYDV, NCMV, MD and WSMV vectors. Spider mites are easily moved by the wind, which enhances spread of plant viruses [5]. It cannot be ruled out that a deeper study of tetranichids will contribute to our understanding of their role as pathogen vectors during viral infections in plants.

Summarizing, we note that in phytocenoses of the southern Russian Far East, 10 viruses have been described that infect cereal plants (Table). Their strains have been deposited and preserved in the Russian collection of viruses in East Asia (Laboratory of virology of the Federal Research Center of Biodiversity of Terrestrial Biota of East Asia, Far East Branch RAS) [7-9].

Thus, in the southern part of the Russian Far East, cereal viruses have been regularly monitored since 1962. The strains isolated during the monitoring are stored in the Russian collection of viruses in East Asia (FSC of Biodiversity of Terrestrial Biota of East Asia, FEB RAS). In this review, we mainly focused on data on the current taxonomic status and ecology of the following viruses infecting cereals in the region: Barley stripe mosaic virus (BSMV), Brome mosaic virus (BMV), Poa semilatent virus (PSLV), Northern cereal mosaic virus (NCMV), Russian oat mosaic virus (ROMV), Rice stripe virus (RSV), Rice spotted mosaic virus (RSMV), Maize dwarf mosaic virus (MDMV), Barley yellow dwarf virus (BYDV), and Wheat streak mosaic virus (WSMV). Prevalence of the viruses and their vectors shows that in the agrocenoses of Primorye, the epiphytotic situation for cereals is relatively favorable, while in the Khabarovsk Territory and in the Amur Region it is much more tense. Special attention should be paid to monitoring of both agro- and wild phytocenoses for preventing the spread of viral diseases.

REFERENCES

1. Timofeeva Y.O., Kosheleva Y., Semal V., Burdukovskii M. Origin, baseline contents, and vertical distribution of selected trace lithofile elements in soils from nature reserves, Russian Far East. *Journal of Soils and Sediments*, 2018, 18(3): 968-982 (doi: 10.1007/s11368-017-1847-5).
2. Kim A., Min K. The state Bohai and its provincial government system (698 A.D.-926 A.D.). *Nuova Rivista Storica*, 2015, 99(2): 421-434.
3. *China under Jurchen rule: Essays on Chin intellectual and cultural history*. H.C. Tillman, S.H. West (eds.). NY, 1995.
4. Shelokhaev V.V. The Stolypin variant of Russian modernization. *Russian Social Science Review*, 2016, 57(5): 350-377 (doi: 10.1080/10611428.2016.1229962).
5. *Rukovodstvo po virusologii. Virusy i virusnye infektsii cheloveka i zhivotnykh* /Pod redaktsiei D.K. L'vova [Virology guide. Viruses and viral infections of humans and animals. D.K. L'vov (ed.)]. Moscow, 2013 (in Russ.).
6. *Plant viruses in Asia*. D. Murayama, H.O. Agrawal, T. Inoue, I. Kimura, E. Shikata, K. Tomaru, T. Tsuchizaki, Triharso (eds.). Bulaksumur, Gadjah Mada University Press, 2000.
7. Shchelkanov M.Yu., Volkov Yu.G., Kakareka N.N., Kozlovskaya Z.N., Sapotskii M.V., Tolkach V.F., Pleshakova T.I., Gapeka A.V., Galkina I.V. *Materialy Mezhdunarodnykh nauchnykh chtenii «Primorskie Zori 2017»* [Proc. Int. Scientific Readings «Primorye Dawns 2017»]. Vladivostok, 2017: 466-470 (in Russ.).
8. Volkov Yu.G., Kakareka N.N., Shchelkanov M.Yu. *Materialy IV Mezhdunarodnoi nauchnoi konferentsii «Ekologicheskaya bezopasnost' zashchity rastenii»* [Proc. IV Int. Conf. «Eco-friendly plant protection»]. Minsk, 2017: 79-84 (in Russ.).
9. Kakareka N.N., Volkov Yu.G., Tolkach V.F., Shchelkanov M.Yu. Russian collection of viruses from East Asia as an element of biological safety ensuring. *Proc. Ist Int. Conf. «North-East Asia Biodiversity»*. Vladivostok, 2018: 114.
10. Lebedeva E.G., D'yakonov K.P., Nemilostiva N.I. *Nasekomye — perenoschiki virusov rastenii na Dal'nem Vostoke* [Plant viral insect vectors in the Far East]. Vladivostok, 1982 (in Russ.).
11. Mamaev P.Yu. V sbornike: *Fitovirusy Dal'nego Vostoka* /Pod redaktsiei R.V. Gnutovoi [Plant viruses of the Far East. R.V. Gnutova (ed.)]. Vladivostok, 1993: 39-57 (in Russ.).
12. Sapotski M.V., Kakareka N.N. Cereal mosaic virus at the Far East (Russia). In: *Viruses and virus diseases of Poaceae (Gramineae)*. H. Lapierre, P. Signoret (eds.). Paris, 2004: 480-483.
13. D'yakonov K.P., Sapotskii M.V. About phytovirological state of cereal crops at Primorsky province. *Russian Agricultural Sciences*, 2004, 30(1): 92-96.
14. Volkov Y.G., Kakareka N.N., Kozlovskaya Z.N., Klykov A.G. Evaluation of plant virus infection of cereal crops and prediction of spread of disease in Primorskii krai. *Russian Agricultural Sciences*, 2011, 37, 392-394 (doi: 10.3103/S1068367411050259).
15. Klykov A.G., Volkov Y.G., Gapeka A.V. Agrobiological features and technologies cultivation of spring barley in Primorsky Krai. In: *Barley: Physical properties, genetic factors and environmental impacts on growth*. K. Hasunuma (ed.). New York, 2014: 21-36.
16. Zarzyńska-Nowak A., Jeżewska M., Hasiyw-Jaroszewska B., Zielińska L. A comparison of ultrastructural changes of barley cells infected with mild and aggressive isolates of Barley stripe mosaic virus. *J. Plant Dis. Prot.*, 2015, 122: 153-160 (doi: 10.1007/BF03356545).
17. Zarzyńska-Nowak A., Hasiyw-Jaroszewska B., Jeżewska M. Molecular analysis of barley stripe mosaic virus isolates differing in their biological properties and the development of reverse transcription loop-mediated isothermal amplification assays for their detection. *Archives of Virology*, 2018, 163(5): 1163-1170 (doi: 10.1007/s00705-018-3725-x).
18. Gapeka A.V., Volkov Yu.G., Kakareka N.N., Pleshakova T.I., Kozlovskaya Z.N., Klykov A.G., Murugova G.A., Bogdan P.M., Konovalova I.V. Efficiency of phytovirological monitoring of cereal crop breeding in Primorsky krai. *Russian Agricultural Sciences*, 2016, 42: 431-434 (doi: 10.3103/S1068367416060069).
19. Kakareka N.N., Volkov Yu.G., Kozlovskaya Z.N., Pleshakova T.I., Gapeka A.V. *Zashchita i karantin rastenii*, 2017, 1: 24-26 (in Russ.).
20. Gapeka A.V., Zelikova A.A., Zhmurkina S.K., Ledneva V.A., Volkov Yu.G., Kakareka N.N., Shchelkanov M.Yu. Barley stripe mosaic virus (Virgaviridae, Hordeivirus) as etiological agent of maize chlorotic stripe disease. *Russian Agricultural Sciences*, 2018, 44(2): 142-145 (doi: 10.3103/S1068367418020052).
21. Smith O., Clapham A., Rose P., Liu Y., Wang J., Allaby R.G. A complete ancient RNA genome: identification, reconstruction and evolutionary history of archaeological Barley Stripe Mosaic Virus. *Scientific Reports*, 2014, 4: 4003 (doi: 10.1038/srep04003).
22. Vavilov N.I. *Teoreticheskie osnovy selektsii. Tom 1* [Theoretical foundations of breesiing. Vol. 1]. Moscow-Leningrad, 1935: 17-73 (in Russ.).
23. Yuan C., Li C., Yan L., Jackson A.O., Liu Z., Han C., Yu J., Li D. A high throughput barley stripe mosaic virus vector for virus induced gene silencing in Monocots and Dicots. *PLoS ONE*, 2011, 6(10): e26468 (doi: 10.1371/journal.pone.0026468).

24. Lee W.S., Hammond-Kosack K.E., Kanyuka K. Barley stripe mosaic virus-mediated tools for investigating gene function in cereal plants and their pathogens: virus-induced gene silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. *Plant Physiology*, 2012, 160(2): 582-590 (doi: 10.1104/pp.112.203489).
25. Sun X., Zhang C. A conserved C-terminal motif is essential for self-interaction of Barley stripe mosaic virus China strain TGB3 protein. *Biochemical and Biophysical Research Communication*, 2012, 426(1): 153-157 (doi: 10.1016/j.bbrc.2012.08.056).
26. Tao Y., Man J., Wu Y. Development of a multiplex polymerase chain reaction for simultaneous detection of wheat viruses and a phytoplasma in China. *Archives of Virology*, 2012, 157(7): 1261-1267 (doi: 10.1007/s00705-012-1294-y).
27. Aigul T., Zibagul I., Saira S. The Great silk road as a cultural phenomenon. *International Journal of Economic Perspectives*, 2016, 10(4): 41-46.
28. Crawford G.W., Lee G.A. Agricultural origins in the Korean peninsula. *Antiquity*, 2003, 77(295): 87-95 (doi: 10.1017/S0003598X00061378).
29. Hatada T., Smith Jr W.W., Hazard B.H. *A history of Korea*. Santa Barbara, 1969.
30. Vlasov Yu.I., Teploukhova T.N. V sbornike: *Tezisy dokladov soveshchaniya «Problemy virusnykh boleznei zernovykh kul'tur i puti ikh resheniya»* [Abstracts of the meeting «Viral diseases of grain crops — problems and solutions»]. Tver', 1993: 7-8 (in Russ.).
31. Panarin I.V. *Zashchita zlakovykh kul'tur ot virusnykh boleznei* [Cereal protection from viral diseases]. Moscow, 1985 (in Russ.).
32. Volkov Yu.G., Kakareka N.N. *Vestnik DVO RAN*, 2017, 2: 43-50 (in Russ.).
33. Slykhuis J.T. Poa semilatent virus from native grasses. *Phytopathology*, 1972, 62(5): 508-513 (doi: 10.1094/Phyto-62-508).
34. Goodin M.M., Min B.E. Virus host protein interaction of plant adapted rhabdoviruses. In: *Rhabdoviruses: molecular taxonomy, evolution, genomics, ecology, host-vector interaction, cytopathology and control*. R.G. Dietzgen, I.V. Kuzmin (eds.). London, 2012: 133-146.
35. Redinbaugh M.G., Whitfield A.E., Ammar E.D. Insect vector interaction and transmission of cereal infecting rhabdoviruses. In: *Rhabdoviruses: molecular taxonomy, evolution, genomics, ecology, host-vector interaction, cytopathology and control*. R.G. Dietzgen, I.V. Kuzmin (eds.). London, 2012: 133-146.
36. Ostapenko K.A. *Ekologiya i geografiya tsikadovykh (Homoptera, Cicadina) yuga Primorskogo kraya. Kandidatskaya dissertatsiya* [Ecology and geography of cicadas (*Homoptera*, *Cicadina*) in the south of Primorsky Krai. PhD Thesis]. Vladivostok, 2010 (in Russ.).
37. *Plant viruses online. Descriptions and lists from the VIDE database*. A.A. Brunt, K. Crabtree, M.J. Dallwitz, A. Gibbs, L. Watson, E. Zurcher (eds.). Cambridge, 1996.
38. Fedotina V.L. *Doklady Akademii nauk SSSR*, 1974, 218(5): 1211-1213 (in Russ.).
39. Sapotski M.V., Kakareka N.N. Oat Russian mosaic. In: *Viruses and virus diseases of Poaceae (Gramineae)*. H. Lapierre, P. Signoret (eds.). Paris, 2004: 498-500.
40. Sapotskii M.V. V sbornike: *Fitovirusy Dal'nego Vostoka* /Pod redaktsiei R.V. Gnutovoi [In: Plant viruses of the Far East. R.V. Gnutova (ed.)]. Vladivostok, 1993: 81-89 (in Russ.).
41. Sapotskii M.V., Artyukova E.V. V sbornike: *Stanovlenie i razvitie fitovirusologii na Dal'nem Vostoke Rossii* /Pod redaktsiei K.P. D'yakonova, Yu.G. Volkova, V.D. Kostina, V.I. Malinovskogo [In: Formation and development of plant virology in the Russian Far East. K.P. D'yakonov, Yu.G. Volkov, V.D. Kostin, V.I. Malinovskii (eds.)]. Vladivostok, 2002: 37-58 (in Russ.).
42. Hogenhout S.A., Ammar E.D., Whitfield A.E., Redinbaugh M.G. Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology,* 2008, 46: 327-359 (doi: 10.1146/annurev.phyto.022508.092135).
43. Raga I.N., Ito K., Matsui M., Okada M. Effect of temperature on adult longevity, fertility, and rate transovarial passage of rice stripe virus in the small brown planthopper *Laodelphax striatellus* Fallen (*Homoptera*: *Delphacidae*). *Applied Entomology and Zoology*, 1988, 23(1): 67-75.
44. *Opredelitel' nasekomykh Dal'nego Vostoka SSSR. T. 2. Ravnokrylye i poluzhestkokrylye* /Otv. red. A.S. Lelei. L., 1988.
45. Moran N.A. The evolution of aphid life cycles. *Annual Review of Ecology and Systematics*, 1992, 37: 321-348 (doi: 10.1146/annurev.en.37.010192.001541).
46. Kieckhefer R.W., Thysell J.R. Host preferences and reproduction of four cereal aphids on 20 triticale cultivars. *Crop Science*, 1981, 21(2): 322-324 (doi: 10.2135/cropsci1981.0011183X002100020029x).
47. Kieckhefer R.W. Cereal aphid (*Homoptera*: *Aphididae*) preferences for and reproduction on some warm-season grasses. *Environmental Entomology*, 1984, 13(3): 888-891 (doi: 10.1093/ee/13.3.888).
48. Peresypkin V.F. *Sel'skokhozyaistvennaya fitopatologiya* [Agricultural plant pathology]. Moscow, 1989.
49. Kakareka N.N., Volkov Yu.G., Gapeka A.V. *Zashchita i karantin rastenii*, 2015, 8: 49-50 (in Russ.).
50. Mozhaeva K.A., Kastal'eva T.B. *Agroekologicheskii zhurnal*, 2002, 1: 28-33 (in Russ.).
51. Mozhaeva K.A., Kastal'eva T.B., Girsova N.V. *Virus zheltoi karlikovosti yachmenya i drugie virusy zernovykh kul'tur na territorii Rossiiskoi Federatsii* [Barley yellow dwarf virus and other grain viruses in the Russian Federation]. Moscow, 2007 (in Russ.).
52. Rosenkranz E., Scott G.E. Effect of plant age at time of inoculation with maize dwarf mosaic

virus on disease development and yield in corn. *Phytopathology*, 1978, 68(12): 1688-1692.
53. Gray S., Gildow F.E. Luteovirus-aphid interactions. *Annual Review of Phytopathology*, 2003, 41: 539-566 (doi: 10.1146/annurev.phyto.41.012203.105815).
54. Wang X., Chang S., Jin Z., Li L., Zhou G. Nucleotide sequences of the coat protein and readthrough protein genes of the Chinese GAV isolate of barley yellow dwarf virus. *Acta Virologica*, 2001, 45(4): 249-252.
55. Jin Z., Wang X., Chang S., Zhou G. The complete nucleotide sequence and its organization of the genome of Barley yellow dwarf virus-GAV. *Science in China Series C Life Sciences*, 2004, 47(2): 175-182 (doi: 10.1360/03yc0076).
56. D'yakonov K.P. *Chteniya pamyati Alekseya Ivanovicha Kurentsova*, 2000, 10: 5-16 (in Russ.).
57. D'yakonov K.P. *Chteniya pamyati Alekseya Ivanovicha Kurentsova*, 2003, 13: 53-60 (in Russ.).
58. Kostin V.D. *Virozy dikorastushchikh rastenii Dal'nego Vostoka Rossii* [Wild plant viral diseases in the Russian Far East]. Vladivostok, 2005 (in Russ.).
59. Arita H., Yamamoto M., Ohkuro T., Tomosyou T. The cost of restoring abandoned paddy fields invaded by grass in a wet soil environment. *Transactions of the Japanese Society of Irrigation, Drainage and Reclamation Engineering*, 2007, 248: 55-60.
60. Uehara-Ichiki T., Shiba T., Matsukura K., Ueno T., Hirae M., Sasaya T. Detection and diagnosis of rice-infecting viruses. *Frontiers of Microbiology*, 2013, 4: 289 (doi: 10.3389/fmicb.2013.00289).
61. Abo M.E., Ali Fadhila H. Epidemiology and management of rice viruses and virus diseases. *Proc. of a Conference Organized by IITA*. Ibadan, 2001: 112-127.
62. Chen H., Wie T., Saotome A., Sakaguchi K., Shikamoto Y., Mizuno H., Akita F., Shimizu T., Uehara-Ichiki T., Omura T. Aggregation ability of virus-specific antibodies is correlated with their capacity to neutralize Rice dwarf virus. *Japan Agricultural Research Quarterly*, 2012, 46(1): 65-71 (doi: 10.6090/jarq.46.65).
63. Omura T., Morinaka T., Inoue H., Saito Y. Purification and some properties of Rice gall dwarf virus, a new phytoreovirus. *Phytopathology*, 1982, 72: 1246-1249 (doi: 10.1094/Phyto-72-1246).
64. Xie L.H., Lin Q.Y., Zhou Q.L. On the bunchy stunt disease of rice. II. The distribution, loss, hosts and overwintering of rice bunchy stunt. *Acta Phytopathologica Sinica*, 1982, 12: 16-20 (in Chinese).
65. Hibino H., Kimura I. Detection of Rice ragged stunt virus in insect vectors by enzyme-linked immunosorbent assay. *Phytopathology*, 1982, 72: 656-659 (doi: 10.1094/phyto-72-656).
66. Zhang P., Mar T.T., Liu W., Li L., Wang X. Simultaneous detection and differentiation of Rice black streaked dwarf virus (RBSDV) and Southern rice black streaked dwarf virus (SRBSDV) by duplex real time RT-PCR. *Virology Journal*, 2013, 10: 24 (doi: 10.1186/1743-422X-10-24).
67. Hibino H., Usugi T., Oraura T., Tsuchizaki T., Shohara K., Iwasaki M. Rice grassy stunt virus: a planthopper-borne circular filament. *Phytopathology*, 1985, 75: 894-899 (doi: 10.1094/Phyto-75-894).
68. Hiraguri A., Hibino H., Hayashi T., Shimizu T., Uehara-Ichiki T., Omura T., Sasaya T. Complete sequence analysis of rice transitory yellowing virus and its comparison to rice yellow stunt virus. *Archives of Virology*, 2010, 155: 243-245 (doi: 10.1007/s00705-009-0557-8).
69. Sharma S., Dasgupta I. Development of SYBR Green I based real-time PCR assays for quantitative detection of Rice tungro bacilliform virus and Rice tungro spherical virus. *Journal of Virological Methods*, 2012, 181(1): 86-92 (doi: 10.1016/j.jviromet.2012.01.018).
70. Chetverikov F.E. *Trudy Zoologicheskogo instituta RAN*, 2016, 320(S4): 349-376 (in Russ.).
71. Ranabhat N.B., Seipel T., Lehnhoff E.A., Miller Z.J., Owen K.E., Menalled F.D., Burrows M.E. Temperature and alternative hosts influence *Aceria tosichella* infestation and wheat streak mosaic virus infection. *Plant Diseases*, 2018, 102(3): 546-551 (doi: 10.1094/PDIS-06-17-0782-RE).
72. Navia D., de Mendonça R.S., Skoracka A., Szydło W., Knihinicki D., Hein G.L., da Silva Pereira P.R., Truol G., Lau D. Wheat curl mite, *Aceria tosichella*, and transmitted viruses: an expanding pest complex affecting cereal crops. *Experimental and Applied Acarology*, 2013, 59(1-2): 95-143 (doi: 10.1007/s10493-012-9633-y).
73. Razvyazkina G.M. *Virusnye zabolevaniya zlakov* [Viral diseases of cereals]. Novosibirsk, 1975 (in Russ.).
74. Mitrofanov V.I., Strunkova Z.I., Livshits I.Z. *Opredelitel' tetranikhovykh kleshchei fauny SSSR i sopredel'nykh stran (Tetranychidae, Bryobiidae)* /Pod redaktsiei V.G. Babaevoi [Key to the tetranic tick fauna (*Tetranychidae*, *Bryobiidae*) of the USSR and neighboring countries. V.G. Babaeva (ed.)]. Dushanbe, 1987 (in Russ.).
75. Robertson N.L., Carroll T.W. Virus-like particles and a spider mite intimately associated with a new disease of barley. *Science*, 1988, 240(4856): 1188-1190 (doi: 10.1126/science.240.4856.1188).
76. Smidansky E.D., Carroll T.W. Factors influencing the outcome of barley yellow streak mosaic virus-brown wheat mite-barley interactions. *Plant Diseases*, 1996, 80: 186-193 (doi: 10.1094/PD-80-0186).

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OPERATIVE AND LONG-TERM FORECASTING OF CROP PRODUCTIVITY BASED ON MASS CALCULATIONS OF THE AGROECOSYSTEM SIMULATION MODEL IN GEOINFORMATION ENVIRONMENT

(review)

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Abstract

In the context of changing socio-economic, natural and climatic conditions, there is a need for effective management tools to adapt agricultural activities. Such tools are farming systems, which are a set of interconnected agrotechnical, reclamation and organizational measures aimed at the efficient use of agrolandscapes, preservation and improvement of soil fertility, and obtaining high crop yields. The efficiency of agricultural production can be improved by using various forecasting methods based on the use of mathematical models. In crop production, statistical and dynamic simulation forecast models have been developed. The latter are more accurate and adaptive and allow you to get an answer to the question about the development of argoecosystems in the conditions of changing climatic conditions and the application of various agricultural measures. The paper provides an overview of methodological approaches for predicting crop productivity based on mass calculations of a simulation model of an agroecosystem in a geoinformation environment that can be used to justify farming systems. The analysis of the state of the problem is carried out, which presents the main current trends in the use of simulation models of agroecosystems in decision support systems for management in agriculture in general and in the support of farming systems in particular. Existing approaches and methods are classified based on spatial coverage into macro-scale, meso-scale, and micro-scale modeling methods. In the general case, these different methods require different methodological approaches are presented in the paper. The relevant basic methods and approaches for creating a universal environment for mass calculations of dynamic models of agroecosystems for different levels of spatial coverage are also presented. The analysis of the very important issue of choosing a set of control (base) points is presented where model calculations will be performed that should belong to real agricultural fields and sufficiently reflect the diversity of soil and climatic conditions of the region under consideration. Also presented are the requirements for a universal modeling environment for carrying out calculations on different models from various suppliers.

Keywords: agroecosystems, simulation modeling, mass calculations, forecasting, geographic information systems, farming systems

The current view on the role of dynamic simulation models of plant production process has changed significantly [1-3]. The scope of application of such models, which has traditionally been limited to scientific research in agroecology, is increasingly expanding towards practical use in decision-making systems [4]. Improved models and theoretical bases of mathematical simulation of processes and phenomena in soil—plant—atmosphere systems allow universal ecophysio-

logical models to be applied to practically unlimited number of crops and soil-climatic conditions [5]. In addition, most modern models of agroecosystems are based on an increasing number of physical and biological determining processes and phenomena [4-7]. Therefore, such models, along with productivity (yield), make it possible to quantitatively evaluate other characteristics of agroecosystems. In model forecasts, there is a transition from single growing seasons to long-term continuous calculations of crop rotations [4]. Also, the idea of a significant variation in the temporal and spatial scale of dynamic models is becoming increasingly popular, in particular, calculations are performed not only for one crop at one geographic point for one growing season, but also for arbitrarily chosen periods of time and territories. In other words, spatial expansion is understood as the simultaneous calculation of a spatially one-dimensional model not only for one selected representative point of the earth's surface with specified soil and terrain properties, but also for a representative set of such points that form a heterogeneous agro-landscape [6, 7]. In contrast to classical one-dimension models of homogeneous sowing, the third-generation dynamic models are applicable to adaptive landscape farming systems [5]. This is due to significant progress in computing technologies, which provide practically unlimited efficiency and speed of calculations of numerical algorithms embedded in the model.

Dynamic models of agroecosystems in decision-making systems. The modeling in decision-making systems requires adequate data input for which all modern tools for on-line monitoring and automated measurements are being introduced in the practice and field experiments, including non-contact methods, remote sensing and portable automatic weather stations [7-11]. Such instrumental methods improve the quality of information for modeling the production process of plants and the reliability of data used for parametric identification and verification of algorithms by assimilating spatially distributed measurement data. Nevertheless, traditional regression models of agroecosystems of varying complexity are currently the most common tool for assessing crop yields on large areas during regional medium- and long-term planning [12-16]. Such models still are basic to regionally assess a potential productivity of the main economic crops for the current growing season. However, in such models the influence of many factors (physical, climatic and technological) may not be fully accounted. Therefore, dynamic simulation models of agroecosystems are a promising forecasting tool that can adequately respond to numerous challenges [17-20]. The methodology of such dynamic model application to regional planning is currently practically absent, and examples of successful medium- and long-term forecasting are few. This is largely due to the fact that adequate spatial-temporal scaling requires adaptation and modification of the internal logic of existing models and the infrastructure of computer experiments [21-23]. Such modification is necessary to provide adequate modeling for long-term crop rotations given the diversity of long-term agro-technological measures [24-27]. The solution can be the use of one model of the production process of agroecosystems for the entire variety of cultivated crops with a common data structure and standard architecture, which will allow application of the model to manage regional farming systems.

The Laboratory for Mathematical Modeling of Agroecosystems, the Research Institute of Agrophysics (St. Petersburg), has been developing and improving the AGROTOOL family of models of production process for 40 years [28, 29]. To date, according to de Wit (30), Agrotool v3.5, a third-level computer model of productivity, has been suggested.

In general, the analysis of the above publications [1-30] allows us to formulate criteria for a model applicable to justify crop rotations in farming systems. The model should regard i) the impact of the predecessor crop, including crop

residues, symbiotic nitrogen fixation by legumes, changes in the agrophysical and agrochemical properties of the soil, etc.; ii) out-of-season abiotic processes ("overwintering"), such as snow cover growth and melt rates, soil freez-ing and warming, etc.; iii) possible explicit indication of doses and timing of agronomic treatments, reactive control modes such as automatic irrigation, sowing date assignment, etc. And finally, the model should be resistant to a lack of factual information. The mentioned AGROTOOL family of models, and, particularly, Agrotool v3.5 fully comply with these criteria.

Also, to expand application of agroecosystem models, stringent criteria should be imposed on the execution environment, i.e. the computer shells [31, 32]. The main requirements are i) a multivariate automated calculation of the model for a large number of alternative options with previously prepared sets of input data and ii) the ability of sequential or simultaneous runs of model calculations. A convenient connection of the execution environment with geoinformation systems (GIS) is also necessary to provide spatial referencing for the soil parameters and a digital model of the relief, and also to visualize modeling results as thematic maps of the indicators [33]. In addition, it should be borne in mind that most forecasting tasks require the procedures for automatic generation of "synthetic" input data (for example, a generator of weather characteristics), which are spatially and temporally coherent [34, 35].

Agronomic dynamic models of productivity are traditionally one-dimensional, or one-point [5]. In other words, each specific version of the model run requires, as input data, information about a specific area of the territory for which the calculation is performed (soil characteristics, relief, weather, etc.). Extending the scope of the model to massive spatial calculations for geographically distributed territories requires the coupling of dynamic agroecosystem models with GIS. Indeed, such a combination provides simultaneous analyses of agroecosystems in time and space [33, 36, 37]. Practical orientation and relevance of such research are obvious. So, prediction of losses of agricultural production due to changes in external conditions, primarily natural, allows practitioners to undertake preventive measures through the choice of appropriate farming system and to maintain a certain level of food security [37, 38]. In risky farming, early warning systems based on remote sensing can help to identify an emerging problem in advance [39, 40]. Often, such systems are based on monitoring weather and agricultural conditions during the growing season and include regionally calibrated crop models for assessing yield uncertainty [41). Yield forecasts can be also made before planting or during the current growing season, and the results can underly managerial decisions to promptly provide alternative options for farming [42].

However, the spatial scalability of a model inevitably leads to the fact that when the scope expands from a point to a territory, the required amount of input information and computing resources increase sharply [43]. Moreover, fundamentally new areas of potential application of relevant technologies in crop and agricultural production have appeared, for example, the use of crop remote sensing data [14, 39, 41, 44, 45], including those for precision farming [10]. To a certain extent, the necessity to obtain all data for mass spatial calculations is avoided in the task of assessing impact of potential climatic changes on productivity and sustainability of an agrolandscape [46, 47]. For a specific calculation option, the required set of input meteorological data (a representative sample of scenarios of weather realizations of the future climate) for an arbitrary spatial grid of control points can be obtained from the IPCC database (Intergovernmental Panel on Climate Change, https://www.ipcc.ch /). However, the methodology has not yet been developed and an adequate toolkit has not been created for the operational model

forecasting of crop productivity for different spatial coverage. Scalability and universalization of the corresponding software and methodological solutions has recently become more and more urgent [48, 49]. Suffice it to say that within the framework of the MACSUR project (Modeling European Agriculture with Climate Change for Food Security, https://www.macsur.eu/), a pan-European information portal on simulation model data support of the European agriculture adaptation to anthropogenic climatic changes, a special working group Scale-It! has been allocated (http://www.scale-it.net/). The group aims to coordinate European research on practical use of crop production models for decision support at various spatial scales [50]. Its scope includes i) organization of ensemble calculations projects, ii) aggregation of specific input data from different geographic locations, iii) substantiation of methods of spatial discretization and spatial interpolation of input data for models with an assessment of the degree of uncertainty of the results obtained, iv) collection of information about personalities, models, approaches, and v) providing operational information on research challenges and achievements on the issue.

The LandCare-DSS environment software (http://www.landcare2020.de/, Leibniz Centre for Agricultural Landscape Research — ZALF, Germany) is one of the most notable applied results of European studies on comprehensive dynamic models of crop yields at regional scale [51]. At present, this software seems to be the only finalized product with an acceptable level of automation [52]. The unique characteristics of LandCare-DSS include its own built-in GIS interface, integration with economic models, and the use of models of different types and spatial detailing within a single architecture, from the YieldStat regression statistical model at a regional level [13] to the dynamic ecophysiological model MONICA for a specific agricultural field [31, 53].

Application of crop yield point models [29] at regional level also requires tools for GIS integration [34, 36, 45, 54]. Suffice it to say that by now almost all most popular models have special shells or extensions for calculations in spatial resolution, for example, the CRAFT platform for the DSSAT family of models [37]. For post-processing (geostatistical and spatial analysis when modeling processes in crops), the Geographic Information System for Agriculture and the Environment (AEGIS; AEGIS/WIN) has been created, which integrates DSSAT models with the geographic mapping tools ArcInfo and ArcView using an object-oriented macro programming language [37]. The GIS-based EPIC model (GEPIC) is another specialized spatial tool that combines the EPIC (Environmental Policy Integrated Climate) biophysical model with GIS to simulate the spatial and temporal dynamics in the soil—plant—atmosphere processes [55]. Back in the 1990s, within the framework of the MARS project coordinated by the Joint Research Center (JRC, European Commission, https://ec.europa.eu/jrc/en), a European crop growth monitoring system (https://ec.europa.eu/jrc/en/research-topic/agricultural-monitoring) and a yield forecasting system based on the BioMA model have been developed [56]. As a result, plant growth and development models became compatible with remote sensing and GIS data [5, 18, 19, 57]. Other solutions in this class include pSIMS, MINK, SIMPLACE and GeoSIM [58-61].

A special task is to use remote sensing data of agricultural lands in dynamic models of agroecosystems [10, 14, 39, 41, 44, 62]. With the advent of remote and even space-based monitoring devices, many soil—plant—atmosphere system characteristics allow direct on-line measurement at almost arbitrarily temporal and spatial resolutions. Moreover, this often does not require any special efforts or expensive measurements, it is enough to have access to partially or completely open and promptly updated databases for processing remote satellite sensing images. The problem, however, is that, despite the development of measuring

instruments, from a mathematical point of view, most agroecological models are still unobservable control systems [63], with only a small part of the state variables available for estimation from observations. Moreover, most of the relevant observations are measurements of indirect quantities, some optical indices, which are undoubtedly related to the essential variables of the model (shoot biomass, soil moisture, chlorophyll content, nitrogen nutrition, etc.), but this relationship is often ambiguous and weakly formalizable [64].

Thus, the analysis of the problem shows that simulation modeling of agroecosystems is a technology based on special studies [54] and currently used in many areas, such as forecasting yield [65], climate change [29, 65, 66)], crop response to various treatments and external conditions [67]. In the very near future, new applications are expected for precision farming and smart agriculture (Smart Agriculture). The world leaders in the area are DSSAT [68] and EPIC [69] (USA), STICS (France) [70], APSIM (Australia) [54], AGROTOOL (Russia) [29], MONICA (Germany) [31], and WOFOST (the Netherlands) [65]. The geography of use of simulation models of agroecosystems is also wide [52, 71]. As in other earth sciences (climatology, hydrology), there is a steady tendency to use not one, but several models (ensemble calculations) for solving specific problems, especially since many of them are freely available [72, 73].

However, it should be noted that simulation models are still little used in applications with long-term forecasts [54, 74], that is, for the development of farming and land use systems, although there are positive examples [75, 76]. The main challenge remains the need to continuously calculate the dynamics of the agroecosystem states for the selected geographic location, taking into account crop changes and non-growing periods. Sometimes for this, a complete description of the crop rotation is directly included in the input data (for example, the MONICA model) [31], an alternative approach is special platforms and shells for the automated transfer of the state of the modeled object from the previous run of the model to the next [76]. Many spatial crop modeling systems have been developed for specific applications and, therefore, have certain requirements and limitations that can significantly complicate their implementation in specific regions. Some of these tools are already outdated (AEGIS) [77], others are very reliable (pSIMS, Mink) [59] and based on scripting languages, so the calculations in these systems must be performed on computational clusters or on high-performance computers. In some models (MARS) [21], it is difficult to solve practical problems of application and maintenance [71], and some solutions are limited in functionality [59]. In this, all researchers point to the need for a convenient and easy-to-use software platform that could become an accessible and adaptable environment for calculating basic models of the crop production to facilitate forecasting the growth and development of plants upon different regional farming systems [39, 54, 78, 79].

Operational and long-term forecasting of crop productivity — a practical overview. A perfect scalable information analytical system for dynamic monitoring and forecasting parameters of an agricultural area should be applicable to develop farming systems at different spatial and temporal detailing levels [80]. For spatial resolution, these tasks are triple. The first group includes macroscale calculations, i.e. assessment of the potential and achievable productivity of the main crops on a national scale for the current conditions (operational time management) and possible climate change (strategic planning in time). The second group is mesoscale calculations, i.e. monitoring of productivity and ecological sustainability of agrolandscapes (operational management) and model-based analysis and optimization of farming systems on a regional scale (strategic management). Finally, the third group includes micro-scale calculations, i.e. analysis (both operational and long-term) of the effectiveness of precision or

coordinate technologies in a farm or in a field. The problems that can be posed and solved using mass calculations of point dynamic models of the agroecosystem at various time and spatial scales, as well as the methods of creating a universal environment for such calculations, are summarized in Table 1 [81].

1. Tasks solved using the simulation model of the agroecosystem at different spatial (SML) and temporal (TML) management levels [81]

TML \ SML	Micro level (a farm, a field)	Meso level (a region, a province, an agricultural holding)	Macro level (country, continent)
Operational monitoring and forecasting	Operational solutions in precision farming Assimilation of remote sensing data into model forecasts	Promptly updating forecast of expected productivity during the current growing season	
Long-term analysis and planning	Farm-level land management projects	Design of regional farming systems strategic impact analysis of new technologies and introduced crops	Assessment of the impact of climate change on agriculture

Mass calculations of the model for a representative set of spatial points should be within a general ideology of batch calculation of all scenarios of the generated computational experiment project, where the scenario's belonging to a specific point is determined by the gradations of "soil" and "terrain" factors [82, 83]. Table 2 shows sources and mechanisms for the on-line data replenishment in calculations of various spatial and temporal granularities [83].

2. Sources and types of input data for modeling productivity of agroecosystems at different spatial detailing (83)

Factor \ SL	Micro level (a farm)	Meso level (a geographic region)	Macro level (country, continent)
Soil	Detailed data on soil sections	Soil maps coupled with cadastral data	Statistic soil maps
Terrain	Coordinates and digital elevation maps	Coordinates	Coordinates
Technology	Operational management: precision farming Strategic planning: automated technology selection	Regionally adapted farming system	Reference regionally adapted farming system
Crop	Crops and varieties of the current crop rotation season	Crops for the analyzed farming system	Basic crops
Weather	Operational management: local automatic weather station Strategic planning: single point weather generator calibrated as per the data of the nearest weather station	Operational management: network of reference weather stations and short-term synoptic forecasts Strategic planning: space-time stochastic weather generator	Operational management: network of reference weather stations and short-term synoptic forecasts Strategic planning: набор эталонных лет-аналогов

N o t e. SL — spatial level.

For a regional scale calculation, it is important to determine a set of control (base) points for model calculations on actual agricultural fields which sufficiently reflect the diversity of soil and climatic conditions of the region. At a regional level, it is reasonable to use not specific, but conditional soil characteristics most typical for the region (e.g., from the Unified State Register of Soil Resources of Russia, http://egrpr.esoil.ru/). Not a specific known option, but an average technology from the recommended farming systems is assigned to the model. There are several variables the values of which determine the initial state of the agroecosystem to which dynamic models are very sensitive during running [84, 85]. Therefore, one should start modeling the current season not from the sowing date, but from a rather long preceding period [76]. In addition, for any geographic location, it is possible to obtain time series of the actually observed weather from the resources of the World Meteorological Organization (WMO, Switzerland) and short-term weather forecasts (https://www.worldweatheronline.com,

https://www.aerisweather.com) for at least 3-5 days [39, 41, 86]. A predictive modeling requires a large number of possible weather scenarios, i.e. so-called analog years [87] or stochastic weather generators [88-90]. Such scenarios have been theoretically developed and successfully integrated in practice into crop computer models. On a regional scale, one can speak of a "good season" and a "bad season" for crop production, which makes it possible to consider a homogeneous region of agricultural production such a geographical area where the interannual variability of productivity significantly exceeds the spatial variability within the region [80].

In Russia, a prototype of an information analytical system has been developed for crop yield model forecasting for different spatial and temporal coverage. The APEX software (Automation of Polivariant EXperiments) [32, 42, 91] was used as the main tool for mass calculations of production process models. Here, multivariate analysis means i) designing and running-up a multidimensional computer case study, ii) performing model runs in batch mode, and iii) applying advanced statistical processing of the results.

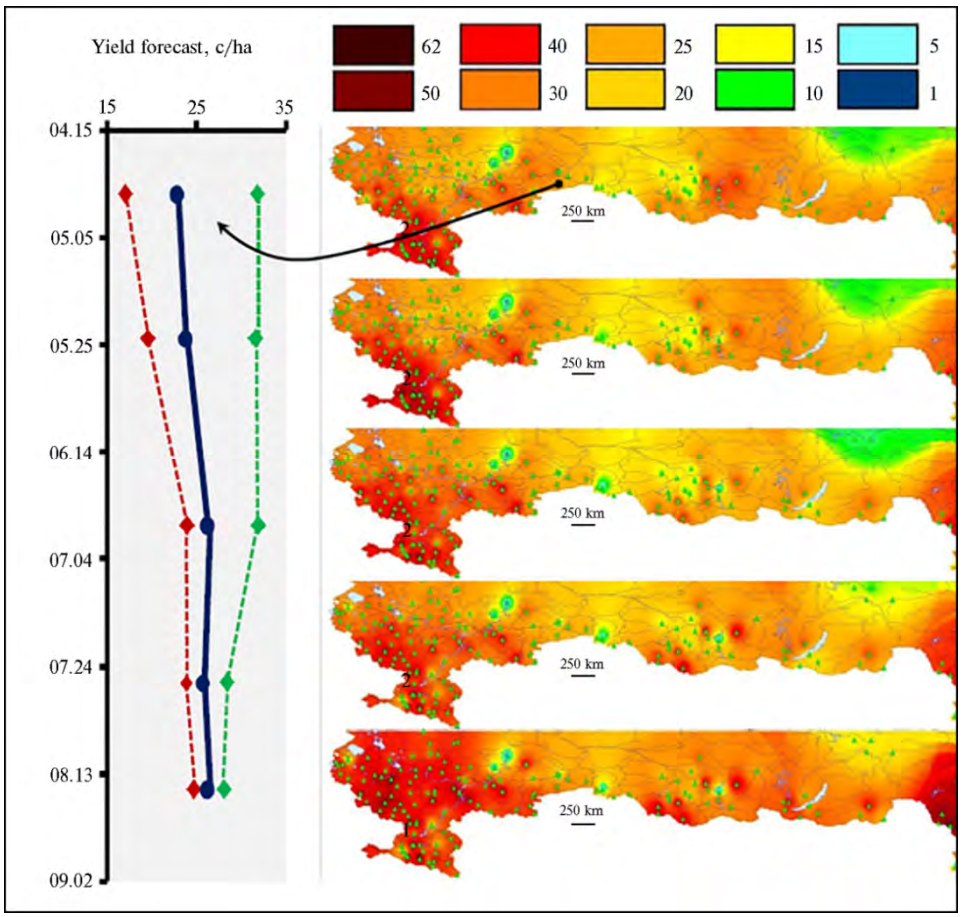

Fig. 1. Dynamically adjusted forecast of the spring wheat yield at different dates in the growing season 2017 for a specific location (left side) **and for Russia** (right side, average values)**.** Green triangles are reference points for calculations [42].

The concept of using dynamic model mass calculations for operational forecasting, proactive management of agroecosystems and satellite sensing data assimilation was theoretically developed in a series of studies [29, 42, 64, 75, 76, 80, 92] and tested, in particular, in Harvest Map tool (https://cropmap.ru/), an electronic map with built-in services for monitoring and forecasting crop production

processes. The dynamic agroecosystem model AGROTOOL allows prediction of the yield of a given crop in a user-selected geographic location [28, 29].

The study of the potential yield of the main crops throughout the territory of the Russian Federation based on a dynamic (promptly refined) forecast of productivity within one current growing season with GIS data visualization is an example of solving the problem of spatial detailing, opposite to those considered above [42]. Selected modeling results are illustrated in Figure 1. The tested approach allows analyzing the evolution of expectations regarding future profitability for the growing season and for all base points together (thematic maps of the predicted average yield on the right side of the figure), as well as the dynamics and interval of the predicted average yield for any specific base point (on the left side of the figure there is a graph for a test point in the Orenburg Province).

The operational and long-term forecasting of spring barley grain yield based on mass calculations of the agroecosystem simulation model in the geoinformation environment was successfully applied in the Republic of Crimea. A digitized electronic map of Crimean soils and scenarios of actual weather conditions from 15 Crimean meteorological stations of the WMO network were used as input data. Calculations for the 2012 and 2014 growing seasons were performed with a grid of 55 selected reference points located in the fields of agricultural enterprises in all regions of the republic. The spatial distribution of the yield of barley and the date of its ripening are presented in Figure 2 (the number of modeling points with the same date of barley ripening is indicated) [80].

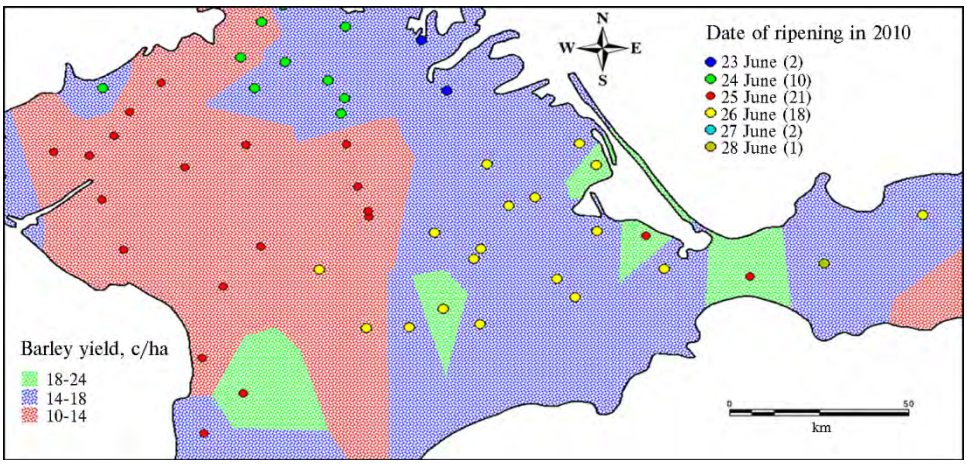

Fig. 2. Spatial distribution of barley yields and ripening dates in the republic of Crimea (the number of modeling points with the same ripening date is indicated in brackets) [80].

We also note a number of studies where the method of multivariate analysis of the dynamics of agroecosystems based on mass calculations of dynamic models of productivity was used for long-term planning [25, 76]. The project of the European Community "Crop growth and soil processes modeling — the use of multi-model ensemble for crop rotations under recent and future climatic conditions" [93-95] is among them. The project aims to apply production process models in assessing possible impact of global climate change on yields and stability of agroecosystems in Eastern Europe, as well as to search for and to analyze the ways to mitigate these negative consequences. As a tool, the researchers chose a popular modern methodology of ensemble calculations instead of one specific model [96]. The fundamental difference between the mentioned project and numerous analogs was the fact that the corresponding ensemble calculations were performed in the context of the selected crop rotation schemes, and the

modification of the traditional practices of crop change was deemed the main investigated mechanism that ensures mitigation of the effects of climate change on agriculture [97-99].

Therefore, modern computing and information technologies create conditions for operational agrotechnological solutions using more accurate and detailed dynamic models. Variable modeling makes it possible to study the effects of various agricultural technologies and assimilate remote sensing data of agricultural areas to select the optimal agrotechnical plan and adjust modeling based on the results of real measurements during the growing season. The number of calculation options and the corresponding model runs are determined by the variation of the following main factors: spatial factor (calculations are carried out in certain locations, each of which is characterized by soil properties and parameters of the simulated crop); meteorological factor (possible weather dynamics for the rest of the growing season is modeled using "options for possible trajectories" consisting of a representative number of synthetic weather scenarios, and for their formation a stochastic weather generator should be used that supports both temporal and spatial correlations); technological factor (for a well-grounded choice of the date, number and parameters of technological operations, it is necessary to analyze the influence of various options in order to choose the best one in the context of the chosen criterion of statistical optimization and to apply a proactive management strategy in crop production); model factor (it is advisable to use not one, but several alternative models of agroecosystems to obtain reliable results).

As a result of simultaneous variation of the above factors with a set of their gradations, the total number of variants of one program-on-model that implement a full factorial computational experiment determines the need to use modern technologies of distributed parallel computing and supercomputer technology. Analysis of the current situation in the design of farming systems allows us to point out the most important role and potential demand of dynamic models of agroecosystems. Decision-makers, in an ideal situation, would like to have a tool that, based on the most diverse sources of information, including remote sensing data, can assess the medium- and long-term consequences of the choice of farming systems. Simulation models of agroecosystems coupled with GIS provide the best environment for solving the optimization of scenarios "what will be if ...". This approach can be a powerful tool in optimizing agricultural land use. Creation of an adequate information analytical system allows farmers and the authorities carrying out planning, monitoring and regulatory functions to make decisions at new level. The tool can be also used as an external intellectual component of state information and analytical resources within the framework of the adopted National Platform for Digital State Management of Agriculture "Digital Agriculture".

Thus, the operational and long-term forecasting of crop productivity based on mass calculations of the simulation model of the agroecosystem in the geoinformation environment plays an important role in the development of farming systems. Empirical regression models based on statistical information are still widely used for regional predictive yield estimates. However, their main drawback is the relatively low accuracy of the results, which makes it impossible to use this approach for operational agrotechnological solutions. The development of computing and information technologies allows us to answer this challenge by applying more accurate and detailed dynamic models of agroecosystems. The choice of optimal agrotechnical plan and adjustment of the modeling process online based on the results of actual measurements during the growing season remain relevant. The main tools are variational modeling, which is used to

analyzed the effects of various modifications of agricultural technologies, and assimilation of remote sensing data, while the best environment is simulation models of agroecosystems coupled with GIS.

REFERENCES

1. Rodriguez D., de Voil P., Rufino M.C., Odendo M., Van Wijk M.T. To mulch or to munch? Big modelling of big data. *Agricultural Systems*, 2017, 153: 32-42 (doi: 10.1016/j.agsy.2017.01.010).
2. Sierra J., Causeret F., Chopin P. A framework coupling farm typology and biophysical modelling to assess the impact of vegetable crop-based systems on soil carbon stocks. Application in the Caribbean. *Agricultural Systems*, 2017, 153: 172-180 (doi: 10.1016/j.agsy.2017.02.004).
3. Jeuffroy M.H., Casadebaig P., Debaeke P., Loyce C., Meynard J.M. Agronomic model uses to predict cultivar performance in various environments and cropping systems. A review. *Agronomy for Sustainable Development*, 2014, 34(1): 121-137. (doi: 10.1007/s13593-013-0170-9).
4. Dury J., Schaller N., Garcia F., Reynaud A., Bergez J. E. Models to support cropping plan and crop rotation decisions. A review. *Agronomy for Sustainable Development*, 2012, 32(2): 567-580. (doi: 10.1007/s13593-011-0037-x).
5. Jones J.W., Antle J.M., Basso B., Boote K.J., Conant R.T., Foster I., Godfray H.C.J., Herrero M., Howitt R.E., Janssen S., Keating B.A., Munoz-Carpena R., Porter C.H., Rosenzweig C., Wheeler T.R. Brief history of agricultural systems modeling. *Agricultural Systems*, 2017, 155: 240-254 (doi: 10.1016/j.agsy.2016.05.014).
6. Morais R., Silva N., Mendes J., Adão T., Pádua L., Lypez-Riquelme J.A., Pavón-Pulido N., Sousa J.J., Peres E. Mysense: A comprehensive data management environment to improve precision agriculture practices. *Computers and Electronics in Agriculture*, 2019, 162: 882-894 (doi: 10.1016/j.compag.2019.05.028).
7. Insua J.R., Utsumi S.A., Basso B. Estimation of spatial and temporal variability of pasture growth and digestibility in grazing rotations coupling unmanned aerial vehicle (UAV) with crop simulation models. *PloS ONE*, 2019, 14(3): e0212773 (doi: 10.1371/journal.pone.0212773).
8. Gebremedhin A., Badenhorst P.E., Wang J., Spangenberg G.C., Smith K.F. Prospects for measurement of dry matter yield in forage breeding programs using sensor technologies. *Agronomy*, 2019, 9(2): 65 (doi: 10.3390/agronomy9020065).
9. Reynolds D., Ball J., Bauer A., Davey R., Griffiths S., Zhou J. CropSight: a scalable and open-source information management system for distributed plant phenotyping and IoT-based crop management. *GigaScience*, 2019, 8(3): giz009 (doi: 10.1093/gigascience/giz009).
10. Hunt E.R. Jr., Daughtry C.S. What good are unmanned aircraft systems for agricultural remote sensing and precision agriculture? *International Journal of Remote Sensing*, 2018, 39(15-16): 5345-5376 (doi: 10.1080/01431161.2017.1410300).
11. Farooque A.A., Chang Y.K., Zaman Q.U., Groulx D., Schuman A.W., Esau T.J. Performance evaluation of multiple ground-based sensors mounted on a commercial wild blueberry harvester to sense plant height, fruit yield and topographic features in real-time. *Computers and Electronics in Agriculture*, 2013, 91: 135-144 (doi: 10.1016/j.compag.2012.12.006).
12. Sankaran S., Khot L.R., Espinoza C.Z., Jarolmasjed S., Sathuvalli V.R., Vandemark G.J., Miklas P.N., Carter A.H., Pumphrey M.O., Knowles N.R.N., Pavek M.J. Low-altitude, high-resolution aerial imaging systems for row and field crop phenotyping: A review. *European Journal of Agronomy*, 2015, 70: 112-123 (doi: 10.1016/j.eja.2015.07.004).
13. Mirschel W., Wieland R., Wenkel K.O., Nendel C., Guddat C. YIELDSTAT — a spatial yield model for agricultural crops. *European Journal of Agronomy*, 2014, 52: 33-46 (doi: 10.1016/j.eja.2013.09.015).
14. Kern A., Barcza Z., Marjanovi H., Árendás T., Fodor N., Bynis P., Bognár P., Lichtenberger J. Statistical modelling of crop yield in Central Europe using climate data and remote sensing vegetation indices. *Agricultural and Forest Meteorology*, 2018, 260: 300-320 (doi: 10.1016/j.agrformet.2018.06.009).
15. Conradt T., Gornott C., Wechsung F. Extending and improving regionalized winter wheat and silage maize yield regression models for Germany: enhancing the predictive skill by panel definition through cluster analysis. *Agricultural and Forest Meteorology*, 2016, 216: 68-81 (doi: 10.1016/j.agrformet.2015.10.003).
16. Gutzler C., Helming K., Balla D., Dannowski R., Deumlich D., Glemnitz M., Sieber S. Agricultural land use changes — a scenario-based sustainability impact assessment for Brandenburg, Germany. *Ecological Indicators*, 2015, 48: 505-517 (doi: 10.1016/j.ecolind.2014.09.004).
17. Grados D., Schrevens E. Multidimensional analysis of environmental impacts from potato agricultural production in the Peruvian Central Andes. *Science of the Total Environment*, 2019, 663: 927-934 (doi: 10.1016/j.scitotenv.2019.01.414).
18. Antle J.M., Jones J.W., Rosenzweig C. Next generation agricultural system models and knowledge products: synthesis and strategy. *Agricultural Systems*, 2017, 155: 179-185 (doi: 10.1016/j.agsy.2017.05.006).

19. Janssen S.J., Porter C.H., Moore A.D., Athanasiadis I.N., Foster I., Jones J.W., Antle J.M. Towards a new generation of agricultural system data, models and knowledge products: information and communication technology. *Agricultural Systems*, 2017, 155: 200-212 (doi: 10.1016/j.agsy.2016.09.017).
20. Fielke S., Taylor B., Jakku E. Digitalisation of agricultural knowledge and advice networks: a state-of-the-art review. *Agricultural Systems*, 2020, 180: 102763 (doi: 10.1016/j.agsy.2019.102763).
21. Lecerf R., Ceglar A., Lypez-Lozano R., Van Der Velde M., Baruth B. Assessing the information in crop model and meteorological indicators to forecast crop yield over Europe. *Agricultural Systems*, 2019, 168: 191-202 (doi: 10.1016/j.agsy.2018.03.002).
22. Ozturk I., Sharif B., Baby S., Jabloun M., Olesen J.E. The long-term effect of climate change on productivity of winter wheat in Denmark: a scenario analysis using three crop models. *The Journal of Agricultural Science*, 2017, 155(5): 733-750 (doi: 10.1017/S0021859616001040).
23. Hannah L., Donatti C.I., Harvey C.A., Alfaro E., Rodriguez D.A., Bouroncle C., Castellanos E., Diaz F., Fung E., Hidalgo H.G., Imbach P. Regional modeling of climate change impacts on smallholder agriculture and ecosystems in Central America. *Climatic Change*, 2017, 141(1): 29-45 (doi: 10.1007/s10584-016-1867-y).
24. Belem M., Saqalli M. Development of an integrated generic model for multi-scale assessment of the impacts of agro-ecosystems on major ecosystem services in West Africa. *Journal of Environmental Management*, 2017, 202: 117-125 (doi: 10.1016/j.jenvman.2017.07.018).
25. Badenko V.L., Topaj A.G., Yakushev V.V., Mirschel W., Nendel C. Crop models as research and interpretative tools. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52: 437-445 (doi: 10.15389/agrobiology.2017.3.437eng).
26. Anten N.P., Vermeulen P.J. Tragedies and crops: understanding natural selection to improve cropping systems. *Trends in Ecology & Evolution*, 2016, 31(6): 429-439 (doi: 10.1016/j.tree.2016.02.010).
27. Lee H., Lautenbach S., Nieto A.P.G., Bondeau A., Cramer W., Geijzendorffer I.R. The impact of conservation farming practices on Mediterranean agro-ecosystem services provisioning — a meta-analysis. *Regional Environmental Change*, 2019, 19: 2187-2202 (doi: 10.1007/s10113-018-1447-y).
28. Poluektov R.A., Fintushal S.M., Oparina I.V., Shatskikh D.V., Terleev V.V., Zakharova E.T. AGROTOOL — a system for crop simulation. *Archives of Agronomy and Soil Science*, 2002, 48(6): 609-635 (doi: 10.1080/0365034021000041597).
29. Badenko V.L., Terleev V.V., Topaj A. G. AGROTOOL software as an intellectual core of decision support systems in computer aided agriculture. *Applied Mechanics and Materials,* 2014, 635-637: 1688-1691 (doi: 10.4028/www.scientific.net/AMM.635-637.1688).
30. de Wit C.T., Van Keulen H. Modelling production of field crops and its requirements. *Geoderma*, 1987, 40(3-4): 253-265 (doi: 10.1016/0016-7061(87)90036-X).
31. Nendel C., Kersebaum K.C., Mirschel W., Wenkel K.O. Testing farm management options as climate change adaptation strategies using the MONICA model. *European Journal of Agronomy*, 2014, 52: 47-56 (doi: 10.1016/j.eja.2012.09.005).
32. Medvedev S., Topaj A. Crop simulation model registrator and polyvariant analysis. *IFIP Advances in Information and Communication Technology*, 2011, 359: 295-301 (doi: 10.1007/978-3-642-22285-6_32).
33. Ramírez-Cuesta J.M., Mirás-Avalos J.M., Rubio-Asensio J.S., Intrigliolo D.S. A novel ArcGIS toolbox for estimating crop water demands by integrating the dual crop coefficient approach with multi-satellite imagery. *Water*, 2019, 11(1): 38 (doi: 10.3390/w11010038)
34. Liben F.M., Wortmann C.S., Tirfessa A. Geospatial modeling of conservation tillage and nitrogen timing effects on yield and soil properties. *Agricultural Systems*, 2020, 177: 102720 (doi: 10.1016/j.agsy.2019.102720).
35. Liben F.M., Wortmann C.S., Yang H., Lindquist J.L., Tadesse T., Wegary D. Crop model and weather data generation evaluation for conservation agriculture in Ethiopia. *Field Crops Research*, 2018, 228: 122-134 (doi: 10.1016/j.fcr.2018.09.001).
36. Hartkamp A.D., White J.W., Hoogenboom G. Interfacing geographic information systems with agronomic modeling: a review. *Agronomy Journal*, 1999, 91(5): 761-772 (doi: 10.2134/agronj1999.915761x).
37. Shelia V., Hansen J., Sharda V., Porter C., Aggarwal P., Wilkerson C.J., Hoogenboom G. A multi-scale and multi-model gridded framework for forecasting crop production, risk analysis, and climate change impact studies. *Environmental Modelling & Software*, 2019, 115: 144-154 (doi: 10.1016/j.envsoft.2019.02.006).
38. Bassoa B., Liua L. Seasonal crop yield forecast: methods, applications, and accuracies. *Advances in Agronomy*, 2018, 154: 201-255 (doi: 10.1016/bs.agron.2018.11.002).
39. Huang J., Gómez-Dans J., Huang H., Ma H., Wu Q., Lewis P., Liang S., Chen Z., Xue J., Wu Y., Zhao F., Wang J., Xie X. Assimilation of remote sensing into crop growth models: Current status and perspectives. *Agricultural and Forest Meteorology*, 2019, 276: 107-109 (doi: 10.1016/j.agrformet.2019.06.008).
40. Machwitz M., Hass E., Junk J., Udelhoven T., Schlerf M. Crop GIS — a web application for the spatial and temporal visualization of past, present and future crop biomass development. *Computers and Electronics in Agriculture*, 2019, 161: 185-193 (doi: 10.1016/j.compag.2018.04.026).

41. Jin X., Kumar L., Li Z., Feng H., Xu X., Yang G., Wang, J. A review of data assimilation of remote sensing and crop models. *European Journal of Agronomy*, 2018, 92: 141-152 (doi: 10.1016/j.eja.2017.11.002).
42. Badenko V.L., Topazh A.G., Medvedev S.A., Zakharova E.T. *AgroEkoInfo*, 2018, 3(33): 68 (in Russ.).
43. Reynolds M., Kropff M., Crossa J., Koo J., Kruseman G., Molero Milan A., Rutkoski J., Schulthess U., Balwinder-Singh, Sonder K., Tonnang H., Vadez V. Role of modelling in international crop research: overview and some case studies. *Agronomy*, 2018, 8(12): 291 (doi: 10.3390/agronomy8120291).
44. Kumhálová J., Matějková Š. Yield variability prediction by remote sensing sensors with different spatial resolution. *International Agrophysics*, 2017, 31(2): 195-202 (doi: 10.1515/intag-2016-0046).
45. Resop J.P., Fleisher D.H., Wang Q., Timlin D.J., Reddy V.R. Combining explanatory crop models with geospatial data for regional analyses of crop yield using field-scale modeling units. *Computers and Electronics in Agriculture*, 2012, 89: 51-61 (doi: 10.1016/j.compag.2012.08.001).
46. Hodson D., White J. GIS and crop simulation modelling applications in climate change research. In: *Climate change and crop production*. CABI Publishers, Wallingford, UK, 2010: 245-262 (doi: 10.1079/9781845936334.0245).
47. Akpoti K., Kabo-bah A.T., Zwart S.J. Agricultural land suitability analysis: state-of-the-art and outlooks for integration of climate change analysis. *Agricultural Systems*, 2019, 173: 172-208 (doi: 10.1016/j.agsy.2019.02.013).
48. Mirschel W., Schultz A., Wenkel K.O., Wieland R., Poluektov R.A. Crop growth modelling on different spatial scales — a wide spectrum of approaches. *Archives of Agronomy and Soil Science*, 2004, 50(3): 329-343 (doi: 10.1080/03650340310001634353).
49. Gaso D.V., Berger A.G., Ciganda V.S. Predicting wheat grain yield and spatial variability at field scale using a simple regression or a crop model in conjunction with Landsat images. *Computers and Electronics in Agriculture*, 2019, 159: 75-83 (doi: 10.1016/j.compag.2019.02.026).
50. Maharjan G.R., Hoffmann H., Webber H., Srivastava A.K., Weihermьller L., Villa A., Coucheney E., Lewan E., Trombi G., Moriondo M., Bindi M., Grosz B., Dechow R., Kuhnert M., Doro L., Kersebaum K.-C., Stella T., Specka X., Nendel C., Constantin J., Raynal H., Ewert F., Gaiser T. Effects of input data aggregation on simulated crop yields in temperate and Mediterranean climates. *European Journal of Agronomy*, 2019, 103: 32-46 (doi: 10.1016/j.eja.2018.11.001).
51. Wenkel K.O., Berg M., Mirschel W., Wieland R., Nendel C., Kцstner B. LandCaRe DSS — an interactive decision support system for climate change impact assessment and the analysis of potential agricultural land use adaptation strategies. *Journal of Environmental Management*, 2013, 127: 168-183 (doi: 10.1016/j.jenvman.2013.02.051).
52. Zhai Z., Martínez J.F., Beltran V., Martínez N.L. Decision support systems for agriculture 4.0: Survey and challenges. *Computers and Electronics in Agriculture*, 2020, 170: 105256 (doi: 10.1016/j.compag.2020.105256).
53. Battisti R., Parker P.S., Sentelhas P. C., Nendel C. Gauging the sources of uncertainty in soybean yield simulations using the MONICA model. *Agricultural Systems*, 2017, 155: 9-18 (doi: 10.1016/j.agsy.2017.04.004).
54. Holzworth D.P., Snow V., Janssen S., Athanasiadis I.N., Donatelli M., Hoogenboom G., White J.W., Thorburn P. Agricultural production systems modelling and software: current status and future prospects. *Environmental Modelling & Software*, 2015, 72: 276-286 (doi: 10.1016/j.envsoft.2014.12.013).
55. Yin Y., Zhang X., Lin D., Yu H., Shi P. GEPIC-VR model: a GIS-based tool for regional crop drought risk assessment. *Agricultural Water Management*, 2014, 144: 107-119 (doi: 10.1016/j.agwat.2014.05.017).
56. Donatelli M., Srivastava A.K., Duveiller G., Niemeyer S., Fumagalli D. Climate change impact and potential adaptation strategies under alternate realizations of climate scenarios for three major crops in Europe. *Environmental Research Letters*, 2015, 10(7): 075005 (doi: 10.1088/1748-9326/10/7/075005).
57. Seguini L., Bussay A., Baruth B. From extreme weather to impacts: the role of the areas of concern maps in the JRC MARS bulletin. *Agricultural Systems*, 2019, 168: 213-223 (doi: 10.1016/j.agsy.2018.07.003).
58. Thorp K.R., Bronson K.F. A model-independent open-source geospatial tool for managing point-based environmental model simulations at multiple spatial locations. *Environmental Modelling & Software*, 2013, 50: 25-36 (doi: 10.1016/j.envsoft.2013.09.002).
59. Elliott J., Kelly D., Chrryssanthacopoulos J., Glotter M., Jhunjhnuwala K., Best N., Wilde M. Foster I. The parallel system for integrating impact models and sectors (pSIMS). *Environmental Modelling & Software*, 2014, 62: 509-516. (doi: 10.1016/j.envsoft.2014.04.008).
60. Gabaldyn-Leal C., Webber H., Otegui M.E., Slafer G.A., Ordócez R.A., Gaiser T., Lorite I.J., Ruiz-Ramos M., Ewert F. Modelling the impact of heat stress on maize yield formation. *Field Crops Research*, 2016, 198: 226-237 (doi: 10.1016/j.fcr.2016.08.013)
61. Huang J., Scherer L., Lan K., Chen F., Thorp K.R. Advancing the application of a model-independent open-source geospatial tool for national-scale spatiotemporal simulations.

Environmental Modelling & Software, 2019, 119: 374-378 (doi: 10.1016/j.envsoft.2019.07.003).
62. Calera A., Campos I., Osann A., D'Urso G., Menenti M. Remote sensing for crop water management: from ET modelling to services for the end users. *Sensors*, 2017, 17(5): 1104 (doi: 10.3390/s17051104).
63. Suchkov A.P. *Sistemy i sredstva informatiki*, 2015, 25(3): 195-205 (doi: 10.14357/08696527150312) (in Russ.).
64. Topazh A.G., Mitrofanov E.P. *Vestnik Sankt-Peterburgskogo universiteta. Prikladnaya matematika. Informatika. Protsessy upravleniya*, 2017, 13(3): 326-338 (doi: 10.21638/11701/spbu10.2017.309) (in Russ.).
65. de Wit A., Boogaard H., Fumagalli D., Janssen S., Knapen R., van Kraalingen D., Supit I., van der Wijngaart R., van Diepen K. 25 years of the WOFOST cropping systems model. *Agricultural Systems*, 2019, 168: 154-167 (doi: 10.1016/j.agsy.2018.06.018).
66. Ewert F., Rцtter R.P., Bindi M., Webber H., Trnka M., Kersebaum K.C., Olesen J.E., van Ittersum M.K., Janssen S., Rivington M., Semenov M.A., Wallach D., Porter J.R., Stewart D., Verhagen J., Gaiser T., Palosuo T., Tao F., Nendel C., Roggero P.P., Bartosová L., Asseng S. Crop modelling for integrated assessment of risk to food production from climate change. *Environmental Modelling & Software*, 2015, 72: 287-303 (doi: 10.1016/j.envsoft.2014.12.003).
67. Asseng S., Ewert F., Rosenzweig C., Jones J.W., Hatfield J.L., Ruane A.C., Boote K.J., Thorburn P.J., Rötter R.P., Cammarano D., Brisson N., Basso B., Martre P., Aggarwal P.K., Angulo C., Bertuzzi P., Biernath C., Challinor A.J., Doltra J., Gayler S., Goldberg R., Grant R., Heng L., Hooker J., Hunt L.A., Ingwersen J., Izaurralde R.C., Kersebaum K.C., Müller C., Naresh Kumar S., Nendel C., O'Leary G., Olesen J.E., Osborne T.M., Palosuo T., Priesack E., Ripoche D., Semenov M.A., Shcherbak I., Steduto P., Stцckle C., Stratonovitch P., Streck T., Supit I., Tao F., Travasso M., Waha K., Wallach D., White J.W., Williams J.R., Wolf J. Uncertainty in simulating wheat yields under climate change. *Nature Climate Change*, 2013, 3(9): 827-832 (doi: 10.1038/nclimate1916).
68. Jones J.W., Hoogenboom G., Porter C.H., Boote K.J., Batchelor W.D., Hunt L.A., Wilkens P.W., Singh U., Gijsman A.J., Ritchie J.T. The DSSAT cropping system model. *European Journal of Agronomy*, 2003, 18(3-4): 235-265 (doi: 10.1016/S1161-0301(02)00107-7).
69. Wang X., Williams J.R., Gassman P.W., Baffaut C., Izaurralde R.C., Jeong J., Kiniry J.R. EPIC and APEX: model use, calibration, and validation. *Transactions of the ASABE*, 2012, 55(4): 1447-1462 (doi: 10.13031/2013.42253).
70. Brisson N., Gary C., Justes E., Roche R., Mary B., Ripoche D., Zimmer D., Sierraa J., Bertuzzi P., Burgera P., Bussière F., Cabidoche Y.M., Cellier P., Debaeke P., Gaudillère J.P., Hénault C., Maraux F., Seguin B., Sinoquet H. An overview of the crop model STICS. *European Journal of Agronomy*, 2003, 18(3-4): 309-332 (doi: 10.1016/S1161-0301(02)00110-7).
71. Leghari S.J., Hu K., Liang H., Wei Y. Modeling water and nitrogen balance of different cropping systems in the North China Plain. *Agronomy*, 2019, 9(11): 696 (doi: 10.3390/agronomy9110696).
72. Jiang R., He W., Zhou W., Hou Y., Yang J.Y., He P. Exploring management strategies to improve maize yield and nitrogen use efficiency in northeast China using the DNDC and DSSAT models. *Computers and Electronics in Agriculture*, 2019, 166: 104-988 (doi: 10.1016/j.compag.2019.104988).
73. Xiang Z., Bailey R.T., Nozari S., Husain Z., Kisekka I., Sharda V., Gowda P. DSSAT-MODFLOW: a new modeling framework for exploring groundwater conservation strategies in irrigated areas. *Agricultural Water Management*, 2020, 232: 106033 (doi: 10.1016/j.agwat.2020.106033).
74. Newbery F., Qi A., Fitt B.D. Modelling impacts of climate change on arable crop diseases: progress, challenges and applications. *Current Opinion in Plant Biology*, 2016, 32: 101-109 (doi: 10.1016/j.pbi.2016.07.002).
75. Badenko V.L., Garmanov V.V., Ivanov D.A., Savchenko A.N., Topazh A.G. *Doklady Rossiiskoi akademii sel'skokhozyaistvennykh nauk*, 2015, 1-2: 72-76 (in Russ.).
76. Badenko V.L., Badenko G., Topaj A.G., Medvedev S., Zakharova E., Terleev V.V. Comparative simulation of various agricultural land use practices for analysis of impacts on environments. *Environments*, 2017, 4(4): 92 (doi: 10.3390/environments4040092).
77. Engel T., Hoogenboom G., Jones J.W., Wilkens P.W. AEGIS/WIN: a computer program for the application of crop simulation models across geographic areas. *Agronomy Journal*, 1997, 89(6): 919-928 (doi: 10.2134/agronj1997.00021962008900060012x).
78. McDonald C.K., MacLeod N.D., Lisson S., Corfield J.P. The Integrated Analysis Tool (IAT) — a model for the evaluation of crop-livestock and socio-economic interventions in smallholder farming systems. *Agricultural Systems*, 2019, 176: 102659 (doi: 10.1016/j.agsy.2019.102659).
79. Zhao G., Bryan B.A., King D., Luo Z., Wang E., Bende-Michl U., Song X., Yu Q. Large-scale, high-resolution agricultural systems modeling using a hybrid approach combining grid computing and parallel processing. *Environmental Modelling & Software*, 2013, 41: 231-238 (doi: 10.1016/j.envsoft.2012.08.007).
80. Badenko V.L., Topazh A.G., Medvedev S.A., Zakharova E.T., Dunaeva E.A. *Tavricheskii vestnik agrarnoi nauki*, 2019, 3: 18-30 (doi: 10.33952/2542-0720-2019-3-19-18-30) (in Russ.).
81. Aref'ev N.V., Badenko V.L., Latyshev, N.K. *Nauchno-tekhnicheskie vedomosti SPbPU.*

Estestvennye i inzhenernye nauki, 2010, 110: 205-210 (in Russ.).
82. Medvedev S.A., Poluektov R.A., Topazh A.G. *Melioratsiya i vodnoe khozyaistvo,* 2012, 2: 10-13 (in Russ.).
83. Topaj A., Badenko V., Medvedev S., Terleev V. V knige: *Novye metody i rezul'taty issledovanii landshaftov v Evrope, Tsentral'noi Azii i Sibiri. Tom III. Monitoring i modelirovanie landshaftov* /Pod redaktsiei V.G. Sycheva, L. Myullera [In: New methods and results of landscape studies in Europe, Central Asia and Siberia. Vol. III. Landscape monitoring and modeling. V.G. Sychev, L. Myuller (eds.)]. Moscow, 2018: 253-257 (doi: 10.25680/3309.2018.28.99.246) (in Russ.).
84. Poluektov R.A., Oparina I.V., Topazh A.G., Mirshel' V. *Matematicheskoe modelirovanie*, 2000, 12(11): 3-16 (in Russ.).
85. Dobor L., Barcza Z., Hlásny T., Árendás T., Spitky T., Fodor N. Crop planting date matters: Estimation methods and effect on future yields. *Agricultural and Forest Meteorology*, 2016, 223: 103-115 (doi: 10.1016/j.agrformet.2016.03.023).
86. Rötter R.P., Hoffmann M.P., Koch M., Müller C. Progress in modelling agricultural impacts of and adaptations to climate change. *Current Opinion in Plant Biology*, 2018, 45: 255-261 (doi: 10.1016/j.pbi.2018.05.009).
87. Bannayan M., Hoogenboom G. Weather analogue: a tool for real-time prediction of daily weather data realizations based on a modified k-nearest neighbor approach. *Environmental Modelling & Software*, 2008, 23(6): 703-713 (doi: 10.1016/j.envsoft.2007.09.011).
88. Semenov M.A. Using weather generators in crop modelling. *Acta Horticulturae*, 2006, 707: 93-100 (doi: 10.17660/ActaHortic.2006.707.11).
89. Peleg N., Fatichi S., Paschalis A., Molnar P., Burlando P. An advanced stochastic weather generator for simulating 2 - D high - resolution climate variables. *Journal of Advances in Modeling Earth Systems*, 2017, 9(3): 1595-1627 (doi: 10.1002/2016MS000854).
90. Maraun D., Huth R., Gutiérrez J.M., Martín D.S., Dubrovsky M., Fischer A., Widmann M. The VALUE perfect predictor experiment: evaluation of temporal variability. *International Journal of Climatology*, 2019, 39(9): 3786-3818 (doi: 10.1002/joc.5222).
91. Medvedev S., Topaj A., Badenko V., Terleev V. Medium-term analysis of agroecosystem sustainability under different land use practices by means of dynamic crop simulation. *IFIP Advances in Information and Communication Technology*, 2015, 448: 252-261 (doi: 10.1007/978-3-319-15994-2_25).
92. Dunaieva I., Mirschel W., Popovych V., Pashtetsky V., Golovastova E., Vecherkov V., Melnichuk A., Terleev V., Nikonorov A., Ginevsky R., Topaj A., Lazarev V. GIS services for agriculture monitoring and forecasting: development concept. *Advances in Intelligent Systems and Computing*, 2019, 983: 236-246 (doi: 10.1007/978-3-030-19868-8_24).
93. Teixeira E.I., de Ruiter J., Ausseil A.G., Daigneault A., Johnstone P., Holmes A., Tait A., Ewert F. Adapting crop rotations to climate change in regional impact modelling assessments. *Science of the Total Environment*, 2018, 616: 785-795 (doi: 10.1016/j.scitotenv.2017.10.247).
94. Tayyebi A., Tayyebi A., Vaz E., Arsanjani J.J., Helbich M. Analyzing crop change scenario with the SmartScape™ spatial decision support system. *Land Use Policy*, 2016, 51: 41-53 (doi: 10.1016/j.landusepol.2015.11.002).
95. Palosuo T., Kersebaum K.C., Angulo C., Hlavinka P., Moriondo M., Olesen J.E., Patil R.H., Ruget F., Rumbaur C., Takáč J., Trnka M., Bindi M., Caldag B., Ewert F., Ferrise R., Mirschel W., Saylan L., Šiška B., Rötter R. Simulation of winter wheat yield and its variability in different climates of Europe: a comparison of eight crop growth models. *European Journal of Agronomy*, 2011, 35(3): 103-114 (doi: 10.1016/j.eja.2011.05.001).
96. Wallach D., Mearns L.O., Ruane A.C., Rötter R.P., Asseng S. Lessons from climate modeling on the design and use of ensembles for crop modeling. *Climatic Change*, 2016, 139(3-4): 551-564 (doi: 10.1007/s10584-016-1803-1).
97. Kollas C., Kersebaum K.C., Nendel C., Manevski K., Müller C., Palosuo T., Conradt T. Crop rotation modelling — a European model intercomparison. *European Journal of Agronomy*, 2015, 70: 98-111 (doi: 10.1016/j.eja.2015.06.007).
98. Raza A., Razzaq A., Mehmood S.S., Zou X., Zhang X., Lv Y., Xu J. Impact of climate change on crops adaptation and strategies to tackle its outcome: a review. *Plants*, 2019, 8(2): 34 (doi: 10.3390/plants8020034).
99. Droutsas I., Challinor A.J., Swiderski M., Semenov M.A. New modelling technique for improving crop model performance — application to the GLAM model. *Environmental Modelling & Software*, 2019, 118: 187-200 (doi: 10.1016/j.envsoft.2019.05.005).

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RISK ASSESSMENT METHODOLOGY FOR AGROECOSYSTEMS IN THE CONDITIONS OF TECHNOGENIC POLLUTION

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Abstract

Currently, the world scientific community is faced with the task of identifying and minimizing environmental risks of the impact of anthropogenic factors on ecosystems, especially agricultural ones (A.A. Muzalevsky et al., 2011). The keystone of environmental risk assessment is estimated probability of the adverse effects of various nature (radiation, chemical and biological agents) and prevention of their negative impact. The most common sources of pollution of agroecosystems are precipitation from industrial and transport emissions, industrial wastewater, sewage sludge, organic and mineral fertilizers and plant protection products, dumps of ash, slag, ores and slime (S.C. Barman et al., 2000; Yu.N. Vodyanitsky et al., 2011; E.C. Rowe et al., 2015). Such risks are usually assessed situationally, and methods used are applicable for specific factor (agent) acting in analyzed case and object of its influence. The purpose of the presented theoretical research was to develop a unified methodology for assessing agroecological risks caused by anthropogenic pollutants. The developed methodology uses mathematical modeling methods and is based on the principles and criteria ensuring the safety of agricultural ecosystems in conditions of man-made pollution. Atmospheric pollutants are the main source of man-made impact. Temporal patterns of their impact vary from acute (e.g. upon accidents) to chronic that should be taken into account. Agroecosystem productivity, as an integral indicator, is a basic criterion for assessing agroecological risks. The methodology includes a four-step algorithm: i) hazard identification based on available agroecosystem data with identification of the sources and nature of the hazard and key affected components; ii) impact assessment by measuring or calculating its intensity, duration, and mode of exposure; iii) a dose-effect assessment by a relationship between the degree of the impact and the probability of its negative consequences; iv) risk characterization, including reliability analysis of the obtained data, estimates of risk from individual factors and their combinations, and calculation of a probability of adverse effect for each agroecosystem component. The choice of a method for assessing agroecological risks (deterministic, probabilistic of the 1st and 2nd type, and integral probabilistic) is substantiated based on the indicator pools, risk criteria and the degree of technogenic impact. Risk characterization includes its classification in terms of an environmentally acceptable level as per maximum allowed concentrations and semi-lethal doses (LD_{50}). For each step, risk uncertainties are accounted. Agroecological risk assessment algorithm includes i) database analysis and selection of agent-specific and exposure-specific values of the effects; ii) estimates of meteorological parameters of pollutant diffusion under specific release conditions; iii) calculated or experimental estimates of pollutant deposition on the ground depending on peculiarities of the impact; iv) calculation or experimental assessment of radionuclide or chemical toxicant contaminations; v) calculation or measurement of the effects of radionuclides or chemical pollutants on the agroecosystem components. The proposed approaches to assessing agroecological risk are applicable to a wide class of environmental problems.

Keywords: agroecosystem, agricultural products, heavy metals, radionuclides, technogenic factor, agroecosystem components, impact level, dose-response relationships, mathematical models

Present status and development of nuclear power industry, metallurgy, transport, chemical and other industries, the activities of which (including man-made accidents) lead to environmental pollution, poses the task to identify and minimize environmental risks associated with their impact on ecosystems,

especially agricultural [1-3]. Currently, environmental risk is the main generalized indicator for making management decisions. This requires adequate calculation methods, which, in turn, necessitates understanding of the basic mechanisms of the organization and functioning of ecosystems [4-6]. Such methods can provide a quantitative assessment of environmental risk, significantly reduce its uncertainty, and help find ways to manage risks at all levels of ecosystems [7-9]. The main role of environmental risk assessment is to determine the likelihood of various effects in ecosystems as a result of the influence of technogenic (radiation, chemical, biological) factors for taking preventive measures [10-12].

Agroecosystems are communities of cultivated plants and animals and their habitats, artificially created by man to obtain foodstuff and row materials. Human health directly depends on the quality of food products [13-15]. This necessitates restriction and prediction of the possible negative impact of technogenic factors of different nature on agricultural land and products [16-18]. A balanced composition of trace elements (including heavy metals) in soils is important for the optimal growth of agricultural plants [19-21], while the increased concentrations of heavy metals negatively affect physiological processes [22-24]. Mostly, the sources of pollution of agroecosystems are aerial fallout from industrial and transport emissions, industrial wastewater, sewage sludge, organic and mineral fertilizers and plant protection products, heaps of ash, slag, ores, sludge [25-27].

The features of the current ecological situation in the agro-sphere are the simultaneous influence of a large number of factors of different (physical, chemical and biological) nature, their low impact and chronic character [28-30]. Increased concentration of pollutants negatively affects the productivity of agroecosystems, which largely determines their stability [31-33]. In this regard, it is necessary to assess the likelihood of reversible or irreversible changes in the structure of the agroecosystem and the functions of its components in response to technogenic impact [34-36].

The qualitative and quantitative indicators characterizing the likelihood of negative effects include environmental (agroecological) risks, which currently serve as priority generalized parameters of technogenic impact on agroecosystems [37-39]. Quantitative indicators can be standardized through the development of standards for risk (acceptable risks) and further used for a comprehensive assessment of the agroecosystem component states [40-42]. Two currently used indicators, MPC/TPC (maximum permissible concentration/tentative permissible concentration), have certain disadvantages, since they do not distinct the contributions of natural and technogenic components, and do not regard the climatic and geochemical features of the region [43-45].

Previously, we have developed the methodology for assessing radiation risks in agricultural ecosystems and used it to calculate the agroecological risk for crops at ^{137}Cs contamination from an accident at a radiation hazardous facility [9]. However, it should be noted that the existing methods for assessing agroecological risks make the results hardly comparable, which hinders to obtain reliable estimates of negative technogenic impact on the components of agroecosystems [46-48]. In addition, technogenic risk assessments are performed, as a rule, situationally for a specific case and by the methods applicable for a certain factor (agent) in specific conditions and for a certain object of the impact [10].

This theoretical study aims to develop a unified methodology for assessing agroecological risks from technogenic pollution.

Stages of assessing agroecological risks from technogenic pollution. Prediction and reduction of negative impact of a technogenic factor on the components of agroecosystems should be based on the risk assessment using generalized quantitative or qualitative criteria. These criteria should

assess the probability of 50% death of plants and a decrease in yields under a certain degree of technogenic pollution. [49-51].

Assessment of agroecological risks to agriculture under man-made pollution usually includes four stages [52-54]. Let's consider these stages given the task of unifying the methodology of risk analysis, regardless of the nature of technogenic pollution.

I. Hazard identification. This stage includes the collection and generalization of available information in order to determine the sources and the nature of pollution (radiation, chemical, biological) and to evaluate the degree of the impact. Particular attention should be paid to the most susceptible components of the agroecosystem, i.e. the "critical" components [55, 56]. The criteria to choose such components are the sensitivity to one or another negative factor and the response to the impact. Plant sensitivity is the ability to respond to external irritation manifested in different forms.

When choosing a priority pollutant or a group of pollutants for agroecological risk quantitative assessment, it is necessary to focus on the criteria characterizing the concentration of pollutants in the "critical" components, the toxic properties of substances, the ability to migrate, and the likelihood of a negative effect due to various exposure conditions. All factors (physical, chemical, etc.) should be taken into account, in particular, the migration of the pollutant, its transformation, the time of exposure [58-60]. When identifying man-made risk factors for the components of an agricultural ecosystem, it is necessary to rely on the design documentation of the pollution source and the results of environmental monitoring or special studies.

1. Dose of γ-irradiation leading to a 50% decrease in the yield of various agricultural crops [57]

Crops	Absorbed radiation dose, Gy
Winter rye, horse beans	5-8
Winter and spring wheat, barley, oats, maize, peas, soy	10-20
Sunflower, rapeseed	20-30
Buckwheat, millet, rice	30-50
Cotton plant, tomatoes	50-80
Cabbage	80-120
Potato	120-150
Sugar and table beets	180-220
Carrot	250-300
Flax	300-400

2. Probable loss of grain yield (%) upon exposure to ionizing radiation at different plant development stages [57]

Stage	Total dose of γ- and β-irradiatin, Gy					
	5	10	20	30	50	100	200
Winter rye							
Tillering	5	15	40	70	95	100	100
Stem extension	25	80	95	100	100	100	100
Heading—flowering	15	40	75	95	100	100	100
Milk ripening	5	6	8	10	15	30	50
Winter and spring wheat, barley, oats							
Tillering	5	8	27	50	95	100	100
Stem extension	9	20	50	75	90	100	100
Heading—flowering	7	15	35	50	75	95	100
Milk ripening	4	5	7	10	15	30	50
Maize							
6-8 leaves	15	25	40	55	85	100	100
Tasseling	30	45	55	70	95	100	100
Milk maturity	4	10	20	30	40	60	80

Hazard identification includes generation of a preliminary scenario of the influence of a technogenic factor on "critical" components which describes the physicochemical properties of the acting substance, the mode, intensity and

duration of exposure. For each identified technogenic factor, a list of indicators (effects) is established, reflecting the violation of the functioning of the components of the agroecosystem. In this, reliable published research data on negative effects can be the information sources. The indicators widely used as the intensity of plant biomass gain (productivity) and the biomass at a certain point in time (yield). Under radiation impact on crops, these parameters are estimated most accurately (Tables 1, 2). For low technogenic impact, the significant indicators are those observed at lower levels of biological organization (organismic, cellular and subcellular) [61, 62].

The integral risk for the components of the agrarian ecosystem should be assessed on the basis of generalized information, including data on the impact of each risk factor. Already at this stage, preliminary decisions on agroecological risk management can be made, including the termination of further analysis due to the insignificant danger or the sufficiency of the initial assessments, a more detailed hazard analysis and risk assessment, and the development of preliminary recommendations to reduce the hazards [9].

II. Impact assessment. The assessment includes measurement and/or calculation of the intensity, duration, and the ways of the impact on the agroecosystem components. The intensity of the radiation factor means the dose of irradiation, for a chemical factor it is the concentration or dose of a chemical substance, for a biological factor it is the number of biological agents entering the body per unit of time. The source of information on the intensity of the technogenic factor is the accumulated scientific data obtained both for the previous period and as a result of the experiments, as well as data from published scientific works and reports. The atmospheric route of spreading radionuclides, chemicals and biological agents is one of the main sources of technogenic impact. In assessing exposure loads, the radiation exposure for less than 2 weeks is identified as acute, up to 7 years as subacute, more than 7 years as chronic [10].

Assessment of the technogenic impact includes a sequence of actions, i.e. i) analysis of generalized information on the impact levels, including the values of the response of agroecosystem components at different levels of technogenic impact; ii) determination of meteorological parameters of the model for the behavior of air impurities for specific conditions of their release (atmosphere stability class, wind speed at the height of release, aerodynamic roughness of the underlying surface as per MU 2.6.5.010-2016) [63]; iii) quantitation of the impurities deposited on the earth surface, depending on specificity of pollution; iv) assessment of the pollution of the aboveground plant biomass and soil with priority substances; v) calculation or measurement of the degree of impact of priority substances on agricultural plants.

The second stage results in a quantitative description of the intensity, frequency and nature of the technogenic impact on the components of the agroecosystem.

III. Assessment of the dose-effect relationship. This reflects the quantitative relationship between the level of technogenic impact on the components of agrarian ecosystem and the likelihood of occurring negative effects of different severity [9].

The dose-effect relationship assessment is based on accumulated scientific data, mathematical models and agroecological safety criteria. This information should characterize the dependence of negative effects towards the agroecosystem components on the specific extend of the technogenic factor impact. The degree of impact should be chosen given negative effects from minimal impact, as well as different duration of exposure (acute, subacute, chronic). The response of biotic components above the acceptable environmental risk is characterized by a median

lethal dose (LD_{50}) justified by survivability or by maximum permissible concentration (MPC) [9, 10].

This stage results in the models of dose-effect relationship which contain quantitative parameters and descriptions of the "critical" components and allow us to assess the likelihood of negative effects of the established technogenic factors.

The choice of methods for agroecological risk models is determined primarily by the level of information support (a set of risk assessment criteria, levels of anthropogenic impact). Deterministic, probabilistic and integral probabilistic methods are mostly used in assessing risk [9]. By deterministic method, the risk indices are assessed, which are the ratio of the dose load to the risk criterion value (the level of acceptable environmental risk). The advantages of the method are relative simplicity and a small amount of input data (only two indicators are required). The disadvantages are the lack of accounting for uncertainties of indicators and, as a consequence, rough estimates [4]. Probabilistic methods are of the 1st and 2nd type. The 1st type method uses a point estimate as a risk criterion. The probabilistic method of the 2nd type is advisable to apply when the identification of the technogenic factor distribution is difficult due to lack of data or the need for a quick assessment [4, 10]. The integral probabilistic method takes into account the uncertainties of the parameters inherent in the object and the environment, which determine the intensity of the impact [9].

IV. Risk characterization. The stage includes an analysis of the hazard data reliability obtained at the previous stages. Based on the calculated quantitative indicators of the dose-effect relationship and comparison with the data of similar studies, a conclusion is made about the degree and probability of environmental risk for the "critical" component or the agroecosystem as a whole. At this stage, the risk is classified and its compliance with the acceptable environmental level is assessed (the use of MPC and LD_{50} values) [9, 10].

The analysis of sources of uncertainty is an integral part of the agroecological risk assessment, which significantly increases the reliability of the obtained results. Uncertainty may be due to i) lack of information about the problem as a whole; ii) the absence or inaccuracy of the data necessary to determine the level of risk; iii) lack of research and theoretical knowledge for an appropriate conceptual or calculation model; iv) lack of knowledge on the true statistical distribution of data [9].

Probable uncertainties of hazard identification include i) an insufficient information about the studied components of the agroecosystem; ii) incorrect parameters of the established risk factors; iii) lack of data on negative response of the agroecosystem components; iv) incorrect formation of the original data set. The main sources of uncertainty in "impact assessment" include i) inappropriate choice of exposure models or input parameters; ii) errors in the choice of ways of exposure. Errors in determining "critical" components and the reliability of negative effects can be sources of uncertainty at the stage of dose-effect assessing [9].

The assessment of the uncertainty of agroecological risk makes it possible to make decisions without constraints (first level), decisions aimed at minimizing risk, or specific decisions on risk management (second level), or provides evaluative data that are used to simulate the situation (third level) [9, 10].

The final results of all stages are the basis for making decisions on the management of agroecological risks from man-made pollution.

Algorithm for assessing agroecological risks from technogenic pollution. The generalized algorithm for assessing agroecological risks is as follows.

The first step is the database (DB) analysis to sample quantitative parameters characterizing the considered negative effects depending on degrees of

technogenic impact.

The second step includes determination of meteorological parameters of the air pollutant behavior model for specific emission conditions (atmospheric stability class, wind speed at the emission height, the aerodynamic roughness of the underlying surface) as per MU 2.6.5.010-2016 [63]. The atmosphere stability class is determined according to Pasquill [64].

The third step includes quantitation of pollutant deposition on the earth surface (by calculation or experimentally), depending on the character of the technogenic impact. It should be noted that at present, the assessment of the inflow of radioactive and chemical substances to the earth surface is most fully developed [65, 66].

The density of the fallout of radioactive or chemical substances is determined by the formula:

$$As_n(x) = Q \cdot (V_g \cdot G(x)), \quad (1)$$

where Q is integral release of radioactive or chemical substances, g or Bq; V_g is the rate of gravitational settling of radioactive or chemical substances, m/s; G(x) is meteorological dispersion factor at a distance of x meters from the emission source, s/m^3 [65].

The parameter of meteorological dispersion at different distances x from the emission source is calculated at the underlying surface level (z = 0) on the axis of the fallout trace (y = 0) [65]:

$$G(x) = \frac{f_d(x) \cdot f_s(x) \cdot f_w(x)}{\pi \cdot \sigma_y(x) \cdot \sigma_z(x) \cdot U} \cdot \exp^{\left(-\frac{H_g^2}{2 \cdot \sigma_z(x)}\right)}, \quad (2)$$

where x is distance to the source of emission, m; U is the wind speed at the emission height, m/s; Hg is the height of the discharge above the ground, m; σ_z, σ_y are standard deviations of the pollutant dispersion of in the ejection cloud to the direction of corresponding coordinate axes, m [67]; f_d or f_{tr}, f_s, f_w are corrections for decay or chemical transformation, deposition and washing out of pollutants from the atmosphere by precipitation.

The expression for the correction for a radionuclide decay or a toxicant chemical transformation is as follows:

$$f_d(x) = \exp(-\lambda \cdot x/u), \quad (3)$$

where λ is the constant of radioactive decay for a specific radionuclide or constant of chemical transformation, 1/s; x/u is the time of cloud movement to the point with a distance x from the ejection site [65].

The correction for gravity settling is calculated as

$$f_s(x) = \exp\left[-\sqrt{\frac{2}{\pi}} \cdot \frac{v_g}{u} \int_0^x \left(\frac{1}{\sigma_z(x) \cdot \exp(0.5 \cdot h^2 \cdot \sigma_z^{-2}(x))}\right) dx\right], \quad (4)$$

where vg is the gravitational settling velocity, m/s (0.001 for aerosols, 0.02 for elemental iodine, 0.0005 for organic iodine, 0 for inert gases) [65].

The correction for the washing out of chemical toxicants or radioactive substances from the atmosphere is as follows:

$$f_w(x) = \exp(-\Lambda \cdot x/u), \quad (5)$$

where Λ is the average annual constant for the removal of impurities from the atmosphere by precipitation, averaged over the year given the type and duration of precipitation during the year, 1/s [65].

At the fourth stage, the content of pollutants in the agroecosystem components is calculation or determined experimentally. The concentration of radionuclides (Bq) or chemical toxicants (g) in the aboveground biomass for any day after the release q(t) is calculated as follows:

$$q(t) = \frac{K_z(t_o) \cdot \exp^{[-(\lambda+\lambda_{ec})(t-t_o)]}}{a \cdot (t - t_{bg}) \cdot 4} \cdot As_n(x), \quad (6)$$

where λ is the decay constant of radionuclides or chemical transformation constant of toxicants, 1/day; λ_{ec} is the rate constant for the loss of radionuclides or chemical toxicants from the aboveground biomass, 1/day; t_0 is the number of days from the beginning of the year to the fallout date; t is the number of days from the beginning of the year to the date to determine the main parameters of the agroecosystem components (e.g. plant height and biomass, the pollutant concentration in the main components, dose characteristics for the aboveground biomass); 4 is the conversion factor to change from air-dry mass to native mass.

The coefficient of initial retention of radionuclides or chemical toxicants by the aboveground plant biomass is calculated as

$$K_z(t_0) = 1 - \exp^{(-\mu\rho(t_0))}, \quad (7)$$

where μ is an empirical constant of the retention of radionuclides or chemical toxicants by vegetation cover (2.8 m^2/kg air-dry mass), $\rho(t_0)$ is the aboveground biomass at the fallout time t_0, kg/m^2.

The plant aboveground biomass at the fallout time is calculated as given in [67]:

$$\rho(t_0) = a \cdot (t_o - t_{bg}), \quad (8)$$

where a is the rate of biomass growth per unit of crop area, kg/(m · day) (Table 3); t_{bg} is the number of days from the beginning of the growing season of certain agricultural plant species (see Table 3).

3. Growing season parameters and the rate of biomass growth for some crops [10]

Crop	Start of vegetation season	Time form the beginning of growth (t_{bg}), days	Rate of biomass gain, kg/(m^2 · day)
Spring wheat	May 15	135	6×10^{-3}
Spring rye	May 15	135	6×10^{-3}
Barley	May 15	135	3×10^{-3}
Oats	May 15	135	3×10^{-3}
Potato	May 25	145	9×10^{-4}
Beet	June 5	155	6.4×10^{-4}
Cabbage	May 20	140	6.4×10^{-4}
Hayfield grass	April 15	105	6.4×10^{-4}

The fifth step of the suggested algorithm includes calculation or measurement of the pollutant impact on the agroecosystem components. In the release of chemicals, the calculated concentration of toxicants (6) is deemed the starting point to determine the effect of pollutants on plants. To assess the effect of radionuclides on agricultural plants, it is necessary to consider two main sources of radiation [68, 69], an infinite source of finite thickness equal to the plant height with a uniform distribution of activity (the source consists of plants contaminated with radionuclides and atmospheric air between plants as a single environment) and an endless source of radiation with a mass thickness of 0.5 g/cm^2 [68] from radionuclides deposited on the soil due to incomplete retention by the aboveground plant parts.

In more detail, methods for assessing the impact of radionuclides from various sources on agricultural plants are described by Perevolotskaya et al. [9].

To conclude, it should be noted that, given the world trends in industry and industrial agriculture, the probability of complex technogenic pollution [11, 14], including potentially hazardous to human health [15, 18, 20], objectively increases. To control the threats of technogenic impact, it is not enough to have a set of methods suitable for analysis of a particular case. The methodology we propose establishes criteria and approaches that provide assessment of agroecological risks from technogenic pollution, regardless of their nature [70, 71]. The described methods for creating assessment models are applicable to a wide range of environmental challenges, including human health protection [72, 73], and can

be the basis for managing man-made risks in the agro-industrial complex, environmental protection, control and forecasting environmental pollution.

Thus, we suggest a unified methodology of assessing risks of various technogenic pollution for agricultural ecosystems. The methodology is based on the principles and criteria for minimizing threats and ensuring the safety of agroecosystems subjected to technogenic impact. Mathematical modeling is used as an analytical tool. The methodology defines approaches and criteria for agroecological risk assessing. A methodological framework is proposed that allows one to study the dynamics of dose-dependent effects of various factors towards each component of the system and to undertake management decisions to minimize agroecological risks.

REFERENCES

1. Katkova M.N., Ivanitskaya M.V. *Radiatsiya i risk*, 2008, 17(4): 67-78 (in Russ.).
2. Franco-Uría A., López-Mateo C., Roca E., Fernández-Marcos M.L. Source identification of heavy metals in pastureland by multivariate analysis in NW Spain. *Journal of Hazardous Materials*, 2009, 165(1-3): 1008-1015 (doi: 10.1016/j.jhazmat.2008.10.118).
3. Rajaganapathy V., Xavier F., Sreekumar D., Mandal P.K. Heavy metal contamination in soil, water and fodder and their presence in livestock and products: a review. *Journal of Environmental Science and Technology*, 2011, 4(3): 234-249 (doi: 10.3923/jest.2011.234.249).
4. Il'yazov R.G., Aleksakhin R.M., Fisinin V.I., Smirnov A.M., Gusmanov U.G. Methodology of investigations and experiments in agroecosphere at different technogenesis types. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2010, 2: 3-17 (in Russ.).
5. Manzoor S., Shah M.H., Shaheen N., Khalique A., Jaffar M. Multivariate analysis of trace metals in textile effluents in relation to soil and groundwaters. *Journal of Hazardous Materials*, 2006, 137(1): 31-37 (doi: 10.1016/j.jhazmat.2006.01.077).
6. Muzalevskiii A.A., Karlin L.N. *Ekologicheskie riski: teoriya i praktika* [Environmental risks: theory and practice]. St. Petersburg, 2011 (in Russ.).
7. Bashkin V.N. *Ekologicheskie riski: raschet, upravlenie, strakhovanie* [Environmental risks: calculation, management, insurance]. Moscow, 2007 (in Russ.).
8. Dalezios N.R., Blanta A., Spyropoulos N.V., Tarquis A.M. Risk identification of agricultural drought for sustainable agroecosystems. *Natural Hazards and Earth System Science*, 2014, 14(9): 2435-2448 (doi: 10.5194/nhess-14-2435-2014).
9. Perevolotskaya T.V., Panov A.V. Metodologiya otsenki agroekologicheskikh riskov, obuslovlennykh posledstviyami radiatsionnykh avarii. *Radiatsiya i risk*, 2018, 27(4): 119-132 (doi: 10.21870/0131-3878-2018-27-4-119-132).
10. Spiridonov S.I., Sanzharova N.I., Teten'kin V.L., Geras'kin S.A., Panov A.V., Solomatin V.M., Epifanova I.E., Karpenko E.I. *Metodologiya otsenki riska vozdeistviya tekhnogennykh faktorov razlichnoi prirody na agroekosistemy*. Obninsk, 2007.
11. Li Z., Ma Z., van der Kuijp T.J., Yuan Z., Huang L. A review of soil heavy metal pollution from mines in China: pollution and health risk assessment. *Science of the Total Environment*, 2014, 468-469: 843-853 (doi: 10.1016/j.scitotenv.2013.08.090).
12. Fraga C.G. Relevance, essentiality and toxicity of trace elements in human health. *Molecular Aspects of Medicine*, 2005, 26(4-5): 235-244 (doi: 10.1016/j.mam.2005.07.013).
13. Vries W., Römkens P.F.A.M., Schütze G. Critical soil concentrations of cadmium, lead, and mercury in view of health effects on humans and animals. In: *Reviews of environmental contamination and toxicology*, vol. 191. Springer, New York, NY, 2007: 91-130 (doi: 10.1007/978-0-387-69163-3_4).
14. Kim J., Lee Y., Yang M. Environmental exposure to lead (Pb) and variations in its susceptibility. *Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews*, 2014, 32(2): 159-185 (doi: 10.1080/10590501.2014.907461).
15. Bortey-Sam N., Nakayama S.M.M., Ikenaka Y., Akoto O., Baidoo E., Yohannes Y.B., Mizukawa H., Ishizuka M. Human health risks from metals and metalloid via consumption of food animals near gold mines in Tarkwa, Ghana: estimation of the daily intakes and target hazard quotients (THQs). *Ecotoxicology and Environmental Safety*, 2015, 111: 160-167 (doi: 10.1016/j.ecoenv.2014.09.008).
16. Miranda M., López-Alonso M., Castillo C., Hernández J., Benedito J.L. Effects of moderate pollution on toxic and trace metal levels in calves from a polluted area of northern Spain. *Environment International*, 2005, 31(4): 543-458 (doi: 10.1016/j.envint.2004.09.025).
17. Cai Q., Long M.L., Zhu M., Zhou Q.Z., Zhang L., Liu J. Food chain transfer of cadmium and lead to cattle in a lead-zinc smelter in Guizhou, China. *Environmental Pollution*, 2009, 157(11):

3078-3082 (doi: 10.1016/j.envpol.2009.05.048).
18. Miranda M., Benedito J.L., Blanco-Penedo I., López-Lamas C., Merino A., López-Alonso M. Metal accumulation in cattle raised in a serpentine-soil area: relationship between metal concentrations in soil, forage and animal tissues. *Journal of Trace Elements in Medicine and Biology*, 2009, 23(3): 231-238 (doi: 10.1016/j.jtemb.2009.03.004).
19. Hänsch R., Mendel R.R. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Current Opinion in Plant Biology*, 2009, 12(3): 259-266 (doi: 10.1016/j.pbi.2009.05.006).
20. Skalny A.V., Salnikova E.V., Burtseva T.I., Skalnaya M.G., Tinkov A.A. Zinc, copper, cadmium, and lead levels in cattle tissues in relation to different metal levels in ground water and soil. *Environmental Science and Pollution Research*, 2019, 26: 559-569 (doi: 10.1007/s11356-018-3654-y).
21. Sánchez-Pardo B., Fernández-Pascual M., Zornoza P. Copper microlocalisation, ultrastructural alterations and antioxidant responses in the nodules of white lupin and soybean plants grown under conditions of copper excess. *Environmental and Experimental Botany*, 2012, 84: 52-60 (doi: 10.1016/j.envexpbot.2012.04.017).
22. Tsygvintsev P.N., Goncharova L.I., Manin K.V., Rachkova V.M. Estimation of the optimal Cu content in different soil types based of the dynamic model for copper accumulation in above ground parts and roots (on the example of barley *Hordeum vulgare* L. plants). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2018, 53(3): 570-577 (doi: 10.15389/agrobiology.2018.3.570rus).
23. Küpper H., Götz B., Mijovilovich A., Küpper F.C., Meyer-Klaucke W. Complexation and toxicity of copper in higher plants. I. Characterization of copper accumulation, speciation, and toxicity in *Crassula helmsii* as a new copper accumulator. *Plant Physiology*, 2009, 151(2): 702-714 (doi: 10.1104/pp.109.139717).
24. Wang X., Ma Y., Hua L., McLaughlin M.J. Identification of hydroxyl copper toxicity to barley (*Hordeum vulgare*) root elongation in solution culture. *Environmental Toxicology and Chemistry*, 2009, 28(3): 662-667 (doi: 10.1897/07-641.1).
25. Barman S.C., Sahu R.K., Bhargava S. K., Chaterjee C. Distribution of heavy metals in wheat, mustard, and weed grown in field irrigated with industrial effluents. *Bulletin of the Environmental Contamination and Toxicology*, 2000, 64: 489-496 (doi: 10.1007/s001280000030).
26. Vodyanitskii Yu.N., Plekhanova I.O., Prokopovich E.V., Savichev A.T. Soil contamination with emissions of non-ferrous metallurgical plants. *Eurasion Soil Science*, 2011, 44: 217-226 (doi: 10.1134/S1064229311020177).
27. Vodyanitskii Yu.N. Contamination of soils with heavy metals and metalloids and its ecological hazards (analytic review). *Eurasion Soil Science*, 2013, 46: 793-801 (doi: 10.1134/S1064229313050153).
28. Antle J.M., Basso B., Conant R.T., Godfray H.C.J., Jones J.W., Herrero M., Howitt R.E., Keating B.A., Munoz-Carpena R., Rosenzweig C., Tittonell P., Wheeler T.R. Towards a new generation of agricultural system data, models and knowledge products: design and improvement. *Agricultural Systems*, 2017, 155: 255-268 (doi: 10.1016/j.agsy.2016.10.002).
29. Anisimov V.S., Anisimova L.N., Frigidova L.M., Sanzharova N.I., Frigidov R.A., Dikarev D.V., Korneev Yu.N. *Vestnik Rossiiskoi sel'skokhozyaistvennoi nauki*, 2016, 4: 14-17 (in Russ.).
30. Ul'yanenko L.N., Filipas A.S., Loi N.N., Stepanchikova N.S., Kruglov S.V. Vliyanie zagryazneniya kadmiem dernovo-podzolistoi pochvy na rost i razvitie rastenii yachmenya. *Agrokhimiya*, 2009, 6: 56-60.
31. Lequeux H., Hermans C., Lutts S., Verbruggen N. Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. *Plant Physiology and Biochemistry*, 2010, 48(8): 673-682 (doi: 10.1016/j.plaphy.2010.05.005).
32. Talukdar D. Studies on antioxidant enzymes in *Canna Indica* plant under copper stress. *Journal of Environmental Biology*, 2013, 34(1): 93-98.
33. Fidalgo F., Azenha M., Silva A.F., de Sousa A., Santiago A., Ferraz P., Teixeira J. Copper-induced stress in *Solanum nigrum* L. and antioxidant defense system responses. *Food and Energy Security*, 2013, 2(1): 70-80 (doi: 10.1002/fes3.20).
34. Demková L., Árvay J., Bobuľská L., Tomáš J., Stanovič R., Lošák T., Harangozo L., Vollmannová A., Bystrická J., Musilová J. Jobbágy J. Accumulation and environmental risk assessment of heavy metals in soil and plants of four different ecosystems in a former polymetallic ore mining and smelting area (Slovakia). *Journal of Environmental Science and Health Part A Toxic/Hazardous Substances & Environmental Engineering*, 2017, 52(5): 479-490 (doi: 10.1080/10934529.2016.1274169).
35. Kisku G.C., Barman S.C., Bhargava S.K. Contamination of soil and plants with potentially toxic elements irrigated with mixed industrial effluent and its impact on the environment. *Water, Air, and Soil Pollution*, 2000, 120(1-2): 121-137 (doi: 10.1023/A:1005202304584).
36. Rowe E.C., Wamelink G.W.W., Smart S.M., Butler A., Henrys P.A., van Dobben H.F., Reinds G.J., Evans C.D., Kros J., de Vries W. Field survey-based models for exploring nitrogen and acidity effects on plant species diversity and assessing long-term critical loads. In: *Critical loads and dynamic risk assessments. Environmental pollution*, vol. 25. Springer, Dordrecht, 2015: 297-326 (doi: 10.1007/978-94-017-9508-1_11).

37. U.S. EPA. *Concepts, methods, and data sources for cumulative health risk assessment of multiple chemicals, exposures and effects*: A resource document (Final report, 2008). U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-06/013F, 2007.
38. Munns J.W.R., Kroes R., Veith G.D., Suter II G.W., Damstra T., Waters M.D. Approaches for integrated risk assessment. *Human and ecological risk assessment: An International Journal*, 2003, 9(1): 267-272 (doi: 10.1080/713609863).
39. Munns W.R. Jr., Poulsen V., Gala W.R., Marshall S.J., Rea A.W., Sorensen M.T., von Stackelberg K. Ecosystem services in risk assessment and management. *Integrated Environmental Assessment and Management*, 2017, 13(1): 62-73 (doi: 10.1002/ieam.1835).
40. Franklin J., Regan H.M., Syphard A.D. Linking spatially explicit species distribution and population models to plan for the persistence of plant species under global change for the persistence of plant species under global change. *Environmental Conservation*, 2014, 41(2): 97-109 (doi: 10.1017/S0376892913000453).
41. Stern B.R. Essentiality and toxicity in copper health risk assessment: overview, update and regulatory considerations. *Journal of Toxicology and Environmental Health, Part A*, 2010, 73(2-3): 114-127 (doi: 10.1080/15287390903337100).
42. White P.J., Brown P.H. Plant nutrition for sustainable development and global health. *Annals of Botany*, 2010, 105(7): 1073-1080 (doi: 10.1093/aob/mcq085).
43. Tsygvintsev P.N., Goncharova L.I., Rachkova V.M. *Uspekhi sovremennogo estestvoznaniya*, 2018, 11(2): 305-310 (in Russ.).
44. Budantsev P.B., Uvarov L.A., Tsyplakov S.E. *Agrokhimiya*, 2011, 4: 74-81 (in Russ.).
45. Gladkov E.A. Estimation of complex phytotoxicity of heavy metals and determination of admissible concentration for zinc and copper. Sel'skokhozyaistvennaya *biologiya* [*Agricultural Biology*], 2010, 45(6): 94-99 (in Engl.).
46. Shao C., Yang J., Tian X., Ju M., Huang L. Integrated environmental risk assessment and whole-process management system in chemical industry parks. *International Journal of Environmental Research and Public Health*, 2013, 10(4): 1609-1630 (doi: 10.3390/ijerph10041609).
47. Chen J., Shafi M., Li S., Wang Y., Wu J., Peng Z.Y.D., Yan W., Liu D. Copper induced oxidative stresses, antioxidant responses and phytoremediation potential of Moso bamboo (*Phyllostachys pubescens*). *Scientific Reports*, 2015, 5: 13554 (doi: 10.1038/srep13554).
48. Bizzo A.L.T., Intorne A.C., Gomes P.H., Suzuki M.S., dos Santos Esteves B. Short-term physiological responses to copper stress in *Salvinia auriculata* Aubl. *Acta Limnologica Brasiliensia*, 2014, 26(3): 268-277 (doi: 10.1590/S2179-975X2014000300006).
49. Goncharova L.I., Manin K.V., Rachkova V.M. *Agrokhimiya*, 2011, 6: 61-67 (in Russ.).
50. Frigidov R.A., Anisimov V.S., Frigidova L.M., Geras'kin S.A., Anisimova L.N., Korneev Yu.N., Sanzharova N.I. *Agrokhimiya*, 2014, 12: 42-54 (in Russ.).
51. Tsygvintsev P.N., Goncharova L.I., Sanzharova N.I., Rachkova V.M. *Agrokhimiya*, 2016, 12: 76-81 (in Russ.).
52. Devos Y., Romeis J., Luttik R., Maggiore A., Perry J.N., Schoonjans R., Streissl F., Tarazona J.V., Brock T.C.M. Optimising environmental risk assessments. Accounting for biodiversity and ecosystem services helps to translate broad policy protection goals into specific operational ones for environmental risk assessments. *EMBO Reports*, 2015, 16(9): 1060-1063 (doi: 10.15252/embr.201540874).
53. Leung K.M.Y., Dudgeon D. Ecological risk assessment and management of exotic organisms associated with aquaculture activities. In: *Understanding and applying risk analysis in aquaculture. FAO Fisheries and Aquaculture Technical Paper 519*. M.G. Bondad-Reantaso, J.R. Arthur, R.P. Subasinghe (eds.). FAO, Rome, 2008: 67-100.
54. Gworek B., Baczewska-Dąbrowska A.H., Kalinowski R., Górska E.B., Rekosz-Burlaga H., Gozdowski D., Olejniczak I., Graniewska M., Dmuchowski W. Ecological risk assessment for land contaminated by petrochemical industry. *PLoS ONE*, 2018, 13(10): e0204852 (doi: 10.1371/journal.pone.0204852).
55. Kapustka L. Limitations of the current practices used to perform ecological risk assessment. *Integrated Environmental Assessment and Management*, 2008, 4(3): 290-298 (doi: 10.1897/IEAM_2007-084.1)
56. Cabell J.F., Oelofse M. An indicator framework for assessing agroecosystem resilience. *Ecology and Society*, 2012, 17(1): 18 (doi: 10.5751/ES-04666-170118).
57. Annenkov B.N., Egorov A.V., Il'yazov R.G. *Radiatsionnye avarii i likvidatsiya ikh posledstvii v agrosfere* /Pod redaktsiei B.N. Annenkova [Radiation accidents and the elimination of their consequences in the agrosphere. B.N. Annenkov (ed.)]. Kazan', 2004 (in Russ.).
58. Kostyuk V.I., Vikhman M.I., Kashulin P.A., Shmakova N.Yu., Zhirov V.K., Kizeev A.N. *Agrokhimiya*, 2005, 12: 51-58 (in Russ.).
59. Loi N.N., Sanzharova N.I., Shchagina N.I., Mironova M.P. The Effect of cadmium toxicity on the development of lettuce plants on contaminated sod-podzolic soil. *Russian Agricultural Science*, 2018, 44: 49-52 (doi: 10.3103/S1068367418010111).
60. Loi N.N., Sanzharova N.I., Mironova M.P. *Agrokhimiya*, 2015, 1: 89-96 (in Russ.).

61. Prakash M.G.N., Chung I.M. Impact of copper oxide nanoparticles exposure on *Arabidopsis thaliana* growth, root system development, root lignification, and molecular level changes. *Environmental Science and Pollution Research*, 2014, 21: 12709-12722 (doi: 10.1007/s11356-014-3210-3).
62. Frigidov R.A., Frigidova L.M., Anisimov V.S., Sanzharova N.I., Anisimova L.N., Dikarev D.V. *Agrokhimiya*, 2017, 3: 69-78 (in Russ.).
63. *MU 2.6.5.010-2016. 2.6.5. Atomnaya energetika i promyshlennost'. Obosnovanie granits i usloviya ekspluatatsii sanitarno-zashchitnykh zon i zon nablyudeniya radiatsionnykh ob"ektov. Metodicheskie ukazaniya* (utv. FMBA Rossii 22.04.2016) [MU 2.6.5.010-2016. 2.6.5. Nuclear power and industry. Justification of the limits and operating conditions of sanitary protection zones and observation zones of radiation facilities. Guidelines (approved by the FMBA of Russia 04/22/2016)]. Moscow, 2016 (in Russ.).
64. Pasquill F. *Atmospheric Diffusion*. 2nd ed. John Wiley & Sons. NY, 1974.
65. *Metodicheskie ukazaniya po raschetu radiatsionnoi obstanovki v okruzhayushchei srede i ozhidaemogo oblucheniya naseleniya pri kratkovremennykh vybrosakh radioaktivnykh veshchestv v atmosferu MPA-98* (utv. prikazom Ministra RF po atomnoi energii 30.12.1998) [Guidelines for calculating the radiation level in the environment and the expected exposure of the population during short-term releases of radioactive substances into the atmosphere MPA-98 (approved by order of the Minister of the Russian Federation for Atomic Energy 12/30/1998)] (in Russ.).
66. *OND-86. Metodika rascheta kontsentratsii v atmosfernom vozdukhe vrednykh veshchestv, soderzhashchikhsya v vybrosakh predpriyatii* (utv. 04.08.1986.) [OND-86. Methodology for calculating air concentrations of harmful substances contained in emissions of enterprises (approved 04.08.1986.)]. Leningrad, 1987 (in Russ.).
67. *DV-98: Rukovodstvo po ustanovleniyu dopustimykh vybrosov radioaktivnykh veshchestv v atmosferu* [DV-98: Guidelines for establishing permissible emissions of radioactive substances into the atmosphere]. Moscow, 1999 (in Russ.).
68. Spiridonov S.I., Fesenko S.V., Geras'kin S.A., Solomatin V.M., Karpenko E.I. *Radiatsionnaya biologiya. Radioekologiya*, 2008, 48(4): 432-438 (in Russ.).
69. Spirin E.V. *Radiatsionnaya biologiya. Radioekologiya*, 2009, 49(3): 338-345 (in Russ.).
70. ICRP, 2008. *Environmental protection — the concept and use of reference animals and plants.* ICRP Publication 108. Ann. ICRP 38 (4-6).
71. WHO/IPCS. *Environmental health criteria 210: principles for the assessment of risks to human health from exposure to chemicals.* World Health Organization, International Program on Chemical Safety, Geneva, 1999.
72. Onishchenko G.G., Novikov S.M., Rakhmanin Yu.A., Avaliani S.L., Bushtueva K.A. *Osnovy otsenki riska dlya zdorov'ya naseleniya pri vozdeistvii khimicheskikh veshchestv, zagryaznyayushchikh okruzhayushchuyu sredu* [Basics for assessment of the public health risk from chemicals polluting the environment]. Moscow, 2002 (in Russ.).
73. WHO/IPCS. *Environmental health criteria 214: human exposure assessment*. Geneva, 2000.

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Bacillus megaterium 501^{rif} AS ANTIDOT OF HERBICIDE PROMETRYN IN CROPS OF OATS AND CORN

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Abstract

The application of the herbicide prometryn (4,6-bis-(isopropylamino)-2-methylthio-1,3,5-triazine) for weed control, makes many agrotechnological and ecological problems due to relatively higher persistence in the environment. It is well known that many microorganisms are capable of decomposing the herbicide. There has been some attempt to use microorganisms for bioremediation of soils. *Bacillus megaterium* is of particular interest because it produces many physiologically active substances that increase the efficiency of photosynthesis, stimulates growth, and accelerates the formation of plants reproductive organs, as well as decomposes some pesticides. In this article, we present new data on the effect of *B. megaterium* 501^{rif} inoculation upon plant resistance to the herbicide prometryn. There was shown that the *B. megaterium* 501^{rif} brings down the phytotoxicity of the herbicide and decomposes it in the rhizospheres of oats and corn. The purposes of the work were to study the survival rate of *B. megaterium* 501^{rif} in the rhizosphere of oats and maize and to estimate its effect on plant resistance to prometryn, as well as ability to effectively decomposition of this herbicide in the soil. *B. megaterium* 501^{rif} was cultured on a rotary shaker for 48 hours at 30 °C, 140 rpm. The bacterial titer was 5×10^8 CFU/ml and included at least 90 % of the alive cells. Seeds of oats (*Avena sativa* L.) cultivar Pobeda and maize (*Zeal mays* L.) cultivar Ross 199 MV were inoculated with a 2-days liquid culture of *B. megaterium* 501^{rif} and were sown in vegetative pots. The soil was soddy-podzolic, medium loam, with an organic matter content of 2.3 %, pH 5.8. An aqueous suspension of wetting powder of prometryn (Panama Agrochemical Inc., Panama) was applied in the quantity of 0.12, 0.22, 0.67, and 1.23 mg/kg in the experiment with oats and 3.4, 6.8 and 20.4 mg/kg in the experiment with corn. In last case there was a variant with prometryn (6.5 mg/kg) but without plants. Plants were grown in the light chamber Phytos-4 (PHYTOS, Russia) at a temperature of 22-25 °C. The dry weight of plants, the quantity of prometryn in the soil, and the number of bacteria in the rhizosphere were determined 30 days after the sprouts appeared. The field experiment was conducted in the experimental field of the All Russia Institute for Agricultural Microbiology (Pushkin, Leningrad region). The soil was soddy-podzolic medium loam, with an organic carbon content of 2.3 %, pH 5.6. The herbicide prometryn was applied to the soil at a dose of 500 mg/m^2, which approximately corresponded to 1,5 mg/kg. The seeds of corn were not inoculated in the control. The dry weight of plants and the quantity of herbicide in the soil were determined 30 days after the sprouts appeared. *B. megaterium* 501^{rif} took root well in the rhizospheres of oats and corn. The number of bacteria were 300 to 500 thousand CFU/g soil, and from 58 to 80 % of them were physiologically active cells. The weight of oats increased by 11 %, corn — by 20 %, when seeds were inoculated with *B. megaterium* 501^{rif} culture. The resistance of plants to herbicide significantly increased and quantity of prometryn decreased 2-3-fold in the soil under oats, and 20-fold under corn. In the field experiment the weight of the corn plant was 11.6 % higher, while the herbicide quantity in the soil was 3 times lower than in the control. We suppose that the higher resistance of plants inoculated with bacteria to the herbicide is due to a positive effect of their metabolites, in particular poly-beta-hidroxibutyrate, produced by bacteria as well as active participation of bacteria in the degradation of the herbicide. Thus, *B. megaterium* 501^{rif} like an antidote takes off the phytotoxic effect of the herbicide on plants and increases their productivity. Corn, when inoculated with bacteria, decomposes prometryn effectively and can be used for bioremediation.

Keywords: *Bacillus megaterium*, protector, antidote, prometryn, herbicide degradation, oats, corn, soil bioremediation

The herbicide prometryn (4,6-bis-(isopropylamino)-2-methylthio-1,3,5-triazine) is widely and effectively used to control annual dicotyledonous and cereal weeds in corn, cotton, soybeans, potatoes, as well as vegetables and green crops [1, 2]. Its persistence (T_{50}) in soil, depending on the dose, soil and climatic conditions, and agrotechnical methods, ranges from several weeks to 18 months [3, 4]. The long-term persistence of the herbicide in the soil causes agrotechnological and ecological problems. From an agronomic point of view, they are primarily associated with the alternation of crops in the crop rotation. There is a significant risk of reduced yield and even death of herbicide-sensitive plants sown after the prometryn-treated precursor. From an ecotoxicological point of view, it should be noted that, despite the relatively low mobility, prometryn and its transformation products are washed out of the soil into the surrounding water bodies, having a negative effect on aquatic vegetation and undermining the food base of aquatic organisms [5, 6].

Microorganisms and physicochemical properties of the soil are the main factors providing degradation of herbicides [6, 7]. Bacteria utilizing prometryn as a source of carbon [8], nitrogen [9, 10], and sulfur [11] were isolated from the soil. According to Kruglov et al. [12], an accelerated decomposition of the herbicide occurred in the soil inoculated with *Bacillus megaterium* 501^{rif}. Some *B. megaterium* strains produce poly-beta-hydroxybutyric acid. It has been found that the treatment of crops with a preparation containing this substance increases plant resistance to stress, including some pesticides [13]. The *B. megaterium*-based preparations have been created that are used in crop production to increase crop yields [14, 15]. However, there are practically no works in which the influence of these microorganisms on plant resistance to herbicides widely and universally used in modern agricultural technologies has been studied.

This paper provides new data on the effects of *B. megaterium* 501^{rif} inoculation on plants with regard to their resistance to the herbicide prometryn. It is shown for the first time that *B. megaterium* 501^{rif} culture reduces phytotoxicity of the herbicide and degrades it in the rhizosphere of oats and corn

The work aimed to study the survival rate of *B. megaterium* 501^{rif} in the rhizosphere of oats and maize plants, and to assess its effect on plant resistance to prometryn and the efficiency of decomposition of this herbicide in soil.

Materials and methods. Pot tests were carried out at the experimental base of the All-Russian Research Institute of Agricultural Microbiology (St. Petersburg—Pushkin).

Mutant rifampicin resistant strain *B. megaterium* 501^{rif} was obtained by gradient selection from the original strain previously isolated by us from ordinary chernozem (Kokchetav region, Kazakhstan). The original strain is deposited in the Collection of Non-Pathogenic Beneficial Microorganisms for Agricultural Purposes (All-Russian Research Institute of Agricultural Microbiology) [16].

B. megaterium 501^{rif} was cultured for 48 h on a rotary shaker (140 rpm, 30 °C). The nutrient medium was as follows (g/l): K_2HPO_4 — 1.6; KH_2PO_4 — 0.4; NH_4NO_3 — 0.5; $MgSO_4$ — 0.2; $CaCO_3$ — 0.05; $FeSO_4$ — 0.025; yeast extract — 0.2; sucrose — 10.0; pH 6.8. The bacterial titer was 5×10^8 CFU/ml (at least 90% of vegetative cells).

Formation of poly-β-hydroxybutyrate intracellular granules in *B. megaterium* 501^{rif} was investigated by phase contrast microscopy (Axio Lab. A1, Carl Zeiss, Germany).

Seeds of oat (*Avena sativa* L.) variety Pobeda and corn (*Zeal mays* L.) variety Ross 199 MB were inoculated with a 2-day liquid culture of *B. megaterium* 501^{rif} and then planted in 2.0 kg pots. The soil is soddy-podzolic medium loamy, with a 2.3% organic matter content, $pH_{sal.}$ 5.8. Prometryn (an

aqueous suspension of a wettable powder, Panama Agrochemical Inc., Panama) was added at 0.0 (no herbicide), 0.12; 0.22; 0.67 and 1.23 mg/kg for oats and 0.0 (without herbicide), 3.4; 6.8 and 20.4 mg/kg for corn. In the latter case, an additional option introduced was bare fallow soil containing prometryn (6.8 mg/kg). The soil was thoroughly mixed and packaged. Similar variants without inoculation served as control.

The plants were grown in a Phitos-4 light chamber (Phitos, Russia) at 22-25 °C and 50-60% soil moisture content of total moisture capacity. The experiment was repeated 3 times.

The dry biomass of plants, the prometryn content in the soil, and the *B. megaterium* 501^{rif} abundance in the rhizosphere of oats and maize were assessed 30 days after the emergence of seedlings. The bacteria were counted by serial dilution method [17] with a mineral salt agar medium of the above composition with the addition of 2.5% agar (Difco, USA) and 0.02 g/l rifampicin. For differentiated determination of bacterial spores, the soil suspension was pasteurized for 10 min at 80 °C prior to plating on the nutrient medium. The herbicide was extracted from the soil with acetone, followed by quantitative determination on a Tsvet-106 gas chromatograph (NPO Khimavtomatika, Russia) with a thermoionic detector [18]. The amount of prometryn extracted from soil was 65-70% of the calculated amount.

In a field trial (experimental field, the All-Russian Research Institute of Agricultural Microbiology, Pushkin, Leningrad region, 2010; 1 m^2 plots in three replicates), the soil was soddy-podzolic medium loamy, with a 2.3% organic carbon content, pH 5.6. Prometryn (500 mg/m^2, which, according to the analysis, corresponded to 1.5 mg/kg) was incorporated into the topsoil at a 0-10 cm depth. Prior to sowing, seeds of maize variety Ross 199 MB were inoculated with liquid culture of *B. megaterium* 501^{rif} (5×10^8 CFU/ml) (no inoculation in control). The aboveground part weight of plants, the herbicide content in the soil, and the bacterial titer were determined 30 days after the emergence of seedlings.

The data were statistically processed using Microsoft Excel software. The mean values of the indicators (*M*) and standard deviations (±SD) were calculated. The reliability of the results was assessed at the $P_{0.95}$ confidence level.

Results. In the pot tests, 0.12 mg/kg prometrine slightly stimulated oat plant growth. The phytomass increased by 11% ($P_{0.05}$) compared to that in plants not treated with the herbicide (Fig. 1). Doses of prometryn above 0.22 mg/kg inhibited plant growth, and 1.23 mg/kg prometryn caused plant death in 2 weeks.

Oat seed inoculation with *B. megaterium* 501^{rif} culture decreased the phytotoxic effect of prometryn and a significantly ($P_{0.95}$) increased the aboveground phytomass as compared to the control (without inoculation), regardless of the herbicide content in the soil. *B. megaterium* 501^{rif} colonized the oat rhizosphere well, and physiologically active vegetative cells comprised from 58 to 80% of bacteria. A month after the emergence of seedlings, the prometryn content in the soil decreased, and, moreover, in the variants with inoculated seeds it was 1.5-3.0 times less than in the control (without inoculation) (Table). Thence, *B. megaterium* 501^{rif} had a protective effect on oat plants, increasing their resistance to the herbicide, and also reducing the herbicide content in the soil, which, in turn, reduced its phytotoxic effect.

Prometryn had a similar effect on corn, but its resistance was an order of magnitude higher than that of oats (see Fig. 1). The herbicide manifested its inhibitory effect at a concentration of 20.4 mg/kg, while lower doses stimulated plant growth. Corn seed inoculation with bacteria completely eliminated the phytotoxic effect of the herbicide. In addition, the phytomass increased 1.5-3.0 times ($P_{0.95}$) as compared to non-inoculated corn plants.

B. megaterium 501^{rif} colonized the corn rhizosphere well, up to 335 thousand CFU g/soil (see Table). Thirty days after germination, the content of the herbicide in the soil under corn plants decreased 20 times, and with bacterial inoculation 60 times compared to bare fallow soil. Therefore, both plants and *B. megaterium* 501^{rif} bacteria from the rhizosphere, were involved in the herbicide degradation.

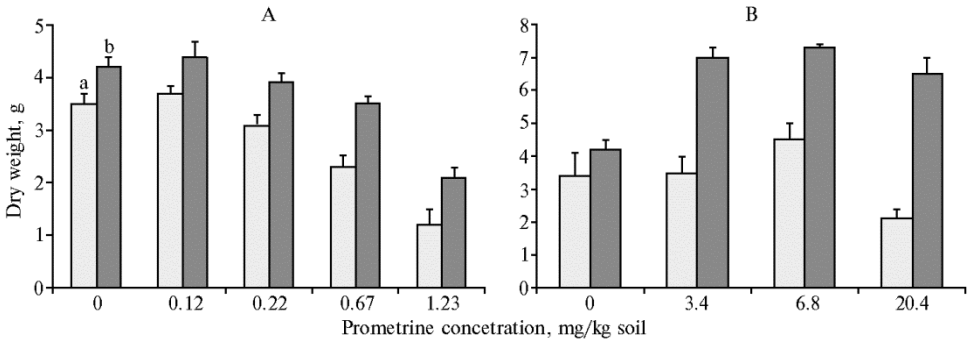

Fig. 1. Dry weight of oat (*Avena sativa* L.) variety Pobeda (A) **and corn (*Zeal mays* L.) variety Ross 199 Mv** (B) **plants as influenced by herbicide** prometryn **upon seed inoculation with *Bacillus megaterium* 501^{rif} culture (30 days after the emergence of seedlings):** a — control (without inoculation), b — inoculation. Test in pots (vertical bars mean root-mean-square deviations).

The soil concentration of prometryn **and abundance of bacterial inoculant *Bacillus megaterium* 501^{rif} in oats (*Avena sativa* L.) variety Pobeda and corn (*Zeal mays* L.) variety Ross 199 MB (30 days after the emergence of seedlings)** (pot tests, $M \pm SD$)

Soil	Prometryn mg/kg			*B. megaterium* 501^{rif}, CFU n 10^3/g	
	initial	day 30			
		without inoculation	inoculation with *B. megaterium* 501^{rif}	total number	spores, % of the total number
Oats	0.22±0.03	< 0.02	< 0.02	500±70	20.0
Oats	0.67±0.04	0.59±0.02	0.18±0.03	550±65	27.0
Oats	1.23±0.03	0.93±0.15	0.58±0.07	350±33	42.0
Corn	6.80±0.05	0.22±0.02	0.07±0.02	335±40	12.0
Soil without corn	6.80±0.05	4.50±0.07			

Given the high efficiency of herbicide degradation under the cover of corn plants, the effect of bacteria on the accumulation of green phytomass and the prometryn degradation in soil was also assessed in plot tests. Thirty days after the emergence of seedlings, the plant weight in the control was 257±15 g/m^2, while under inoculation with *B. megaterium* 501^{rif} it was 287±20 g/m^2, or 12% more than in the control. There was practically no difference between the variants of the experiment. The soil concentration of the herbicide upon inoculation decreased almost 3 times compared to the control, 0.15±0.03 vs. 0.45±0.10 mg/kg, which is consistent with the pot test data.

Considering the reasons for the positive effect of *B. megaterium* 501^{rif}, one should pay attention to the fact that in plants inoculated with bacteria, the architecture of the root system changes significantly due to the more intensive development of lateral roots [19], the generative organ formation is accelerated [20], and the total concentration of photosynthetic pigments in leaves rises [21].

It was found that these microorganisms produce auxins and B vitamins [22], as well as poly-beta-hydroxybutyric acid [23-26] which had a positive effect on root formation and photosynthesis in plants. The content of poly-beta-hydroxymatic acid in *B. megaterium* cells ranged from 10 to 80% of the mass of bacteria, depending on the strain and cultivation conditions [23-25]. The strain *B. megaterium* 501^{rif}, obtained by us, during its growth in a liquid medium also intensively synthesized and accumulated poly-beta-hydroxybutyric acid granules

in vegetative cells (Fig. 2).

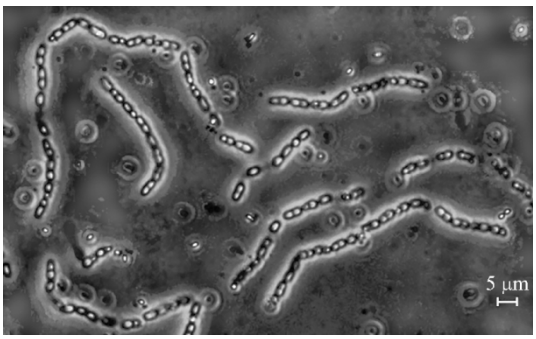

Fig. 2. ***Bacillus megaterium*** **501^{rif} cells filled with poly-beta-hydroxybutyric acid beads** (Axio Lab. A1 microscope, Carl Zeiss, Germany, phase contrast).

The trial of Albit biological (Scientific and Production Company Albit LLC, Russia), the main active ingredient of which is poly-beta-hydroxybutyric acid, has shown its high efficiency in a number of agricultural crops as compared to vatious chemical herbicides [13, 27]. Zlotnikov et al. [13, 27] consider Albit as a universal anti-stress preparation with antidote properties towards pesticides used in agriculture. According to the authors, the mechanism of protective action is primarily associated with the positive effect of poly-beta-hydroxybutyrate on photosynthesis. Based on this, it can be assumed that the protective effect of *B. megaterium* 501^{rif} we observed is associated, with a high degree of probability, with the positive effect of bacterial products, primarily poly-beta-hydroxybutyric acid.

The results for corn are of particular interest. It is known that corn absorbs and degrades triazine herbicides. In this case, the enzymes involved in the hydroxylation play a significant role [28]. It was found that during the transformation of prometryn by plants and microorganisms, significant amounts of metabolic products are formed among which sulfoxide, sulfone, hydroxypropazine occupy the main place, and, with the subsequent transformation of hydroxypropazine, the production of ameline, amelide and cyanuric acid is possible. The products of hydrolysis and N-dealkylation of sim-triazines form conjugates with glutathione and sugars [6, 28].

Until now, the main attention of researchers has been focused on the study of the herbicidal effect of prometryn and the products of its transformation [29]. There is very little evidence of their beneficial effect on plants. Thus, Lebedev reported [30, 31] that small doses of prometryn increased the net productivity of photosynthesis and the absorption of nitrogen and phosphorus by Scots pine and Siberian cedar seedlings. Nadar et al. [32] observed a stimulating effect of sim-triazine herbicides, including methylthio derivates (prometryn and ametryne, on the growth of callus and protein synthesis in sorghum, which allowed the authors to concluded about the hormonal kinin-like action of low concentrations of sim-triazines on plants. Triazine herbicides affect the ionic balance of plants and, thence, the synthesis of DNA, proteins, and enzymes [33]; therefore, it is possible that the high efficiency of corn plant inoculation with *B. megaterium* 501^{rif} upon prometryn application is due to the synergistic effect of the bacterial excretions, the herbicide and products of its transformation.

Thus, inoculation of oat and maize seeds with *Bacillus megaterium* 501^{rif} has a positive effect on plant growth. Moreover, the efficiency of inoculation is higher upon prometryn application than in the control. Also, the plant resistance to prometryn increases and its decomposition in the rhizosphere accelerates. Consequently, the *B. megaterium* 501^{rif} and its metabolites, in particular poly-beta-hydroxybutyric acid, serve as protectors, or antidotes, removing the phytotoxic effect of prometryn . The pot tests and plot trials showed that the corn plants inoculated with *B. megaterium* 501^{rif} possesses properties of a biomeliorant, effectively removing herbicide prometryn from soil. This opens up prospects for the use of corn inoculated with *B. megaterium* in bioremediation of the soils

contaminated by prometryn.

REFERENCES

1. *Gosudarstvennyi katalog pestitsidov i agrokhimikatov, razreshennykh k primeneniyu na territorii Rossiiskoi Federatsii* [State catalog of pesticides and agrochemicals approved for use on the territory of the Russian Federation]. Moscow, 2013 (in Russ.).
2. Novozhilov K.V., Dolzhenko V.I. *Sredstva zashchity rastenii* [Plant protection products]. Moscow, 2011 (in Russ.).
3. Koskinen W.C., Banks P.A. Soil movement and persistence of triazine herbicides. In: *The triazine herbicides: 50 years revolutionizing agriculture*. H.M. LeBaron, J.E. McFarland, O.C. Burnside (eds.). Elsevier BV, Amsterdam, Boston, Heidelberg, London, New York, Paris, San Diego, San Francisco, Sydney, Tokio, 2008: 355-385 (doi: 10.1016/B978-044451167-6.50027-1).
4. Scott M. Herbicide residues in soil and water. *SMART train chemical notes* 3, 2008.
5. *Risks of prometryn use to federally threatened California red-legged frog* (*Rana aurora draytonii*). *Pesticide effects determination environmental fate and effects. Division Office* of *Pesticide Programs Washington*, *D.C.* 20460 6.17.2009.
6. Mel'nikov N.N., Volkov A.I., Korotkova O.A. *Pestitsidy i okruzhayushchaya sreda* [Pesticides and the environment]. Moscow, 1977 (in Russ.).
7. Charles S. Soil residual herbicides: science and management. In: *Topics in Canadian weed science*. R.C. Van Acker (ed.). Sainte-Anne-de Bellevue, Québec, Canadian Weed Science Society — Société canadienne de malherbologie, 2005, 3: 3-22.
8. *Ignatovets O.S., Leont'ev V.N. Mekhanizm degradatsii prometrina bakteriyami roda Pseudomonas. Doklady NAN Belarusi*, 2008, 52(3): 82-86(in Russ.).
9. Myśków W., Lasota T., Stachyra A. Cyanuric acid--a s-triazine derivate as a nitrogen source for some soil microorganisms. *Acta Microbiologica Polonica*, 1983, 32(2): 80-85.
10. Cook A.M., Hütter R. Triazines as nitrogen sources for bacteria. *J. Agric. Food Chem.*, 1981, 29(6): 1135-1143 (doi: 10.1021/jf00108a009).
11. Cook A.M., Hütter R. Ametryne and prometryne as sulfur sources for bacteria. *Applied Environmental Microbiology,* 1982, 43(4): 781-786 (doi: 10.1128/aem.43.4.781-786.1982).
12. Kruglov Yu.V., Lisina T.O. *Bacillus megaterium* 501^{rif} introduced into the soil: factors affecting the rate of survival, sporulation and decomposition of the herbicide prometryn. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2014, 5: 107-112 (doi: 10.15389/agrobiology.2014.5.107eng).
13. Zlotnikov A.K. *Razrabotka i kompleksnaya kharakteristika polifunktsional'nogo preparata al'bit dlya zashchity rastenii ot boleznei i stressov. Avtoreferat doktorskoi dissertatsii* [Development and comprehensive characterization of the multifunctional drug albit to protect plants from diseases and stress. DSc Thesis]. Pushchino—Ramon', 2011 (in Russ.).
14. Dorosinskii L.M. *Bakterial'nye udobreniya — dopolnitel'noe sredstvo povysheniya urozhaya* [Bacterial fertilizers — an additional mean of increasing yields]. Moscow, 1965 (in Russ.).
15. Chumthong A., Kanjanamaneesathian M., Pengnoo A., Wiwattanapatapee R. Water-soluble granules containing *Bacillus megaterium* for biological of rice sheath blight: formulation, bacterial viability efficacy testing. *World J. Microbiol. Biotechnol.*, 2008, 24(11): 2499-2507 (doi: 10.1007/s11274-008-9774-7).
16. Gavrilova E.A., Lisina T.O., Kruglov Yu.V. *Shtamm B. megaterium, prednaznachennyi dlya destruktsii fosfororganicheskikh pestitsidov. A.s. 1735359 A1. (RF). Vsesoyuznyi nauchno-issledovatel'skii institut sel'skokhozyaistvennoi mikrobiologii (RF). № 4781535. Zayavl. 11.01.90. Opubl. 23.05.92. Byul. 19* [Strain *B. megaterium* designed to destruct organophosphorus pesticides. A.s. 1735359 A1. (RF). All-Russian Research Institute for Agricultural Microbiology. № 4781535. Appl. 11.01.90. Publ. 23.05.92. Bull. № 19] (in Russ.).
17. *Metody pochvennoi mikrobiologii i biokhimii* /Pod redaktsiei D.G. Zvyagintseva [Methods of soil microbiology and biochemistry. D.G. Zvyagintsev (ed.)]. Moscow, 1991 (in Russ.).
18. *Metody opredeleniya mikrokolichestv pestitsidov v produktakh pitaniya, kormakh i vneshnei srede* /Pod redaktsiei M.A. Klisenko [Trace-level determination of pesticides in food, feed and the environment. M.A. Klisenko (ed.)]. Moscow, 1983 (in Russ.).
19. López-Bucio J., Campos-Cuevas J.C., Hernández-Calderón E., Velásquez-Becerra C., Farías-Rodríguez R., Macías-Rodríguez L.I., Valencia-Cantero E. *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-independent signaling mechanism in Arabidopsis thaliana. *Molecular Plant Microbe Interaction*, 2007, 20(2): 207-217 (doi: 10.1094/MPMI-20-2-0207).
20. Lisina T.O., Garan'kina N.G., Kruglov Yu.V. *Prikladnaya biokhimiya i mikrobiologiya*, 2001, 37(3): 374-378 (in Russ.).
21. Subbotin A.M., Narushko M.V., Petrov S.A. *Shtamm mikroorganizmov Bacillus megaterium 2-06-TS1 v kachestve aktivatora fotosinteza i energii prorastaniya rastenii. Pat. 2627608 S1 (RF). FGBUN Tyumenskii nauchnyi tsentr SO RAN (RF). № 2016125604. Zayavl. 27.06.2016. Opubl. 09.08.2017,*

Byul. № 22 [*Bacillus megaterium* strain 2-06-TS1 as an activator of photosynthesis and plant germination energy. Patent 2627608 S1 (RF). FGBUN Tyumen Scientific Center SB RAS (RF). № 2016125604. Appl. 27.06.2016. Publ. 09.08.2017. Bull. № 22] (in Russ.).
22. Voznyakovskaya Yu.M. *Mikroflora rastenii i urozhai* [Plant microflora and harvest]. Leningrad, 1969 (in Russ.).
23. Otari S.V., Ghosh J.S. Production and characterization of the polymer polyhydroxy butyrate-co-polyhydroxy valerate by *Bacillus megaterium* NCIM 2475. *Current Research Journal of Biology Sciences*, 2009, 1(2): 23-26.
24. Mohapatra S., Maity S., Dash H.R., Das S., Pattnaik S., Rath C.C., Samantaray D. Bacillus and biopolymer: prospects and challenges. *Biochemistry and Biophysics Reports*, 2017, 12: 206-213 (doi: 10.1016/j.bbrep.2017.10.001).
25. Mona K., Gouda A., Swellam A., Omar S. Production of PHB by a *Bacillus megaterium* strain, using sugarcane molases and corn steep liquor as sole carbon and nitrogen source. *Microbiology Research*, 2001, 156(3): 201-207 (doi: 10.1078/0944-5013-00104).
26. Kofroňová O., Ptáčková L., Chaloupka J. Poly(3-hydroxybutyrate) granules of *Bacillus megaterium*. *Folia Microbiologica*, 1994, 39(2): 166-167 (doi: 10.1007/BF02906816).
27. Zlotnikov A.K., Zlotnikov K.M., Podvarko A.T., Balakhonenkov B.E., Khryukina E.I. *Zemledelie*, 2010, 3: 40-41 (inRuss.).
28. Simoneaux B.J., Gould T.J. Plant uptake and metabolism of triazine herbicides. In: *The triazine herbicides. 50 years revolutionizing agriculture*. H.M. LeBaron, J.E. McFarland, O.C. Burnside (eds.). Elsevier BV, Amsterdam, Boston, Heidelberg, London, NY, Paris, San Diego, San Francisco, Sydney, Tokio, 2008: 73-99 (doi: 10.1016/B978-044451167-6.50010-6).
29. Trebst A. The mode of action of triazine herbicides in plants. In: *The Triazine herbicides. 50 years revolutionizing agriculture*. H.M. LeBaron, J.E. McFarland, O.C. Burnside (eds). Elsevier BV, Amsterdam, Boston, Heidelberg, London, NY, Paris, San Diego, San Francisco, Sydney, Tokio, 2008: 101-110 (doi: 10.1016/B978-044451167-6.50011-8).
30. Lebedev E.V. *Izvestiya Orenburgskogo agrarnogo universiteta*, 2011, 4(32): 21-23 (in Russ.).
31. Lebedev E.V. *Izvestiya Orenburgskogo agrarnogo universiteta*, 2012, 1(33): 23-26 (in Russ.).
32. Nadar H.M., Clegg M.D., Maranville J.W. Promotion of sorghum callus growth by the s-triazine. *Plant Physiology*, 1975, 56(6): 747-751 (doi: 10.1104/pp.56.6.747).
33. Ebert E., Dumford S.W., Van Assche C.J., Warwick D.D. Effects of triazine herbicides on the physiology of plants. In: *Residue reviews. Residues of pesticides and other contaminants in the total environment (sontinuation of residue reviews), vol. 65*. F.A. Gunther, J.D. Gunther (eds.). Springer, New York, NY, 1976 (doi: 10.1007/978-1-4613-9413-6_1).

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IDENTIFICATION OF THE ANCESTRAL CHARACTERISTICS IN THE GENOME OF *Rhizobium leguminosarum* bv. *trifolii*

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Abstract

Nodule bacteria of the species *Rhizobium leguminosarum* are symbiotic N_2-fixers that divide into two biotypes: *viciae* and t*rifolii* (D.C Jordan. et al., 1984). Symbiotic genes, the evolution of which depends on host plants, are responsible for the function of symbiotic nitrogen fixation (J.P.W. Young et al., 1989). Recently it was shown that according to the type of organization of the symbiotic regions of the genomes, rhizobia isolated from the *Vavilovia formosa* (Stev.) Fed. are close to the protosymbiont of the tribe *Fabeae R. leguminosarum bv. viceae* (E.R. Chirak et al., 2019). However, in the evolution of *R. leguminosarum*, there was another earlier divergence between the biotypes *viceae* and *trifolii*, the starting point of which was the protosymbiont of the entire species *R. leguminosarum*, which existed before its separation into biovars. In this work we present the results of genomes sequencing of a group of *Rhizobium leguminosarum* bv. *trifolii* and comparisons of the structure of their symbiotic regions with the corresponding regions of the genomes of *Rhizobium leguminosarum* bv. *viciae*, related to the ancestral and "advanced" types. In the program CLC Genomics Workbench 7.5.1, we compared the obtained genome-wide sequences of the strains *R. leguminosarum* bv. *trifolii* (Oxford Nanopore sequencing technique) with reference strains of *R. leguminosarum* bv. *viceae*, related to ancestral and "advanced" types. It was shown that in the genomes of strains of clover symbionts, four of five ancestral characters are found: an increased size of intergenic regions in the symbiotic region, the presence of the *nodX* gene in the nod-operon, the absence of the *nodT* gene in the *sym*-region, and only one copy of the *fixNOPQ* operon located on the pSym. Based on the results obtained, we suggest that the protosymbiont *R. leguminosarum* could be close to clover rhizobia.

Keywords: *Rhizobium leguminosarum* bv. *trifolii*, symbiosis evolution, symbiotic genes, protosymbiont, genome-wide sequences

Nodule bacteria *Rhizobium leguminosarum*, the most widespread symbiotic nitrogen fixators in temperate latitudes, comprise two biotypes contrasting in host specificity, bv. *viciae* (symbionts of vetch, pea, rank, lentil and vavilovia) and bv. *trifolii* (clover symbionts) [1]. Symbiotic genes (*sym*-genes) are responsible for the function of symbiotic nitrogen fixation, the evolution of which is largely determined by the host plants [2].

Three main groups of *sym*-genes distinguished in rhizobia are *nod* (syn-

thesis of lipochitooligosaccharide signaling Nod factors that induce nodule development) [3-5], *nif* (synthesis of nitrogenase) [5] and *fix* (energy supply for nitrogenase and regulation of *nif* genes) [5, 6]. The evolution of the symbiotic complex first occurred through the assembly of groups of genes encoding various signaling and metabolic properties that ensure the functioning of symbiosis in primary rhizobia. The primary rhizobia originated from non-symbiotic diazotrophs, followed by the transfer of the assembled symbiotic constructs into the so-called secondary (derivative) species [7]. Primary rhizobia, the relatives of the modern genus *Bradyrhizobium*, were close to the free-living phototroph *Rhodopseudomonas* and acquired the ability to fix nitrogen by recruiting some genes that control photosynthesis into the *fix* system [8]. This reorganization led to the emergence of photosynthetically active Bradyrhizobium spp., nodulating the stems of tropical legumes without the use of *nod* genes. The ability to synthesize Nod factors for which *nod* genes are responsible was probably first acquired by bacteria of the genus *Bradyrhizobium* in which phototrophy was replaced by the ability to use photosynthetic products of plant. These heterotrophic rhizobia usually retain the expression of ex planta *nif* genes, but they are not capable of diazotrophic growth due to low nitrogenase activity [9]. The most studied secondary rhizobia are the members of genera *Rhziobium*, *Sinorhizobium*, *Mezorhizobium*, and *Neorhizobium*. These bacteria are devoid of photosynthetic systems and cannot express ex planta nitrogenase genes; their appearance was the result of horizontal transfer of *sym* genes from primary rhizobia to various soil heterotrophic bacteria [10].

Structural and functional organization of *sym*-gene regions has been studied in detail in *Rhizobium leguminosarum* bv. *viceae*, the strains of which vary significantly in the specificity towards different host plants. It has recently been shown that, according to the type of organization of symbiotic regions (*sym*-regions) of rhizobial genomes, *R. leguminosarum* bv. *viceae* can be divided into two groups [10]. The first group isolated from *Vavilovia formosa* (Stev.) Fed. (a plant, presumably close to the last common ancestor of the entire tribe *Fabeae*) [11] and possessing a complex of ancestral features of the genome, is characterized by i) an extended *sym*-region, sometimes divided between two symbiotic plasmids (pSym), ii) the presence of *nodX* and *fixW* genes in plasmid *sym*-operons, iii) the lack of chromosomal copies of *fixNOPQ*, and iv) the location of *nodT* gene outside the operons of *nod* genes. In the second, derived (or evolutionarily "advanced") group, the *sym*-region is more compact, there are chromosomal copies of *fixNOPQ*, *nodT* is integrated into the *nod* operon between *nodN* and *nodO*, and the *nodX* and *fixW* genes are lost.

The transition from the ancestral form to the advanced one is associated with general (structural and functional) compaction of the genome, an increase in the intensity of nitrogen fixation and a narrowing of the host specificity. Thus, it has been shown that the rhizobia isolated from *V. formosa* are close to *R. leguminosarum* bv. *viceae*, the protosymbiont of the tribe *Fabeae*. However, in the evolution of rhizobia of this group, there was another earlier divergence between *R. leguminosarum* bv. *viceae* and *R. leguminosarum* bv. *trifolii*, the starting point of which was the protosymbiont of the entire species *R. leguminosarum*, which existed before *R. leguminosarum* separation with the formation of biovars *viceae* and *trifolii*. And although today nothing is known either about the host plant of this protosymbiont, much less about the organization of its genome, it is obvious that comparing the symbiotic operons of the *R. leguminosarum* bv. *viceae* ancestral variants and corresponding regions in the *R. leguminosarum* bv. *trifolii* genomes will be very helpful for understanding the evolutionary construction of the protosymbiont.

In this work, we present the first results of sequencing genomes of the *Rhizobium leguminosarum* bv. *trifolii* (Rlt) group of the ancestral type to collate the structure of their symbiotic regions with the corresponding regions in *R. leguminosarum* bv. *viciae* (Rlv) of the advanced type.

The investigation aimed to search for ancestral symbiotic characters in the genome of *Rhizobium leguminosarum* bv. *trifolii*.

Materials and methods. Nodule bacteria were isolated from 50 samples of soil from rhizosphere of three clover plants (species *Trifolium pratense* L., *T. repens* L., and *T. hybridum* L.) collected in the village. Vyritsa (Leningrad Province, Gatchinsky District). To collect samples, we selected areas of compact growth of flowering plants (not farther than 0.2-0.3 m from each other), the distance between the sampling sites was at least 5 m.

Soil suspensions were prepared from each sample, which were used to inoculate sterile seedlings of red clover (*T. pratense*) and white clover (*T. repens*).

The plants were grown in pots in gnotobiotic condition on a nitrogen-free Krasilnikov-Korenyako medium. One nodule was taken from each green plant, which was sterilized in 96% alcohol and washed twice with sterile water. The nodules were destroyed with a glass rod in an eppendorf; 0.1 ml of an aqueous suspension was plated on agar medium 79 [12]. On day 3 of growth, individual colonies were subcultured in tubes on bean agar for storage [12]. A total of 37 clover rhizobia isolates were obtained, of which five were selected and grown in 5 ml of liquid medium 79 for 1 day at 28 °C. The cultures were used to isolate genomic DNA according to a standard technique [13].

For whole genome sequencing, the libraries were constructed according to the 1D native barcoding genomic DNA protocol, recommended by the manufacturer, with EXP-NBD104, EXP-NBD114, and SQK-LSK109 kits (Oxford Nanopore, Great Britain). The libraries were sequenced (a MinION nanopore sequencer, Oxford Nanopore, UK) according to the manufacturer's instructions on well R9.4. Basecalling of fast5 raw files resulted from sequencing was performed with Albacore v. 1 software (https://rubygems.org/gems/albacore/ver-sions/2.3.1). We used Deepbiner v. 0.2.0 software [14] to demultiplex the reads, Porechop v. 0.2.3 software (https://github.com/rrwick/Porechop) for cleaning sequence reads. The reads were assembled in Flye v. 2.6 (https://github.com/fenderglass/Flye). The resulting assemblies were corrected with the use of Racon v. 1.3.2 software (https://github.com/lbcb-sci/racon; -m 8 -x -6 -g -8 -w 500 options), as well as in Medaka v. 0.10.0 software (https://github.com/nanoporetech/medaka). Genome annotation was performed using the Prokka program [15]. Genomes were deposited in GenBank (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA611463, the PRJNA611463 bioproject).

Sequence extraction, concatenation, and other manipulations with genomes during information processing were performed in CLC Genomics Workbench v. 7.5.1 (https://secure.clcbio.com/helpspot/in-dex.php?pg=kb.printer.friendly&id=15). The sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA) X program (https://www.megasoftware.net) [18]. The construction of phylogenetic trees by the maximum likelihood method with a bootstrap (1000 repeats) was carried out with PhyML v. 3.3 software(http://www.atgc-montpellier.fr/phyml/) [19]. The choice of the distribution model was automatically determined using the least BIC (Bayesian information criterion) method [20]. The resulting dendrograms were visualized in the online application iTOL (https://itol.embl.de) [21].

Results. Genome analysis was performed for five local isolates (3B, 9B,

22B, 23B, and 31B) and five strains of *R. leguminosarum* (Table 1).

1. *Rhizobium leguminosarum* strains used

Strain	Region	Host plant	GenBank accession number	Reference
Rhizobium leguminosarum bv. *trifolii* (Rlt)				
3B	Pgt Vyritsa, Leningrad Province, Russia	*Trifolium repens* L.	PRJNA611463	This work
9B	Pgt Vyritsa, Leningrad Province, Russia	*Trifolium pratense* L.	PRJNA611463	This work
22B	Pgt Vyritsa, Leningrad Province, Russia	*Trifolium pratense* L.	PRJNA611463	This work
23B	Pgt Vyritsa, Leningrad Province, Russia	*Trifolium pratense* L.	PRJNA611463	This work
31B	Pgt Vyritsa, Leningrad Province, Russia	*Trifolium pratense* L.	PRJNA611463	This work
WSM1689	Greece	*Trifolium uniflorum* L.	CP007045-CP007050	[17]
Rhizobium leguminosarum bv. *viciae* (Rlv)				
Vaf10	North Ossetia, Russia	*Vavilovia formosa* (Stev.) Fed.	CP016286-CP016293	[10]
Vaf108	Dagestan, Russia	*Vavilovia formosa* (Stev.) Fed.	CP018228-CP018236	[10]
TOM	Turkey	*Pisum sativum* L.	AQUC01000001-AQUC01000006	[16]
248	England	*Vicia faba* L.	ARRT01000001-ARRT01000007	[16]

We sequenced the genomes of five Rlt isolates (3B, 9B, 22B, 23B, 31B) to collate them with the genomes of the Rlv strains (see Table 1). The strains to compare were Vaf108 and Vaf10, the symbionts of *Vavilovia formosa* [22] which is probably the closest living relative of the common ancestor of the tribe *Fabeae* [23], TOM which is a symbiont of pea (*Pisum sativum* L.) Afghan cultivars [24], 248, a symbiont of *Vicia faba* L., and WSM1689, a symbiont of *T. uniflorum* (see Table 1). Since the divergence of *R. leguminosarum* biovars is determined by symbiotic genes, while the chromosome background of these biovars is common [25], we focused on the symbiotic regions of the genomes.

Fig. 1. Differences in the organization of *sym*-operons in the genomes of *Rhizobium leguminosarum* isolates. 3B, 9B, 22B, 23B, 31B, and WSM1689 are *R. leguminosarum* bv. *trifolii* (Rlt) strains, Vaf10, Vaf108, TOM, and 248 are *R. leguminosarum* bv. *viciae* (Rlv) strains. The *nod*-operons are marked in blue, *nif* is green, and *fix* in yellow. The top scale is the length of sym-regions, bp.

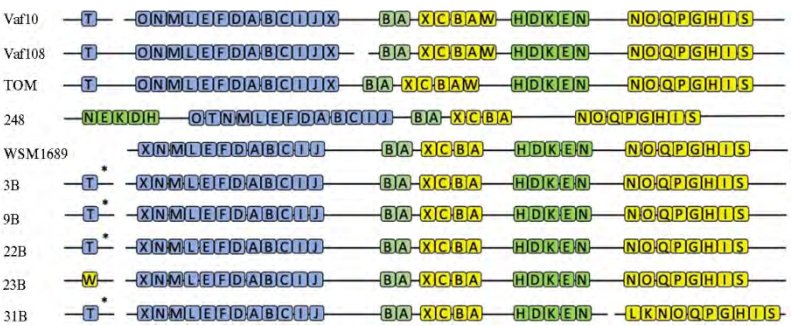

Fig. 2. Schematic structure of *sym*-operons in *Rhizobium leguminosarum* isolates. 3B, 9B, 22B, 23B, 31B, and WSM1689 are *R. leguminosarum* bv. *trifolii* (Rlt) strains, Vaf10, Vaf108, TOM, and 248 are *R. leguminosarum* bv. viciae (Rlv) strains. The *nod* operons are marked in blue, *nif* in green, and fix in yellow. An asterisk (*) marks a gene located on a chromosome.

Structure of *sym*-operons. Comparing *sym*-regions of the genomic sequences of Rlt 3B, 9B, 22B, 23B, and 31B isolates with the *sym*-regions of Rlt WSM1689 and Rlv Vaf10, Vaf108, TOM, and 248 strains revealed differences be-

tween Rlt and Rlv strains in the arrangement and location of *sym* genes (Fig. 1). In strains 3B, 9B, 22B, and 23B, the *sym* genes are organized into *sym* operons located on the pSym. Strain 31B is somewhat different due to the *fixNOQPGHIS* operon location on the chromosome (Fig. 2) and two additional genes (*fixLK*) not detected in other strains (see Fig. 2).

The *nodT* gene was also found on the chromosomes of Rlt strains 3B, 9B, 22B, and 31B, but there was no *fixW* gene. The *nodT* gene was not identified in 23B strain, but of all the studied Rlt strains, only 23B has the *fixW* gene separately located on a plasmid. In the *nod* operons of all Rlt strains, the *nodX* gene was found, but the *nodO* gene was absent.

Genomic distribution of *sym*-regions. In Rlt strains, the structure of *sym*-regions varied (see Fig. 1). In 31B, in contrast to the other strains under consideration, the *fixNOQPGHIS* operon is located on the chromosome. In strains 3B, 9B, and 22B, the distance between *nifHDKE* and *fixNOQPGHIS* operons is greatly increased, while in strain 23B it is noticeably smaller (Fig. 3).

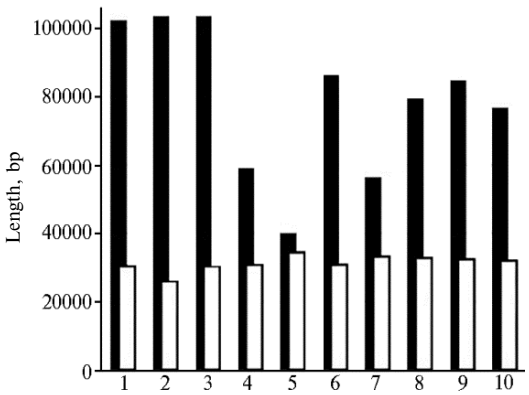

Fig. 3. The proportions between the sizes of *sym*-regions (black bars) **and *sym*-genes** (white bars) **in the genomes of *Rhizobium leguminosarum* bv. *trifolii* (Rlt) strains** (1 — 3B, 2 — 9B, 3 — 22B, 4 — 23B, 5 — 31B, 6 — WSM1689) **and *R. leguminosarum* bv. viciae (Rlv)** (7 — 248, 8 — TOM, 9 — Vaf10, 10 — Vaf108).

In addition, the *sym*-region clusters in strain 23B is the most compact and comparable in size to that in strain 248, a *V. faba* symbiont. The sizes of the *sym*-region in strains 23B and 31B correspond to those characteristic of the evolutionarily advanced group, for which a compact arrangement of *sym*-genes is typical, while strains 3B, 9B, and 22B, in which the *sym*-region is expanded, can be attributed to the ancestral evolutionary group.

Phylogenetic analysis of *sym*-genes. In strains Rlt and Rlv, in addition to the revealed structural features of the symbiotic region, we analyzed the nucleotide polymorphism of three gene groups, *fix*, *nif*, and *nod*. Figure 4 shows the phylogenies of the corresponding concatenates. The grouping of clover symbionts in a relatively compact cluster occurred in two gene groups, *nif* and *nod*, while Rlv strains grouped in a compact cluster only for *fix* genes. Noteworthy is the fact that, in the phylogeny for the *nif* and *nod* genes, the advanced symbionts Rlv (248 and TOM) appear, with reliable statistical support, in one cluster with clover rhizobia, while for *fix* genes, there are two Rlt strains (WSM1689 and 31B) fall into a relatively compact cluster with the Rlv group, which includes both advanced and ancestral Rlv strains.

The biovars of *R. leguminosarum* are represented by symbionts of two very different leguminous tribes. The *R. leguminosarum* separation into *viciae* and *trifolii* biovars has a long evolutionary history, and strains of these biovars do not nodulate legumes from the tribes *Trifolieae* and *Fabeae* upon cross-inoculation. In biovar *viciae*, symbionts of Vavilovia are distinguished, possessing a number of ancestral characters, and it is assumed that they are closest to the protosymbiont of the tribe *Fabeae*, the common ancestor of the biovar *R. leguminosarum* bv. *viceae* [10]. In the presented study, when comparing the Vavilovia rhizobia genomes sequenced earlier and the genomes of clover rhizobia studied in this work, we obtained data concerning the protosymbiont common for the entire

species *R. leguminosarum*, which existed evolutionary earlier, i.e. before the separation into biovars *viceae* and *trifolii*.

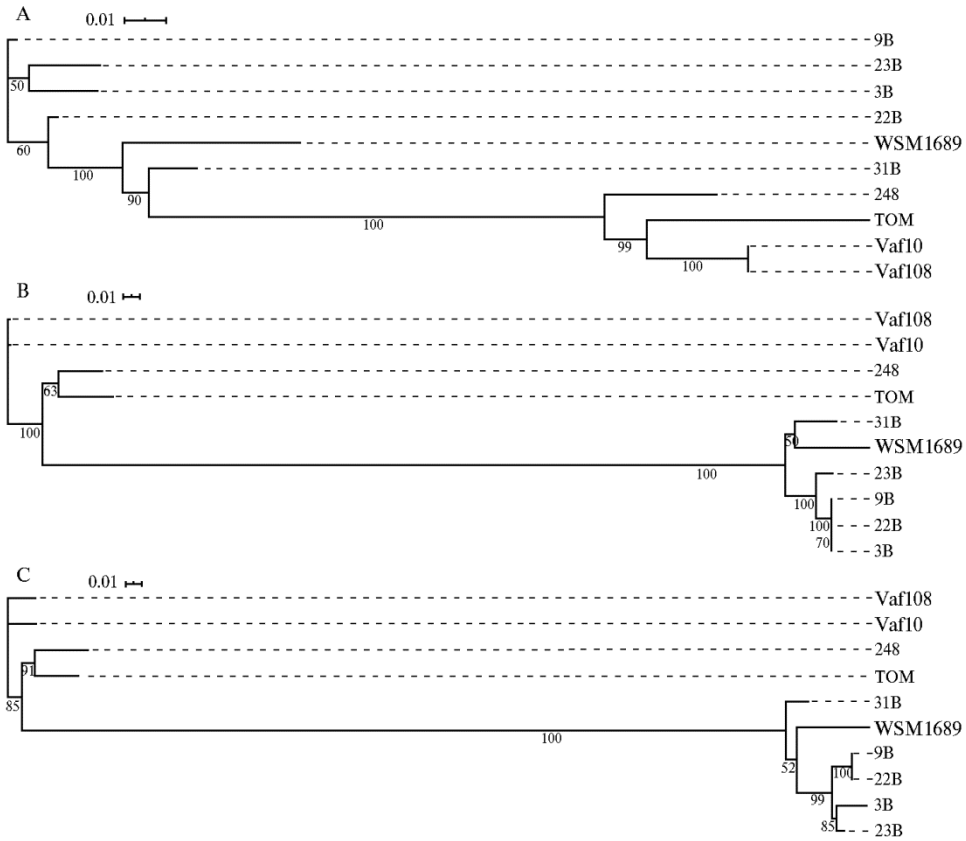

Fig. 4. Dendrogram of *Rhizobium leguminosarum* strains based on concatenated sequences of genes *fixABCGHINOPQ* (A)**, *nifABDEHKN*** (B), and ***nodABCDEFIJLMN*** (C). 3B, 9B, 22B, 23B, 31B, and WSM1689 are *R. leguminosarum* bv. *trifolii* (Rlt) strains, Vaf10, Vaf108, TOM, and 248 are *R. leguminosarum* bv. *viciae* (Rlv) strains.

A significantly larger size of intergenic regions in the symbiotic region, due to the primary "rough" assembly at the early stages of evolution, is an ancestral character. Later in evolution, these regions have compacted [10]. An important result of our studies is that we have identified in the Rlt strains 3B, 9B, and 22B a sym-region with the size which is much larger than in symbionts of Vavilovia (see Fig. 1, Table 2).

2. Summarized ancestral genomic characters of in *Rhizobium leguminosarum* biotypes (based on genome-wide sequencing of 10 strains)

Rhizobium biovbar	Extended *sym*-region	Absence of *nodT* in *nod*-operon	Presence of *nodX*	Presence of *fixW*	Absence of chromosomal copy of *fixNOPQ*
R. leguminosarum bv. *trifolii*	+	+	+	–	+
R. leguminosarum bv. *viciae*	+	+	+	+	+

N o t e. «+» or «–» — the trait is present or absent, respectively. The studied strains are 3B, 9B, 22B, 23B, 31B, and WSM1689 (*R. leguminosarum* bv. *Trifolii*, Rlt), Vaf10, Vaf108, TOM, and 248 (*R. leguminosarum* bv. *viciae*, Rlv).

All Rlt strains are characterized by the presence of the *nodX* gene in *nod*-operon, which in Rlv strains also serves as a trait that marks ancestral genotypes [25]. The significance of *nodX* gene for symbiosis with clover has not been studied, and its loss in advanced Rlv strains is associated with a narrowing of the host specificity and an increase in the activity of nitrogen fixation [26].

Another ancestral feature is the absence of the *nodT* gene which encodes the efflux system ensuring effective release of the nod factor from the rhizobial cell [27]. As shown in Rlv strains, a probable evolutionary scenario is associated with the recruitment of this gene from the chromosome into a symbiotic cluster through duplication, neofunctionalization, and transfer. In Rlt strains, *nodT* gene is present in one copy only on the chromosome (the exception is isolate 31B in which *nodT* was not detected at all). Thus, according to this trait, the genomes of clover rhizobia demonstrate correspondence to even earlier stages of the evolution of the symbiotic gene cluster.

In Rlv strains, ancestral traits associated with functional redundancy of ancestral genotypes also include the presence of *fixW* gene. In the studied clover symbionts, *fixW* gene was found only in strain 31B, but not in the *fix* operon on pSym as in Rlv, but in a separate nonsymbiotic contig. The *fixW* function, as suggested earlier, may be associated with deep differentiation of bacteroids characteristic of rhizobia. The *fixW* manifestation and significance for symbiosis have not been studied in detail, but, most likely, *fixW* does not affect the host specificity [28].

Finally, strains Rlt 3B, 9B, 22B, and 23B, like strains Rlv Vaf10 and Vaf108, have only one copy of *fixNOPQ* genes per pSym (the operon is absent in the chromosome), in contrast to other members of Rlv which have two *fixNOPQ* copies in their genome, i.e. in pSym and in the chromosome. In strain 31B, one copy of *fixNOPQ* was detected, but in the chromosome. The *fixNOQP* genes and their homologues in Gram-negative nitrogen-fixing bacteria encode a high-affinity terminal cytochrome oxidase of the cbb3 type which provides respiration under microaerophilic conditions [29], for example, in nodule symbiosis. Most likely, the duplication of *fixNOQP* cluster and its transfer to the chromosome occurs during the late evolution of *R. leguminosarum* [30].

The differences that we revealed in the phylogenetics topology of *fixABCGHINOPQ*, *nifABDEHKN*, and *nodABCDEFIJLMN* concatenates indicate an independent evolution of the groups of genes that control various functions. The data obtained make it possible to extend the assumptions made earlier about the independent evolution of these groups within the *viceae* biovar [10] to the whole species *R. leguminosarum*.

So, the analysis of whole genome sequencing data showed that at least three (3B, 9B, and 22B) of the studied *Rhizobium leguminosarum* bv. *trifolii* strains possess a large part of the ancestral features (extended *sym*-region, absence of a chromosomal copy of *fixNOPQ* and *nodT* gene in the *nod*-operon, and the presence of *nodX*) found in rhizobia of *Vavilovia formosa* (Stev.) Fed. However, the evolutionary interpretation of the obtained data is complicated by the fact that the mechanisms of *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* evolution, undoubtedly, are determined not only by the host plants and their phylogenesis, but also by the history of adaptation of these plants to various ecological and geographical zones. It is possible that characters identified in one group of rhizobia as ancestral in another group may have a different evolutionary meaning. Despite these constraints, one of the significant results of our study, is, in our opinion, the assumption that clover rhizobia, together with *Vavilovia* rhizobia, may be close to the protosymbiont of *R. leguminosarum*. We consider this assumption as one of the working hypotheses for further research.

REFERENCES

1. Jordan D.C. Family III. *Rhizobiaceae.* In: *Bergey's manual of systematic bacteriology.*

N.R. Krieg, J.G. Holt (eds.). Williams and Wilkins, Baltimore, 1984: 234-242.
2. Young J.P.W., Johnston A.W.B. The evolution of specificity in the legume-rhizobium symbiosis. *Trends in Ecology & Evolution*, 1989, 4(11): 341-349 (doi: 10.1016/0169-5347(89)90089-X).
3. Göttfert M. Regulation and function of rhizobial nodulation genes. *FEMS Microbiology Reviews*, 1993, 10(1-2): 39-63 (doi: 10.1111/j.1574-6968.1993.tb05863.x).
4. Debellé F., Moulin L., Mangin B., Dénarié J., Boivin C. Nod genes and Nod signals and the evolution of the *Rhizobium* legume symbiosis. *Acta Biochimica Polonica*, 2001, 48(2): 359-365. (doi: 10.18388/abp.2001_3921).
5. Shamseldin A. The role of different genes involved in symbiotic nitrogen fixation. *Global Journal of Biotechnology & Biochemistry*, 2013, 8(4): 84-94 (doi: 10.5829/idosi.gjbb.2013.8.4.82103).
6. Fischer H.M. Genetic regulation of nitrogen fixation in rhizobia. *Microbiological Reviews*, 1994, 58(3): 352-386.
7. Provorov N.A., Andronov E.E. Evolution of root nodule bacteria: reconstruction of the speciation processes resulting from genomic rearrangements in a symbiotic system. *Microbiology*, 2016, 85: 131-139 (doi: 10.1134/S0026261716020156).
8. Rey F.E., Harwood C.S. FixK, a global regulator of microaerobic growth, controls photosynthesis in *Rhodopseudomonas palustris*. *Molecular Microbiology*, 2010, 75(4): 1007-1020 (doi: 10.1111/j.1365-2958.2009.07037.x).
9. Wongdee J., Boonkerd N., Teaumroong N., Tittabutr P., Giraud E. Regulation of nitrogen fixation in *Bradyrhizobium* sp. strain DOA9 involves two distinct NifA regulatory proteins that are functionally redundant during symbiosis but not during free-living growth. *Frontiers in Microbiology*, 2018, 9: 1644 (doi: 10.3389/fmicb.2018.01644).
10. Chirak E.R., Kimeklis A.K., Karasev E.S., Kopat V.V., Safronova V.I., Belimov A.A., Aksenova T.S., Kabilov M.R., Provorov N.A., Andronov E.E. Search for ancestral features in genomes of *Rhizobium leguminosarum* bv. *Viciae* strains isolated from the relict legume *Vavilovia formosa*. *Genes*, 2019, 10(12): 990 (doi: 10.3390/genes10120990).
11. Mikič A., Smýkal P., Kenicer G., Vishnyakova M., Sarukhanyan N., Akopian J., Vanyan A., Gabrielyan I., Smýkalová I., Sherbakova E., Zorić L., Atlagić J., Zeremski-Škorić T., Ćupina B., Krstić P., Jajić I., Antanasović S., Porđević V., Mihailović V., Ivanov A., Ochatt S., Ambrose M. The bicentenary of the research on 'beautiful' vavilovia (*Vavilovia formosa*), a legume crop wild relative with taxonomic and agronomic potential. *Bot. J. Linn. Soc.*, 2013, 172(4): 524-531 (doi: 10.1111/boj.12060).
12. *Biologicheskoe raznoobrazie kluben'kovykh bakterii v ekosistemakh i agrotsenozakh. Teoreticheskie osnovy i metody (monografiya)* /Pod redaktsiei M.L. Rumyantsevoi, B.V. Simarova [The biological diversity of nodule bacteria in ecosystems and agrocenoses. Theoretical foundations and methods (a monograph). M.L. Rumyantseva, B.V. Simarova (eds.)]. St. Petersburg, 2011 (in Russ.).
13. Somasegaran P., Hoben H.J. Isolating and purifying genomic DNA of *Rhizobia* using a large-scale method. In: *Handbook for Rhizobia: methods in legume-rhizobium technology*. Springer, New York, 1994: 279-283 (doi: 10.1007/978-1-4613-8375-8_31).
14. Wick R.R., Judd L.M., Holt K.E. Deepbinner: Demultiplexing barcoded Oxford Nanopore reads with deep convolutional neural networks. *PLoS Computational Biology*, 2018, 14(11): e1006583 (doi: 10.1371/journal.pcbi.1006583)
15. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*, 2014, 30(14): 2068-2069 (doi: 10.1093/bioinformatics/btu153).
16. Reeve W., Ardley J., Tian R., Eshragi L., Yoon J.W., Ngamwisetkun P., Seshadri R., Ivanova N.N., Kyrpides N.C. A genomic encyclopedia of the root nodule bacteria: assessing genetic diversity through a systematic biogeographic survey. *Standards in Genomic Sciences*, 2015, 10: 14 (doi: 10.1186/1944-3277-10-14).
17. Terpolilli J., Rui T., Yates R., Howieson J., Poole P., Munk C., Tapia R., Han C., Markowitz V., Tatiparthi R., Mavrommatis K., Ivanova N., Pati A., Goodwin L., Woyke T., Kyrpides N., Reeve W. Genome sequence of *Rhizobium leguminosarum* bv. *trifolii* strain WSM1689, the microsymbiont of the one flowered clover *Trifolium uniflorum*. *Standards in Genomic Sciences*, 2013, 9: 527-539 (doi: 10.4056/sigs.4988693).
18. Kumar S., Stecher G., Li M., Knyaz C., Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 2018, 35(6): 1547-1549 (doi: 10.1093/molbev/msy096).
19. Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W., Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, 2010, 59(3): 307-321 (doi: 10.1093/sysbio/syq010).
20. Schwarz G. Estimating the dimension of a model. *Annals of Statistics*, 1978, 6(2): 461-464 (doi: 10.1214/aos/1176344136).
21. Letunic I., Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, 2016, 44(W1): W242-W245 (doi:

10.1093/nar/gkw290).
22. Safronova V.I., Kimeklis A.K., Chizhevskaya E.P., Belimov A.A., Andronov E.E., Pinaev A.G., Pukhaev A.R., Popov K.P., Tikhonovich I.A. Genetic diversity of rhizobia isolated from nodules of the relic species *Vavilovia formosa* (Stev.). Fed. *Antonie van Leeuwenhoek*, 2014, 105: 389-399 (doi: 10.1007/s10482-013-0089-9).
23. Makasheva R.Kh., Drozd A.M., Adamova O.P., Golubev A.A. *Sbornik trudov po prikladnoi botanike, genetike i selektsii*, 1973, 51(1): 44 (in Russ.).
24. Ma S.W., Iyer V.N. New field isolates of *Rhizobium leguminosarum* biovar *Viciae* that nodulate the primitive pea cultivar Afghanistan in addition to modern cultivars. *Applied and Environmental Microbiology*, 1990, 56(7): 2206-2212.
25. Kimeklis A.K., Chirak E.R., Kuznetsova I.G., Sazanova A.L., Safronova V.I., Belimov A.A., Onishchuk O.P., Kurchak O.N., Aksenova T.S., Pinaev A.G., Andronov E.E., Provorov N.A. Rhizobia isolated from the relict legume *Vavilovia Formosa* represent a genetically specific group within *Rhizobium leguminosarum* biovar *viciae*. Genes, 2019, 10(12): 991 (doi: 10.3390/genes10120991).
26. Provorov N., Tikhonovich I. Genetic resources for improving nitrogen fixation in legume-rhizobia symbiosis. *Genetic Resources and Crop Evolution*, 2003, 50: 89-99 (doi: 10.1023/A:1022957429160).
27. Alvarez-Ortega C., Olivares J., Martínez J.L. RND multidrug efflux pumps: What are they good for? *Frontiers in Microbiology*, 2013, 4: 7 (doi: 10.3389/fmicb.2013.00007).
28. Hontelez J.G., Lankhorst R.K., Katinakis P., van den Bos R.C., van Kammen A. Characterization and nucleotide sequence of a novel gene *fixW* upstream of the *fixABC* operon in *Rhizobium leguminosarum*. *Mol. Gen. Genet.*, 1989,218: 536-544 (doi: 10.1007/BF00332421).
29. Talbi C., Sanchez C., Hidalgo-Garcia A., González E.M., Arrese-Igor C., Girard L., Bedmar E.J., Delgado M.J. Enhanced expression of *Rhizobium etli* cbb3 oxidase improves drought tolerance of common bean symbiotic nitrogen fixation. *Journal of Experimental Botany*, 2012, 63(14): 5035-5043 (doi: 10.1093/jxb/ers101).
30. Kopat V.V., Chirak E.R., Kimeklis A.K., Safronova V.I., Belimov A.A., Kabilov M.R., Andronov E.E., Provorov N.A. Evolution of *fixNOQP* genes encoding cytochrome oxidase with high affinity to oxygen in Rhizobia and related bacteria. *Russ. J. Genet.*, 2017, 53: 766-774 (doi: 10.1134/S1022795417070067).
31. Berrada H., Fikri-Benbrahim K. Taxonomy of the rhizobia: current perspectives. *British Microbiology Research Journal*, 2014, 4(6): 616-639 (doi: 10.9734/BMRJ/2014/5635).

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GENOME VARIABILITY OF RUSSIAN POTATO CULTIVARS: AFLP-ANALYSIS DATA

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Abstract

Plant breeding success largely depends on knowledge of the genetic diversity and pedigree of cultivars, which is important for determining parental pairs for crossbreeding, donor genotypes of valuable traits and intraspecific homogeneity. AFLP is one of the popular methods for detecting genomic polymorphism and genotyping plant accessions, cultivars and lines. In addition to solving taxonomic and phylogenetic problems, the AFLP method is widely used to determine the variability, homogeneity, and the introgression and hybridity degree of *S. tuberosum* cultivars, reconstruct their pedigrees, and also to search for markers linked to various traits. Despite the importance of cultivar certification and inter-cultivar genomic variability assessment, in the Russian Federation, there are few studies on molecular marking of the potato domestic and foreign cultivars farmed in Russia. In the present work, the nuclear genome variability of 60 potato cultivars and five perspective clones was evaluated using the multilocus AFLP analysis. With primer combinations E35/M40 and E41/M35, 218 AFLP fragments were detected, 189 (86.7%) of which were polymorphic and 19 were unique for individual cultivars. Each of the 65 analyzed accessions was characterized by a specific AFLP spectrum. The genetic distances between the analyzed accessions varied widely from 0.37 to 0.77 with an average value of GD = 0.61. The species *Solanum stoloniferum*, used as an outgroup genotype, was most similar to the cv. Fioletovyi (GD = 0.59), and the greatest difference was to the cv. Aurora (GD = 0.80). Statistical analysis of the obtained AFLP data resulted in statistically insignificant clustering. On dendrograms constructed using the PAST and Structure v. 2.3.4 software, there was a tendency toward clustering (with low bootstrap support) of cultivars from the Lorch Potato Research Institute, and accessions with resistance to late blight, cyst nematode or PVY, as well as with yellow-coloured tuber peel. The high polymorphism level of the analyzed cultivars, the lack of their clear clustering and their "unstable" position at the dendrograms may be due to the current intensive exchange of breeding material, as well as to the increasing popularity of using wild potatoes in the potato breeding programs.

Keywords: *Solanum tuberosum*, potato, Russian cultivars, foreign cultivars, genomic polymorphism, tuber skin color, tuber flesh color, resistance, potato blight, cyst nematode, PVY, AFLP-clustering

A success of any breeding program largely depends on knowledge of the genetic diversity and pedigrees of plant cultivars, which is important for identifying parental pairs for crossing, donors of valuable traits and intravarietal homogeneity. Modern methods of molecular analysis make it possible to characterize the genotype, as well as to determine the degree of diversity within a cultivar and between cultivars of different geographical and breeding origin [1].

Currently, DNA genotyping of plants by assessing the polymorphism of both the entire genome and its functional regions (gene families, individual loci and genes) is becoming more and more relevant. In plants, DNA markers based on polymorphic DNA sequences obtained by molecular analysis methods are used to identify valuable genotypes, specific genes and chromosomal loci, as well as for the certification of cultivars and lines. DNA markers are not influenced by the environment and can be detected at any stage of development [2, 3]; therefore, their use makes it possible to overcome the disadvantages of protein markers in a number of breeding issues, including cultivars certification.

AFLP (amplified fragment length polymorphism) is a popular technique to detect genomic polymorphism and to genotype plant accessions, cultivars, and lines [4]. AFLP analysis makes it possible to assess the genome variability without determining the specific loci sequence, as well as to study an extensive, predominantly selectively neutral, part of the genome represented by unique and moderately repetitive DNA sequences [4]. High efficiency of AFLP markers has been shown in determining genetic distances and phylogenetic relationships at various taxonomic levels [5]. The frequency of its use evidences about the effectiveness of the method. AFLP is actively and successfully used to assess intervarietal variability in many agricultural crops, including wheat [6[, barley [7], peas [8, 9], and pepper [10, 11].

In potato research, AFLP technique was used to assess genetic diversity in existing world collections, for example, in wild species *Solanum microdontum* (GenBank USDA, USA) [12], *S. acaule* and *S. demissum* (CGN GenBank, the Netherlands) [13]. AFLP was used to revise 619 accessions of 13 wild potato species from the CPC (Great Britain) and NRSP6 GenBanks — The US Potato GenBank (USA) [14]. The method allowed assessing the levels of polymorphism of representative accessions of wild and cultivated potato species differing in geographic origin, ploidy and breeding system [14]. A number of taxonomic issues in the genus *Solanum* were solved using the AFLP method, e.g. the grouping of potato species in a series, previously proposed by Hawkes [15], has been revised; the effectiveness of AFLP for studying phylogeny of the genus *Solanum* and potato cultivars was shown [16], and the differences between taxa *S. americanum* and *S. nodiflorum* were confirmed [17].

In addition to addressing taxonomic and phylogenetic problems, the AFLP method is widely used to determine variability, homogeneity, degree of introgression and hybridity of *S. tuberosum* cultivars, to reconstruct their pedigrees, and also to search for markers associated with various traits. Thus, AFLP genotyping of 20 local Chilean cultivars was performed [18]. An analysis of 32 potato varieties cultivated in Scandinavian countries (NGB — Nordic Gene Bank, Sweden) showed that the collection consists of genetically and morphologically different clones, without any grouping by geographic origin [19]. AFLP analysis of 54 potato cultivars from the SASA GenBank (Great Britain) identified a group of 7 cultivars that were recommended for use in breeding programs in southern Italy [20].

Despite the significance of certification and assessment of intervarietal genomic variability, for potato cultivars of domestic and foreign origin cultivated in Russia, little is known about analysis and development of systems for molecular genotyping [21-24] or the determination of gene allelic variants for pathogen resistance [25-28].

The research aimed to assess the genomic variability of 60 potato cultivars and five perspective breeding clones of domestic and foreign breeding by the

AFLP method, as well as the effectiveness of AFLP analysis in genotyping potato varieties cultivated in Russia.

Materials and methods. Sixty domestic and foreign cultivars and five promising breeding clones of potato *S. tuberosum* (provided by the Lorkh All-Russian Research Institute of Potato Farming — VNIIKH, Moscow Province, Russia) were analyzed (a related species *S. stoloniferum* was an outgroup accession). Of the 60 varieties, 59 (or 90.77%) are included in the State Register of Breeding Achievements Allowed for Use (RF, 2020; http://reestr.gossortrf.ru/reestr/culture/159.html). The tubers were germinated in standard greenhouse conditions (23 °C/25 °C and 16 h/8 h day/night).

Genomic DNA was extracted from freshly harvested seedlings by the CTAB method [21, 29].

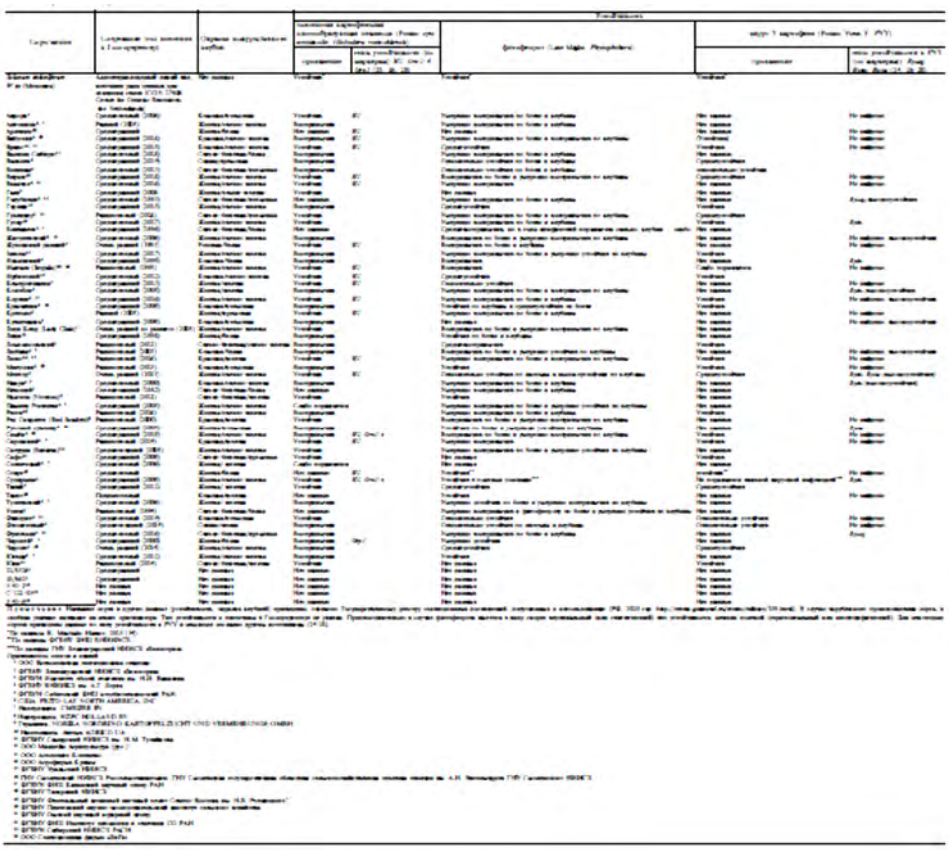
AFLP analysis was carried out according to a standard technique with hydrolysis of 350 ng of each accession genomic DNA with EcoRI and MseI restriction enzymes followed by ligation with EcoRI and MseI adapters [4] Selective amplification was carried out in two stages. The first step was a pre-amplification (denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 1 min; 24 cycles) using adapter primers EcoRI+1 and MseI+1 [4] with a 3′-end selective nucleotide A. The second step was amplification using primers EcoRI+3 and MseI+3 with three selective nucleotides at the 3′-end. The results were visualized in a denaturing 6% polyacrylamide (a LI-COR 4300 gel analyzer, LI-COR operator manual, LI-COR, USA).

The obtained AFLP fragments were entered in MS Excel for calculation as binary matrices. Based on the constructed spectra and matrices, the variety-specific DNA markers were identified, the coefficients of pairwise genetic similarity between the accessions (GS) and the genetic distances (GD = 1 – GS) were calculated; cluster analysis was performed (by Neighbor Joining method and principal coordinates analysis), and groups of genetically similar accessions were identified using PAST software [30]. The genomic structure of the studied accessions was analyzed with Structure v. 2.3.4 (https://web.stanford.edu/group/pritchardlab/home.html) which allows identification of common genetic blocks and their ratio in each accession [31, 32].

Results. The description of the cultivars used in the study (originators, ripening dates, year of entry into the State Register, color of the tuber skin and flesh, resistance to golden potato cyst nematode, late blight, potato virus Y (PVY), resistance genes) are given in the Table 1 (see at http://www.agrobiology.ru).

The primer/enzyme combinations testing for multilocus AFLP genomic analysis of *S. tuberosum* cultivars. The restriction endonucleases EcoRI and MseI were used to digest DNA of the potato accessions, since it was previously shown that these enzymes provide the highest efficiency of AFLP analysis [10, 13, 18]. At the second step of amplification, seven combinations of EcoRI+3/MseI+3 primers differing in the composition of selective nucleotides at the 3′-end were tested using five cultivars (from different breeding centers), namely E35/M40 (E-ACA/M-AGC), E41/M35 (E-AGG/M-ACA), E41/M48 (E-AGG/M-CAC), E41/M45 (E-AGG/M-ATG), E12/M50 (E-AC/M-CAT), E32/T55 (E-AAC/M-CGA), and E32/T61 (E-AAC/M-CTA). Only two combinations, E35/M40 and E41/M35, could generate the polymorphic, clearly differentiated profiles with an optimal number of fragments and were subsequently used for AFLP labeling of 60 cultivars and five breeding clones of *S. tuberosum*.

1. Potato varieties and lines subjected to AFLP analysis (see at http://www.agrobiology.ru)



AFLP analysis of potato cultivars and breeding clones. AFLP analysis of 60 cultivars and five breeding clones of *S. tuberosum* and accession of wild species *S. stoloniferum* (an outgroup) detected 218 fragments (80-450 bp), 189 (86.7%) of which were polymorphic (Table 2). The E41/M35 primer combination was the most effective with 122 out of 139 obtained fragments variable (see Table 2). For some cultivars, unique fragments were found (19 in total).

2. AFLP analysis results for 65 potato cultivars and breeding clones

Primer combination	Number of fragments			
	total	polymorphic		unique
		total	%	
E35/M40	79	67	84,8	7
E41/M35	139	122	87,8	12
Total	218	189	86,7	19

The combinations E35/M40 and E41/M35 revealed the polymorphism of cultivars with greater efficiency than in a number of other studies. For example, AFLP analysis of 32 potato cultivars from the NGB GenBank with five EcoRI+3/MseI+3 combinations revealed 21-26 fragments of which only 4-18 were polymorphic [19]. Labeling of 22 potato cultivars (Chile) with five EcoRI+3/MseI+3 combinations showed only 26-71 polymorphic fragments out of 34-77 described [18]. AFLP genotyping of 25 potato cultivars from Iran with 16 primer combinations PstI+3/MseI+3 identified only 16-52 polymorphic fragments out of 19-53 [33]. It was previously reported that up to 80% of a standard AFLP pattern can serve as markers for detecting genetic polymorphisms at

restriction sites or within an excised fragment and, as a consequence, for determining the population structure and reconstructing the species phylogeny [4]. At the same time, careful selection of primer combinations can significantly increase the number of detected polymorphic bands. Thus, in some studies [18, 33], the percentage of polymorphic fragments in AFLP analysis of potato accessions is almost as high as in this study (75-100%) (see Table 2) while in other reports it varies from 17.4 to 78.3% [19].

Thus, it is obvious that the polymorphism revealed by the E35 M40 and E41/M35 primer combinations is so high that even one of the combinations would be sufficient for genotyping the analyzed potato accessions. As a result of AFLP analysis using E35/M40 and E41/M35 primers, each of 60 analyzed cultivars and five breeding potato clones was characterized by a specific AFLP pattern.

AFLP data statistical analysis. The analysis of the obtained data showed that genetic distances of the analyzed cultivars vary within wide limits, from 0.37 (between cv. Tanay and Yugan) to 0.77 (between cv. Aurora and Nakra) with an average of 0.61. *S. stoloniferum*, used as an outgroup, shows the greatest similarity with the cv. Fioletovii (GD = 0.59), and the greatest difference with the cv. Aurora (GD = 0.80).

Based on the AFLP analysis data, a dendrogram was obtained using the PAST program, where group 1, separated with low bootstrap support, comprised 14 cultivars of which half were of VNIIKH breeding origin, four (cv. Lady Claire, Red Scarlett, Impala and Saturn) were of Dutch breeding and three (cv. Aurora, Elizaveta and Charodei) were originated by other breeding centers (Fig. 1, see Table 1). Group 2 consisted of three cultivars of foreign (Gala) and domestic (Zhigulevskii and Safo) breeding (see Fig. 1). The cultivars of VNIIKKH (Meteor, Golubizna, Pamyami Rogacheva, Nakra and Velikan), Ural Research Institute of Agriculture (Gornyak), Tatar Research Institute of Agriculture (Reggi) and those bred in the USA (Newton) grouped in the third implicit cluster (group 3). It was the sister group to the outgroup accession, which, together with *S. stoloniferum*, included the cv. Fioletovii (see Fig. 1). All other analyzed cultivars formed a highly polymorphic cluster without reliable segregation into subclusters (see Fig. 1). Interestingly, the cv. Fioletovii had a high similarity (GD = 0.54-0.59) with nine cultivars, Fritella, Krasavchik, Lyuks, Irbitskii, Lina, Kortni, Virazh, Tanai, and Sarovskii. However, cv. Fioletovii grouped with the wild species *S. stoloniferum*.

When trying to group the cultivars by agronomic traits, country of origin or originator (see Table 1), we did not identify statistically significant groups.

However, it should be noted that not seeing the clustering by country of origin in the analyzed accession set may be because mainly the domestic cultivars were studied.

The outer group (cv. Fioletovii and *S. stoloniferum*) turned out to be resistant to late blight and PVY. Cv. Velikan, Gornyak, and Newton from group 3, which are closest to the outer group, are also resistant to late blight, and the subgroup that unites cv. Velikan and Gornyak is resistant to PVY. Group 2 cultivars are completely susceptible to late blight. In a large cluster, the subgroup of cv. Tanai, Yugana and Lina is resistant to late blight, and three other subgroups (subgroup 1 — cv. Favorit and Bravo; subgroup 2 — cv. Kolobok, Irbitskii, Start and Kortni, and subgroup 3 — cv. Lomonosovskii and Charoit) are resistant to PVY. The cultivars resistant to nematode *Globodera rostochiensis* show a slight trend to form a cluster. For the remaining subgroups of the large cluster and group 1, we did not find common traits.

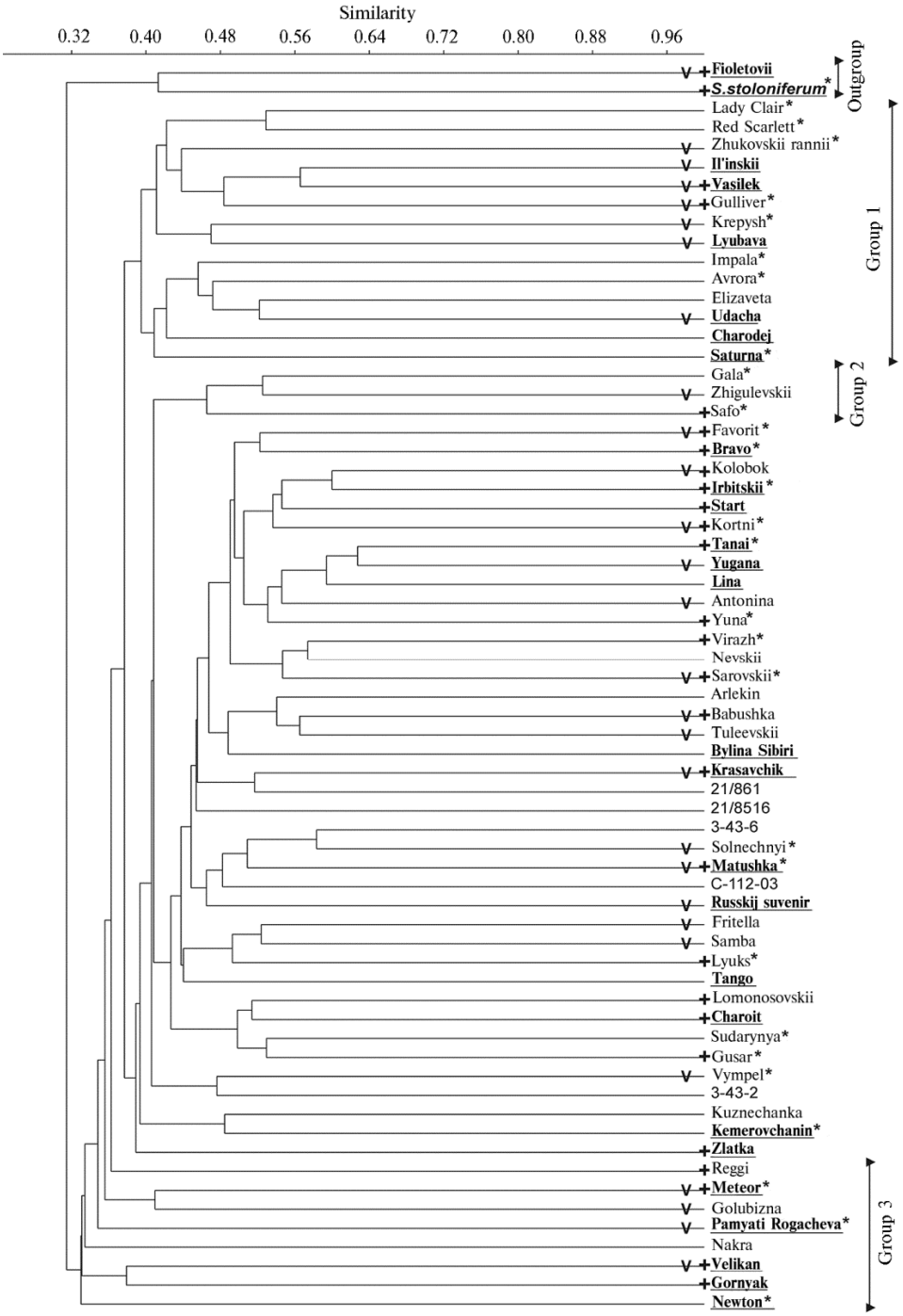


Fig. 1. Genetic diversity among 60 cultivars and five breeding clones of potatoes (AFLP analysis, Neighbor Joining method, PAST software). The cultivars resistant to late blight are highlighted in bold and underlined, the cultivars resistant to potato virus Y are marked with (+), to golden cyst nematode with (∗). The originator of the cultivars marked with (v) is Lorkh All-Russian Research Institute of Potato Farming.

Such a high polymorphism between the analyzed cultivars and the absence of statistically significant clustering may be due to the growing popularity of the wild potato species as genetic donors in recent decades. A total of 57 accessions, i.e. most of those we used in the study, are promising breeding clones and cultivars

(the entries in the Russian State Register of Varieties since 2000). The majority of these clones and cultivars are complex interspecific hybrids, in which wild potato species are often donors of economically valuable traits, e.g. resistance to pathogens, abiotic factors, etc. [34]. This is also evidenced by recent studies of potato cultivars originated from Russia and neighboring countries, which showed a relationship between a constant increase in the number of cultivars with rare and unique SSR loci alleles, on the one hand, and the use of interspecific hybridization, on the other hand [22].

The dendrogram obtained shows a tendency to clustering cultivars from Lorkh All-Russian Research Institute of Potato Farming (see Fig. 1). Earlier, SSR analysis of 41 domestic and foreign potato cultivars and 26 breeding accessions revealed clustering of the cultivars of related origin [23]. On the other hand, according to the AFLP analysis results, the cultivars Udacha and Lyubava, which have a common origin [22], belong to different clusters (see Fig. 1). In addition to cultivars, five lines from three originators (see Table 1) used in the study clustered with the cultivars of other originators (see Fig. 1). This may be the result of an intensive exchange of breeding material between breeding centers.

SSR analysis carried out by Kolobova et al. [23] demonstrated the possibility of potato cultivars clustering according to the tuber color. However, in the present work, such clustering (with low bootstrap values) was observed only for the cultivars with yellow tuber skin or flesh (see Fig. 1, Table 1). The cultivars Fioletovii and Vasilek with blue-violet tuber skin (the flesh of cv. Fioletovii tubers is also blue-violet) are distant from each other on the dendrogram (see Fig. 1).

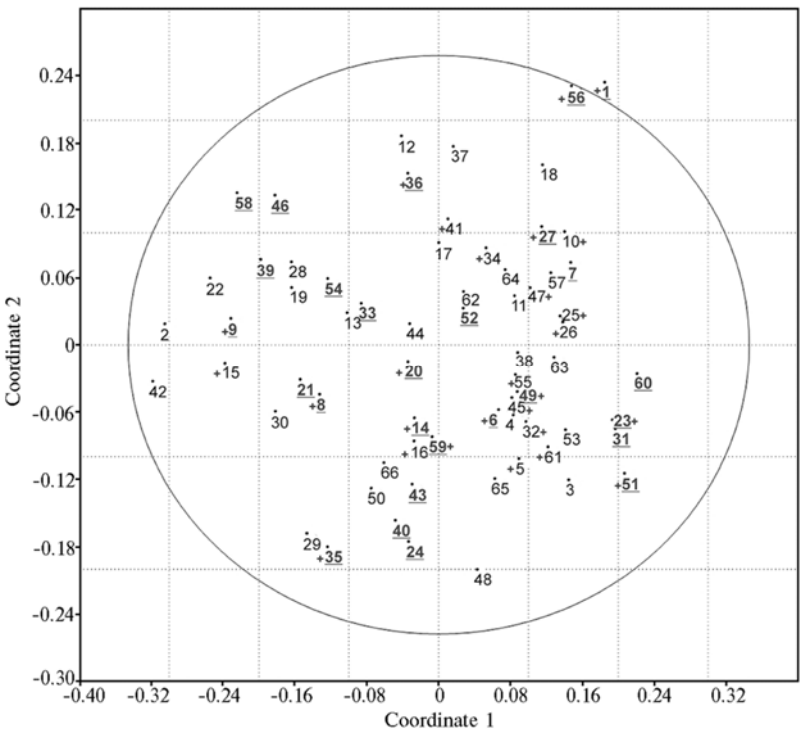


Fig. 2. PCA graph of AFLP analysis data for 60 cultivars and five breeding clones of potatoes. The numbering is as in Table 1. The outgroup comprises *Solanum stoloniferum* (1) and cv. Fioletovii (20). The cultivars resistant to late blight are highlighted in bold and underlined, those resistant to potato virus Y are marked with (+).

On the PCA graph, the analyzed cultivars form a single diffuse pool of genotypes, in which the same external group is distinguished as on the dendrogram,

however, the clustering of cultivars occurs in a slightly different way, although there is a noticeable convergence of the accessions resistant to late blight or to PVY (Fig. 2). It is interesting to note that a cultivar resistance, as it is described by the originator in the State Register does not always coincide with the research data. An example is the study of Klimenko et al. [26]. This is very probably due to the difficulty of visual determination of infection symptoms [26]. *S. stoloniferum* (the outgroup) is located on the PCA plot quite close to the rest of the *S. tuberosum* cultivars and accessions. The explanation may be that *S. stoloniferum* members have been quite often used in breeding programs as donors of resistance to various stresses [34].

The rather high general polymorphism of the analyzed cultivars, the absence of clear clustering and the "unstable" position of the accessions most likely result from the intensive exchange of breeding material, which is currently going on. When selecting parental pairs, breeders include accessions from various world breeding centers, which is confirmed by many studies. For example, SSR analysis of 113 domestic potato cultivars (80 accessions cultivated in Russia and 33 accessions from neighboring countries), including 12 cultivars that were studied in our work, showed no country-based clustering [22]. Even a morphologically little polymorphic collection of 32 potato cultivars grown in Scandinavian countries was not grouped according to the countries of origin as per the AFLP analysis results [19].

We also determined the genomic structure of potato accessions with Structure v. 2.3.4 software. Genomic structure analysis makes it possible to identify common genetic blocks and the ratio of such blocks in each accession to distribute the accessions into subgroups. In comparing the number of subgroups (k) from 2 to 15, the best result (LnLike = –23219.2) was obtained for k = 3.

The resulting graph shows the genomic structure of the studied 65 cultivars and breeding clones as different ratios of three blocks (Fig. 3). Any clear correlation between the ratios of the blocks and any of the considered features (see Table 1) was not revealed. There is some weakly expressed tendency of grouping cultivars resistant to nematodes (groups 1, 2, 3) and PVY (group 3), and cultivars having the same tuber color, namely those with yellow tuber skin, which is probably due to the prevalence of yellow tuber cultivars in the accession set) (see Fig. 3, Table 1).

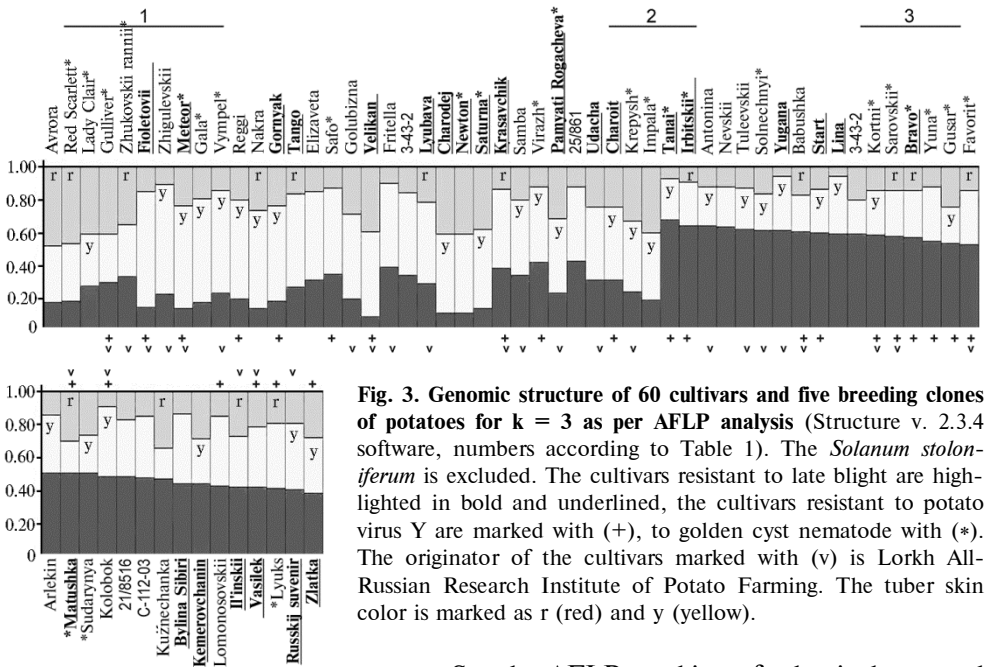


Fig. 3. Genomic structure of 60 cultivars and five breeding clones of potatoes for k = 3 as per AFLP analysis (Structure v. 2.3.4 software, numbers according to Table 1). The *Solanum stoloniferum* is excluded. The cultivars resistant to late blight are highlighted in bold and underlined, the cultivars resistant to potato virus Y are marked with (+), to golden cyst nematode with (∗). The originator of the cultivars marked with (v) is Lorkh All-Russian Research Institute of Potato Farming. The tuber skin color is marked as r (red) and y (yellow).

So, the AFLP marking of selectively neutral

regions in the genomes of 65 cultivars and promising breeding clones of potatoes, including modern domestic cultivars, revealed a high level of genomic polymorphism. No clear clustering was detected according to cultivar origin (a certain breeding center) or morphological traits. It has been shown that the AFLP analysis with the primer combinations we have chosen is promising for genotyping potato cultivars during the initial screening in collections and the primary selection for target traits for subsequent in-depth analysis.

REFERENCES

1. Russell J.R., Fuller J.D., Macaulay M., Hatz B.G., Jahoor A., Powell W., Waugh R. Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theoretical and Applied Genetics*, 1997, 95: 714-722 (doi: 10.1007/s001220050617).
2. Semagn K., Bjørnstad E., Ndjiondjop M.N. An overview of molecular marker methods for plants. *African Journal of Biotechnology*, 2006, 5(25): 2540-2568.
3. Khlestkina E.K. *Vavilovskii zhurnal genetiki i selektsii*, 2013, 17(4/2): 1044-1053 (in Russ.).
4. Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Friters A., Pot J., Paleman J., Kuiper M., Zabeau M. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 1995, 23(21): 4407-4414 (doi: 10.1093/nar/23.21.4407).
5. Kardolus J.P., van Eck H.J., van den Berg R.G. The potential of AFLPs in biosystematics: a first application in *Solanum* taxonomy (*Solanaceae*). *Plant Systematics and Evolution*, 1998, 210(1-2): 87-103 (doi: 10.1007/BF00984729).
6. Hassan F.S.C., Solouki M., Fakheri B.A., Nezhad N.M., Masoudi B. Mapping QTLs for physiological and biochemical traits related to grain yield under control and terminal heat stress conditions in bread wheat (*Triticum aestivum* L.). *Physiology and Molecular Biology of Plants*, 2018, 24(6): 1231-1243 (doi: 10.1007/s12298-018-0590-8).
7. El-Esawi M.A., Alaraidh I.A., Alsahli A.A., Ali H.M., Alayafi A.A., Witczak J., Ahmad M. Genetic variation and alleviation of salinity stress in barley (*Hordeum vulgare* L.). *Molecules*, 2018, 23(10): 2488 (doi: 10.3390/molecules23102488).
8. D'yachenko E.A., Ryzhova N.N., Vishnyakova M.A., Kochieva E.Z. Molekulyarno-geneticheskoe raznoobrazie gorokha (*Pisum sativum* L.) iz kollektsii VIR na osnove dannykh AFLP-analiza. *Genetika*, 2014, 50(9): 1040-1049 (doi: 10.7868/s0016675814090045).
9. El-Esawi M.A., Al-Ghamdi A.A., Ali H.M., Alayafi A.A., Witczak J., Ahmad M. Analysis of genetic variation and enhancement of salt tolerance in French pea (*Pisum sativum* L.). *International Journal of Molecular Sciences*, 2018, 19(8): 2433 (doi: 10.3390/ijms19082433).
10. Kochieva E.Z., Ryzhova N.N. *Genetika*, 2003, 39(12): 1589-1593 (in Russ.).
11. Kochieva E.3., Ryzhova N.N. *Doklady Akademii nauk*, 2009, 425(2): 256-258 (in Russ.).
12. Bamberg J.B., del Rio A.H. Selection and validation of an AFLP marker core collection for the wild potato *Solanum microdontum*. *American Journal of Potato Research*, 2014, 91(4): 368-375 (doi: 10.1007/s12230-013-9357-5).
13. McGregor C.E., van Treuren R., Hoekstra R., van Hintum T.J. Analysis of the wild potato germplasm of the series Acaulia with AFLPs: implications for ex situ conservation. *Theoretical and Applied Genetics*, 2002, 104(1): 146-156 (doi: 10.1007/s001220200018).
14. Bryan G.J., McLean K., Waugh R., Spooner D.M. Levels of Intra-specific AFLP diversity in tuber-bearing potato species with different breeding systems and ploidy levels. *Frontiers in Genetics*, 2017, 8: 119 (doi: 10.3389/fgene.2017.00119).
15. Hawkes J.G. The potato: evolution, biodiversity and genetic resources. *American Potato Journal*, 1990, 67(10): 733-735 (doi: 10.1007/BF03044023).
16. Jacobs M.M., van den Berg R.G., Vleeshouwers V.G., Visser M., Mank R., Sengers M., Hoekstra R., Vosman B. AFLP analysis reveals a lack of phylogenetic structure within *Solanum* section Petota. *BMC Evolutionary Biology*, 2008, 8: 145 (doi: 10.1186/1471-2148-8-145).
17. Manoko M.L.K., van den Berg R.G., Feron R.M.C., van der Weerden G.M., Mariani C. AFLP markers support separation of *Solanum nodiflorum* from *Solanum americanum* sensu stricto (*Solanaceae*). *Plant Systematics and Evolution*, 2007, 267(1-4): 1-11 (doi: 10.1007/s00606-007-0531-4).
18. Solis J.S., Ulloa D.M., Rodŕguez L.A. Molecular description and similarity relationships among native germplasm potatoes (*Solanum tuberosum* ssp. *tuberosum* L.) using morphological data and AFLP markers. *Electronic Journal of Biotechnology*, 2007, 10(3): 436-443 (doi: 10.2225/vol10-issue3-fulltext-14).
19. Vetelainen M., Gammelgard E., Valkonen J.P.T. Diversity of Nordic landrace potatoes (*Solanum tuberosum* L.) revealed by AFLPs and morphological characters. *Genetic Resources and Crop Evolution*, 2005, 52(8): 999-1010 (doi: 10.1007/s10722-003-6129-y).
20. Cicatelli A., Baldantoni D., Iovieno P., Carotenuto M., Alfani A., De Feis I., Castiglione S. Genetically biodiverse potato cultivars grown on a suitable agricultural soil under compost amendment or mineral fertilization: yield, quality, genetic and epigenetic variations, soil properties.

Science of the Total Environment, 2014, 493: 1025-1035 (doi: 10.1016/j.scitotenv.2014.05.122).
21. Gavrilenko T., Antonova O., Shuvalova A., Krylova E., Alpatyeva N., Spooner D., Novikova L. Genetic diversity and origin of cultivated potatoes based on plastid microsatellite polymorphism. *Genetic Resources and Crop Evolution*, 2013, 60(7): 1997-2015 (doi: 10.1007/s10722-013-9968-1).
22. Antonova O.Yu., Shvachko N.A., Novikova L.Yu., Shuvalov O.Yu., Kostina L.I., Klimenko N.S., Shuvalova A.R., Gavrilenko T.A. *Vavilovskii zhurnal genetiki i selektsii*, 2016, 20(5): 596-606 (doi: 10.18699/VJ16.181) (in Russ.).
23. Kolobova O.S., Malyuchenko O.P., Shalaeva T.V., Shanina E.P., Shilov I.A., Alekseev Ya.I., Velishaeva N.S. *Vavilovskii zhurnal genetiki i selektsii*, 2017, 21(1): 124-127 (doi: 10.18699/VJ17.230) (in Russ.).
24. Gavrilenko T.A., Klimenko N.S., Antonova O.Yu., Lebedeva V.A., Evdokimova Z.Z., Gadzhiev N.M., Apalikova O.V., Alpat'eva N.V., Kostina L.I., Zoteeva N.M., Mamadbokirova F.T., Egorova K.V. *Vavilovskii zhurnal genetiki i selektsii*, 2018, 22(1): 35-45 (doi: 10.18699/VJ18.329) (in Russ.).
25. Klimenko N.S., Antonova O.Yu., Kostina L.I., Mamadbokirova F.T., Gavrilenko T.A. *Trudy po prikladnoi botanike, genetike i selektsii*, 2017, 178(4): 66-75 (doi: 10.30901/2227-8834-2017-4-66-75) (in Russ.).
26. Klimenko N.S., Antonova O.Yu., Zheltova V.V., Fomina N.A., Kostina L.I., Mamadbokirova F.T., Gavrilenko T.A. Screening OF Russian POTATO CULTIVARS (*Solanum tuberosum* L.) with DNA markers linked to the genes conferring extreme resistance to potato virus Y. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(5): 958-969 (doi: 10.15389/agrobiology.2019.5.958eng).
27. Biryukova V.A., Shmyglya I.V., Zharova V.A., Beketova M.P., Rogozina E.V., Mityushkin A.V., Meleshin A.A. *Rossiiskaya sel'skokhozyaistvennaya nauka*, 2019, 5: 17-22 (doi: 10.31857/S2500-26272019517-22) (in Russ.).
28. Biryukova V.A., Shmyglya I.V., Abrosimova S.B., Zapekina T.I., Meleshin A.A., Mityushkin A.V., Manankov V.V. *Zashchita kartofelya*, 2015, 1: 3-7 (in Russ.).
29. Puchooa D. A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). *African Journal of Biotechnology*, 2004, 3(4): 253-255.
30. Hammer O., Harper D.A.T., Ryan P.D. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 2001, 4(1): 1-9.
31. Pritchard J.K., Stephens M., Donnelly P. Inference of population structure using multilocus genotype data. *Genetics*, 2000, 155(2): 945-959.
32. Hubisz M.J., Falush D., Stephens M., Pritchard J.K. Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, 2009, 9(5): 1322-1332 (doi: 10.1111/j.1755-0998.2009.02591.x).
33. Esfahani S.T., Shiran B., Balali G. AFLP markers for the assessment of genetic diversity in European and North American potato varieties cultivated in Iran. *Crop Breeding and Applied Biotechnology*, 2009, 9(1): 75-86 (doi: 10.12702/1984-7033.v09n01a11).
34. Machida-Hirano R. Diversity of potato genetic resources. *Breed Science*, 2015, 65(1): 26-40 (doi: 10.1270/jsbbs.65.26).

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A MULTIPLEX MICROSATELLITE PCR METHOD FOR DETECTION OF *Brassica* L. A, B AND C GENOME FRAGMENT INTROGRESSIONS UPON INTERSPECIFIC HYBRIDIZATION

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Abstract

The genus *Brassica* L. is a source of oilseeds, vegetables, spices, fodder and ornamental crops widely cultivated around the world. The six most cultivated species of the genus *Brassica* comprise allotetraploid species *B. juncea* (L.) Czern. (2*n* = 36, genome AABB), *B. napus* L. (2*n* = 38, genome AACC) and *B. carinata* A. Braun (2*n* = 34, genome BBCC), which are natural hybrids of corresponding diploid species *B. rapa* L. (2*n* = 20, genome AA), *B. nigra* L. (2*n* = 16, genome BB), and *B. oleracea* L. (2*n* = 18, genome CC). An effective way to increase the genetic diversity and improve the agronomic traits of *Brassica* crops, such as high yields, resistance to diseases, and abiotic stresses is to introduce traits of interest by the interspecific hybridization. To control the introgression of genomic material upon the hybridization, the development and implementation of genetic markers are necessary. This paper proposes an effective approach for controlling the introgression of A, B, and C genomes of *Brassica* in intraspecific hybrids. The investigation aimed to develop a high-throughput technology based on multiplex PCR analysis of genome-specific microsatellite markers for controlling the introgression of A-, B-, and C-genomes in *Brassica* intraspecific hybrids. Control samples were obtained from the Center for Genetic Resources CGN (Netherlands) and the All-Russian Institute of Plant Genetic Resources N.I. Vavilov (VIR, St. Petersburg). Plant material for the genomic material introgression study were obtained from the Timofeev Breeding Station (Moscow). Genomic DNA was extracted by sorbent method. PCR was run with specific primers for the Na10-D09, Na12-A02, Na12-F12, Ni2-B02, Ni2-F02, Ni3-G04B, Ol12-A04, Ra2-E12, BRMS-043, BN6A2 loci. Fluorescently labelled PCR products were analyzed by high-resolution electrophoresis using a Nanofor-05 genetic analyzer (Syntol — The Institute for Analytical Instrumentation, Russia). The length of the amplified DNA fragments was determined using the DNA Fragment Analysis software (The Institute for Analytical Instrumentation, Russia). A multiplex PCR technique was developed based on the six microsatellite loci Na12-A02, BRMS-043, Na10-D09, Ol12-A04, Ni2-F02, BN6A2, allowing us to determine the markers of three *Brassica* genomes in one run. A, B, and C genome-specific markers were identified during multiplex PCR analysis of control samples of six *Brassica* species with known taxonomic attributions and genome compositions: *B. rapa* (AA), *B. nigra* (BB), *B. oleracea* (CC), *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC). The length of marker fragments was determined by high resolution electrophoresis using a genetic analyzer with an accuracy of one nucleotide. A-genome specific markers were identified at the loci Na12-A02 (178 bp, 180 bp, 182 bp), BRMS-043 (303 bp, 307 bp, 313 bp), and Na10-D09 (283 bp, 285 bp, 291 bp, 293 bp, 299 bp). B-genome specific markers were detected at the loci Na12-A02 (196 bp, 198 bp, 200 bp, 202 bp, 204 bp, 212 bp, 214 bp, 216 bp), Ol12-A04 (125 bp, 127 bp, 129 bp), Ni2-F02 (198 bp, 200 bp ., 202 bp, 204 bp, 208 bp), and BN6A2 (222 bp). C-genome specific markers were detected at the loci Na12-A02 (164 bp, 168 bp, 170 bp) and Ni2-F02 (164 bp, 166 bp, 168 bp, 186 bp). The developed multiplex PCR system reveals introgressions of fragments of genomes A, B and C in the genetic profiles of interspecific hybrids

(Et2 × KK)2 × Tsv9, (Et2 × KK)1, Green × FBLM(1), JR × Agr2ki, BK × ZM1-1(6), BK × ZM1-1(8), BK, and KB. The method also confirmed the presence of the corresponding genomes in the studied samples with a known breeding history. Due to the automation, analysis allows the large-scale screening of plant samples. The proposed technology can be used in breeding practice as a tool for controlling the introgression of A, B and C genome material upon the interspecific hybridization, as well as controlling its inheritance in subsequent generations.

Keywords: *Brassica*, U triangle, *Brassica* genomes, interspecific hybridization, introgression, microsatellites, genome-specific markers

Among *Brassicaceae* family, the genus *Brassica* L. is of the greatest economic value as a source of oilseeds, vegetables, spices, fodder and ornamental crops widely cultivated all over the world. The genus *Brassica* includes 39 species [1]. Most of the cultivated plants belong to *B. napus* L. (oilseed rape, rutabaga), *B. rapa* L. (Asian collard and head cabbage, turnip and turnip), *B. oleracea* L. (head, Brussels sprouts and cauliflower, broccoli, kohlrabi), *B. juncea* Czern. (Sarepta mustard), *B. carinata* A. Braun (Ethiopian mustard), and *B. nigra* (L.) K. Koch (black mustard).

Morphological, cytogenetic and molecular relationships between cultivated *Brassica* species have been extensively studied. There are six cytogenetic groups of *Brassica* [2]. According to the U's evolutionary model [3], natural hybridization of three diploid species, the *B. rapa* ($2n = 20$, AA genome), *B. nigra* ($2n = 16$, BB), and *B. oleracea* ($2n = 18$, CC), resulted in appearance of amphidiploid species *B. juncea* ($2n = 36$, AABB), *B. napus* (2n = 38, AACC), and *B. carinata* (2n = 34, BBCC). A comparative analysis of the three *Brassica* genomes reveals significant conservatism, which indicates that all three genomes completely inherited from the common ancestor, were significantly rearranged [4]. The difference in the number of chromosomes presumably arose as a result of fusion/fragmentation of chromosomes during species divergence [5]. Analysis of chloroplast, mitochondrial, and nuclear genomes shows two evolutionary pathways of *Brassica* species, the *B. nigra* (B genome) and *B. rapa*/*B. oleracea* (A/C genomes) [6]. According to genetic mapping data, genomes A and C, despite the difference in the number of chromosomes, are highly colinear [7]. Divergence between *B. nigra* and *B. rapa*/*B. oleracea* presumably occurred 7.9 million years ago, *B. rapa* and *B. oleracea* diverged about 4 million years ago. The hybridization of the species that gave rise to *B. napus* apparently took place 10 thousand years ago [6]. Also, some differences were revealed in the structure of subgenomes of allotetraploid species as compared to the corresponding genomes of diploid species [8, 9]. Differentiation of subgenomes during interspecific hybridization and long-term domestication of polyploid *Brassica* species could occur due to translocation, inversion, deletion, duplication, and homeologous recombination [8].

Distant hybridization is an effective way to improve the agronomic traits of *Brassica* crops, such as high yield, resistance to diseases and adverse environmental conditions [10]. In crosses of *Brassica* species with each other and with other related members of the *Brassicaceae* family, the barrier of interspecies or intergeneric incompatibility is successfully overcome by embryo rescue or somatic hybridization techniques [11, 12].

Wild members of *Brasicaceae* family can be donors of potentially useful agronomic traits for crop improvement [12, 13], e.g. *Brassica maurorum* Durieu and *Eruca versicaria* ssp. *sativa* (Mill.) Thell. resistant to white rust *Albugo candida* Pers.) [14], *Raphanus sativus* L. resistant to nematode *Heterodera schachtii* Schmidt [15], Sinapis alba L. resistant to nematode *H. schachtii* and blackleg *Leptosphaeria maculans* (Sowerby) P. Karst. [16], *Sinapis arvensis* L. resistant to blackleg *L. maculans*) [17], *Sinapis incana* L. and *Diplotaxis* L. as sources of cytoplasmic male sterility [18].

Cultivated *Brassica* species also possess useful agronomic traits, for

example, *B. oleracea* shows resistance to powdery mildew caused by *Hyaloperonospora parasitica* (Pers.) Constant [12], *B. rapa*, *B. oleracea* and *B. napus* are resistant to clubroot disease (*Plasmodiophora brassicae* Woronin) [19], *B. carinata* to vascular disease caused by *Xanthomonas campestris* (Pammel) Dowson [20], and *B. juncea* possesses tolerance to heavy metals [21]. Brassica species with B genome are genetic donors of resistance to blackleg and unfavorable environmental conditions [22, 23].

Interspecific hybridization is a natural phenomenon which results in appearance of new species or introgression of adaptive traits. In *Brassica* allotetraploids resynthesized from diploid parental species the genetic diversity increases. Allotetraploids *B. juncea*, *B. napus*, and *B. carinata* were de novo assembled in order to expand their genetic basis [24-26]. The *Raphanus* genome was used to produce synthetic nematode-resistant allotetraploids [12]. *Brassica* allohexaploids ($2n$ = 54, AABBCC) derived from crossing *B. napus* × *B. nigra* and *B. carinata* × *B. rapa* [27]. In hybridization of *B. carinata* and *B. rapa*, meiotically stable allohexaploids were obtained [28-31]. For a long time, meiotic aberrations due to uncontrolled pairing between three genomes hindered creation of stable allohexaploids with the expected number of chromosomes [32]. Synthetic polyploids are involved into crosses as sources of new agronomic traits

Mechanisms underlying relationships between genomes A, B and C are of practical importance. A capability of economically important *Brassica* crops to acquire traits from *B. nigra* and related members of genera *Sinapis* and *Raphanus* may depend on the degree of genomic collinearity between *B. nigra* and widely cultivated *B. oleracea* and *B. rapa* species. Detection of homologous regions in genomes will improve the methodology of genetic determinant transfer into resynthesized hybrids via homeologous recombination followed by marker-assisted selection of forms with the desired hybrid chromosomes.

Modern breeding technologies that allow distant hybridization required effective breeding control based on genetic analysis. Along with cytogenetic methods, such as fluorescence in situ hybridization technique (FISH) [31] and genomic in situ hybridization (GISH) [32, 33] techniques, DNA markers, e.g. RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) [34], SSR (simple sequence repeats) [35-37], and SNP (single nucleotide polymorphism) [38], are widely used to study genetic relationships between *Brassica* species, evolutionary changes in genomes and to control chromosome inheritance when creating digenomic and trigenomic hybrids.

Microsatellite markers are effective for studying introgressions in interspecific hybrids. Due to uniform distribution over the genome, codominant inheritance and high polymorphism, the microsatellite markers are a good tool to estimate homozygosity or heterozygosity of loci. Since the genomes of related *Brassica* species are highly linear, they are characterized by homology of flanking sequences of microsatellite loci. Due to conservativeness of flanking sequences, loci found in one species can in most cases be used to study related species.

For genus *Brassica*, microsatellite markers were developed independently by several research teams [39-41]. Linkage groups and locations on the genetic map have been established for many of these loci. A number of works have shown advantages of microsatellite loci in assessment of interspecific and intraspecific diversity of *Brassica* [42, 43], distinctness, uniformity, and stability of cultivars [44, 45], and in use as markers of diseases resistance genes, for example, upon clubroot [46, 47] and vascular disease [48].

In this research study, we have identified effective A-, B-, and C-genome specific microsatellite markers of *Brassica* and determined their length by a high-resolution electrophoresis with an accuracy of one nucleotide. The obtained results show the possibility of using these markers for the analysis of breeding samples upon interspecific hybridization.

Our goal was to develop an efficient methodology based on multiplex micro-satellite PCR analysis of genome-specific microsatellite markers, which would be suitable for monitoring the introgression of the A, B and C genomes in *Brassica* plants during distant hybridization.

Materials and methods. The control plants were obtained from the CGN Center for Genetic Resources (the Netherlands) and Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg). Plants for the introgression study were provided by the LLC. Timofeev Breeding Station (Moscow).

Genomic DNA was extracted by adsorption method on a sorbent in accordance with instructions for the Fitosorb kit (OOO NPF Sintol, Russia). Five plants of each denomination were used. Microvolumes of solutions were dispensed automatically (Lenpipet, Russia), the sedimentation was carried out using a Centrifuge 5415D (Eppendorf, Germany). The plant biomass was lysed (a Termit thermostat, NPO DNA-Tekhnologiya, Russia). A Microspin FV-2400 mini-vortex centrifuge (SIA Biosan, Latvia) was used to mix and sediment DNA samples.

Plant DNA was amplified by PCR (a CFX-96 thermal cycler, Bio-Rad, USA) in a 25 μl reaction mixture of 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 2.5 mM MgCl2; 5 units/μl of Taq-DNA polymerase (NPO DNA-Technology LLC, Russia), 25 mM dNTP (Medigen LLC, Russia), 5-20 pmol of each primer, depending on the fluorescence intensity (LLC NPF Syntol, Russia), and 10 ng of DNA template. The primers were specific to the loci Na10-D09, Na12-A02, Na12-F12, Ni2-B02, Ni2-F02, Ni3-G04B, Ol12-A04, Ra2-E12 [39], BRMS-043 [40], and BN6A2 [41]. The PCR protocol was as follows: 5 min at 95 °C; 30 s at 94 °C, 30 s at 48 °C, 30 s at 72 °C (30 cycles); 5 min at 72 °C. To confirm amplification, the products were electrophoresed on 2% agarose gel stained with ethidium bromide.

Fluorescently labeled PCR fragments were analyzed by capillary electrophoresis under denaturing conditions in a Nanofor-05 genetic analyzer (OOO NPF Sintol, FGBNU Institute for Analytical Instrumentation RAS — IAI RAS, Russia) according to the instructions for the instrument (Shared-Use Equipment Center Biotechnology, All-Russian Research Institute of Agricultural Biotechnology). For fragment analysis, 1 μl of the PCR product mixed with 1 μl of molecular weight marker S-450 (OOO NPF Syntol, Russia) and 8 μl of Super DI formamide (MCLab, United States), were denatured for 5 min at 95 °C.

The PCR fragment length was determined using a DNA Fragment Analysis software tool (FGBNU IAI RAS, Russia).

Results. In previous study, using an 8% polyacrylamide gel electorophoresis, we revealed the most polymorphic loci suitable for *B. rapa*, *B. nigra*, *B. oleracea*, *B. napus*, *B. juncea*, and B. *carinata* species differentiation [49-51]. Since most of these loci have conserved flanking sequences in the A, B, and C genomes, their amplification occurs in all six *Brassica* species, which allows comparative analysis and identification of genome-specific markers. As a result, 10 microsatellite loci were selected. Length polymorphism of the microsatellite fragments was assessed in the control samples of six species, the *B rapa* (AA), *B. nigra* (BB), *B. oleracea* (CC), *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC), with known species attribution and genomic composition (Table 1).

1. Members of six *Brassica* L. species and interspecies hybrids with studied microsatellite fragment length polymorphism

Name	Description
Control samples	
1-02 (VIR), Ural (VIR), VikRos (VIR)	*B. napus* L. ($2n$ = 38, genome AACC)
CGN06832, CGN06818, 114 (VIR), 107 (VIR)	*B. rapa* L. ($2n$ = 20, genome AA)
CGN06619, CGN006634, CGN02656	*B. nigra* L. ($2n$ = 16, genome BB)
CGN03950, CGN03952	*B. carinata* A. Braun ($2n$ = 34, genome BBCC)
CGN15778, CGN06998, CGN07004, CGN07022	*B. oleracea* L. ($2n$ = 18, genome CC)
CGN04588, CGN04594, CGN015193	*B. juncea* (L.) Czern. ($2n$ = 36, genome AABB)
Breeding samples	
Nos. 63, 69, 70,]97,]98,]106	Regenerants derived from in vitro microspore culture of BC_2 plants upon interspecific hybridization *B. oleracea* and *B. carinata*: {[(*B. oleracea* × *B. carinata*) × *B. oleracea*] × *B. oleracea*}
(Et2 × KK)2 × Tsv9	Progeny BC_1 from interspecific hybridization (*B. oleracea* × *B. rapa*) × *B. oleracea*
Tsv9	Inbred line of *B. oleracea*
KK	Inbred line of *B. rapa*
(Et2 × KK)1	Interspecific hybrid F_1 (*B. oleracea* × *B. rapa*)
Grin × FBLM(1)	Interspecific hybrid F_1 (*B. oleracea* × *B. juncea*)
FBLM	Inbred line of *B. juncea*
JR	Inbred line of *B. rapa*
JR × Agr2ki	Interspecific hybrid F_1 (*B. rapa* × *B. oleracea*)
PR3	Inbred line of *B. oleracea*
ZM tetr	Tetraplois line of *B. oleracea*
BK × ZM1-1(6), BK × ZM1-1(8)	Progeny BC_1 from interspecific hybridization *B. oleracea* and *B. carinata*: [(*B. oleracea* × *B. carinata*) × *B. oleracea*)]
B. carinata 1	Inbred line of *B. carinata*
BK	Interspecific hybrid F_1 (*B. oleracea* × *B. carinata*)
B. carinata 2	Inbred line of *B. carinata*
KB	Interspecific hybrid F_1 (*B. carinata* × *B. oleracea*)

Note. CGN — Centre for Genetic Resources (the Netherland), VIR — Vavilov All-Russian Institute of Genetic Resources (St. Petersburg). The breeding samples are produced by OOO Timofeev Breeding Station (Moscow).

Fragments of a certain length identified in plants of the species *B. rapa* (AA) and absent in control samples of *B. nigra* (BB) and *B. oleracea* (CC) were taken as markers of the A genome material (A-genome-specific). Fragments identified in *B. nigra* (BB) but not in *B. rapa* (AA) and *B. oleracea* (CC) were deemed markers of the B genome (B-genome-specific). Fragments identified in B. oleracea (CC) and not found in *B. rapa* (AA) and *B. nigra* (BB) were markers of the C genome (C-genome-specific).

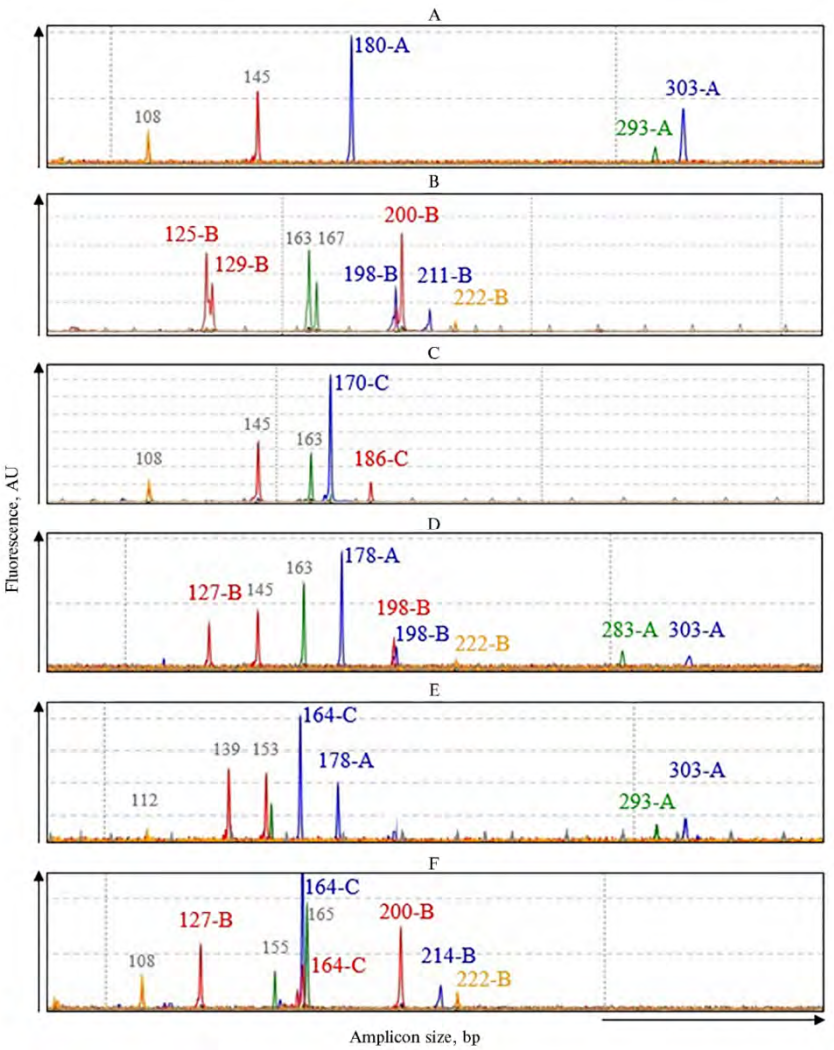
PCR analysis of Na12-A02 locus in *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) plants revealed the A-, B-, and C-genome-specific fragments. Also, a codominant combination of the corresponding fragments was identified in amphidiploid species *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC). Locus BRMS-043 was amplified only in plants with A genome, i.e. *B. rapa* (AA), *B. juncea* (AABB), and *B. napus* (AACC), which allows us to distinguish them from plants with B and C genomes. For Na10-D09 locus, A-genome-specific fragments were found. PCR products were synthesized in the studied samples of all six species. However, only the DNA fragments found in *B. rapa* (AA) plants significantly differed in length from those in *B. nigra* (BB) and *B. oleracea* (CC) plants. B-genome-specific fragments were identified for loci Ol12-A04 and BN6A2. PCR analysis of Ni2-F02 locus revealed B- and C-genome-specific fragments. In loci NI2-B02, RA2-E12, NA12-F12, fragments of similar length were found in control samples of *B. rapa*, *B. nigra*, and *B. oleracea*. That is, these loci were unsuitable for studying the A-, B-, and C-genome introgressions and not further used.

Based on investigation of the control samples, six microsatellite loci (Na12-A02, BRMS-043, Na10-D09, Ol12-A04, Ni2-F02, and BN6A2) were selected to develop a multiplex PCR system that allows assay for all loci simultaneously (Table 2). For amplification of microsatellite loci in one PCR run, a

single optimal annealing temperature for primer pairs was selected (T_m = 48 °C). In the multiplex system, of each microsatellite locus was amplified with a pair of specific primers, one of which was labeled with a certain fluorescent dye (FAM, R6G, ROX, and Sy630). This allows for separate assay of PCR fragments for each locus through the corresponding detection channel (see Table 2). The loci were selected in such a way that the ranges of the lengths of their fragments did not overlap when detected through the same channel. The PCR fragment length was measured with a single nucleotide accuracy due to use of high-resolution capillary electrophoresis with fluorescence detection technique (an automatic genetic analyzer Nanofor-05).

2. Parameters of the multiplex PCR system for analysis of A, B and C genome introgressions in *Brassica* L. species based on microsatellite loci

Locus	Genome specificity	Stain	Amplicon size, bp
Na12-A02	ABC	FAM	164-216
BRMS-043	A	FAM	303-313
Na10-D09	A	R6G	155-299
OI12-A04	B	ROX	125-153
Ni2-F02	BC	ROX	164-200
BN6A2	B	Sy630	108-222



Genetic profiling of control plants of six *Brassica* L. species by a multiplex PCR analysis of microsatellite

loci: A — CGN06832 (*B. rapa*, genome AA), B — CGN006634 (*B. nigra*, genome BB), C — CGN15778 (*B. oleracea*, genome CC), D — CGN015193 (*B. juncea*, genome AABB), E — ВИР ВикРос (*B. napus*, genome AACC), F — CGN03950 (*B. carinata*, genome BBCC). The color of the peak (fragment) corresponds to the detection channel on the Nanofor-05 device (OOO NPF Syntol —FGBNU Institute for Analytical Instrumentation RAS, Russia) and indicates the locus the fragment belongs to, i.e. blue (FAM) for Na12-A02 and BRMS-043, green (R6G) for NA10-D09, red (ROX) for Ol12-A04 and Ni2-F02, and orange (SY630) for BN6A2. Non-discriminatory fragments amplified simultaneously in two or three genomes are shown in gray. For a description of the samples, see Table 1.

The developed multiplex system generates output data as digitized genetic profiles of *Brassica* samples, in which each peak corresponds to a PCR fragment of a certain length (Fig.). Microsatellite analysis of the control plants identified fragments specific for genomes A, B, and C. In the genetic profile of the control sample CGN06832 (*B. rapa*) there were 180 bp (locus Na12-A02), 303 bp (locus BRMS-043), and 293 bp (locus Na10-D09) fragments characteristic of the genome A (see Fig., A). The profile of the sample CGN006634 (*B. nigra*) contained distinctive markers of the B genome with a length of 198 bp and 211 bp (locus Na12-A02), 125 bp and 129 bp (locus OL12-A04), 200 bp (locus NI2-F02), and 222 bp (locus BN6A2) (see Fig., B). In sample CGN15778 (*B. oleracea*), the 170 bp (locus Na12-A02) and 186 bp (locus Ni2-F02) C-genome-specific fragments were found (see fig., B). Genetic profiles of the allotetraploid species showed a codominant combination of fragments of the corresponding diploid genomes (see Fig. D-F).

From the profiling data, we identified the allele composition of each microsatellite locus and the markers specific for genomes A, B and C (Table 3).

3. A-, B- and C-genome-specific markers revealed in six *Brassica* L. species by a multiplex PCR analysis of microsatellite loci

Locus	Genome	Microsatellite fragment length, bp		
		A-specific	B-specific	C-specific
Na12-A02	ABC	178, 180, 182	196, 198, 200, 202, 204, 212, 214, 216	164,168,170
BRMS-043	A	303, 307, 313	–	–
Na10-D09	A	283, 285, 287, 291, 293, 299	–	–
Ol12-A04	B	–	125, 127, 129	–
Ni2-F02	BC	–	198, 200, 202, 204, 208	164, 166, 168, 186
BN6A2	B	–	222	–

N o t e. Dashes mean the absence of a corresponding genome-specific marker in the loci.

4. Genome-specific markers (bp) **revealed in lines and interspecific hybrids of *Brassica* L. by a multiplex PCR analysis of microsatellite loci:**

Sample	Locus						Genome
	Na12-A02	BRMS-043	Na10-D09	Ol12-A04	Ni2-F02	BN6A2	
KK	180^{A}	303^{A}	293^{A}	145	–	108	AA
JR	180^{A}	311^{A}	285^{A} 341^{A}	143	–	114	AA
No. 63	164^{C}	–	155	–	166^{C}	108	CC
No. 69	164^{C}	–	155	–	166^{C}	108	CC
No. 70	164^{C}	–	155	–	166^{C}	108	CC
No. 97	164^{C}	–	155	–	166^{C}	108	CC
No. 98	164^{C}	–	155	–	166^{C}	108	CC
No. 106	164^{C}	–	155	–	166^{C}	108	CC
Tsv9	164^{C}	–	155	–	166^{C}	108	CC
PR3	164^{C}	–	–	–	166^{C} 186^{C}	108	CC
ZM tetr	164^{C}	–	–	–	166^{C} 186^{C}	108	CC
BK × ZM1-1(6)	164^{C} 168^{C}	–	167	–	–	108	CC
BK × ZM1-1(8)	164^{C} 168^{C}	–	155	–	166^{C}	108	CC
(Et2 × KK)2 × Tsv9	170^{C} 180^{A}	303^{A}	155 293^{A}	145	166^{C}	108	AACC
(Et2 × KK)1	170^{C} 180^{A}	303^{A}	293^{A}	145	166^{C}	108	AACC

Continued Table 4

JR × Agr2ki	164^{C}	311^{A}	287^{A}	145	166^{C}	108	AACC
	180^{A}					114	
FBLM	178^{A}	313^{A}	163	127^{B}	198^{B}	222^{B}	AABB
	198^{B}		285^{A}	145			
Grin × FBLM(1)	164^{C}	313^{A}	163	127^{B}	166^{C}	108	AABBCC
	178^{A}		287^{A}	145	198^{B}	222^{B}	
	198^{B}						
B. carinata 1	16	–	155	127^{B}	164^{C}	108	BBCC
			167		200^{B}		
BK	164^{C}	–	155	127^{B}	166^{C}	108	BBCC
	214^{B}		167		200^{B}	222^{B}	
B. carinata 2	164^{C}	–	155	127^{B}	166^{C}	108	BBCC
	214^{B}		167		200^{B}	222^{B}	
KB	164^{C}	–	155	127^{B}	166^{C}	108	BBCC
	168^{C}		167		200^{B}	222^{B}	
	214^{B}						

N o t e. Superscripts (^{A}, ^{B}, ^{C}) indicate the genomic specificity of the marker fragment. Dashes indicate the absence of the corresponding genome-specific marker of the indicated locus. For a description of the samples, see Table 1.

We also used the developed PCR system to identify A-, B-, and C-genome-specific fragments in breeding samples (see Table 1), obtained their genetic profiles and found genome-specific fragments (Table 4). Consequently, the multiplex system used makes it possible to reliably detect the of A, B, and C genome fragment introgressions in species of the genus *Brassica*. Codominant combinations of genomic fragments in the genetic profiles of breeding samples (Et2 × KK)2 × Tsv9 obtained from hybridization (*B. oleracea* × *B. rapa*) × *B. oleracea*, (Et2 × KK)1 (F_1 *B. oleracea* × *B. rapa*), Grin × FBLM (1) (F_1 *B. oleracea* × *B. juncea*), JR × Agr2ki (F_1 *B. rapa* × *B. oleracea*), BK (F_1 *B. oleracea* × *B. carinata*) and KB (F_1 *B. carinata* × *B. oleracea*) confirmed introgressions as a result of interspecific hybridization. The data obtained by microsatellite analysis correspond to the breeding history of the samples.

The developed system for multiplex analysis based on six microsatellite loci specific for genus Brassica A, B, and C genomes reliably differentiates plants of six species of the U triangle and also can evaluate genetic diversity, since each genomic marker possesses several allelic variants. Each genome is defined by at least two markers, which serve as internal controls for each other.

This multiplex system also provides tracing an introgression of certain regions of the *Brassica* genome, since linkage groups for the loci used in it have been determined (http://www.brassica.info/resource/markers/ssr-exchange.php). For example, it was shown that the BRMS-043 locus is associated with resistance to vascular diseases of *B. rapa* (48).

The effectiveness of DNA markers in genotyping new forms and detection of genomic material transfer during plant breeding has been demonstrated in a number of works. The analysis of potential genetic changes in 25 synthesized allohexaploids (H1 *B. rapa* × *B. carinata*, AABBCC, $2n = 54$) was carried out using 162 combinations of A-, B-, and C-genome-specific SSR primers [31]. To assess the genetic variability of the new form of *B. napus* obtained by crossing *B. rapa* with a hexaploid (*B. napus* × *B. oleracea*, AACCCC), 153 combinations of primers for microsatellite loci were used [32]. With 34 SSR markers, homeological recombination of chromosomes was detected in the offspring of *B. napus* × *B. carinata* (ABCC) trigenomic hybrids derived from microspores [37].

The Illumina Infinium *B. napus* 60K SNP (Illumina, Inc., USA) array of SNP markers for *B. napus* allotetraploid was developed to identify *Brassica* species and assess genetic diversity [52]. Also, a multiplex PCR analysis (MPCR) of A-, B-, and C-genome-specific sequences using five combinations of primers was proposed for rapid identification of *Brassica* species of the U triangle [35]. The MPCR

results were validated on 120 genetically characterized *Brassica* samples. Due to the direct detection of specific fragments in a 2% agarose gel, the MPCR assay is useful as an affordable and rapid diagnostic technique that can be easily applied in a conventional laboratory. However, as noted by the authors themselves, this method has a rather low throughput and should be adapted for high throughput real-time screening.

In contrast to expensive and more complicated methods that require special data processing (for example, when using the Illumina Infinium *B. napus* 60K SNP DNA chip), the technology we propose is more accessible and convenient to use. Electrophoretic analysis is carried out automatically using high-precision equipment. This significantly increases the reliability of the obtained data and thir interpretation (un-like the empirical assessment in the gel without precise determination of the lengths of the analyzed DNA fragments) [35, 45, 48]. Due to the automation of all stages in a 96-well plate format, the proposed approach allows large-scale screening of selection samples. The multiplex system can be used to assess the introgression of *Brassica* genomes during interspecific hybridization and control the inheritance of genomic material in subsequent generations.

Thus, we have developed a system for multiplex PCR analysis of six *Brassica* microsatellite loci (Na12-A02, BRMS-043, Na10-D09, Ol12-A04, Ni2-F02, BN6A2) for detecting A, B, and C genome fragments. The reliability of the system is confirmed with the control samples of known genomic composition and taxonomic attribution. The developed markers allowed us to identified the A-, B- and C-genome-specific fragments and to determine the genomic composition of a number of breeding samples. The results of the investigation can be used to detect sequences specific for genomes A, B, C in *Brassica* plants and to control the inheritance of genetic material during distant hybridization.

REFERENCES

1. Warwick S.I., Francis A., Al-Shehbaz I.A. *Brassicaceae*: Species checklist and database on CD-ROM. *Plant Systematics and Evolution*, 2006, 259(2-4): 249-258 (doi: 10.1007/s00606-006-0422-0).
2. Morinaga T. Interspecific hybridization in *Brassica*. IV. The cytology of F1 hybrid of *B. juncea* and *B. nigra*. *Cytologia*, 1934, 6: 62-67 (doi: 10.1508/cytologia.6.62).
3. U N. Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese Journal of Botany*, 1935, 7: 389-452.
4. Lagercrantz U. Comparative mapping between *Arabidopsis thaliana* and *Brassica nigra* indicates that *Brassica* genomes have evolved through extensive genome replication accompanied by chromosome fusions and frequent rearrangements. *Genetics*, 1998, 150(3): 1217-1228.
5. Kowalski S.P., Lan T.H., Feldmann K.A., Paterson A.H. Comparative mapping of *Arabidopsis thaliana* and *Brassica oleracea* chromosomes reveals islands of conserved organization. *Genetics*, 1994, 138(2): 499-510.
6. Rana D., van den Boogaart T., O'Neill C.M., Hynes L., Bent E., Macpherson L., Park J.Y., Lim Y.P., Bancroft I. Conservation of the microstructure of genome segments in *Brassica napus* and its diploid relatives. *Plant Journal*, 2004, 40(5): 725-733 (doi: 10.1111/j.1365-313X.2004.02244.x).
7. Lagercrantz U., Lydiate D. Comparative genome mapping in Brassica. *Genetics*, 1996, 144(4): 1903-1910.
8. Zou J., Hu D., Liu P., Raman H., Liu Z., Liu X., Parkin I.A.P., Chalhoub B., Meng J. Co-linearity and divergence of the A subgenome of *Brassica juncea* compared with other *Brassica* species carrying different A subgenomes. *BMC Genomics*, 2016, 17: 18 (doi: 10.1186/s12864-015-2343-1).
9. Jiang C., Ramchiary N., Ma Y., Jin M., Feng J., Li R., Wang H., Long Y., Choi S.R., Zhang C., Cowling W.A., Park B.S., Lim Y.P., Meng J. Structural and functional comparative mapping between the *Brassica* A genomes in allotetraploid *Brassica napus* and diploid *Brassica rapa*. *Theoretical and Applied Genetics*, 2011, 123(4): 927-941 (doi: 10.1007/s00122-011-1637-1).
10. Mason A.S., Batley J. Creating new interspecific hybrid and polyploid crops. *Trends in Biotechnology*, 2015, 33(8): 436-441 (doi: 10.1016/j.tibtech.2015.06.004).
11. Snowdon R.J. Cytogenetics and genome analysis in *Brassica* crops. *Chromosome Research*, 2007, 15(1): 85-95 (doi: 10.1007/s10577-006-1105-y).

12. Katche E., Quezada-Martinez D., Katche E.I., Vasquez-Teuber P., Mason A.S. Interspecific hybridization for *Brassica* crop improvement. *Crop Breeding, Genetics and Genomics*, 2019, 1: e190007 (doi: 10.20900/cbgg20190007).
13. Siemens J. Interspecific hybridisation between wild relatives and *Brassica napus* to introduce new resistance traits into the oilseed rape gene pool. *Czech Journal of Genetics and Plant Breeding*, 2002, 38(3-4): 155-157 (doi: 10.17221/6258-CJGPB).
14. Chrungu B., Verma N., Mohanty A., Pradhan A., Shivanna K.R. Production and characterization of interspecific hybrids between *Brassica maurorum* and crop Brassicas. *Theoretical and Applied Genetics*, 1999, 98(3-4): 608-613 (doi: 10.1007/s001220051111).
15. Lelivelt C.L.C., Krens F.A. Transfer of resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) into the *Brassica napus* L. gene pool through intergeneric somatic hybridization with *Raphanus sativus* L. *Theoretical and Applied Genetics*, 1992, 83(6-7): 887-894 (doi: 10.1007/BF00226712).
16. Lelivelt C.L.C., Leunissen E.H.M., Frederiks H.J., Helsper J.P.F.G., Krens F.A. Transfer of resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) from *Sinapis alba* L. (white mustard) to the *Brassica napus* L. gene pool by means of sexual and somatic hybridization. *Theoretical and Applied Genetics*, 1993, 85(6-7): 688-696 (doi: 10.1007/BF00225006).
17. Snowdon R.J., Winter H., Diestel A., Sacristán M.D. Development and characterisation *of Brassica napus - Sinapis arvensis addition lines exhibiting resistance to Leptosphaeria maculans. Theoretical and Applied Genetics*, 2000, 101(7): 1008-1014 (doi: 10.1007/s001220051574).
18. Yamagishi H., Bhat S.R. Cytoplasmic male sterility in *Brassicaceae* crops. *Breeding Science*, 2014, 64(1): 38-47 (doi: 10.1270/jsbbs.64.38).
19. Piao Z., Ramchiary N., Lim Y.P. Genetics of clubroot resistance in *Brassica* species. *Journal of Plant Growth Regulation*, 2009, 28(3): 252-264 (doi: 10.1007/s00344-009-9093-8).
20. Sharma B.B., Kalia P., Singh D., Sharma T.R. Introgression of black rot resistance from *Brassica carinata* to cauliflower (*Brassica oleracea botrytis* group) through embryo rescue. *Frontiers in Plant Science*, 2017, 8: 1255 (doi: 10.3389/fpls.2017.01255).
21. Belimov A.A., Safronova V.I., Demchinskaya S.V., Dzyuba O.O. Intraspecific variability of cadmium tolerance in hydroponically grown Indian mustard (*Brassica juncea* (L.) Czern.) seedlings. *Acta Physiologiae Plantarum*, 2007, 29(5): 473-478 (doi: 10.1007/s11738-007-0057-y).
22. Plieske J., Struss D., Röbbelen G. Inheritance of resistance derived from the B-genome of *Brassica* against *Phoma lingam* in rapeseed and the development of molecular markers. *Theoretical and Applied Genetics*, 1998, 97: 929-936 (doi: 10.1007/s001220050973).
23. Navabi Z.K., Parkin I.A.P., Pires J.C., Xiong Z., Thiagarajah M.R., Good A.G., Rahman M.H. Introgression of B-genome chromosomes in a doubled haploid population of *Brassica napus* × *B. carinata*. *Genome*, 2010, 53(8): 619-629 (doi: 10.1139/g10-039).
24. Abel S., Möllers C., Becker H. Development of synthetic *Brassica napus* lines for the analysis of "fixed heterosis" in allopolyploid plants. *Euphytica*, 2005, 46(1-2): 157-163 (doi: 10.1007/s10681-005-3364-7).
25. Srivastava A., Mukhopadhyay A., Arumugam N., Gupta V., Verma J.K., Pental D., Pradhan A.K. Resynthesis of *Brassica juncea* through interspecific crosses between *B. rapa* and *B. nigra*. *Plant Breeding*, 2004, 123(2): 204-206 (doi: 10.1046/j.1439-0523.2003.00933.x).
26. Narasimhulu S.B., Kirti P.B., Prakash S., Chopra V.L. Resynthesis of *Brassica carinata* by protoplast fusion and recovery of a novel cytoplasmic hybrid. *Plant Cell Reports*, 1992, 11(8): 428-432 (doi: 10.1007/BF00234376).
27. Chen S., Nelson M.N., Chèvre A.M., Jenczewski E., Li Z., Mason A.S. Trigenomic bridges for *Brassica* improvement. *Critical Reviews in Plant Sciences*, 2011, 30(6): 524-547 (doi: 10.1080/07352689.2011.615700).
28. Monakhos G.F., Ignatov A.N., Dzhalilov F.S. *Izvestiya TSKHA*, 2001, 4: 56-68 (in Russ.).
29. Malek M.A., Rahman L., Das M.L., Hassan L., Rafii M.Y., Ismail M.R. Development of hexaploid *Brassica* (AABBCC) from hybrids (ABC) of *Brassica carinata* (BBCC) × *B. rapa* (AA). *Ausralian Journal of Crop Science*, 2013, 7(9): 1375-1382.
30. Zhou J., Tan C., Cui C., Ge X., Li Z. Distinct subgenome stabilities in synthesized *Brassica* allohexaploids. *Theoretical and Applied Genetics*, 2016, 129(7): 1257-1271 (doi: 10.1007/s00122-016-2701-7).
31. Gupta M., Atri C., Agarwal N., Banga S.S. Development and molecular-genetic characterization of a stable *Brassica* allohexaploid. *Theoretical and Applied Genetics*, 2016, 129(11): 2085-2100 (doi: 10.1007/s00122-016-2759-2).
32. Li Q., Mei J., Zhang Y., Li J., Ge X., Li Z., Qian W. A large-scale introgression of genomic components of *Brassica rapa* into *B. napus* by the bridge of hexaploid derived from hybridization between *B. napus* and *B. oleracea*. *Theoretical and Applied Genetics*, 2013, 126(8): 2073-2080 (doi: 10.1007/s00122-013-2119-4).
33. Ma N., Li Z.-Y., Cartagena J. A., Fukui K. GISH and AFLP analyses of novel *Brassica Napus* lines derived from one hybrid between *B. napus* and *Orychophragmus Violaceus*. *Plant Cell Reports*, 2006, 25(10): 1089-1093 (doi: 10.1007/s00299-006-0171-0).

34. Monakhos S.G. *Integratsiya sovremennykh biotekhnologicheskikh metodov v selektsii ovoshchnykh kul'tur. Doktorskaya dissertatsiya*. [Integration of modern biotechnological methods in the vegetable crop breeding technologies. DSc Thesis]. Moscow, 2015 (in Russ.).
35. Koh J.C.O., Barbulescu D.M., Norton S., Redden B., Salisbury P.A., Kaur S., Cogan N., Slater A.T. A multiplex PCR for rapid identification of *Brassica* species in the triangle of U. *Plant Methods*, 2017, 13(1): 49 (doi: 10.1186/s13007-017-0200-8).
36. Thakur A.K., Singh K.H., Singh L., Nanjundan J., Khan Y.J., Singh D. SSR marker variations in *Brassica* species provide insight into the origin and evolution of *Brassica* amphidiploids. *Hereditas*, 2017, 155: 6 (doi: 10.1186/s41065-017-0041-5).
37. Mason A.S., Nelson M.N., Castello M.C., Yan G., Cowling W.A. Genotypic effects on the frequency of homoeologous and homologous recombination in *Brassica napus* × *B. carinata* hybrids. *Theoretical and Applied Genetics*, 2011, 122(3): 543-553 (doi: 10.1007/s00122-010-1468-5).
38. Clarke W.E., Higgins E.E., Plieske J., Wieseke R., Sidebottom C., Khedikar Y., Batley J., Edwards D., Meng J., Li R., Lawley C.T., Pauquet J., Laga B., Cheung W., Iniguez-Luy F., Dyrszka E., Rae S., Stich B., Snowdon R.J., Sharpe A.G., Ganal M.W., Parkin I.A. A high-density SNP genotyping array for *Brassica napus* and its ancestral diploid species based on optimised selection of single-locus markers in the allotetraploid genome. *Theoretical and Applied Genetics*, 2016, 129(10): 1887-1899 (doi: 10.1007/s00122-016-2746-7).
39. Lowe A.J., Moule C., Trick M., Edwards K.J. Efficient large-scale development of microsatellites for marker and mapping applications in *Brassica* crop species. *Theoretical and Applied Genetics*, 2004, 108(6): 1103-1112 (doi: 10.1007/s00122-003-1522-7).
40. Suwabe K., Iketani H., Nunome T., Kage T., Hirai M. Isolation and characterization of microsatellites in *Brassica rapa* L. *Theoretical and Applied Genetics*, 2002, 104(6-7): 1092-1098 (doi: 10.1007/s00122-002-0875-7).
41. Kresovich S., Szewc-McFadden A.K., Bliek S.M., McFerson J.R. Abundance and characterisation of simple-sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* L. (rapeseed). *Theoretical and Applied Genetics,* 1995, 91(2): 206-211 (doi: 10.1007/BF00220879).
42. Plieske J., Struss D. Microsatellite markers for genome analysis in *Brassica*. I. Development in *Brassica napus* and abundance in *Brassicaceae* species. *Theoretical and Applied Genetics*, 2001, 102(5): 689-694 (doi: 10.1007/s001220051698).
43. Hasan M., Seyis F., Badani A.G., Pons-Kühnemann J., Friedt W., Lühs W., Snowdon R.J. Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. *Genetic Resources and Crop Evolution*, 2006, 53(4): 793-802 (doi: 10.1007/s10722-004-5541-2).
44. Tommasini L., Batley J., Arnold G.M., Cooke R.J., Donini P., Lee D., Law J.R., Lowe C., Moule C., Trick M., Edwards K.J. The development of multiplex simple sequence repeat (SSR) markers to complement distinctness, uniformity and stability testing of rape (*Brassica napus* L.) varieties. *Theoretical and Applied Genetics*, 2003, 106(6): 1091-1101 (doi: 10.1007/s00122-002-1125-8).
45. Dubina E.V., Koroleva S.V., Garkusha S.V., Yurchenko S.A., Esaulova L.V. *Dostizheniya nauki i tekhniki APK*, 2016, 30(8): 49-51 (in Russ.).
46. Suwabe K., Tsukada H., Iketani H., Hatakeyama K., Fujimura M., Nunome T., Fukuoka H., Matsumoto S., Hirai M. Identification of two loci for resistance to clubroot (*Plasmodiophora brassicae* Wornin) in *Brassica rapa* L. *Theoretical and Applied Genetics*, 2003, 107(6): 997-1002 (doi: 10.1007/s00122-003-1309-x).
47. Kato T., Hatakeyama K., Fukino N., Matsumoto S. Fine mapping of the clubroot resistance gene *CRb* and development of a useful selectable marker in *Brassica rapa*. *Breeding Science*, 2013, 63(1): 116-124 (doi: 10.1270/jsbbs.63.116).
48. Artem'eva A.M., Ignatov A.N., Volkova A.I., Kocherina N.V., Konopleva M.N., Chesnokov Yu.V. Physiological and genetic components of black rot resistance in double haploid lines of *Brassica rapa* L. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2018, 53(1): 157-169 (doi: 10.15389/agrobiology.2018.1.157eng).
49. Shilov I.A. V sbornike: *Problemy agrobiotekhnologii* [In: Problems of agrobiotechnology]. Moscow, 2012: 140-162 (in Russ.).
50. Aniskina Yu.V. *Tekhnologiya genotipirovaniya kul'turnykh i dikorastushchikh form Brassica na osnove analiza polimorfizma mikrosatellitov. Avtoreferat kandidatskoi dissertatsii* [Genotyping technology for cultivated and wild forms of *Brassica* based on the analysis of microsatellite polymorphism. PhD Thesis]. Moscow, 2006 (in Russ.).
51. Aniskina Yu.V., Velishaeva N.S., Shilov I.A., Khavkin E.E. *Genotipirovanie paslenovykh i krestotsvetnykh rastenii metodom mikrosatellitnogo analiza. Metodicheskie rekomendatsii* [Genotyping of nightshade and cruciferous plants by microsatellite analysis. Methods]. Moscow, 2005 (in Russ.).
52. Mason A.S., Zhang J., Tollenaere R., Vasquez Teuber P., Dalton-Morgan J., Hu L., Edwards D., Redden R., Batley J. High-throughput genotyping for species identification and diversity assessment in germplasm collections. *Molecular Ecology Resources*, 2015, 15(5): 1091-1101 (doi: 10.1111/1755-0998.12379).

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COMPARATIVE ANALYSIS OF THE *VvMybA1* LOCUS ALLEAL STATE IN SOME INDIGENOUS AND INTRODUCENT GRAPEVINE VARIETIES

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Abstract

European cultivated grape *Vitis vinifira* L. is one of the most common agricultural crops grown by man since ancient times. Of course, the usual companion of the cultivation of this culture is a traditional hybridization and clonal selection, which allows you to select spontaneous mutants. Nevertheless, the study of such an important agrobiological trait as anthocyanin coloration and, in particular, the genes that determine it, is one of the most important problems both in modern grape genetics and in studies of plant metabolic pathways in general. The study of this problem can not only provide the basis for subsequent fundamental research on the functioning of both individual genes and the genome as a whole, but also create a basis for the selection of varieties for economically valuable traits. The *VvMybA* family of grape transcription factors is responsible for anthocyanin accumulation in berries of cultivated and wild grapes. In the present work, alleles of the *VvMybA1* gene were first identified in native Russian grape varieties. Theses alleles in the colored and uncolored grape varieties were found out to be the same in size. Sequence alignment showed the characteristic features of alleles for each of the studied genotypes. The purpose of our study, we set a description of the four alleles of the *VvMybA1* gene in the Chardonnay, Sibirkovskii, Cabernet Cortis and Sypun cherny varieties. Two introduced and well-known varieties and two indigenous varieties also cultivated in the Krasnodar Territory were investigated. The leaves for DNA extraction was collected at the Anapa zonal experimental station. DNA was isolated by CTAB method with mercaptoethanol. PCR was performed using primers and amplification parameters published in the literature. For PCR amplification and isolation of *VvMybA1* gene alleles we used markers which allow simultaneous identification of two alleles, *VvMybA1b* and *VvMybA1c*. We sequenced and compared with each other and with the GenBank NCBI database the sequences of the *VvMybA1* gene alleles of these varieties. Alignment of sequences in the ClustalO program revealed structural features of the allele nucleotide sequences. In particular, a single nucleotide insert was found in white-berry varieties and nucleotide substitutions in different places in varieties with colored and uncolored berries. Further, comparing with the GenBank NCBI database found that the alleles of colored varieties have a structure characteristic for varieties with a pronounced color of berries, while uncolored varieties apparently have a specific reason for the loss of color. Thus, it was found that the Chardonnay variety has an allele with the insertion of *Gret-1* transposon which blocks the normal expression of *VvMybA1* gene. It was also revealed that the Sibirkovky variety also has the allele of *VvMybA1* gene which is not functional due to the blocking by *Gret-1* transposon. As shown in previous studies by other authors, this allele is also present in other uncolored varieties, and, therefore, this is the reason for blocking gene expression. A study of the amino acid sequence also revealed differences between the groups of colored and uncolored varieties. These differences can be obviously divided into those characteristic of colored and uncolored varieties. However, a

mutation was detected in Sypun cherny variety, which affected the replacement of the amino acid isoleucine with valine, but did not affect the overall color of the berries. When searching for the amino acid sequence in the GenBank NCBI database, it was revealed that this mutation is not unique in nature, as it was found in the Alphonse Lavallée variety, as well as in interspecific hybrids.

Keywords: *Vitis vinifera* L., indigenous varieties, introduced varieties, allele, gene, *VvMybA1*, sequencing, anthocyanin, mutations, amino acids, transposons

European wine grape (*Vitis vinifera* L.) is one of the most widespread and economically important agricultural crops. The color of its berries is one of the main characteristics when describing existing varieties and creating new forms. The trait depends on the amount and composition of anthocyanins, which the colored varieties accumulate in the berries, while the uncolored ones do not synthesize [1].

Methods of cultivating grapes and processing the grape vine harvest depend on which hybrid, clone and stock are used in production, that is, on the ampelographic properties [2]. Vegetative reproduction preserves the desired traits, but significantly influenced the frequency of spontaneous somatic mutations observed in vineyards [3, 4]. Thence, many traits of grape plants were acquired not only by hybridization, but also due to clonal selection, for example, yield per bush, shape and compactness of a bunch, size and color of berries [5-7]. That is, the varieties intermediate in berry color (pink, red, yellow, etc.) appeared as a result of hybridization and mutations. The ripening and berry formation are also influenced by the environment [8-10], however, berry color is determined by genes. The metabolic pathways of anthocyanin color are primary regulated by the group of *MYB* genes [11-13].

VvMybA family of grape transcription factors is responsible for the content of anthocyanins in the berries of cultivated and wild grapes. Previous studies have shown that uncolored grapes arose due to the insertion of a retroelement in *VvMybA1* [14-16] and a single nucleotide polymorphism mutation in *VvMybA2* [17, 18]. That is, a gene cluster located on chromosome 2 is responsible for most of the color change, and the phenotype is due to the joint work of the *VvMybA* genes [19]. This locus consists of three *MYB* genes, among which *VvMybA1* and *VvMybA2* are functionally involved in berry pigmentation [13, 14]. It was shown that the appearance of a genotype characteristic of white berry varieties depends exactly on *VvMybA1* and *VvMybA2* [11, 13, 15]. Loss of *VvMybA1* gene function occurs due to the insertion of the *Gret1* transposon [16, 20], while *VvMybA2* can have a single nucleotide polymorphism (SNP) K980 in the coding sequence, which modifies the putative α-helix of the recognition domain R2R3 and leads to the loss of the allele functionality [15, 21].

It should be noted that in our country, well-known western introduced varieties have earlier been studied intensively, while the native varieties of the Black Sea basin, which have a huge potential for breeding new hybrids and selecting clones, remain unexplored. In particular, the structures of their genes, for example, *VvMybA1*, remains unknown, information about which is important for identifying the unique genetic structure of alleles, understanding particular cases of phenotypic diversity, and studying *Vitaceae* family evolution.

In this work is the first to identify *VvMybA1* gene alleles in native Russian grape varieties. The size of the alleles in colored and uncolored varieties is the same, but alignment of the sequences showed characteristic features of the allele structure for each of the studied genotypes

Our investigation aimed to reveal and identify structural features and to compare the *VvMybA1* gene alleles in two native and two introduced grape varieties.

Materials and methods. Two colored (Cabernet Cortis and Sypun black) and two uncolored (Chardonnay and Sibirkovy) grape varieties (respectively, native and introduced) were compared. Plant apical leaves were collected in Anapa zonal ampelographic collection SKFNTSSVV (AZOSViV). DNA was extracted by a modified CTAB method with mercaptoethanol [22]. For PCR optimization, the DNA concentration was measured (an Implen NP80 nanophotometer, Implen GmbH, Germany) and adjusted to 20 ng/μl with deionized water.

To perform classical PCR and identify target alleles of *VvMybA1* gene, we used markers described by Azuma et al. [23], which allow identification of two alleles, *VvMybA1b* and *VvMybA1c*, at once in a single PCR run. A 25 μl PCR mixture contained 20 ng of a template DNA, 200 mM dNTP, 0.2 mM of each primer [23] synthesized by JSC Evrogen (Russia), and one unit Taq-DNA polymerase (JSC Evrogen, Russia). PCR was performed in the following mode: 3 min at 95 °C (initial denaturation); 30 s at 94 °C, 30 s at 65 °C, 30 s at 72 °C (35 cycles); 10 min at 72 °C (DT-322, DNA-Technology LLC, Russia). The PCR results were checked for compliance with the expected fragment lengths in 6% PAGE with 1× TBE buffer; amplification products were separated in a VE-20 vertical electrophoresis chamber (Helicon, Russia) for 3 h (7 V/cm) (molecular weight marker M12, NPO SibEnzyme, Russia).

The amplicons were separated in 2% agarose gel with 0.5× TAE buffer at 5 V/cm, then excised from the gel plate and extracted using Cleanup Standard kit (Evrogen, Russia). The DNA concentration in the sample was measured (an Implen NP80 nanophotometer), and the volume was adjusted to 6 μl with deionized water (the purified amplified fragment concentration was 30 ng/μl).

Sequencing was performed with forward and reverse PCR primers [23] to ensure greater reliability (equipment of ZAO Evrogen, Russia).

A compliance with the expected amplicon size was assessed by searching the sequence in the GenBank NCBI database (https://www.ncbi.nlm.nih.gov/genbank) with BLAST, blastx, and CD-search web tools [24]. The obtained DNA and protein sequences were aligned according to standard parameters of the ClustalO program using the VIENNA (for fasta alignment) and ClustalW (for subsequent analysis) formats [25]. MView interface as applied to analyze the aligned sequences [26]. Phylogenetic relationships between the studied amino acid sequences were established using MEGA7 program [27] by the Maximum Likelihood method [28] with the Jones-Taylor-Thornton model [29] (999 bootstraps).

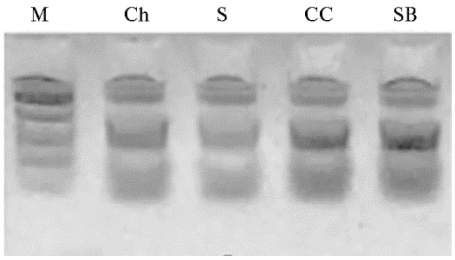

Fig. 1. Separation of amplified DNA fragments of *VvMybA1* alleles in colored (Cabernet Cortis CC and Sypun black SB) and uncolored (Chardonnay Ch and Sibirkovy S) **varieties of European wine grape (*Vitis vinifera* L.)** (M — molecular weight marker M12, NPO SibEnzim, Russia; Anapa zonal ampelographic collection SKANCSVV).

Results. After separation of the amplification products in agarose gel (Fig. 1), it was found that all four grape samples had alleles of the *VvMybA1* gene of the same size (approximately 850 bp). Similar works on grapes have already demonstrated the similarity of DNA sequences obtained by PCR not only in size, but also in primary structure [14, 16, 30]. However, in addition to this, it was shown that areas of the same size can be polymorphic and differ in structure, which also affects the color trait [16, 21]. We sequenced the amplified DNA regions to establish their nucleotide sequences in alleles of the same size and obtained the following results (Fig. 2):

Fig. 2. Sequencing of 850 bp amplicons of *VvMybA1* alleles in colored (Cabernet Cortis and Sypun black) **and uncolored** (Chardonnay and Sibirkovy) **varieties of European wine grape (*Vitis vinifera* L.)** (Anapa zonal ampelographic collection SKANCSVV) (for the figure, see http://www.agrobiology.ru).

The obtained nucleotide sequences were compared with each other using the ClustalO program (Fig. 3):

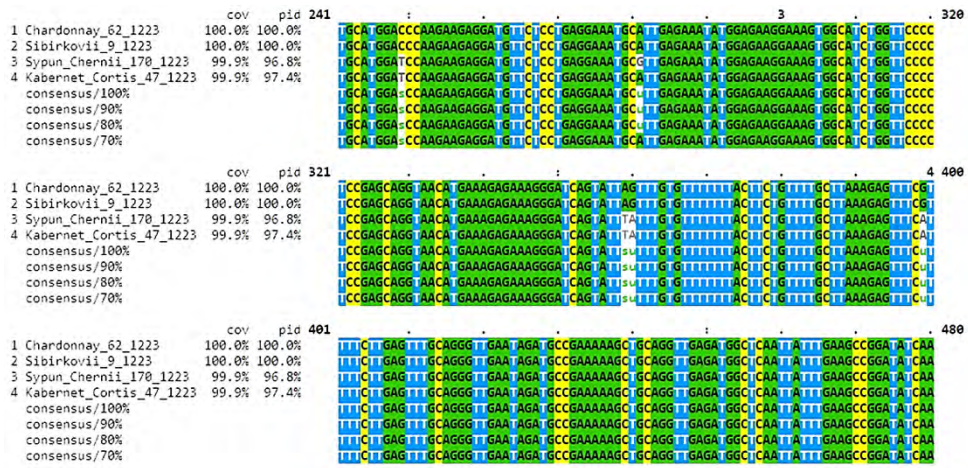

Fig. 3. Analysis of *VvMybA1* gene allele regions in four native and introduced varieties of European wine grape (*Vitis vinifera* L.) (colored Cabernet Cortis and Sypun black and uncolored Chardonnay and Sibirkovy) (ClustalO program; Anapa zonal ampelographic collection SKANCSVV). Nucleotide polymorphisms identified by alignment of the analyzed sequences in four genotypes are highlighted as in white. A unique nucleotide substitution at nucleotide position 281 in Sipun black variety is marked by white background. For the figure, see http://www.agrobiology.ru.

Sequence alignment revealed nucleotide substitutions that made it possible to distinguish between white and red berry varieties, for example, nucleotides 4-10. These differences were revealed in both pairs of the varieties we studied, the colored (Sypun black and Cabernet Cortis) and uncolored (Chardonnay and Sibirkovy). The same was confirmed by a search among the corresponding sequences deposited in the GenBank NCBI databases where the 100% similarity was found (except for the Sipun black variety).

The nucleotide sequences of the analyzed alleles in the Chardonnay and Sibirkovy cultivars were identical and coincided with that of the allele characteristic of the white berry cultivars with the *Gret-1* insert. This retrotransposon, as previously reported [16, 31], causes loss of the *VvMybA1* allele function. Consequently, it is the presence of such an insert in the specified gene that suppresses the synthesis of anthocyanins which provide berry coloration in the Sibirkovy variety. Despite the fact that we identified in the Sipun black cultivar a unique mutation in nucleotide 281 (see Fig. 3), in general, the nucleotide sequence of this allele (as in the Cabernet

Cortis cultivar) was characteristic of the colored cultivars (in particular KY406230.1, GU145121.1 and GU145120.1 accession numbers). Interestingly, a similarity appeared with the *VvMybA1*^{SUB} allele which was present but not expressed in uncolored varieties Sultanina, Pirovano 166A, and others [16], that is, did not play a decisive role in berry coloration.

Cabernet Cortis

Score	Expect	Method	Identities	Positives	Gaps
164 bits(415)	2e-50	Composition-based stats.	81/111(73%)	81/111(72%)	29/111(26%)

```
Query  1    MESLGVRKGAWIQEEDVLLRKCIEKYGEGKWHLVPLRAGNMKEKGISIYLCFFTSVLLKE  60
            MESLGVRKGAW QEEDVLLRKCIEKYGEGKWHLVPLRAG
Sbjct  1    MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAG----------------------  39

Query  61   FHFLEFAGLNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  111
                    LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR
Sbjct  40   --------LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  82
```

Sypun black

Score	Expect	Method	Identities	Positives	Gaps
163 bits(413)	4e-50	Composition-based stats.	80/111(72%)	81/111(72%)	29/111(26%)

```
Query  1    MESLGVRKGAWIQEEDVLLRKCVEKYGEGKWHLVPLRAGNMKEKGISIYLCFFTSVLLKE  60
            MESLGVRKGAW QEEDVLLRKC+EKYGEGKWHLVPLRAG
Sbjct  1    MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAG----------------------  39

Query  61   FHFLEFAGLNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  111
                    LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR
Sbjct  40   --------LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  82
```

Chardonnay

Score	Expect	Method	Identities	Positives	Gaps
166 bits(419)	6e-51	Composition-based stats.	82/111(74%)	82/111(73%)	29/111(26%)

```
Query  1    MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAGNMKEKGISISLCFFTSVLLKE  60
            MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAG
Sbjct  1    MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAG----------------------  39

Query  61   FRFLEFAGLNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  111
                    LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR
Sbjct  40   --------LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  82
```

Sibirkovy

Score	Expect	Method	Identities	Positives	Gaps
166 bits(419)	6e-51	Composition-based stats.	82/111(74%)	82/111(73%)	29/111(26%)

```
Query  1    MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAGNMKEKGISISLCFFTSVLLKE  60
            MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAG
Sbjct  1    MESLGVRKGAWTQEEDVLLRKCIEKYGEGKWHLVPLRAG----------------------  39

Query  61   FRFLEFAGLNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  111
                    LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR
Sbjct  40   --------LNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNR  82
```

Fig. 4. A search for amino acid sequences translated from *VvMybA1* gene alleles in four native and introduced varieties of European wine grape (*Vitis vinifera* L.) (colored Cabernet Cortis and Sypun black and uncolored Chardonnay and Sibirkovy) (blastx algorithms; Anapa zonal ampelographic collection SKANCSVV). When compared with the reference sequences in NCBI Protein (https://www.ncbi.nlm.nih.gov/protein/), an insert was identified which is highlighted by dashes (most likely, it is the intron sequence not deleted by blastx tool during translation).

After assignment of the sequences to the sought alleles, we used blastx tool to search among the corresponding amino acid sequences. For all four sequences, the presumptive structure of the amino acid chain was established (Fig. 4). The results of the search showed 100% coincidence in all analyzed samples, with the exception of a large insert in the middle of the sequences which were turned out to be characteristic of all genotypes and resulted from translation of the intron region not excluded by the algorithm for some reason. This statement is also supported by a search in EnsemblPlants [32] and CD-search [33].

Having shown single nucleotide polymorphisms, we aligned the amino acid sequences in ClustalO program (Fig. 5). Differences between the white berry and

red berry varieties were found for amino acids 12, 49, and 62. A unique substitution of isoleucine for valine was found in Sypun black variety (amino acid 23). In addition, two of the four polymorphisms were located in the coding part of the sequence. One of them was typical for white and red berry genotypes, and amino acid 23 in Sipun black variety was unique. As per CD-search, this substitution was located in a conservative region. It is noteworthy that no differences were found in this position from the variety Alphonse Lavallée [16]. However, both varieties are brightly colored. Therefore, the change in amino acid sequence did not affect the expression of this trait in any way. The search for the amino acid sequence of Cabernet Cortis in NCBI Protein confirmed that it is often found among interspecific hybrids that have a similar sequence of amino acids encoded by *VvMybA1* [11, 13], but differ from the Sipun black genotype by replacing valine to isoleucine.

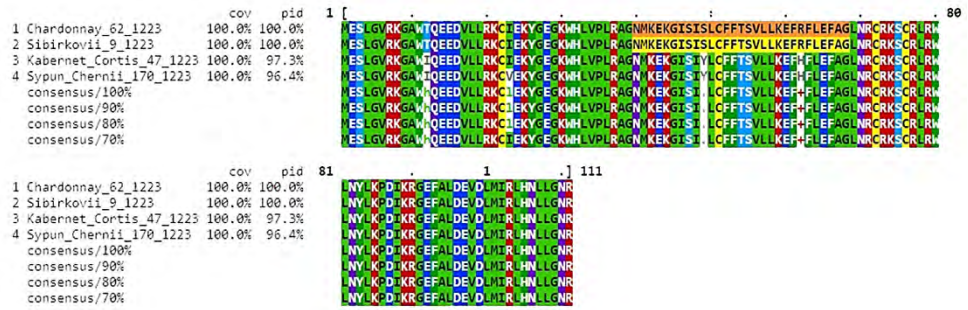

Fig. 5. Alignment of amino acid sequences (with an intron insertion marked) **translated from *VvMybA1* gene alleles in four native and introduced varieties of European wine grape (*Vitis vinifera* L.)** (colored Cabernet Cortis and Sypun black and uncolored Chardonnay and Sibirkovy) (ClustalO program; Anapa zonal ampelographic collection SKANCSVV). In Sypun black variety, in the position 23 (highlighted in white background) there is an amino acid substitution (isoleucine characteristic of the other three varieties is replaced to valine). For the figure, see http://www.agrobiology.ru.

As a whole, our results were in line with the expected ones, since it was revealed that the studied varieties are similar in nucleotide sequences of *VvMybA1* gene alleles to the known genotypes. An unexpected result was the similarity of the amino acid sequence encoded by the *VvMybA1* gene allele in the Sibirkovy variety and the colored varieties Benitaka, Cabernet Sauvignon, and Roditis [16, 34]. This can be explained by the fact that Benitaka variety is a spontaneous mutant of the white-berry variety Italia [31], whereas varieties Cabernet Sauvignon and Roditis are hybrids harboring both *VvMybA1b* and *VvMybA1c* alleles [16, 34, 35].

To compare the sequences of genes that determine colored and uncolored berries, we clustered them based on amino acid sequences (Fig. 6). The genotype *Rosa chinensis* (XP_024179665.1) of *Rosaceae* family was involved as an outgroup.

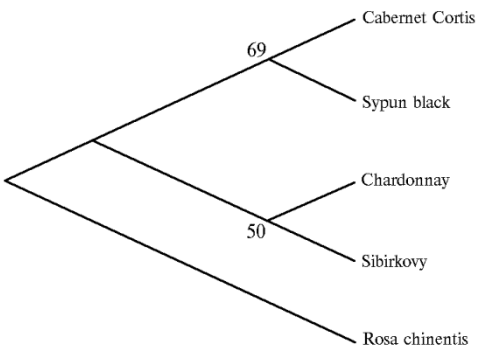

Fig. 6. Clustering of four native and introduced varieties of European wine grape (*Vitis vinifera* L.) (colored Cabernet Cortis and Sypun black and uncolored Chardonnay and Sibirkovy) **based on amino acid sequences translated from the sequenced *VvMybA1* gene alleles** (Anapa zonal ampelographic collection SKANCSVV). Bootstrap values over 50 are indicated.

In clustering, genotypes formed three groups (the outgroup, colored and uncolored varieties). Interestingly, on the generated tree the white-berry varieties were located separately from the main

branch between the colored varieties and the outgroup. These data are confirmed by other studies. Indeed, some uncolored varieties, such as Pinot blanc and Pinot Gris, appeared as a result of mutation [36], while the reverse process was also observed, i.e. the appearance of berry coloration in the Benitaka variety, that is, a transition from white berry color to pink [31].

Thus, in the introduced and native Russian grape varieties, the *VvMybA1* gene alleles are identified, the size of which does not differ in colored and uncolored varieties. The alleles reveal unique single nucleotide substitutions inherent in the specific genotypes we studied, including a unique nucleotide substitution in the Sypun black variety, as well as an insert in the uncolored Chardonnay and Sibirkovy varieties. A search for these sequences in the GenBank NCBI database using BLAST algorithms showed that three genotypes (varieties Cabernet Cortis, Sypun black, and Chardonnay) have alleles characteristic of white and black berry forms. In the Siberian variety, the *VvMybA1* gene allele is similar to the *VvMybA1a* allele with *Gret-1* transposon insertion. Obviously, the *VvMybA1* allele expression in the Sibirkovy variety is turned off by this very factor. A similar result was obtained for the Chardonnay variety (the presence of the *Gret-1* transposon which suppresses functional activity of the *VvMybA1* allele).

REFERENCES

1. Boss P.K., Davies C., Robinson S.P. Expression of anthocyanin biosynthesis pathway genes in red and white grapes. *Plant Molecular Biology*, 1996, 32(3): 565-569 (doi: 10.1007/BF00019111).
2. Swanepoel J.J., Southey J.M. The influence of rootstock on the rooting pattern of the grapevine. *South African Journal of Enology and Viticulture*, 1989, 10(1): 23-28 (doi: 10.21548/10-1-2295).
3. Forneck A. Plant breeding: clonality — a concept for stability and variability during vegetative propagation. In: *Progress in botany, vol. 66*. K. Esser, U. Lüttge, W. Beyschlag, J. Murata (eds.). Springer, Berlin, Heidelberg, 2005: 164-183 (doi: 10.1007/3-540-27043-4_8).
4. Pelsy F., Hocquigny S., Moncada X., Barbeau G., Forget D., Hinrichsen P., Merdinoglu D. An extensive study of the genetic diversity within seven French wine grape variety collections. *Theoretical and Applied Genetics*, 2010, 120(6): 1219-1231 (doi: 10.1007/s00122-009-1250-8).
5. Rühl E., Konrad H., Lindner B., Bleser E. Quality criteria and targets for clonal selection in grapevine. *Acta Horticulturae*, 2004, 652: 29-33 (doi: 10.17660/ActaHortic.2004.652.1).
6. Torres N., Goicoechea N., Morales F., Antolín M.C. Berry quality and antioxidant properties in *Vitis vinifera* cv. Tempranillo as affected by clonal variability, mycorrhizal inoculation and temperature. *Crop and Pasture Science*, 2016, 67(9): 961-977 (doi: 10.1071/CP16038).
7. Van Leeuwen C., Roby J.P., Alonso-Villaverde V., Gindro K. Impact of clonal variability in *Vitis vinifera* Cabernet franc on grape composition, wine quality, leaf blade stilbene content, and downy mildew resistance. *Journal of Agricultural and Food Chemistry*, 2013, 61(1): 19-24 (doi: 10.1021/jf304687c).
8. Anesi A., Stocchero M., Dal Santo S., Commisso M., Zenoni S., Ceoldo S., Tornielli G.B., Siebert T.E., Herderich M., Pezzotti M., Guzzo F. Towards a scientific interpretation of the terroir concept: plasticity of the grape berry metabolome. *BMC Plant Biology*, 2015, 15(1): 191 (doi: 10.1186/s12870-015-0584-4).
9. Dal Santo S., Zenoni S., Sandri M., De Lorenzis G., Magris G., De Paoli E., Di Gaspero G., Del Fabbro C., Morgante M., Brancadoro L., Grossi D., Fasoli M., Zuccolotto P., Tornielli G.B., Pezzotti M. Grapevine field experiments reveal the contribution of genotype, the influence of environment and the effect of their interaction (G×E) on the berry transcriptome. *The Plant Journal*, 2018, 93(6): 1143-1159 (doi: 10.1111/tpj.13834).
10. Guidoni S., Ferrandino A., Novello V. Effects of seasonal and agronomical practices on skin anthocyanin profile of Nebbiolo grapes. *American Journal of Enology and Viticulture*, 2008, 59(1): 22-29.
11. Azuma A., Kobayashi S., Mitani N., Shiraishi M., Yamada M., Ueno T., Kono A., Yakushiji H., Koshita Y. Genomic and genetic analysis of *Myb*-related genes that regulate anthocyanin biosynthesis in grape berry skin. *Theoretical and Applied Genetics*, 2008, 117(6): 1009-1019 (doi: 10.1007/s00122-008-0840-1).
12. Azuma A., Udo Y., Sato A., Mitani N., Kono A., Ban Y., Yakushiji H., Koshita Y., Kobayashi S. Haplotype composition at the color locus is a major genetic determinant of skin color variation

in Vitis×labruscana grapes. *Theoretical and Applied Genetics*, 2011, 122(7): 1427-1438 (doi: 10.1007/s00122-011-1542-7).
13. Kobayashi S., Ishimaru M., Hiraoka K., Honda C. *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. *Planta*, 2002, 215(6): 924-933 (doi: 10.1007/s00425-002-0830-5).
14. Kobayashi S., Goto-Yamamoto N., Hirochika H. Retrotransposon-induced mutations in grape skin color. *Science*, 2004, 304(5673): 982 (doi: 10.1126/science.1095011).
15. Walker A.R., Lee E., Bogs J., McDavid D.A., Thomas M.R., Robinson S.P. White grapes arose through the mutation of two similar and adjacent regulatory genes. *The Plant Journal*, 2007, 49(5): 772-785 (doi: 10.1111/j.1365-313X.2006.02997.x).
16. Lijavetzky D., Ruiz-García L., Cabezas J.A., De Andrés M.T., Bravo G., Ibáñez A., Carreño J., Cabello F., Ibáñez J., Martínez-Zapater J.M. Molecular genetics of berry colour variation in table grape. *Molecular Genetics and Genomics*, 2006, 276(5): 427-435 (doi: 10.1007/s00438-006-0149-1).
17. Giannetto S., Velasco R., Troggio M., Malacarne G., Storchi P., Cancellier S., De Nardi B., Crespan M. A PCR-based diagnostic tool for distinguishing grape skin color mutants. *Plant Science*, 2008, 175(3): 402-409 (doi: 10.1016/j.plantsci.2008.05.010).
18. De Lorenzis G. Investigation of *VvMybA1* and *VvMybA2* berry color genes in 'Aglianico' biotypes. In: *Progress in Vitis vinfera diversity evaluation and use, vol. 54*. G. De Lorenzis, D. Carrasco, R. Arroyo Garcia, M. Rossoni, G.S. Di Lorenzo, O. Failla (eds.). Bundesanstalt fur Zuchtungsforschung an Kulturpflanzen, Geilweilerhof, 2015: 43-44.
19. Fournier-Level A., Le Cunff L., Gomez C., Doligez A., Ageorges A., Roux C., Bertrand Y., Souquet J., Cheynier V., This P. Quantitative genetic bases of anthocyanin variation in grape (*Vitis vinifera* L. ssp. *sativa*) berry: a quantitative trait locus to quantitative trait nucleotide integrated study. *Genetics*, 2009, 183(3): 1127-1139 (doi: 10.1534/genetics.109.103929).
20. This P., Lacombe T., Cadle-Davidson M., Owens C.L. Wine grape (*Vitis vinifera* L.) color associates with allelic variation in the domestication gene *VvmybA1*. *Theoretical and Applied Genetics*, 2007, 114(4): 723-730 (doi: 10.1007/s00122-006-0472-2).
21. Fournier-Level A., Lacombe T., Le Cunff L., Boursiquot J.M., This P. Evolution of the *VvMybA* gene family, the major determinant of berry colour in cultivated grapevine (*Vitis vinifera* L.). *Heredity*, 2010, 104(4): 351-362 (doi: 10.1038/hdy.2009.148).
22. Porebski S., Bailey L.G., Baum B.R. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter*, 1997, 15(1): 8-15 (doi: 10.1007/BF02772108).
23. Azuma A., Kobayashi S., Yakushui H., Yamada M., Mitani N., Sato A. *VvmybA1* genotype determines grape skin color. *Vitis*, 2007, 46(3): 154-155.
24. McGinnis S., Madden T.L. BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Research*, 2004, 32(suppl_2): 20-25 (doi: 10.1093/nar/gkh435).
25. Sievers F., Higgins D.G. Clustal Omega, accurate alignment of very large numbers of sequences. In: *Multiple sequence alignment methods, vol. 1079*. D. Russel (ed.). Humana Press, Totowa, NJ, 2014: 105-116 (doi: 10.1007/978-1-62703-646-7_6).
26. Brown N.P., Leroy C., Sander C. MView: a web-compatible database search or multiple alignment viewer. *Bioinformatics,* 1998, 14(4): 380-381 (doi: 10.1093/bioinformatics/14.4.380).
27. Kumar S., Stecher G., Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 2016, 33(7): 1870-1874 (doi: 10.1093/molbev/msw054).
28. Murshudov G.N., Vagin A.A., Dodson E.J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallographica*, 1997, 53(3): 240-255 (doi: 10.1107/S0907444996012255).
29. Jones D.T., Taylor W.R., Thornton J.M. A model recognition approach to the prediction of all-helical membrane protein structure and topology. *Biochemistry*, 1994, 33(10): 3038-3049 (doi: 10.1021/bi00176a037).
30. Ferreira V., Pinto-Carnide O., Arroyo-García R., Castro I. Berry color variation in grapevine as a source of diversity. *Plant Physiology and Biochemistry*, 2018, 132: 696-707 (doi: 10.1016/j.plaphy.2018.08.021).
31. Azuma A., Kobayashi S., Goto-Yamamoto N., Shiraishi M., Mitani N., Yakushiji H., Koshita Y. Color recovery in berries of grape (*Vitis vinifera* L.) 'Benitaka', a bud sport of 'Italia', is caused by a novel allele at the *VvmybA1* locus. *Plant Science*, 2009, 176(4): 470-478 (doi: 10.1016/j.plantsci.2008.12.015).
32. Bolser D.M., Staines D.M., Perry E., Kersey P.J. Ensembl plants: integrating tools for visualizing, mining, and analyzing plant genomic data. In: *Plant genomics databases. Methods in Molecular Biology, V. 1533*. A. van Dijk (ed.). Humana Press, New York, NY, 2017: 1-31 (doi: 10.1007/978-1-4939-6658-5_1).
33. Marchler-Bauer A., Bryant S.H. CD-Search: protein domain annotations on the fly. *Nucleic Acids Research*, 2004, 32(2): 327-331 (doi: 10.1093/nar/gkh454).
34. Walker A.R., Lee E., Robinson S.P. Two new grape cultivars, bud sports of Cabernet Sauvignon

bearing pale-coloured berries, are the result of deletion of two regulatory genes of the berry colour locus. *Plant Molecular Biology*, 2006, 62(4): 623-635 (doi: 10.1007/s11103-006-9043-9).
35. Bowers J.E., Meredith C.P. The parentage of a classic wine grape, Cabernet Sauvignon. *Nature Genetics*, 1997, 16(1): 84-87 (doi: 10.1038/ng0597-84).
36. Vezzulli S., Leonardelli L., Malossini U., Stefanini M., Velasco R., Moser C. Pinot blanc and Pinot gris arose as independent somatic mutations of Pinot noir. *Journal of Experimental Botany*, 2012, 63(18): 6359-6369 (doi: 10.1093/jxb/ers290).

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INTRA-CALLUS VARIABILITY OF RICE DOUBLED HAPLOIDS GENERATED THROUGH in vitro ANDROGENESIS

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Abstract

In vitro androgenesis is among the leading methods in creating source material for crop breeding. Many breeders a priori consider the seed progeny of any doubled haploid a new line, regardless of which callus the line was obtained from. In practice, it often turns out that in field conditions the lines from one callus are outwardly identical, so the breeders discard them, leaving two or three of ones for further work. The validity of such a controversial approach requires experimental confirmation or refutation of polymorphism and genetic variability of doubled haploids of the same callus line. About 100 genes of rice resistance to *Pyricularia oryzae* Cav. [*Magnaporthe grisea* (Hebert Barr.)] are known of which *Pi-ta* и *Pi-ta*^{2} are the most relevant for the Russian Far East. This paper is the first to report intracallus morphological polymorphism and genetic variability for *Pi-ta* и *Pi-ta*^{2} genes due to gametoclonal and somaclonal variability of rice *Oryza sativa* L. doubled haploids derived from a hybrid plant via in vitro androgenesis. For the first time, a monomorphism in the absence of genetic variability due to callus cell clonal reproduction (mitotic division) was revealed among doubled haploids produced by the same callus line. Our work aimed to study the intracallus morphological and genetic variability of *Oryza sativa* L. doubled rice haploids derived in vitro androgenetically from a hybrid plant. Experiments were performed in 2017-2018 at the Federal Research Center of Agricultural Biotechnology of the Far East (Russia) and the Crop Resources Institute, Heilongjiang Academy of Agricultural Sciences, (China). A F_1 Don 4237 × Dolinnyi rice hybrid was used. A total of 386 anthers of this hybrid were cultured in vitro with the callus formation rate of 17.1%. Six of eighteen callus lines producing green regenerants were selected for further study. Two seeds of each doubled haploid line were sown in soil and grown in plastic pots (a culture room, 24 °C, 5000 lux, 16 hours/8 hours day/night). One plant of each pair was cut 90 days after sowing to assess the presence/absence of anthocyanin coloration. Plant growth stages (late tillering, stem extension, heading, and flowering) were recorded. Doubled haploids that reached the first two of these stages were assigned to a later group, and those that reached the heading and flowering stages were combined into an early group. The presence or absence of awns, their length and color were estimated after maturation. DNA was extracted from fresh leaves by CTAB method. The DNA quality and quantity were estimated electrophoretically using a 1.0 % agarose gel. Alleles of the *Pi-ta* and *Pi-ta*^{2} genes which determine blast resistance were detected by PCR method. Plants of the Chinese monogenic variety K12 were a positive control for *Pi-ta* gene, and varieties K27 for *Pi-ta*^{2} gene. The doubled haploids of two callus lines, 7.2.2 and 21.2.1, are

monomorphic without genetic variability for both resistance genes, *Pi-ta* and *Pi-ta*^{2}. Plant seeds of each callus line (7.2.2 and 21.2.1) will be further combined into two separate breeding lines to intensify breeding due to a larger number of seeds. Doubled haploids of the callus lines 1.2.1, 4.1.2, 8.2.1, 36.2.3 are polymorphic in awn formation, plant growth stage and anthocyanin pigmentation of stem cuts. The callus line 8.2.1 is genetically variable (ten plants carried alleles of both resistance genes, *Pi-ta* and *Pi-ta*^{2}, eight plants possessed none of the alleles, and in 26 plants only *Pi-ta*^{2}allele was detected). Thus, upon callus formation via in vitro androgenesis, the two opposite processes occurred are somaclonal variation and cell cloning. Somaclonal variation leads to polymorphism of callus cells and plant-regenerants, while cell cloning determines intra-callus uniformity, as well as the uniformity of some regenerants (and, in many cases, all regenerants derived from the same anther). Monomorphic doubled haploids, after a preliminary assessment for morphological traits and molecular characteristics, comprises a single selection sample.

Keywords: *Oryza sativa*, in vitro androgenesis, intra-callus variability, rice blast, resistance, *Pi-ta*, *Pi-ta*^{2}

In vitro androgenesis is one of the leading methods for creating breeding material of many agricultural crops [1-3], including rice *Oryza sativa* L. [4, 5]. Under appropriate conditions, it is relatively easy to obtain a significant number of doubled haploids, constant in morphological and genetic characteristics, with no segregation due to their homozygosity, thus the selection period becomes several years shorter [1, 4, 6]. When characterizing doubled haploids, researchers usually reveal their variability between varieties and hybrids [5, 7]. The issues of intravarietal/intrahybrid and intracallus variability remain poorly understood. According to the complex of quantitative characters in the rice cultivar Kaskad, significant differences were shown between the doubled haploids of two callus lines obtained from two anthers [9].

It is theoretically believed that one anther cultured in vitro can produce more than 1000 haploid plants [10)] which are clones [11, 12]. On one callus line of rice, tens [13] and sometimes more than a hundred doubled haploids appear. In this case, all doubled haploids of one callus line could be combined into one breeding line, which would speed up the breeding process by increasing seed pool per sample. Wheat as an example has convincingly proved the origin of androgenic embryoids from one cell [14]. It is known that there is genomic variability of rice regenerants (haploids—doubled haploids—tetraploids) within one callus line of derived from one anther [13]; therefore, genetic variability among doubled haploids is also probable. Many breeders a priori deem the seed progeny of any doubled haploid to be a new line [8, 15], regardless of which callus it was derived from. However, in the field, lines from one callus often look the same and are discarded, and only two or three of them are finally involved in further work. The validity of such a contradictory approach requires experimental confirmation or refutation of polymorphism and genetic variability of doubled haploids of one callus line.

About 100 genes of *Pi* family are known in rice for resistance to fungal pathogen *Pyricularia oryzae* Cav. [Magnaporthe grisea (Hebert Barr.)] [16] of which many are involved in breeding programs worldwide correspondingly to the pathogen race specificity. *Pi-ta* and *Pi-ta*^{2} genes are the most relevant for the Russian Far East [17]. Molecular markers allow their identification in rice plants [18-20].

This paper is the first to reveal intracallus morphological polymorphism and genetic variability for *Pi-ta* и *Pi-ta*^{2} genes due to gametoclonal and somaclonal variability of rice *Oryza sativa* L. doubled haploids derived from a hybrid plant via in vitro androgenesis. Also, there is the first demonstration of monomorphism of the doubled haploids of the same callus line without genetic variability, due to

clonal reproduction (mitotic division) of callus cells.

The work aimed to assess intracalus morphological and genetic variability of rice *Oryza sativa* L. doubled haploids generated via a hybrid plant in vitro androgenesis.

Materials and methods. Experiments were carried out in the Chaika Federal Research Center of Agricultural Biotechnology of the Far East (Russia) and the Crop Resources Institute, Heilongjiang Academy of Agricultural Sciences (China) in 2017-2018. The plant used to produce doubled rice haploids was Don 4237 × Dolinny F_1 hybrid in which a significant intracalus awnedness variability of doubled haploids was preliminaryly revealed in the R_1 regenerants. The Dolinny rice variety harbors alleles of *Pi-ta* and *Pi-ta*^{2} genes for the rice blast resistance [21, 22]. The technique for doubled haploid production was described earlier [23]. In each callus line (6 lines in total), from 9 to 44 seeds of doubled haploids of the first generation R_1 (106 lines of doubled haploids in total) were collected. For each line, two seeds per plastic pots with soil were planted and grown in a culture room at 24 °C, 5000 lx and 16 h (day)/8 h (night) mode. In the R_2 regenerants emerged from these seeds, the variability of morphological characters and growth phases were assessed.

One plant from each line of doubled haploids R_2 (106 plants in total) was cut off 90 days after planting, and the color of the cut indicative of the presence/absence of anthocyanin pigment was recorded. Leaves were collected from the same plant for DNA isolation. For another plant of the line, the following phases of growth were noted: late tillering (a straw without a flag leaf, the panicle is not visible inside the straw), stem extension (a straw with a flag leaf, the panicle is visible inside the straw), heading (5-6 upper panicle flowers), flowering (the panicle is fully visible). Double haploids that reached the first two stages were assigned to the late group, and those that reached heading and flowering were assigned to the early group. After maturation, discrete traits were assessed, that is, the presence or absence of awns, their length (short when less than 1 cm in size or as spines on some caryopses of panicles, long when more than 2 cm) and color (white black).

DNA was extracted from fresh leaves by the CTAB method [24]. The DNA quality and concertation were determined electrophoretically in 1.0% agarose gel with DNA of known concentration as a standard. Alleles of the *Pi-ta* and *Pi-ta*^{2} genes, encoding resistance, were identified by polymerase chain reaction (PCR) with the primers 5′-AGCAGGTTATAAGCTAGGCC-3′ and 5′-CTACCAACAAGTTCATCAAA-3′ for *Pi-ta* [18] and 5′-CAGCGAACTCCTTCGCATACGCA-3 and 5′-CGAAAGGTGTATGCACTATAGTATCC-3′ for *Pi-ta*^{2} [20]. The reaction mixture (20 μl) contained 2× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 μl each of forward and reverse primers, 1 unit of Taq DNA polymerase (Takara, Japan) and 70-120 ng of template DNA. Amplification was run in 3-fold repetition (a Veriti 96-Well Thermal Cycler, Applied Biosystems, USA). The following modes were used: 5 min at 94 °C (initial denaturation); 1 min at 94 °C (denaturation), 1 min at 56 °C (primer annealing), 1 min at 72 °C (elongation) (35 cycles); 10 min at 72 °C (final elongation) for *Pi-ta*, and 5 min at 95 °C (initial denaturation); 1 min at 95 °C (denaturation), 1 min at 60 °C (primer annealing), 1 min at 72 °C (elongation) (35 cycles); 10 min at 72 °C (final elongation) for *Pi-ta*^{2}.

Amplification products were separated electrophoretically in 1.0% agarose gel with 0.5× TBE buffer (a Sub Cell Model 192 chamber, Bio-Rad, USA; a

PowerPac Basic power supply, Bio-Rad, USA), stained with a 1.0% solution of ethidium bromide and visualized in ultraviolet light (the Gel Doc XR + gel documentation system, Bio-Rad, USA). The presence of the resistance allele was recognized only in the case of bright staining of DNA samples.

The Chinese monogenic cultivar K12 was a positive control for *Pi-ta*, and the cultivar K27 was a positive control for *Pi-ta²*.

Results. In vitro culture was generated from 386 hybrid anthers. Calli were formed in 17.1% of the anthers. Eighteen callus lines (27.3%) produced green regenerants. Six callus lines with numerous doubled haploids were finally selected for the experiment (Table 1).

1. Regeneration capacity of rice (*Oryza sativa* L.) callus lines derived from a Don 4237 × Dolinny F1 hybrid plant via in vitro androgenesis

Callus line	Green regenerants, *n*	Doubled haploids		Sample size (R_2), *n*
		n	%	
1.2.1	138	24	17	12
4.1.2	34	28	82	9
7.2.2	16	15	94	11
8.2.1	69	57	83	44
21.2.1	24	19	79	15
36.2.3	76	71	93	15

Molecular marking revealed that electrophoretic patterns of amplification products in the paternal cultivar Dolinny and the initial hybrid plant have a band characteristic of the *Pi-ta* allele (1042 bp), whereas in the maternal cultivar Don 4237 this DNA fragment is absent (Fig. 1).

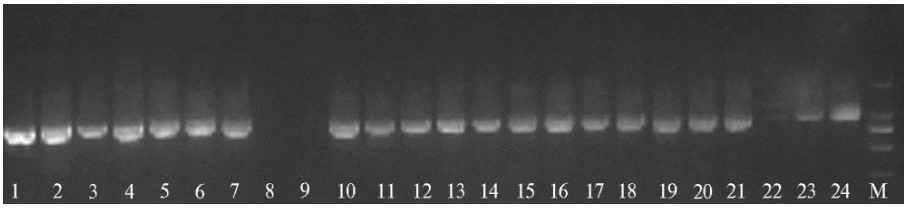

Fig. 1. Electrophoretic identification of amplicons characteristic of blast resistance gene *Pi-ta* in the parental rice (*Oryza sativa* L.) Don 4237 and Dolinny varieties, their F1 hybrid and doubled haploids R_2 of six callus lines derived from the F1 hybrid plant: 1, 2 — 1.2.1; 3, 4 — 4.1.2; 5, 6 — 7.2.2; 7-16 — 8.2.1, 17, 18 — 21.2.1; 19, 20 — 36.2.3; 21 — Dolinnyi variety, 22 — Don 4237 variety, 23 — Don 4237 × Dolinnyi F1 hybrid, 24 — positive control (1042 bp, the monogenous line K12), 25 — a molecular-weight size marker D2000 («TIANGEN», China).

The *Pi-ta²* allele was found in both parental forms and in the hybrid. Doubled haploids of five callus lines (1.2.1, 4.1.2, 7.2.2, 21.2.1, and 36.2.3) had alleles for both *Pi-ta* and *Pi-ta²* resistance genes (Table 2). Doubled haploids derived from the callus line 8.2.1 showed genetic variability, i.e. 10 plants harbored alleles of both resistance genes, in 8 plants both alleles were absent, and in the remaining 26 plants only the *Pi-ta²* allele was revealed. The peculiarities of differentiation of the callus line 8.2.1-derived doubled haploids had little effect on the presence of detectable amplification products. Thus, out of 69 green regenerants formed (see Table 1), plants with alleles of both resistance genes were found among the R_2 doubled haploids Nos. 1-44, of only *Pi-ta²* among the R_2 doubled haploids Nos. 22-68. Note, alleles of both resistance genes were absent in a part of the doubled haploids Nos. 2-69, thence, no definite regularity was observed.

An analysis of phenotypic variability showed that the doubled haploids of callus lines 7.2.2 and 21.2.1 are monomorphic. Polymorphism was observed among the doubled haploids of four callus lines. The callus line 8.2.1 showed the greatest morphological variability, and this variability was not associated with the genetic

variability of doubled haploids (see Table 2).

2. Genotypes and phenotypes of rice (*Oryza sativa* L.) doubled haploids R_2 of six callus lines derived from a Don 4237 × Dolinny F_1 hybrid plant via in vitro androgenesis

Callus line	Doubled haploids, *n*	Stem cut color	Awns, size	Type of development	Genes	
					Pi-ta	*Pi-ta²*
1.2.1	2	Anthocyanin	Short	Early	+	+
	1	Anthocyanin	Absent	Early	+	+
	6	Anthocyanin	Short	Late	+	+
	3	Anthocyanin	Long	Late	+	+
4.1.2	2	Anthocyanin	Short	Early	+	+
	4	Anthocyanin	Short	Late	+	+
	1	Anthocyanin	Absent	Early	+	+
	2	Anthocyanin	Absent	Late	+	+
7.2.2	11	No anthocyanin	Long	Late	+	+
8.2.1	3	Anthocyanin	Short	Early	+	+
	2	Anthocyanin	Long	Late	+	+
	1	No anthocyanin	Long	Late	+	+
	3	Anthocyanin	Absent	Early	+	+
	1	No anthocyanin	Absent	Early	+	+
	1	Anthocyanin	Short	Early	−	+
	1	Anthocyanin	Long	Early	−	+
	18	Anthocyanin	Short	Late	−	+
	1	Anthocyanin	Long	Late	−	+
	5	No anthocyanin	Short	Late	−	+
	1	Anthocyanin	Short	Early	−	−
	5	Anthocyanin	Short	Late	−	−
	1	No anthocyanin	Short	Early	−	−
	1	No anthocyanin	Short	Late	−	−
21.2.1	15	Anthocyanin	Absent	Early	+	+
36.2.3	2	Anthocyanin	Absent	Early	+	+
	13	Anthocyanin	Absent	Late	+	+

N o t e. «+» — presence of the resistance gene; «−» — absence of the resistance gene.

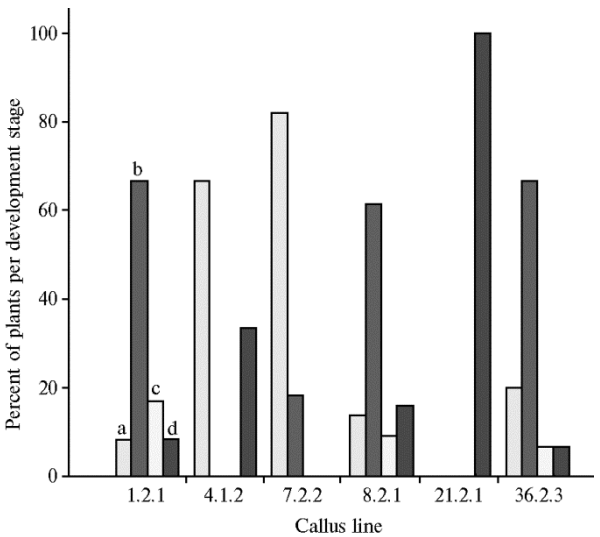

Fig. 2. Distribution of rice (*Oryza sativa* L.) doubled haploids R_2 of six callus lines derived from a Don 4237 × Dolinnyi F_1 hybrid plant via in vitro androgenesis according to the growth stage 90 days after planting: a — tillering, b — stem extension, c — heading, d — flowering.

Figure 2 shows the detailed distribution of doubled haploids according to the stage of growth. All plants of callus line 21.2.1 flowered, no variability in other traits was observed either. Plants of line 4.1.2 were clearly divided into two groups, three early (in the flowering stage) and five late (in the tillering stage), among those and others were those awned and awnless (see Table 2). Plants of line 7.2.2 were late in the timing of both tillering and stem extension stages and monomorphic in other traits. In three of the six callus lines (1.2.1, 8.2.1, and 36.2.3), all four growth stages were observed in doubled haploids. Of these, line 36.2.3 is completely awnless with anthocyanin coloration of the straw cut, that is, variable only in early maturity, line 1.2.1 is polymorphic in two traits, the awn formation and growth stages. In line 8.2.1, doubled haploids exhibited both genetic and phenotypic variability, and among the doubled haploids, a different combination of morphological and genetic traits was revealed (see Table 2). We did not observe variability in the color of

awns among doubled haploids of the same callus line, i.e. in line 7.2.2, the awns of caryopses were white, in lines 1.2.1, 4.1.2, and 8.2.1 they were black

The anther of rice contains about 1,000 pollen grains [25] which can be induced into callus. The callus line from one anther derived from callus aggregates, which can be formed by several immature microspores. This leads to both polymorphism and genetic variability among doubled haploids and to genomic variability of regenerates of one callus line (haploids, doubled haploids, and tetraploids) [13]. However, clonal reproduction of regenerants in vitro is also not excluded. Thus, for some morphotypes and genotypes, there are from a small number to several tens of doubled haploids, i.e. 18 plants in the callus line 8.2.1 (see Table 2). Earlier, we detected the formation of up to 7-18 tetraploid plants of one callus line. Although the polymorphism of tetraploids of one callus line has not been studied, their massive formation on one callus most likely occurs due to mitotic the replication of 4*n* callus cells [26]. The doubled haploids of callus lines 7.2.2 and 21.2.1, which are monomorphic, can be considered as identical plants and combined into two separate sets during selection. Similar methodology is obviously applicable for groups of identical plants within other callus lines.

The callus line 8.2.1 is of particular interest due to emergence of various regenerant types, i.e. those with alleles of both resistance genes, *Pi-ta* and *Pi-ta*^{2}, having only *Pi-ta*^{2} and not carrying alleles of any of these genes. There are several possible explanations for such a segregation of the line 8.2.1 regenerants. One of the parents (variety Dolinnyi) is a carrier of the *Pi-ta* allele which determines resistance, and his hybrid inherited this gene in a heterozygote. As a result, some of the doubled haploids had the *Pi-ta* allele, and some did not (see Fig. 1). Both parents and the hybrid possessed the *Pi-ta*^{2} allele, but it was absent in 8 doubled haploids (see Table 2). The primers we used allow detection of the presence or absence of an allele of the resistance gene but do not reveal the allelic state of the gene. It can be assumed that one of the parents (variety Don 4234) is heterozygous, and the other (variety Dolinnyi) is homozygous for *Pi-ta*^{2} gene, while the hybrid is heterozygous for this gene. Figure 3 shows a possible scheme of dihybrid crossing which leads to genetic variability among the doubled haploids of callus line 8.2.1. Only the second variant of crossing provides for three types of combinations of resistance alleles in regenerated plants. *AB*, *aB*, and *ab* microspores induced callus formation, followed by spontaneous chromosome doubling and regeneration. Microspores of the fourth type *Ab* either did not generate a callus, or only produced haploids.

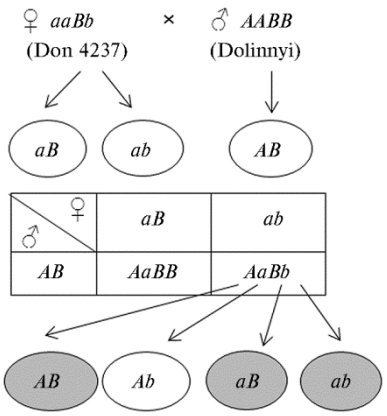

Fig. 3. A possible scheme for the dihybrid crossing of rice (*Oryza sativa* L.) parental varieties, followed by callus formation from microspores to produce doubled haploids with two alleles of the blast resistance genes *Pi-ta* and *Pi-ta*^{2}, with one allele (only for *Pi-ta*^{2}) and without alleles of both resistance genes: A — *Pi-ta* allele which determines resistance, a — *Pi-ta* allele which determines susceptibility, B — *Pi-ta*^{2} allele which determines resistance, b — *Pi-ta*^{2} allele which determines susceptibility.

Wang et al. [27] consider the *Pi-ta* and *Pi-ta*^{2} genes to be allelic or closely linked to each other. According to Oryzabase [28], this is the same gene of 7281 nucleotides in size detected by different primers that amplify different parts of the gene. *Pi-ta* gene allele polymorphisms have been studied in several rice species and varieties. Only 99% of alleles are found to be similar in

nucleotide sequence and show not identical level of divergence [29]. True, the phenotypic manifestation of *Pi-ta* and *Pi-ta²* genes, revealed with the use of differentiating varieties, differs significantly [17, 20, 30]. In the studied plants, one could expect either the presence or the complete absence of resistance alleles for both genes. However, we have revealed in 26 plants only the *Pi-ta²* gene allele which determines resistance.

The phenomenon of somaclonal variability is widespread in cell and tissue cultures in vitro, it causes an increase in genetic variability and often does not affect the viability of regenerants derived from calli [31-33]. In our experiment, genetic mutations caused by in vitro culture are not excluded. These changes could have occurred in the DNA region of the *Pi-ta* gene, which is not identical to the *Pi-ta²* gene, in callus cells even before the spontaneous doubling of chromosomes followed by formation of doubled haploids. It is impossible to exclude mutations in DNA regions that led to the absence of resistant alleles of both genes in 8 doubled haploids. This is supported by the presence of the resistant alleles of *Pi-ta* and *Pi-ta²* among the doubled haploids of 5 other callus lines. Possibly, due to somaclonal variability that arose after spontaneous doubling, several samples could harbor any of the studied genes in a heterozygous state, which cannot be detected using the primers chosen.

The revealed polymorphism may indicate that in the anther, which was used to obtain line 8.2.1, callus formation was induced in at least 14 immature microspores, followed by chromosome doubling and regeneration. However, a high frequency of physiological and quantitative changes among somaclones was previously noted, such as the time of flowering (panicle emergence), ripening time, presence or absence of awns, plant height [32, 34]. Morphological, biochemical, and molecular genetic analysis showed that during somaclonal variation, already at early culture stage, similar genetic transformations occur, leading to the appearance of common characters in different groups of somaclones of the same cultivated species [35]. The coincident variability can be explained by transposition explosions [34]. Among the tobacco and rapeseed dihaploids, morphological mutants segregate, which must be completely homozygous [34]. If such variability occurs in the doubled haploids of the four callus lines obtained by us (1.2.1, 4.1.2, 8.2.1, 36.2.3), genetic changes are possible even at the stage of haploid cells with subsequent spontaneous doubling. The other two callus lines not affected by this variability are probably more stable (7.2.2, 21.2.1). Therefore, it can be assumed that the ability to accumulate mutational changes in cell in vitro culture is significantly influenced not only by the variety of the original plant, which was established by Kuznetsova et al. [33], but also by the genotype of the pollen grain used for in vitro androgenesis.

In vitro androgenesis allows production of a huge variety of doubled haploid lines for breeding programs. In particular, the combination of monomorphic doubled haploids of one callus line into a single breeding line accelerates breeding due to the increased number of seeds per sample. It is also possible to use the diversity of doubled haploids observed in some *O. sativa* callus lines, considering each of them as a separate breeding line. This is especially important for genotypes with a hindered androgenesis in vitro: even upon callus formation induced only in one of the many immature anthers, the derived callus line allows one to obtain several polymorphic lines of doubled haploids.

Next, we plan to study the resistance of the obtained early-maturing leafless lines of doubled rice haploids carrying alleles of both genes, *Pi-ta* and *Pi-ta²*, upon artificial infection with *P. oryzae* strains circulating in the Far Eastern rice

growing zone.

Thus, we have revealed for the first time intracallus morphological polymorphism and genetic variability for *Pi-ta* and *Pi-ta²* blast resistance genes in rice (*Oryza sativa* L.) doubled haploid lines obtained via in vitro androgenesis from one hybrid plant. The doubled haploids of callus lines 7.2.2 and 21.2.1 are monomorphic and do not show genetic variability for both resistance genes, *Pi-ta* and *Pi-ta²*. The doubled haploids of four other callus lines (1.2.1, 4.1.2, 8.2.1, and 36.2.3) are polymorphic in awnedness, plant development rate, and anthocyanin coloration of cuts. The callus line 8.2.1 is genetically variable and represented by three types of plants, those with alleles of both *Pi-ta* and *Pi-ta²* genes, only the *Pi-ta²* gene allele, or without alleles of both genes. Callus formation during in vitro androgenesis involves two-direction events. The first is the well-studied scenario of somaclonal variation which causes polymorphism of cells or regenerants, and the second is cell cloning which leads to intracallus homogeneity, the homogeneity of some of the regenerants, and in many cases of all the regenerants derived from one anther. The monomorphic doubled haploids, after a preliminary morphological and molecular genetic characterization, are grouped into one sample to be further involved in the breeding program.

REFERENCES

1. Germana M.A. Anther culture for haploid and doubled haploid production. *Plant Cell Tiss. Organ. Cult.,* 2011, 104(3): 283-300 (doi: 10.1007/s11240-010-9852-z).
2. Ferrie A.M.R., Caswell K.L. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. *Plant Cell Tiss. Organ. Cult.*, 2011, 104(3): 301-309 (doi: 10.1007/s11240-010-9800-y).
3. Dunwell J.M. Haploids in flowering plants: origins and exploitation. *Plant Biotechnology Journal*, 2010, 8(4): 377-424 (doi: 10.1111/j.1467-7652.2009.00498.x).
4. Mishra R., Rao G.J.N. In-vitro androgenesis in rice: advantages, constraints and future prospects. *Rice Science*, 2016, 23(2): 57-68 (doi: 10.1016/j.rsci.2016.02.001).
5. Sarao N.K., Gosal S.S. In vitro androgenesis for accelerated breeding in rice. In: *Biotechnologies of crop improvement. Vol. 1.* S.S. Gosal, S.H. Wani (eds.). Springer, Cham, 2018: 407-435 (doi: 10.1007/978-3-319-78283-6_12).
6. Datta S.K. Androgenic haploids: factors controlling development and its application in crop improvement. *Current Sci.*, 2005, 89(11): 1870-1878.
7. Mishra R., Rao G.J.N., Rao R.N., Kaushal P. Development and characterization of elite doubled haploid lines from two indica rice hybrids. *Rice Science*, 2015, 22(6): 290-299 (doi: 10.1016/j.rsci.2015.07.002).
8. Lapitan V.C., Redoña E.D., Abe T., Brar D. Molecular characterization and agronomic performance of DH lines from the F1 of *indica* and *japonica* cultivars of rice (*Oryza sativa* L.). *Field Crops Research*, 2009, 112(2-3): 222-228 (doi: 10.1016/j.fcr.2009.03.008).
9. Ilyushko M.V., Romashova M.V. Variability of rice haploids obtained from in vitro anther culture. *Russian Agricultural Sciences*, 2019, 45(3): 243-246 (doi: 10.3103/S1068367419030108).
10. Sibikeeva Yu.E., Sibikeev S.N. *Genetika*, 2014, 50(7): 831-839 (doi: 10.7868/S0016675814070169) (in Russ.).
11. Kruglova N.N. *Agrarnaya Rossiya*, 2009, 1: 34-38 (in Russ.).
12. Heberle-Bors E. In vitro haploid formation from pollen: a critical review. *Theoret. Appl. Genetics*, 1985, 71: 361-374 (doi: 10.1007/BF00251175).
13. Ilyushko M.V., Romashova M.V. *Argarnyi vestnik Primor'ya*, 2018, 1(9): 5-8 (in Russ.).
14. Sel'dimirova O.A., Kruglova N.N. *Uspekhi sovremennoi biologii*, 2014, 134(5): 476-487 (in Russ.).
15. Mishra R., Rao G.J.N., Rao R.N., Kaushal P. Development and characterization of elite doubled haploid lines from two *indica* rice hybrids. *Rice Science,* 2015, 22(6): 290-299 (doi: 10.1016/j.rsci.2015.07.002).
16. Wu Y., Xiao N., Chen Y., Yu L., Pan C., Li Y., Zhang X., Huang N., Ji H., Dai Z., Chen X., Li A. Comprehensive evaluation of resistance effects of pyramiding lines with different broad-spectrum resistance genes against *Magnaporthe oryzae* in rice (*Oryza sativa* L.). *Rice*, 2019, 12: 11 (doi: 10.1186/s12284-019-0264-3).
17. Sankin A.Yu., Lelyavskaya V.N., Sun I.T. *Uspekhi sovremennoi nauki,* 2017, 2(10): S. 26-28 (in Russ.).
18. Dai X.-J., Yan Y.-Z., Zhou L., Liang M.-Z., Fu X.-C., Chen L.-B. Distribution research of blast

resistance genes *Pita*, *Pib*, *Pi9* and *Pikm* in blast-resistant rice resources. *Life Sci. Research*, 2012, 16(4): 340-356 (doi: 10.16605/j.cnki.1007-7847.2012.04.009).
19. Orasen G., Greco R., Puja E., Pozzi C., Stile M.R. Blast resistance *R* genes pyramiding in temperate japonica rice. *Euphytica*, 2020, 216: 40 (doi: 10.1007/s10681-020-2575-2).
20. Wang J.C., Correll J.C., Jia Y. Characterization of rice blast resistance genes in rice germplasm with monogenic lines and pathogenicity assays. *Crop Protection*, 2015, 72: 132-138 (doi: 10.1016/j.cropro.2015.03.014).
21. Ilyushko M.V., Fisenko P.V., Sunitskaya T.V., Guchenko S.S., CHzhan TS., Den L.-V., Kostylev P.I. *Zernovoe khozyaistvo Rossii*, 2017, 4: 41-45 (in Russ.).
22. Ilyushko M.V., Romashova M.V., Fisenko P.V., Sunitskaya T.V., Guchenko S.S., Lelyavskaya V.N. *Vestnik zashchity rastenii,* 2019, 1(99): 36-39 (doi: 10.31993/2308-6459-2019-1(99)-36-39) (in Russ.).
23. Ilyushko M.V., Romashova M.V. *Dal'nevostochnyi agrarnyi vestnik,* 2017, 4(44): 37-45 (in Russ.).
24. *Gennaya inzheneriya rastenii. Laboratornoe rukovodstvo* /Pod redaktsiei Dzh. Dreipera, R. Skotta, F. Armitidzha, R. Uoldena [Genetic engineering of plants. Laboratory manual]. Moscow, 1991 (in Russ.).
25. Farrell T.C., Fox K.M., Williams R.L., Fukai S. Genotypic variation for cold tolerance during reproductive development in rice: screening with cold air and cold water. *Field Crops Research,* 2006, 98(2-3): 178-194 (doi: 10.1016/j.fcr.2006.01.003).
26. Ilyushko M.V., Romashova M.V. *Rossiiskaya sel'skokhozyaistvennaya nauka*, 2020, 3: 14-17 (doi: 10.31857/S2500262720030047) (in Russ.).
27. Wang Z., Jia Y., Fjellstrom R.G. The relationship between the rice blast resistance genes *Pi-ta* and *Pi-ta*^{2}. *Journal of Zhejiang wanly university,* 2004, 17(2): 91-92.
28. *Integrated Rice Science Database*. Available: http://shigen.nig.ac.jp/rice/oryzabase/gene/detail/947. Accessed: 30.08.2018.
29. Ramkumar G., Madhav M.S., Rama Devi S.J.S., Manimaran P., Mohan K.M., Prasad M.S., Balachandran S.M., Neeraja C.N., Sundaram R.M., Viraktamath B.C. Nucleotide diversity of *Pita*, a major blast redistance gene and identification of its minimal promoter. *Gene*, 2014, 546(2): 250-256 (doi: 10.1016/j.gene.2014.06.001).
30. Ma J.-T., Zhang G.-M., Xin A.-H., Zhang L.-Y., Deng L.-W., Wang Y.-L., Wang Y., Ran Y., Gong X.-J., Ge X.-L., Yang X.-F. Comparison of pathogenicity of *Pyricularia oryzae* under different genetic backgrounds. *Acta Agronomica Sinica*, 2015, 41(12): 1791-1801 (doi: 10.3724/SP.J.1006.2015.01791).
31. Yamamoto T., Soeda Y., Nishikawa A., Hirohara H. A study of somaclonal variation for rice improvement induced by three kinds of anther-derived cell culture techniques. *Plant Tissue Culture Letters*, 1994, 11(2): 116-121 (doi: 10.5511/plantbiotechnology1984.11.116).
32. Ezhova T.A., Bagrova A.M., Khartina G.A., Gostimskii S.A. *Genetika*, 1989, XXV(5): 878-885 (in Russ.).
33. Kuznetsova O.I., Ash O.A., Gostimskij S.A. The effect of duration of callus culture on the accumulation of genetic alteration in pea *Pisum sativum* L. *Russian Journal of Genetics*, 2006, 42(5): 555-562 (doi: 10.1134/s1022795406050139).
34. Scowcroff W.R. Somaclonal variation: the myth of clonal uniformit. In: *Genetic flux in plant*. B. Hohn, E.S. Dennis (eds.). Wien, New York: Springer-Verlas, 1985: 217-245 (doi: 10.1007/978-3-7091-8765-4).
35. Osipova E.S., Koveza O.V., Gostimskij S.A., Troitskij A.V., Dolgikh Yu.L., Shamina Z.B. Analysis of specific RAPD and ISSR fragments in maize (*Zea mays* L.) somaclones and development of SCAR markers on their basis. *Russian Journal of Genetics,* 2003, 39(12): 1412-1419 (doi: 10.1023/B:RUGE.0000009156.74246.bc).

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OPTIMAL PARAMETERS OF MODEL BROAD BEAN CULTIVAR FOR THE CENTRAL PART OF THE DANUBE PLAIN, BULGARIA

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Abstract

In order to increase the productive potential of crop plants, a selection of economically valuable traits is needed, the combination of which in the breeding process will lead to the development of the desired genotype. The purpose of the present study was to establish the impact of main quantitative traits on broad bean productivity (in organic production conditions) and to select parental components with increased adaptability and stability. The experimental activity was carried out in the Institute of Forage Crops (Pleven, Bulgaria), during the period 2016-2018, with source collection of 17 accessions of broad bean. A linear equation of regression was used to determine the effect of quantitative traits on seed productivity. The optimal parameters of model broad bean plant with high seed weight was characterized by the following traits: plant height — 69 cm to 90 cm, 1st pod height — 22 cm to 36 cm, pod number per plant — 9 to 14, seed number — 22 to 35, pod length — 7 to 11 cm, pod width — 15 to 19 mm, and 100 seeds weight — from 80 g to 120 g. The obtained results showed that the traits of seed number (R = 0.72) per plant, pod number (R = 0.48), 100-seed weight (R = 0.28) and 1st pod height (R = 0.25) had the greatest and statistically significant impact on the seed productivity. Accessions FbH 14, FbH 16, BGE 032012, FbH 15, and Fb 2486 were distinguished as highly adaptable regarding seed productivity, and Fb 1903 and BGE 041470 were determined as low adaptive. Accessions Fb 1896, Fb 2486, BGE 032012 and BGE 043776 combined good adaptability with low variability and were suitable as parental components for use in the combinative breeding.

Keywords: model, broad bean, regression, traits, plant height, pod size, pod number, 100-seed weight, seed productivity

Broad bean is an annual legume plant known in botany as *Vicia faba* L. It is known by different names (Horse bean, Broad bean, Faba bean, Fava bean, Windsor bean, Tick beans), most of which refer to a separate subgroup rather than the whole species [1, 2]. Broad bean is one of the oldest crops, worldwide it is third most important forage grain legume after peas and soybeans [3]. The long history of growing, wide spread in diverse environments, and different breeding methods have turned the broad bean into one of the most variable species, possessing a wide range of variations in plant architecture, leaf shape and size, seed size, color and shape [4, 5].

Studies in the field of legume breeding have made essential progress in recent years with regard to the creation of new varieties with increased productivity and ecological adaptability [6]. Despite successes in this line, the need for further improvement and development of new varieties continues to be a major task due to the changing environmental conditions and the lack of suitable varieties for them [7]. New challenges present the requirements that impose on cultivars the production systems with reduced levels of inputs (and in particular the organic and biodynamic production systems) and the main problem with these

systems is low productivity.

Crop breeding is a prolonged process that is dependent on a number of factors [8]. The ideotypic breeding distincts from the traditional one in that the breeders mean to change exactly certain traits in order to increase the productivity and adapt the plants to the particular environment of growing [9].

In order to enhance the productive potential of crop plants, a selection of economically valuable characteristics is needed. Their combination in the breeding process will lead to the creation of the desired genotype [10]. Of particular importance for enhancing the efficiency of the selection activity is information on the interrelationships among the quantitative signs in the plant population. One of the commonly used methods for clarifying dependencies is through applying correlation and regression analysis [10, 11].

This study compares the variability of quantitative traits in a number of breeding varieties of forage beans of different origins and local populations and determines optimal parameters of a highly productive variety for organic technologies. The most promising donors are identified for creating a variety corresponding to the model for these conditions.

The purpose of the study was to establish optimal parameters of model broad bean cultivar for the central part of the Danube plain and to select parental components with increased adaptability for the needs of combinative breeding.

Materials and methods. Seventeen accessions of broad bean (*Vicia faba* L.) (FbH 13, FbH 14, FbH 15, FbH 16, BGP, Fb 1896, Fb 1903, Fb 1929, Fb 2481, Fb 2486, Fb 3270, BGE 002106, BGE 029055, BGE 032012, BGE 041470, BGE 043776, BGE 046721) with different origin were objectives of this study (collection of the Institute of Forage Crops, Pleven, Bulgaria). The introduced accessions were local cultivars and the Bulgarian ones were local populations. A starting point in the formation of the initial collection was the choice of samples with high seed productivity. The experimental activity was carried out in the Institute of Forage Crops (Pleven) during the period 2016-2018. The variants were located in randomized block method [12], with a size of the experimental plot of 4 m^2 and a threefold repetition of the variants. The sowing was manual, with a sowing rate of 30 seeds m^2. Accessions were cultivated under organic farming conditions, without application of fertilizers and pesticides. The biometric characteristics at harvesting included the following traits: 1st pod height (cm), plant height (cm), pods number per plant, pod length (cm), pod width (cm), seeds number per plant, seed weight per plant (g), 100 seeds mass (g).

The data were processed by regression analysis of variance for each trait for determining the influence of factors of genotype (accession) and environment. The phenotypic variability of the broad bean traits was evaluated by the nonparametric method [13], and the general adaptability (A) was calculated [14]. All experimental data were processed statistically with using the computer software GENES 2009.7.0 for Windows XP (ftp://ftp.ufv.br/dbg/biodata/) [15].

Results. The studied samples (breeding varieties and local populations) are listed in Table 1.

1. Broad bean *Vicia faba* L. accessions included in the study (Institute of Forage Crops, Pleven, Bulgaria, 2016-2018)

Accessions	Status	Origin
Fb 1896	Cultivar	Portugal
Fb 1903	Cultivar	Portugal
Fb 1929	Cultivar	Portugal
Fb 2481	Cultivar	Portugal
Fb 2486	Cultivar	Portugal
Fb 3270	Cultivar	Portugal
BGE 002106	Cultivar	Spain
BGE 029055	Cultivar	Spain

Continued Table 1

BGE 032012	Cultivar	Spain
BGE 041470	Cultivar	Spain
BGE 043776	Cultivar	Spain
BGE 046721	Cultivar	Spain
FbH 13	Landrace	Bulgaria
FbH 14	Landrace	Bulgaria
FbH 15	Landrace	Bulgaria
FbH 16	Landrace	Bulgaria
BGP	Landrace	Bulgaria

A task of each breeding program is to approximate the biometric values of the plant to theoretically calculated and optimal parameters. The results of the regression analysis (Table 2) showed that the linear component in the regression of seed productivity with respect to the studied quantitative indicators was significant.

2. Regression analysis of seed productivity regarding main quantitative traits in broad bean *Vicia faba* L. cultivars and landraces (Institute of Forage Crops, Pleven, Bulgaria, 2016-2018)

Source	Sum of Square (SS)	Degrees of freedom (df)	Mean Square (MS)	*F*-ratio	p-value
Model	5.31038	7	0.758625	27.43	0.00001
Residual	4.01082	145	0.027661		
Total	9.32120	152			

3. Regression coefficients (R) of seed productivity regarding main quantitative traits in broad bean *Vicia faba* L. cultivars and landraces (Institute of Forage Crops, Pleven, Bulgaria, 2016-2018)

Parameters	Estimate (R)	Standard error	*t*-statistic	p-value
Constant	−29.72	1.9816	−14.9986	0.0000
Plant height	−0.09*	0.0282	−3.2561	0.0014
1st pod height	0.25**	0.0571	4.3878	0.0000
Pods number	0.48*	0.1360	3.5531	0.0005
Seeds number	0.72**	0.0487	14.7781	0.0000
Pod length	0.44	0.2265	1.9346	0.0550
Pod width	0.27	1.4198	0.1876	0.8514
100 seeds mass	0.28**	0.0138	20.6404	0.0000

* and ** Regression coefficients are statistically significant at p = 0.01 and p = 0.001, respectively.

Based on the data from the biometric analysis of the collection of accessions, a statistical model of broad bean plant under the climatic conditions of Central Northern Bulgaria was developed. A regression equation (Table 3) was presented, which expresses the impact of each individual trait on seed productivity:

$$Y = 0{,}883 - 0{,}09X_1 + 0{,}25X_2 + 0{,}48X_3 + 0{,}72X_4 + 0{,}44X_5 + 0{,}27X_6 + 0{,}28X_7,$$

where Y is seed productivity, X_1 is plant height, X_2 is 1st pod height, X_3 is pods number per plant, X_4 is seeds number per plant, X_5 is pod length, X_6 is pod width, X_7 is 100 seeds mass.

The graphical representation of the relationships between the seed productivity and studied quantitative components allowed statistical results to be obtained (with sufficient approximation) and main dependencies between the quantitative traits to be established (Fig. 1). Of the investigated characteristics, only plant height had a negative impact (R = –0.09) on productivity. The negative interaction of this characteristic was very low and expressed at values lower than 69 cm and higher than 90 cm.

The increase in the height of 1st pod placement had a slight positive effect ($R = +0.25$, $p < 0.001$) on seed productivity of the broad bean, with the optimal limits of this trait in the study conditions between 22 and 36 cm. The

influence of the pods number (R = +0.48, p < 0.01) was also statistically significant and favorable, especially when ranging from 9 to 14 pods.

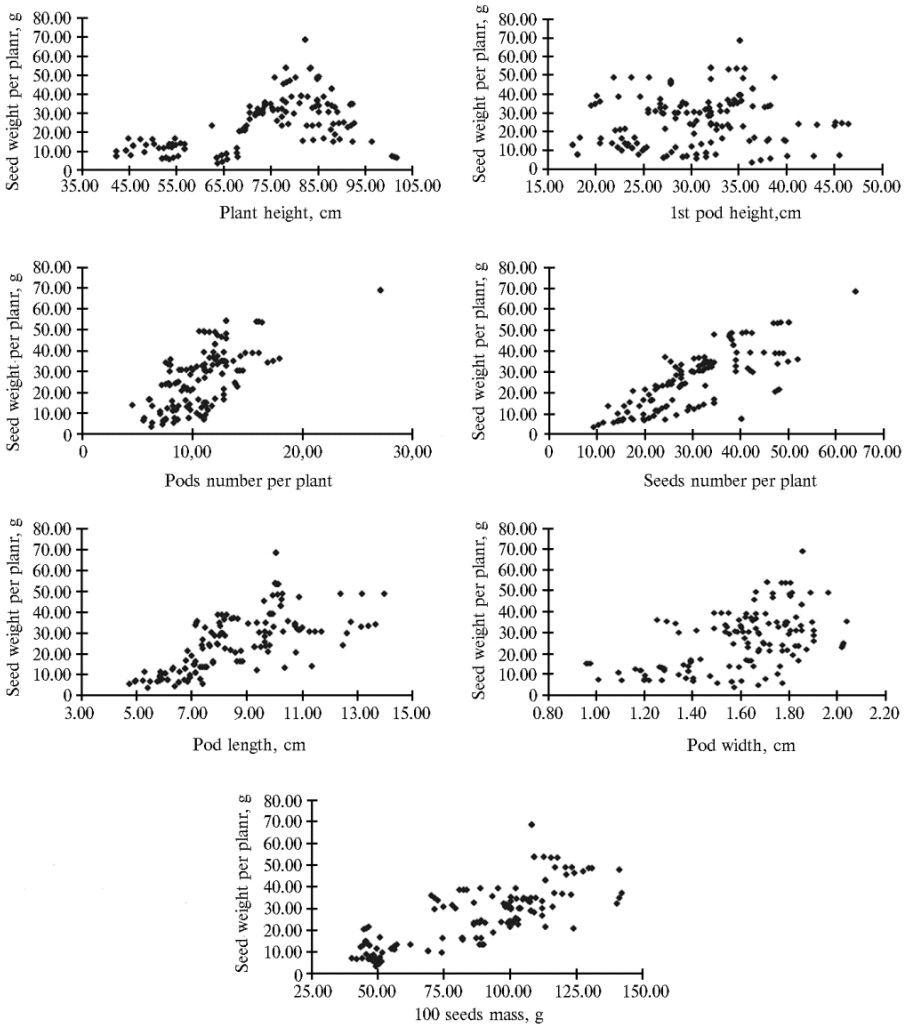

Fig. 1. Dependencies (R) **between seed productivity per plant and quantitative traits in broad bean *Vicia faba* L. cultivars and landraces** (Institute of Forage Crops, Pleven, Bulgaria, 2016-2018).

The impact of the seeds number was significantly positive and with the greatest heaviness (R = +0.72, p < 0.001), suggesting that increasing the number of seeds will be crucial for the seed weight of the plant. Its optimal values, which had a favorable effect on seed productivity, ranged from 22 to 35 seeds.

Other traits that had a positive impact on productivity were the length and width of pods. The regression coefficients for both traits (R = 0.44, R = 0.27) were insignificant as the pod width was almost twice smaller than that of pod length. According to the data obtained, it could be assumed that these signs will not play an essential role in the expression of the seed weight. Seed productivity would decrease if the plants form pods with a length and a width beyond the range 7-11 cm and 15-19 mm, respectively.

Another characteristic, with significant influence (R = +0.28, p < 0.001) on the broad bean productivity, was the mass of 100 seeds. The beneficial impact of this indicator occurs at values ranging from 80 to 120 g. Seed productivity will decrease with a reduction or increase in the mass of 100 seeds beyond the indi-

cated limits.

According to the analysis carried out, the model of a broad bean plant for the climatic conditions of Central Northern Bulgaria for the purpose of realizing high seed productivity should contain parameters within the following limits: plant height — from 69 to 90 cm, 1st pod height — from 22 to 36 cm, pods number per plant — from 9 to 14, seeds number — from 22 to 35, pod length — from 7 to 11 cm, pod width — from 15 to 19 mm, and 100 seeds mass — from 80 to 120 g.

It should be noted, however, that the full performance of productive potential depended not only on the individual influence of each parameter on the seed weight but also on the complex interrelationships between the individual traits and their combination in the plant [16].

Modern principles of modeling of varieties should be applied differentiated and to be adapted to the specifics of the respective soil-climatic conditions [17]. For example, the development of a model in lupine required studying the morpho-physiological nature of indicators influencing the productivity and sustainability to stress factors. The analysis conducted by the author showed that plant productivity depends to a large extent on the number of seeds and pods per plant. The studies of some authors [16] related to cultivar model in chick pea showed that the plant should have the following main characteristics: height — 38-43 cm; pods number — 25-45; seeds number — 25-45 и mass of 1000 seeds — 28-45 g. A similar investigation in fodder peas [18] found that the plant model was characterized by an average height of 60-70 cm, 8-10 pods, 30-40 seeds and 160-260 g regarding the mass per 1000 seeds. The author stated that the number of seeds per plant, height of 1st pod and 1000 seeds mass had the greatest impact on the grain yield of peas.

Adaptability is defined by the ability of plants to form high yields under different environmental conditions. Adaptive potential of species and cultivars is based on modifying genotypic variability and is characterized by the terms “plasticity” and “stability” [19]. To evaluate the adaptability in the present study conditions, the coefficient of general adaptability (Fig. 2) was calculated according to the values of which the production capabilities of accessions can be estimated. Selective value represents those that have a high general adaptation (coefficient A has maximum values) and at the same time, they exhibit low variability of the trait. The obtained data showed that almost all broad bean accessions have positive coefficients of general adaptability. Accessions with the highest adaptation can be arranged in the following order: FbH 14 > FbH 16 > BGE 032012 > FbH 15 > Fb 2486 > BGE 046721. Fb 1903 and BGE 041470 are characterized by the lowest (poorly negative) adaptive ability.

An important condition for ensuring sustainable and organic forage production is the use of cultivars with increased stability of the yield [20]. The conditions under organic farming are much more diverse than in traditional farming, so varieties must be much more adaptable, and yield stability is as important as its magnitude [21]. Some of the varieties that have been developed in conventional conditions have stable yields also in the organic farming system, but they have to be tested for selecting such ones with stable yields, suitable for the conditions of organic farming [20]. The stability assessment in the present study (see Fig. 2), according to the parameter of Kang [13], showed that accessions BGE 043776, Fb 2486, BGE 002106, Fb 2481 and Fb 3270 exhibited good stability and low variability (YS*i* varied from 14 to 20). In contrast, FbH 14, BGE 029055 and FbH 13 occupied the last positions, defining them as the most un-

stable in productivity.

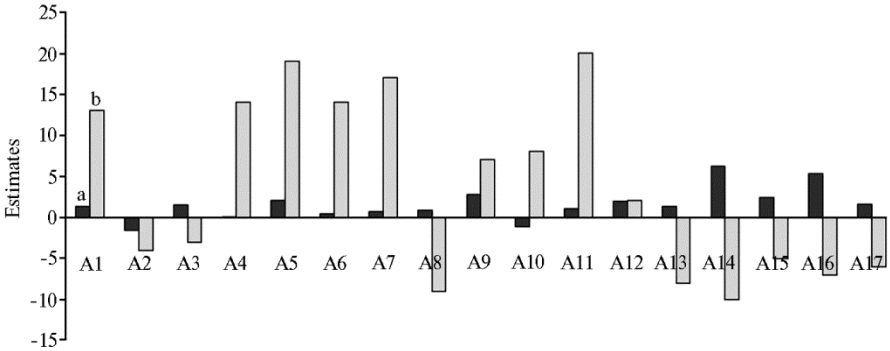

Fig. 2. Coefficient of general adaptability (a) **and index of stability** (YS*i*, b) **regarding seed productivity in broad bean *Vicia faba* L. accessions**: from left to right Fb 1896, Fb 1903, Fb 1929, Fb 2481, Fb 2486, Fb 3270, BGE 002106, BGE 029055, BGE 032012, BGE 041470, BGE 043776, BGE 046721, FbH 13, FbH 14, FbH 15, FbH 16, BGP (A1-A17, respectively) (Institute of Forage Crops, Pleven, Bulgaria, 2016-2018). The a and b values are for A1 — 1.30 and 13, respectively, for A2 — –1.6 and –4; for A3 — 1.53 and –3; for A4 — 0.05 and 14; for A5 — 2.03 and 19; for A6 — 0.39 and 14; for A7 — 0.69 and 17; for A8 — 0.82 and –9; for A9 — 2.75 and 7; for A10 — –1.17 and 8; for A11 — 1.08 and 20; for A12 — 1.90 and 2; for A13 — 1.34 and –8; for A14 — 6.23 and –10; for A15 — 2.42 and –5; for A16 — 5.31 and –7; for A17 — 1.61 and –6.

Thus, a linear equation of regression was used to determine the effect of quantitative traits on seed productivity in broad bean accessions. The optimal parameters of model broad bean plant with high productivity in organic production conditions was characterized by the following traits: plant height — from 69 to 90 cm, 1st pod height — from 22 to 36 cm, pods number per plant — from 9 to 14, seeds number — from 22 to 35, pod length — from 7 to 11 cm, pod width — from 15 to 19 mm, and 100 seeds mass — from 80 to 120 g. The obtained results showed that the traits of seeds number (R = 0.72) per plant, pods number (R = 0.48), mass of 100 seeds (R = 0.28) and 1st pod height (R = 0.25) had the greatest and statistically significant impact on the seed productivity. The accessions distinguished in high adaptability regarding seed productivity were FbH 14, FbH 16, BGE 032012, FbH 15, Fb 2486, and Fb 1903, and BGE 041470 were determined as low adaptive. Accessions Fb 1896, Fb 2486, BGE 032012 and BGE 043776 combined good adaptability with low variability and were suitable as parental components for use in the combinative breeding.

REFERENCES

1. Singh A.K., Bhat B.P., Sundaram P.K., Gupta A.K., Singh D. Planting geometry to optimize growth and productivity faba bean (*Vicia faba* L.) and soil fertility. *Journal of Environmental Biology*, 2013, 34(1): 117-122.
2. Singh A.K., Bharati R.C., Manibhushan N.C., Pedpati A. An assessment of faba bean (*Vicia faba* L.) current status and future prospect. *African Journal of Agricultural Research*, 2013, 8(50): 6634-6641.
3. Mihailovic V., Mikic A., Cupina B., Eric P. Field pea and vetches in Serbia and Montenegro. *Grain Legumes*, 2005, 44: 25-26.
4. Maalouf F., Nawar M., Hamwieh A., Amri A., Xuxiao Z., Shiying B., Tao Y. Faba bean. In: *Genetic and genomic resources of grain legume improvement*. M. Singh, H.D. Upadhyaya, I.S. Bisht (eds.). Elseiver Insight, London, 2013: 113-136 (doi: 10.1016/B978-0-12-397935-3.00005-0).
5. Maalouf F., Hu J., O'Sullivan D.M., Zong X., Hamwieh A., Kumar S., Baum M. Breeding and genomics status in faba bean (*Vicia faba*). *Plant Breeding*, 2019, 138(4): 465-473 (doi: 10.1111/pbr.12644).

6. *Biology and breeding of food legumes.* A. Pratap, J. Kumar. CABI, Wallingford, 2011.
7. Sood M., Kalia P.–Gene action of yield-related traits in garden pea (*Pisum sativum* Linn.). *SABRAO Journal of Breeding and Genetics*, 2006, 38(1): 1-17.
8. Genchev D.I., Kiryakov I. A breeding model of common dry bean. *Field Crop Studies*, 2014, 9(2): 265-272.
9. Kelly J.D., Adams M.W. Phenotypic recurrent selection in idiotype breeding of pinto beans. *Euphytica*, 1987, 36(1): 69-80 (doi: 10.1007/BF00730649).
10. Kalapchieva S. Model for breeding of high productive garden pea varieties. *Bulgarian Journal of Crop Science*, 2013, 50: 73-76.
11. Misnikova N.V., Kornev A.P. *Kormoproizvodstvo*, 2012, 5: 38-39 (in Russ.).
12. Barov V. *Analysis and schemas of the field trial.* Sofia, 1982.
13. Kang M. S. Simultaneous selection for yield and stability and yield-statistic. *Agronomy Journal*, 1993, 85(3): 754-757 (doi: 10.2134/agronj1993.00021962008500030042x).
14. Valchinkov S. Method for ranking genotypes with relatively high and stable yield. *Scientific reports of AU-Plovdiv*, 1990, 4: 161-165.
15. Cruz C.D. *Programa Genes: Biometria. version 7.0.* University of Federal Viçosa, Viçosa, Brazil, 2009.
16. Petrova S.D. *Maintenance, evaluation and use of plant genetic resources of chickpeas.* Ph Thesis, Sadovo, 2015 (in Bul.).
17. Misnikova N.V. Printsipy modelirovaniya pri sozdanii sortov lyupina zheltogo. *AGRO XXI*, 2008, 10-12: 20-21.
18. Kosev V. Model of high-productive varieties in forage pea. *Journal of Central European Agriculture*, 2015, 16(2): 172-180 (doi: 10.5513/JCEA01/16.2.1606).
19. Dragavtsev V.A. Problems of genetic and molecular approaches to the hereditary improvement of the qualitative characters of crop. In: *Integration of biodiversity and genome technology for crop improvement (Proceedings of the Joint International Workshop — 2000 International Workshop on Molecular Plant Breeding and Utilization of Genetic Resources & 8th MAFF International Workshop on Genetic Resources)*. K. Oono, T. Komatsuda, K. Kadowaki, D. Vaughan (eds.). National Institute of Agrobiological Resourses, Tsucuba, Japan, 2000: 93-95.
20. Uhr Z., Ivanov G. Opportunities for increased yields in condition of biological farming system in wheat. *New Knowledge Journal of Science*, 2015, 4(4): 35-41.
21. Bozhanova V., Koteva V., Savova T., Marcheva M., Panayotova G., Nedyalkova S., Rachovska G., Kostov K., Mihova G. Choice of appropriate cereals varieties and seed production for the needs of organic farming in Bulgaria — problems and answers. *Proc. National Conference* «Organic Farming, Livestock and Food». Troyan, Bulgaria, 2014: 68-77.

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BREEDING OF SPELT WHEAT (*Triticum spelta* L.) FOR PRODUCTIVITY AND GRAIN QUALITY

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Abstract

Spelt (*Triticum spelta* L.) is an ancient species of wheat demand for which is currently increasing. Along with positive characteristics of spelt (high protein, gluten and amino acids content, high adaptive potential, presence of a powerful stem and high resistance to lodging) it is significantly inferior to soft wheat *T. aestivum* L. in terms of yield capacity. However, new perspective genotypes with improved quantitative traits can be obtained from crosses of spelt and soft wheat due to introgression of genetic material of *T. aestivum* into *T. spelta* genome. In present research in result of hybridization of spelt wheat and soft wheat we obtained new forms which differ from each other in terms of morphobiological and economically valuable traits. The research aimed to create new initial material of spelt with high quality of grain based on hybridization of *Triticum aestivum* and *T. spelta* and its introduction to the breeding scheme for creating high productive varieties of the crop. Samples of spelt wheat obtained by the method of remote hybridization followed by multiple individual selection. Spelt samples of local breeding from foothill regions of the Carpathians and regionalized varieties of winter wheat Favoritka, Kharus, Panna, Ermak, Podolianka, Kryzhynka, Farandol, Kopilovchanka, Krasnodarskaia 99 were used as initial breeding material. Derived F_1 hybrids were self-pollinated or back-crosses with parental forms. Hybrid progenies F_{2-5} were analyzed in terms of morphobiological and economically valuable traits (plant height, shape of the bush, wax coating on plants, pubescence on the stem and spike, length, color and awnedness of the spike, number of spikelets in the spike, density of the spike, threshing of grain, grain shape and color, weight of the grain from the main spike, number of productive stems per plant, 1000-grain weight, gluten and protein content, grain hardness, productivity). In fifth generation (F_5) when segregation was no longer observed, considering productivity and quality of previous years, 18 best samples of spelt wheat were selected. Their field testing was conducted during 2012-2018 (F_{5-11}) (a research field of Uman National University of Horticulture, right-bank Forrest-steppe of Ukraine, Cherkassy region). Gluten and protein contents were determined by infrared spectroscopy (Laboratory of genetics, breeding and seed production, a device Infratec^{TM} Nova, FOSS Analytical, Sweden**).** Biometric traits **(**plant height, ear length, number of grains per ear, grain weight from head ear) were determined on 50 plants selected from each plot in two non-adjoin repetitions. Grain threshing was performed, and yield capacity was determined. From crossing, the collection of spelt initial material which include more than 200 samples was created. Obtained forms were divided into four groups according to plant height: semi-dwarf, low-growing, medium-growing and high-growing. In each group the best samples were selected which were analyzed for grain quality, yield and productivity structure. A possibility was proved of breeding improvement of spelt based on interspecific hybridization with soft wheat. It was established that eight samples significantly exceeded standard for yields. In the same time samples Nos. 76, 155, 1695 и 1725 had improved grain threshing (80-90 %), samples Nos. 76 and 1817 were characterized by high quality, in particular, 25.2 and 22.0 % protein content, respectively, and 52.1 and 44.7 % gluten level. Samples Nos. 40 and 13 were the best in terms of gluten and protein content with 30.1 and 27.2 % for protein, and 63.2 and 56.5 % for gluten, respectively. As a result of the study, spelt wheat forms were selected which possess several valuable traits, i.e. the sample No. 124 is distinguished due to low plant height (92 cm) and high quality traits (1000-grain weigh of 53.8 g, protein content of 17.9 %, I group gluten level of 37.4 %), while the sample No. 155 shows high

productivity (5.36 t/ha) and an improved grain threshing out from the ear (92 %). A winter spelt variety Europe derived from spelt and soft wheat hybridization has been listed to State register of plants suitable for growing in Ukraine from 2015.

Keywords: spelt wheat, soft wheat, hybridization, yield capacity, grain threshing, protein content, gluten content

Spelt wheat (*Triticum spelta* L.) is one of the most ancient cultivated wheat species, which was known as early as the 7th-8th millennia BC. Spelt originates from Southeast Asia, from where it spread to North and Central Europe. The Asian spelt subspecies are most likely result from spontaneous hybridization of *T. turgidum* ssp. *dicoccon* and *Aegilops tauschii* ssp. *strangulata*, and later from the spelt the naked hexaploid species were derived, including *T. aestivum* L. Spelt wheat is identical to common wheat in genomic composition and chromosome structure of some genomes [1, 2].

Spelt wheat was grown in ancient times, but gradually it disappeared from crops because of low productivity. Currently, the demand for spelt is growing due to the high protein content in grains and the presence of a number of nutrients and amino acids not found in animal products [3-6]. In protein content spelt plants exceed *T. aestivum* by 8-10%, *T. sphaerococum* Persiv. by 3-8%, *T. petropavlovskyi* Udacz. et Migusch by 2-6% [7-10]. In tryptophan level, it surpasses soft wheat by 10-15%, hard wheat by 15-20%, and also spelt grain contains 16-20% more general gluten (prolamines and glutenins) than soft wheat. Moreover, spelt grain contains less gluten fraction than soft wheat, barley, and oats, which allows spelt to be used in dietary nutrition [11, 12].

Recent market offers to grain producers a small number of spelt varieties, and their diversity is limited mainly by local forms of folk selection. In this regard, spelt cannot compete with soft wheat [3, 13]. An urgent task of spelt breeding is to increase productivity while maintaining a high content of protein and grain fibrin [14, 15]. New hybrids with improved quality parameters can be obtained by crossing *T. spelta* with *T. aestivum*.

Many researchers engaged in the breeding improvement of spelt by crossing spelt with soft wheat indicate a positive effect from hybridization, in particular, a significant expansion of genetic diversity and the production of new transgressive forms [14, 16-18]. However, according to Fishing [19], such crosses are undesirable, since they lead to grain quality deterioration in spelt, and to difficult grain threshing and ear fragility in soft wheat.

Spelt wheat breeding in Ukraine focuses on increased productivity, reduced plant height, and improved grain threshing. Large-scale research was launched at the Yuriev Institute of Plant Industry NAAN (Kharkov), Remeslo Mironov Institute of Wheat NAAN (settlement Central, Kiev region), All-Ukrainian Research Institute of Breeding (VNIS) (Kiev) and Uman National University of Horticulture (UNUS) (Uman) [20-22]. Joint efforts of scientists from UNUS and VNIS created the first two varieties of winter spelt wheat, Zarya Ukraine and Europe, which are included in the State register of plants suitable for growing in Ukraine [23]. However, spelt is still a little widespread crop that requires breeding improvement.

In this work, as a result of hybridization of soft wheat with spelt, we obtained a number of new forms that differ from each other in morphobiological and economically valuable traits.

Our research aimed to initially improve grain quality of spelt wheat by hybridization of *Triticum aestivum* and *T. spelta* and then to use the obtained forms in breeding spelt wheat for high grain quality and yields.

Materials and methods. Spelt wheat samples derived from distant hybridization were subjected to multiple individual selection. The creation of the collection began in 2006 under the leadership of F.N. Parii. Spelt wheat samples of local selection from the foothill regions of the Carpathians and zoned varieties of winter soft wheat Favoritka, Kharus, Panna, Ermak, Podolyanka, Kryzhinka, Farandol, Kopilovchanka, Krasnodarskaya 99, etc. (about 60 varieties in total) were used as the initial material. Hybridization was carried out by manual castration of flowers on the maternal plants and their forced pollination with the paternal pollen. Upon reaching full maturity of the grain, the crop was harvested and evaluated.

Hybrid offspring F_{2-5} were analyzed for the following morphobiological and economically valuable traits: plant height, shape, waxy coating on plants and pubescence on stem and spike, spike length, color and awnedness, number of spikelets per spike, spike density, grain threshing, grain shape and color, grain weight per main spike, number of productive stems per plant, 1000-grain weight, protein and gluten content in grain, grain hardness and yield. In the fifth generation (F_5), when segregation was no longer observed, 18 best spelt wheat samples were selected for the research given the productivity and grain quality of previous years. The sample testing on the experimental field of the Uman National University of Horticulture (right-bank forest-steppe of Ukraine, Cherkasy region) lasted for 2012-2018 (F_{5-11}).

The content of grain fibrin and protein was determined by infrared spectroscopy (an Infratectm Nova instrument, FOSS Analytical, Sweden). The height of the plants was measured in the field before harvesting according to the method of the State Scientific and Technical Expertise of Varieties [24]. The samples were grouped by plant height as per Dorofeev et al. [25]. Test 10 m^2 plots were systematically distributed. The samples were grown in blocks with a crop density of 400,000 plants/ha. The experiment was repeated 5 times. Biometric indicators (plant height, spike length, number of grains per spike, grain weight per main spike) were determined for 50 plants selected from each plot in 2 distant replicates. Grain threshing was carried out and the yield was determined. The threshing ability was estimated as a percentage ratio of the amount of threshed grain to the total amount of grain from the site.

The data were statistically processed using Microsoft Excel 2010. When determining the means (M), their standard errors ($\pm$SEM) and the relative standard errors (experimental error, Sx, %) were calculated. The least significant difference ($LSD_{0.99}$ for the grain fibrin and protein content, $LSD_{0.95}$ for other indicators) and the coefficient of variation (Cv, %) were calculated as per Ermantraut and Gudz [26].

Results. The original plants of spelt wheat were high (more than 120 cm), had a long (20 cm), loose, narrow, fragile, hulled, awnless ear of white color without wax coating. An ear, when ripe, splits into separate segments with grain which is difficult to thresh due to thick coarse spikelet hulls. In the initial form, grain productivity was 4.15 t/ha, with 25% protein content, and 50-52% grain fibrin. The parental soft wheat varieties were semi-dwarf (Kopilovchanka, Panna, Ermak, Kharus) or with low stem height (Favoritka, Podolyanka, Krasnodarskaya 99, Kryzhinka, Farandol). All varieties are bare-grain, with optimal threshing ability, differing from each other in approbation traits (wax coating, pubescence of plants and spikes, plant, spike and grain color, shape, density and length of spike, size and shape of spikelet scales, plant

shape, awnedness-awnless, grain shape and size) and economically valuable traits (yield, grain fibrin and protein content).

The resulting F_1 hybrids were self-pollinated or re-crossed with the parental forms. The significant genetic diversity that was involved in hybridization provided intensive generation of varying forms. At the same time, special attention was paid to the detailed study of plants during the initial stages of breeding, since only recombination variability in the F_{2-4} generations ensures the production of new transgressive forms with respect to economically valuable traits [27].

1. Yield structure in spelt wheat samples (F_{5-11}) derived from hybridization of *Triticum aestivum* L. × *Triticum spelta* L. (*M*±SEM, Ukraine, Cherkasy region, 2012-2018)

Genotype	Maternal parent	Grain weigh per main ear, g	Ear length, cm	Grains per ear	Ear density, grains per 10 cm	Plant height, cm
		Semi-dwarf (60-84 cm)				
Average value per group		2.04±0.051	15.1±0.20	44±0.3	15.2±0.14	78±0.7
1786	Favoritka	2.02±0.063	15.5±0.22	44±0.4	15.5±0.17	82±0,8
1817	Khrust	2.56±0.090	18.0±0.28	46±0.4	14.5±0.13	75±0,7
$LSD_{0.95}$		0.07	0.6	2	0.6	3
Cv, %		9.28	10.8	3	3.9	5
Sx, %		3.41	3.9	4.5	3.9	3.8
		Undersized (85-104 cm)				
Average value per group		1.52±0.081	14.2±0.15	45±0.3	14.6±0.15	96±0.9
13	Panna	1.41±0.062	13.6±0.18	42±0.3	15.2±0.13	100±0,9
124	Ermak	1.88±0.089	12.9±0.15	45±0.3	14.3±0.10	92±0,8
179	Podolianka	1.47±0.050	13.1±0.16	46±0.3	15.6±0.15	103±0,9
1559	Kryzhinka	2.40±0.122	18.0±0.24	48±0.4	15.8±0.16	87±0,8
1674	Farandol	2.02±0.113	14.4±0.20	45±0.3	15.0±0.14	89±0,8
1694	Farandol	1.74±0.102	18.3±0.23	44±0.3	14.1±0.15	98±0,8
1755	Panna	2.33±0.131	17.1±0.22	46±0.4	14.7±0.15	98±0,9
$LSD_{0.95}$		0.06	0.5	2	0.6	4
Cv, %		13.45	14.3	3	18.9	7
Sx, %		3.94	3.5	4.4	3.8	4.2
		Medium-sized (105-119 cm)				
Average value per group		1.54±0.062	14.8±0.20	44±0.3	14.4±0.17	110±0.9
40	Panna	1.24±0.028	14.5±0.21	39±0.3	14.4±0.16	113±0,9
76	Kryzhinka	1.81±0.080	15.2±0.24	42±0.3	14.7±0.18	110±0,9
155	Panna	1.85±0.071	16.5±0.28	45±0.3	13.9±0.10	109±0,9
1721	Panna	1.58±0.049	17.4±0.31	42±0.3	13.9±0.11	106±0,9
1725	Kopilovchanka	1.53±0.052	17.3±0.29	45±0.4	13.7±0.09	110±0,9
$LSD_{0.95}$		0.06	0.5	2	0.6	4
Cv, %		6.34	12.6	2	4.7	3
Sx, %		3.8	3.6	4.5	3.7	3.6
		Tall (> 120 cm)				
Average value per group		1.75±0.060	14.1±0.20	44±0.3	13.2±0.10	125±1.1
86	Panna	1.89±0.091	13.2±0.16	45±0.3	14.7±0.15	122±1,1
1691	Krasnodarskaya 99	2.05±0.132	15.8±0.23	43±0.3	14.1±0.13	120±1,1
1695	Panna	2.70±0.149	16.1±0.25	45±0.4	15.3±0.18	129±1,5
1730	Favoritka	1.76±0.064	15.7±0.22	41±0.3	13.7±0.09	127±1,2
$LSD_{0.95}$		0.06	0.5	2	0.5	5
Cv, %		12.25	9.5	2	11.6	2
Sx, %		3.4	3.5	4.5	3.9	4.0

Note. *Cv* — coefficient of variation, Sx — relative standard error. The paternal form of all genotypes was spelt wheat from the foothills of the Carpathians. Genotype numbers are listed according to the field trial records.

Spelt wheat samples characterized by a significant diversity in morphobiological features were isolated among the offspring by individual and family selection. The obtained collection includes early maturing forms small in height, with high winter and frost resistance and other valuable traits. Some forms exceeded the original varieties in terms of yield, grain protein and fibrin content, and grain weight per ear (Table 1).

Spelt wheat is distinguished by its tallness [28, 29]; therefore, it is important to reduce the stem height while maintaining a high content of protein and fibrin. Our collection includes forms that differ significantly in plant height. The range of variability for this trait was 52-129 cm.

According to some reports [15, 30, 31], the hybrids obtained by crossing different wheat types, occupy an intermediate position between the parents in terms of plant height. However, the scientific literature describes the facts of dominance and overdominance of a tall parent [14, 32, 33]. Selective (due to additive gene interaction) and hybrid (due to complementary interaction of genes) dwarfism are also recorded in the offspring [34].

A comparative study of more than 200 accessions of spelt wheat and the original forms indicates a different pattern of plant height inheritance, from typical intermediate to heterosis and dominant dwarfism. The created samples were divided into tall (over 120 cm in height), medium-sized (105-119 cm in height), undersized (85-104 cm in height), semi-dwarf (60-84 cm in height) and dwarf (less than 60 cm in height) according to the classification of Dorofeev et al. [25]. The most numerous and productive were the undersized and medium-sized groups.

Published research information contains few reports on the genetic control of plant height in *T. spelta*. In generations F_5 from crosses of tall forms, tall offspring were obtained, which suggests that tall stems are characteristic of spelt plants [1]. Upon spelt hybridization with common wheat varieties carrying dominant or recessive dwarf genes, there are various types of gene interactions (complementary, epistatic, polymeric) and the formation of offspring with a wide range of variability in plant height.

Spelt wheat has a long, loose ear, which leads to low grain number per ear. In breeding, it is important to increase ear density, which, in turn, will improve productivity.

The speltoid shape of the ear of hexaploid wheat species is controlled by *S* gene, the dominant allele of which affects ear length and density. In addition, *C* gene influences spike length, that is, the dominant allele causes its shortening [34]. When spelt wheat with dominant alleles of *S* gene is crossed with soft wheat having *C* gene, the ear length and density are mainly inherited in generations according to an intermediate type. Thus, the samples obtained by us occupied the intermediate position between the initial forms in the spike length, and were closer to the spelt wheat in terms of the spike density (less than 16 spikelets per 10 cm of the spike length).

The undersized and tall specimens showed a moderate variation in grain weight per the main spike (Cv = 12.25-13.45%). All studied samples showed a significant increase in this parameter, with the exception of Nos. 13 and 40. The sample No. 1559 significantly exceeded the group standard with regard to the grain number per spike (48 grains). For other forms, except for Nos. 13 and 40, the values were comparable to the standard.

In hybridization of soft wheat with spelt wheat, it is important to maintain grain protein and fibrin content at a high level. When a parental form of soft wheat rich in grain protein and fibrin is crossed with spelt wheat samples poor in these substances, the offspring inherit the traits of the worst parent [16, 19]. To create spelt wheat varieties, the hard wheat varieties also valuable in other grain quality parameters should be involved in hybridization. In our studies, such a variety was winter wheat variety Panna. The offspring from crosses of this variety with spelt wheat had the highest grain protein and fibrin levels, in particular, for No. 40 (medium-sized) and No. 13 (undersized), the grain protein content reached 30.1 and 27.2%, respectively, with the fibrin content of 63.2 and 56.5%, respectively, which significantly exceeded the average value per group (Table 2).

2. Grain quality and yields of the best spelt wheat samples (F_{5-11}) derived from hybridization of *Triticum aestivum* L. × *T. spelta* L. (*M*±SEM, Ukraine, Cherkasy region, 2012-2018)

Genotype	Maternal parent	1000-grain weight, g	Bushel weight, g/l	Vitreousness, %	Protein content, %	Gluten		Yield, t/ha	±to averaged value per group	Grain threshing, %
						%	quality group			
Semi-dwarf (60-84 cm)										
Average value per group		49.8±0.20	655±15.1	74±0.6	18.2±0.14	38.5±0.28	–	5.75±0.231	–	78
1786	Favoritka	51.5±0.35	650±16.2	82±0.9	20.4±0.09	41.6±0.21	III	5.78±0.250	+0.03	75
1817	Khrust	50.0±0.38	675±15.0	87±1.0	22.0±0.12	44.7±0.24	II	6.47±0.281	+0.72	70
$LSD_{0.95-0.99}$		2.2	28	3	0.1	0.3	–	0.22	–	3
Cv, %		2.0	2	2.78	2.67	2.54	–	4.05	–	9
S_X, %		4.0	3.6	4.1	0.5	0.8	–	3.81	–	3.4
Undersized (85-104 cm)										
Average value per group		50.2±0.25	660±15.2	75±0.6	16.8±0.18	34.2±0.38	–	5.12±0.201	–	77
13	Panna	50.6±0.21	650±14.9	89±0.4	27.2±0.07	56.5±0.16	II	4.42±0.184	–0.70	80
124	Ermak	53.8±0.18	660±13.3	74±0.5	17.9±0.19	37.4±0.37	I	5.05±0.242	–0.07	84
179	Podolianka	47.5±0.28	640±15.2	85±0.4	22.7±0.13	47.8±0.25	II	4.56±0.221	–0.56	78
1559	Kryzhinka	64.4±0.14	660±12.1	82±0.5	21.0±0.14	43.7±0.29	I	6.27±0.290	+1.15	72
1674	Farandol	55.2±0.18	680±14.8	66±0.5	16.2±0.22	34.7±0.40	III	5.74±0.248	+0.62	75
1694	Farandol	50.7±0.20	660±12.2	74±0.5	18.3±0.14	38.5±0.28	II	5.12±0.209	0.0	77
1755	Panna	50.7±0.19	660±12.4	75±0.5	18.3±0.15	38.5±0.32	II	5.87±0.261	+0.75	78
$LSD_{0.95-0.99}$		2.1	29	3	0.1	0.3	–	0.20	–	3
Cv, %		13.1	5	9	11.4	14.2	–	11.8	–	10
S_X, %		3.8	3.9	3.8	0.6	0.9	–	3.9	–	3.5

Continued Table 2

Medium-sized (105-119 cm)										
Average value per group		49.3±0.25	650±15.2	78±0.5	18.8±0.16	37.2±0.34	–	–	–	76
40	Panna	49.1±0.24	650±16.0	92±0.3	30.1±0.05	63.2±0.11	II	4.26±0.183	–0.61	78
76	Kryzhinka	50.3±0.20	650±15.1	85±0.4	25.2±0.07	52.1±0.15	II	5.15±0.208	+0.28	80
155	Panna	52.0±0.15	675±12.9	81±0.5	20.2±0.12	41.5±0.22	II	5.36±0.251	+0.49	92
1721	Panna	44.5±0.29	650±17.2	84±0.4	21.6±0.10	47.6±0.19	II	4.79±0.222	–0.08	77
1725	Kopilovchanka	48.7±0.27	670±12.1	75±0.5	16.2±0.21	36.7±0.38	I	5.70±0.271	+0.83	90
$LSD_{0.95-0.99}$		2.0	28	3	0.1	0.3	–	0.18	–	3
Cv, %		4.7	3	11	14.8	15.9	–	9.85	–	12
Sx, %		3.8	3.7	4.1	0.5	0.8	–	3.7	–	3.8
Tall (> 120 cm)										
Average value per group		48.2±0.26	660±15.7	75±0.5	18.7±0.15	38.7±0.32	–	5.87±0.257	–	75
86	Panna	45.2±0.25	660±15.1	87±0.4	26.2±0.08	54.0±0.15	I	5.21±0.228	–0.66	78
1691	Krasnodarskaya 99	55.1±0.17	665±14.0	82±0.5	22.3±0.06	47.8±0.13	II	5.77±0.262	–0.10	80
1695	Panna	50.3±0.19	670±12.1	74±0.5	19.0±0.13	40.5±0.25	I	6.45±0.311	+0.58	80
1730	Favoritka	45.8±0.23	680±11.2	62±0.6	15.2±0.23	37.2±0.38	I	4.86±0.190	–1.01	78
$LSD_{0.95-0.99}$		1.9	30	3	0.1	0.3	–	0.23	–	3
Cv, %		7.7	3	12	10.6	11.7	–	7.17	–	6
S_X, %		3.5	4.1	3.7	0.5	0.8	–	3.9	–	3.8

Note. *Cv* — coefficient of variation, S_X — relative standard error. The paternal form of all genotypes was spelt wheat from the foothills of the Carpathians. Genotype numbers are listed according to the field trial records. The significance level of $P_{0.99}$ was used when processing data on protein and gluten content. Dashes indicate that statistics and the group mean were not determined.

For bread-making quality of grain, qualitative rather than qualitative gluten parameters are decisive. Physical parameters such as color, stretch, elasticity and deformation index (GDI) greatly influence the dough formation and bread yield. According to GSTU 3768:2010 [35)], gluten of quality group I should be light gray or gray in color, elastic, with extensibility within 10-20 cm and gluten deformation index (GDI) of 45-85 units of Gluten deformation meter VDK-M (Ukraine). The quality group can be revised if the deformation index goes beyond the limits acceptable for group I, since it is the GDI that is the most important indicator of the gluten quality. In our study, six samples have gluten of quality group I (see Table 2). Especially noteworthy is the No. 86 of the tall group, which also stood out for its high gluten content (54.0%).

Vitreousness is an important trait that determines the suitability of grain for various end products and influences the processing and milling. The grain vitreousness of the spelt wheat samples studied by us ranged from 62 to 92%. The highest it was in Nos. 40 (92%), 13 (89%) and 1817 (87%). The wheat grain is considered vitreous for the values exceeding 70%, half-vitreous for 50-69%, semi-mealy for 21-49%, and mealy for < 20%. The grain of all samples in the experiment was vitreous, with the exception of Nos. 1674 and 1730, which had a semi-vitreous endosperm consistency.

The 1000-grain weight in the studied samples ranged from 44.5-64.4 g. A significant increase in this indicator as compared to the average group standard was recorded in samples Nos. 124 (53.8 g), 1559 (64.4 g), 1674 (55.2 g), 155 (52.0 g) and 1691 (55.1 g). The grain yield of Nos. 76, 155, 1559, 1674, 1695, 1725, 1755 and 1817 significantly exceeded the standard. Worth noting is the No. 1695 of the tall group, which combines a productivity of 6.52 t/ha with high grain quality (1000-grain weight of 50.3 g, grain protein content of 19.0%, 40.5% of gluten of the quality group I) and the No. 155 of the medium-sized group, in which all quality and productivity indicators significantly exceeded the group's standards.

Spelt wheat grain is difficult to thresh because of the spikelet fragility and the presence of coarse spikelet hulls. Unlike soft wheat, spelt wheat harbors the recessive allele *q* of the *Q* gene, which controls the character of grain threshing (easy or difficult). The homozygotes for the *q* allele have spikes of a speltoid type (long, fragile and loose) which are poorly threshed [36]. In addition, the threshing is influenced by the type of spikelet, which in hexaploid wheat species is controlled by the *Tg* gene recessive allele *tg* in a homozygous state [36]. Since spelt wheat has dominant alleles of this gene, its spikes possess coarse spikelet hulls, which negatively affects threshing. In the offspring from crossing soft wheat with spelt wheat, various combinations of alleles of *Q* and *Tg* genes appeared. In this regard, both different threshing capacity and unequal structure of the spikelet hulls were recorded. Probably Nos. 76, 155, 1695, and 1725 have the *QQtgtg* genotype, which improves grain threshing (80-90%).

The growing season of spelt wheat lasts 7-10 days longer compared to soft wheat. Among the samples studied by us, forms were distinguished, the periods of heading and ripening of which were comparable to those of early maturing varieties of common wheat. Nos. 1674 and 155 had a 280-285-day growing season, while their yield (5.74-5.86 t/ha) significantly exceeded the standard.

In 2013 and 2015, spelt wheat crops were affected by brown rust (*Puccinia recondita* Rob. ex Desm f. sp. *tritici*). Nos. 13 and 124 showed high resistance to the pathogen. The lesion intensity was less than 5% of the leaf surface, which corresponds to 8-9 points on the resistance scale. It is obvious that these samples inherited the resistance trait from the parental forms (the common wheat varieties Ermak and Panna, as well as the original form of spelt wheat),

which exhibit high resistance to brown rust. The genes for resistance to this disease in common wheat are in a heterozygous state, and therefore not all plants of the offspring of the same parents were resistant to the disease.

The tests of the breeding samples obtained by us are still ongoing. Among them, the search for new donors of valuable traits is being successfully carried out. As a result, the winter spelt wheat variety Europe was created, which is listed in the State register of plants suitable for growing in Ukraine (since 2015). Nos. 124 and 155 after reproduction will be subjected to the State Scientific and Technical Expertise.

Variety Europe (breeding sample No. 1725) is an awned form of spelt wheat, in which 90% of the grain is separated from the hulls during threshing. The variety derived from hybridization of Kopilovchanka winter bread wheat and spelt wheat upon multiple individual selection. This is a winter type variety with a plant height of 110 cm, a grain protein content of 17%, a gluten content (the quality group I) of 38%, a 1000-grain weight of 47 g, and a bushel weight of 670 g/l. During the period of the State Scientific and Technical Expertise (2012-2015), the variety had an average yield in the forest-steppe zone of 5.82 t/ha. The variety is resistant to brown rust, powdery mildew, snow mold and tolerant to yellow spot, fusarium head blight and root rot. Breeding sample No. 124 was created by hybridization of soft winter wheat variety Ermak with spelt wheat. This is a winter type undersized sample with a plant height of 92 cm. The growing season lasts 290-295 days (a mid-season type). The spikes are awnless, long, loose. The sample shows high grain quality, in particular, the 1000-grain weight of 53.8 g, the protein content of 17.9%, the gluten content (the quality group I) of 37.4%, the productivity is 5.05 t/ha. Selection sample Nos. 155 was obtained by hybridization of winter soft wheat variety Panna with spelt wheat. The sample is of a winter type, medium-sized (109 cm in height), mid-season (290-295-day growing season), has an awnless, long (16.5 cm), loose spike, combines high grain quality (1000-grain weight of 52.0 g, bushel weight of 675 g/l, protein content of 20.2%, gluten content of 41.5%) with a productivity of 5.36 t/ha and threshing capacity of 92%.

Thus, more than 200 spelt wheat samples derived from distant hybridization of soft winter wheat and spelt wheat has been created. This collection includes unique recombinant forms that differ in economically valuable, morphobiological and biochemical traits. Spelt wheat sample No. 124 possesses a combination of valuable traits, in particular, the plants are of 92 cm in height and produce high quality grain (1000-grain weight of 53.8 g, protein content of 17.9%, quality group I gluten content of 37.4%). Spelt wheat sample No. 155 stands out for its high productivity (5.36 t/ha) and improved grain threshing capacity (92%). The variety Europe of winter spelt wheat created within the framework of this breeding program has been included in the State register of plants suitable for growing in Ukraine.

REFERENCES

1. Packa D., Załuski D., Graban Ł., Lajszner W. An evaluation of spelt crosses for breeding new varieties of spring spelt. *Agronomy*, 2019, 9(4): 167 (doi: 10.3390/agronomy9040167).
2. Matsuoka Y. Evolution of polyploid *Triticum* wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. *Plant and Cell Physiology*, 2011, 52(5): 750-764 (doi: 10.1093/pcp/pcr018).
3. Dvorak J., Deal K.R., Luo M.-C., You F.M., von Borstel K., Dehghani H. The origin of spelt and free-threshing hexaploid wheat. *Journal of Heredity*, 2012, 103(3): 426-441 (doi: 10.1093/jhered/esr152).
4. Ninieva A.K., Kozub N.O., Sozinov I.O., Ribalka O.I., Leonov O.Yu., Tverdokhlib O.V., Boguslavs'kii R.L. *Visnik ukraïns'kogo tovaristva genetikiv i selektsioneriv*, 2013, 11(1): 96-105.

5. Lacko-Bartošová M., Korczyk-Szabó J., Ražný R. *Triticum spelta* — a specialty grain for ecological farming systems. *Research Journal of Agricultural Science*, 2010, 42(1): 143-147.
6. Ruibal-Mendieta N.L., Delacroix D.L., Mignolet E., Pycke J.M., Marques C., Rozenberg R., Petitjean G., Habib-Jiwan J.-L., Meurens M., Quetin-Leclercq J., Delzenne N.M., Larondelle Y. Spelt (*Triticum aestivum* spp. *spelta*) as a source of bread making flours and bran naturally enriched in oleic acid and minerals but not phytic acid. *Journal of Agricultural and Food Chemistry*, 2005, 53(7): 2751-2759 (doi: 10.1021/jf048506e).
7. Diordiieva I., Riabovol L., Riabovol I., Serzhuk O., Novak A., Kotsiuba S. The characteristic of wheat collection created by *Triticum aestivum* L./*Triticum spelt*a L. hybridization. *Agronomy Research*, 2018, 16(5): 2005-2015 (doi: 10.15159/AR.18.181).
8. Geisslitz S., Longin C.F.H., Scherf K.A., Koehler P. Comparative study on gluten protein composition of ancient (einkorn, emmer and spelt) and modern wheat species (durum and common wheat). *Foods*, 2019, 8(9): 409 (doi: 10.3390/foods8090409).
9. Sobczyk A., Pycia K., Stankowski S., Jaworska G., Kuzniar S. Evaluation of the rheological properties of dough and quality of bread made with the flour obtained from old cultivars and modern breeding lines of spelt (*Triticum aestivum* ssp. *spelta*). *Journal of Cereal Science*, 2017, 77: 35-41 (doi: 10.1016/j.jcs.2017.07.013).
10. Arzani A., Ashraf M. Cultivated ancient wheats (*Triticum* spp.): a potential source of health-beneficial food products. *Comprehensive Reviews in Food Science and Food Safety*, 2017, 16(3): 477-488 (doi: 10.1111/1541-4337.12262).
11. Gospodarenko G.M, Lyubich V.V., Polyanets'ka I.O. *Visnik Umans'kogo natsional'nogo universitetu sadivnitstva*, 2016, 2: 44-48.
12. Gospodarenko G.M., Kostogriz P.V., Lyubich V.V., Parii M.F., Poltorets'kii S.P., Polyanets'ka I.O., Ryabovol YA.S., Ryabovol L.O., Sukhomud O.G. *Pshenitsya spel'ta. Monografiya*. Kiïv, 2016.
13. Mitrofanova O.P. *Vavilovskii zhurnal genetiki i selektsii*, 2012, 16(1): 10-20 (in Russ.).
14. Sichkar S.M., Morgun V.V., Dubrovna O.V. *Faktori eksperimental'noï evolyutsiï organizmiv*, 2016, 18: 149-153.
15. Liu G., Xu S.B., Ni Z.F., Xie C.J., Qin D.D., Li J., Lu L.H., Zhang J.P., Peng H.R., Sun Q.X. Molecular dissection of plant height QTLs using recombinant inbred lines from hybrids between common wheat (*Triticum aestivum* L.) and spelt wheat (*Triticum spelta* L.). *Chinese Science Bulletin*, 2011, 56(18): 1897-1903 (doi: 10.1007/s11434-011-4506-z).
16. Nemeth C., Yang C., Kasprzak P., Hubbart S., Scholefield D., Mehra S., Skipper E., King I., King J. Generation of amphidiploids from hybrids of wheat and related species from the genera *Aegilops*, *Secale*, *Thinopyrum*, and *Triticum* as a source of genetic variation for wheat improvement. *Genome*, 2015, 58(2): 71-79 (doi: 10.1139/gen-2015-0002).
17. Babenko L.M., Hospodarenko H.M., Rozhkov R.V., Pariy Y.F., Pariy M.F., Babenko A.V., Kosakivska I.V. *Triticum spelta*: Origin, biological characteristics and perspectives for use in breeding and agriculture. *Regulatory Mechanisms in Biosystems*, 2018, 9(2): 250-257 (doi: 10.15421/021837).
18. Osokina N., Liubych V., Novak L., Pushkarova-Bezdil T., Priss O., Verkholantseva V., Hryhorenko O., Pusik V., Pusik L. Elucidation of the mechanism that forms bread baking properties of the spelt grain. *Eastern European Journal of Enterprise Technologies*, 2018, 2/11(92): 39-47 (doi: 10.15587/1729-4061.2018.126372).
19. Ribalka O.I. *Yakist' pshenitsi ta ii polipshennya. Monografiya* [Wheat quality and its improvement. A monograph]. Kiïv, 2011.
20. Parii F.M., Sukhomud O.G., Lyubich V.V. *Nasinnitstvo*, 2013, 5: 5-6.
21. Diordieva I.P. *Genetichni resursi roslin*, 2018, 23: 25-34.
22. Tverdokhlib O.V., Golik O.V., Ninieva A.K., Boguslavskii R.L. *Posibnik Ukraïns'kogo khliboroba*, 2013, 2: 154-155.
23. *Derzhavnii reestr sortiv roslin, pridatnikh dlya poshirennya v Ukraïni v 2019 r.* Kiïv, 2019 (in Russ.).
24. *Metodika Derzhavnoï naukovo-tekhnichnoï ekspertizi sortiv roslin. Metodi viznachennya pokaznikiv yakosti produktsiï roslinnitstva* [Methods of State scientific and technical examination of plant varieties. Methods for determining quality of crop products]. Kiïv, 2015.
25. Dorofeev V.F., Udachin R.A., Semenova L.V. *Pshenitsy mira* [Wheats of the world]. Leningrad, 1987 (in Russ.).
26. Ermantraut E.R., Gudz" V.P. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Sovremennye problemy opytnogo dela»* [Proc. Int. Conf. «Modern problems of experimental research»]. St. Petersburg, 2000, 2: 13-134 (in Russ.).
27. Kochmars'kii V.S. *Tavriis'kii naukovii visnik*, 2012, 78: 33-38 (in Russ.).
28. Longin C.F.H., Ziegler J., Schweiggert R., Koehler P., Carle R., Würschum T. Comparative study of hulled (einkorn, emmer, and spelt) and naked wheats (durum and bread wheat): agronomic performance and quality traits. *Crop Science*, 2016, 56(1): 302-311 (doi: 10.2135/cropsci2015.04.0242).
29. Rapp M., Beck H., Gütler H., Heilig W., Starck N., Römer P., Cuendet C., Kurz H., Uhlig F., Würschum T., Friedrich C., Longin F. Spelt: agronomy, quality, and flavor of its breads from 30 varieties tested across multiple environments. *Crop Science*, 2017, 57(2): 739-

747 (doi: 10.2135/cropsci2016.05.0331).
30. Tverdokhleb E.V., Boguslavskii R.L. *Visnik Kharkivs'kogo natsional'nogo agrarnogo universitetu*, 2010, 2(20): 88-95 (in Russ.).
31. Ivanovska S., Kraljević-Balalić M., Stojkovski C. Diallel analysis for plant height in winter wheat. *Genetika*, 2003, 35(1): 11-19 (doi: 10.2298/GENSR0301011I).
32. Sichkar S.M., Morgun V.V., Dubrovna O.V. Uspadkuvannya morfologichnikh oznak u gibridiv F_1-F_2 *T. spelta* × *T. aestivum*. *Fiziologiya rastenii i genetika*, 2016, 48(4): 344-355.
33. Gulkanyan V.O. *Biologicheskii zhurnal Armenii AN Armenii*, 1971, 23(4): 41-49 (in Russ.).
34. Grant N.P., Mohan A., Sandhu D., Gill S.K. Inheritance and genetic mapping of the reduced height (*Rht18*) gene in wheat. *Plants*, 2018, 7(3): 58-67 (doi: 10.3390/plants7030058).
35. *Pshenitsa. Tekhnicheskie usloviya: GSTU 3768:2010*. Kiev, 2010.
36. Faris J.D., Simons K.J., Zhang Z., Gill B.S. The wheat super domestication gene *Q*. *Frontiers of Wheat Bioscience*, 2005, 100: 129-148.

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EVALUATION OF WINTER CAMELINA (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) CULTIVARS FOR ENVIRONMENTAL ADAPTABILITY

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Abstract

Winter camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) is a promising oilseed crop that enjoys great popularity in the world because of high oil content and a wide range of uses. From the environmental point of view, winter camelina possesses phenotypic plasticity and seems to be particularly adapted to a wide range of soil and climatic conditions. Camelina is a cold-resistant and drought-tolerant plant. Spread of winter camelina cultivation and its productivity improvement depends on the genetic diversity of cultivars adapted to the conditions of the region of its cultivation, as well as on the genotype-environment interaction. In the presented work, it was first established that in the conditions of the Middle Volga and Steppe Crimea, winter camelina specimens are adaptable, stable and able to produce high yield of good quality. This allows us to involve these forms in breeding new varieties adapted for cultivation in both regions. The aim of the work is to evaluate the cultivars of winter camelina for environmental adaptability and stability in two contrasting agroecological regions, the Middle Volga Region (Penza Agricultural Research Institute) and the steppe zone of the Crimea (Research Institute of Agriculture of Crimea) in 2015-2017. Varieties of *Camelina sylvestris* of various ecogeographical origins were investigated. Cultivar Penzyak (Penza Agricultural Research Institute) served as a standard. This cultivar has been cultivated in the Crimea on an industrial scale since 2015. Winter hardiness of the studied cultivars in the fields of Penza was high and ranged between 89.5 % to 96.7 %, in the Crimean Peninsula territory it was 92.3-96.9 %. Various hardiness groups were identified: samples with high cold tolerance (more than 98 %), samples with the middle level of winter hardiness (90-95 %), and samples with low winter-tolerance (less than 90 %). Winter hardiness of the same samples varied within the range of 92.3-96.9 % under the Crimean environment. The highest level of winter hardiness was noted for the cultivar Baron and individual selection line 4156, which exceeded standard cultivar Penzyak by 0.8-1.0 %, respectively. The productivity of varieties in the Crimea was 1.64-1.83 t/ha. Seed oil content varied from 35.61 to 43.90 %. The highest content of fat in seeds was noted in varieties Baron and Kozyr (43.90 and 43.60 %). Realization of yield potential of winter camelina cultivars and samples in the forest-steppe zone of the Middle Volga and steppe zone of the Crimea was relatively high, 70.9-88.9% and 71.1-86.3%, respectively. The highest level of yield realization was identified for the cultivar Dikiy (86.3 % and 88.9 %). Two samples (Dikiy and individual selection line 3290) were the most effective in terms of productivity. Their productivity was 1.83-1.97 when cultivated in Penza Agricultural Research Institute, and 1.73-1.85 t/ha in Research Institute of Agriculture of Crimea. On average, cultivar Dikiy (88.9 g/m^2) and individual selection line 3290 (85.4 g/m^2) showed the highest level of adaptability to the conditions of both territories. The lowest adaptability criterion was identified for the individual selection line 4172 from Sverdlovsk and individual selection line 4175 from Czechoslovakia, the fitness coefficients amounted 56.3 and 59.6 g/m^2, respectively. High adaptability of the samples (b_i = 0.98-0.99 for Dikiy and bi = 0.96-0.95 for individual selection line 3290) allows their cultivation both in the Middle Volga and in Steppe Crimea. Low adaptability was noted for the individual selection line 2219 (b_i = 0.89) in the Penza region; in the Crimea, for the aforementioned individual selection line b_i was 1.15.

Keywords: winter camelina, productivity, cultivation, region, Middle Volga, Crimea, oil content

Negative environmental factors, including temperature fluctuations and excessive or insufficient moisture, adversely affect plants during their growth and development. Each plant has a genotypically determined ability to adapt to changing environmental conditions. According to Zhuchenko [1], plant adaptability and resistance to unfavorable factors are the fundamental criteria in adaptive breeding. At the same time, the adaptability of new varieties to specific soil and climatic conditions characterizes their agroecological targeting [1].

Winter camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) is an oilseed crop that is becoming more and more popular due to its high oil content and multi-purpose use [2, 3]. The potential yield of camelina seeds is over 2.0 t/ha, and the oil content is over 40% [4, 5]. Camelina oil is an edible oil used directly in food due to good taste, but it is also a raw material for production of drying oil, synthetic lipids, green soap and other technical products [6-8], and also is used in cosmetics, aromatherapy, and medicine [9, 10]. In addition, the camelina is promising source for production of biodiesel and bio kerosene which show excellent physical and chemical characteristics and operational parameters [10-12].

The uniqueness of winter camelina lies in its low demands on growing conditions due to its high adaptability [14-16]. From an ecological point of view, winter camelina is a fairly plastic plant that easily adapts to various soil and climatic conditions [17, 18]. The distinctive features of camelina are high winter hardiness [19], early maturation and drought resistance [20, 21].

According to the FAO (Food and Agriculture Organization) reports, winter camelina is currently cultivated in a number of regions of the Russian Federation on an area of 142 thousand hectares [22]. However, for Crimea, this is a new crop the study of which began in 2015.

An increase in the acreage of winter camelina crops and an increase in its productivity depend on the genetic diversity of varieties and specimens adapted to local conditions, as well as on the genotype—environment interaction [23, 24]. Modern methods of plant breeding, including for oilseeds, aim not only to increase seed productivity and quality, but also to improve potential adaptive capabilities of the genotype when exposed to biotic and abiotic environmental factors [25, 26]. Estimates of adaptability and stability are mandatory in characterization of new highly productive varieties of winter camelina intended for practical use.

This work, summarizing results of long-term research, shows that the varieties of winter camelina studied for the first time in the conditions of the Middle Volga region and the steppe Crimea, are well-adaptable, stable and capable of high yielding and producing quality seeds of high quality. Thence, these varieties can be involved in breeding to derive varieties adapted to cultivation in both regions.

Our objective was to evaluate the ecological adaptability and stability of winter camelina varieties in contrasting agroecological conditions.

Materials and methods. Winter camelina varieties Penzyak, Kozyr, Baron and breeding lines Dikii, i.o.-4172, i.o.-1357, i.o.-2219, i.o.-4155, i.o.-4164, i.o.-4156, i.o.-4175, i.o.-3290, i.o.-4165 of various ecological and geographical origin were used in experiments carried out in 2015-2017 in two agroecological regions with contrasting climatic conditions, the Middle Volga (Penza Research Institute of Agriculture, settlement Lunino, Penza Province) and the steppe Crimea (Research Institute of Agriculture of Crimea, Klepinino village, Krasnogvardeiskii District). The Penzyak variety originated by the Penza Research Institute of

Agriculture served as the standard.

The soil of the first experimental site (the Penza Research Institute of Agriculture) is leached chernozem with a humus content in the arable layer of 6.4% and 81.3, 136.7 and 164.9 mg/kg nitrogen, phosphorus and potassium, respectively; pH 5.5. The soil of the second experimental site (the Research Institute of Agriculture of Crimea) is southern low-humus chernozem with 2.4-2.6% humus and 2.2, 4.5 and 39.0 mg/kg nitrogen, phosphorus and potassium, respectively; pH 7.3

In 2009, in the Penza Research Institute of Agriculture (PRIA), the initial breeding material was obtained by individual selection method using accessions of the PRIA collection of camelina varieties. In 2014, the resulting lines were subjected to environmental tests at the Research Institute of Agriculture of Crimea.

The cultivation of camelina in the crop rotation, field experiment design, observations and measurements were carried out as per the recommendations for oilseeds [27]. Sowing was carried out at the optimal time for a particular region in an ordinary way with 15 cm row spacing and a seeding rate of 8.0 million/ha Winter hardiness was assessed by the data of autumn and spring estimates of the crop states in each repetition in both zones. The yield was measured in 1 m^2 plots.

Statistical processing was performed according to Dospekhov [28] using Microsoft Excel 2010 and a Statistica 8 software package (StatSoft, Inc., USA). The mean values (M*)* and their standard errors (±SEM) were calculated. The significance of differences between the options was assessed by the methods of parametric statistics (Student's *t*-test). Ecological stability and adaptability (b_i) were determined as described by Kilchevsky and Khotyleva [29] based on the regression coefficient. Yield potential realization was expressed as the percentage ratio of the maximum yield to the average yield according to Nettevich [30]. The plant fitness criterion (K_0) was determined according to the method described by Belenkevich [31] which is based on the calculation of averaged parameters of plant productivity and its structural components.

Results. The climate of the Middle Volga region is temperate continental with annual precipitation from 350 to 750 mm. During the years of our survey, the climatic conditions differed in moisture conditions: growing season of 2016 was characterized as arid (the hydrothermal coefficient HTC 0.74), while 2015 was highly humidified (HTC 1.37). The year 2017 turned out to be optimal with regard to water supply (HTC 1.10).

The second experimental site (the Research Institute of Agriculture of Crimea) belongs to the zone of the steppe Crimea, the climate is characterized as continental, the amount of precipitation per year averages 428 mm. On average for 2015-2017, from 334.7 to 606.9 mm precipitation fell during the growing season of winter camelina. The driest year was 2017 (HTC 0.61), 2016 was characterized as insufficiently humidified (HTC 0.82), and the optimal water supply was in 2015 (HTC 1.11).

Winter hardiness is an important biological trait that determines the ability of plants to withstand low temperatures and other stressors in winter and early spring. Wither camelina differs from other winter cabbage crops (for example, rapeseed and winter rocket) in high frost and winter resistance [18, 20]. Under the conditions of Penza region, winter hardiness of the camelina varieties ranged from 89.5 to 96.7%. There were samples of a high (95.3-96.7% for Kozyr, Baron, i.o.-4164, i.o.-3290, and i.o.-2219), moderate (93.2-94.8% for Penzyak, Dikii, i.o.-4165, i.o.-1357, i.o.-4175) and low (89.5-89.9% for i.o.-4172, i.o.-4155, and i.o.-4156) winter hardiness (Table 1).

1. Winter hardiness of camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) varieties and breeding lines cultivated in different regions (*M*±SEM, 2015-2017)

Variety, line	Origin	Region of cultivation	
		the Middle Volga (settlement Lunino, Penza Province)	the steppe Crimea (Klepinino village, Krasnogvardeiskii District
Panzyak (st)	Penza	94.8±0.98	95.9±0.33
Kozyr	Penza	95.3±0.98	95.6±0.33
Baron	Penza	96.5±0.98	96.7±0.33
Dikii	Astrakhan	93.9±0.98	93.3±0.33
i.o.-4172	Sverdlovsk	89.9±0.98	93.9±0.33
i.o.-1357	France	94.2±0.98	92.3±0.33
i.o.-2219	Ukraine	95.9±0.98	93.1±0.33
i.o.-4155	Dagestan	89.7±0.98	92.9±0.33
i.o.-4164	Sweden	96.3±0.98	95.7±0.33
i.o.-4156	Mari El Republic	89.5±0.98	96.9±0.33
i.o.-4175	Czechoslovakia	93.9±0.98	96.3±0.33
i.o.-3290	Altai	96.7±0.98	95.0±0.33
i.o.-4165	Germany	93.2±0.98	94.2±0.33
LSD_{05}		1.08	0.99

N o t e. Panzyak variety is the standard (st), p = 0.05.

In Crimea, the resistance of camelina cultivars to overwintering conditions was quite high (92.3-96.9%). The highest value was observed in the Baron variety and i.o.-4156 breeding line, which exceeded the standard variety by 0.8 and 1.0%, respectively.

The yield of the studied varieties and lines varied depending on the region of cultivation. However, the Dikii and i.o.-3290 lines were much more effective than the control: their productivity in the Middle Volga region was 1.85 and 1.97 t/ha, respectively, in the Crimea 1.73 and 1.83 t/ha, respectively (Table 2), which indicates the highest adaptability, plasticity and stability of these varieties in stressful conditions. In addition, in the conditions of the steppe Crimea, the line i.o.-1357 (France) stood out in terms of yield, exceeding the control by 0.10 t/ha.

2. Total seed yield and oil yield of camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) varieties and breeding lines cultivated in different regions (*M*±SEM, 2015-2017)

Variety, line	Origin	Middle Volga (settlement Lunino, Penza Province)		Steppe Crimea (Klepinino village, Krasnogvardeiskii District)	
		yield, t/ha	oil content, %	yield, t/ha	oil content, %
Panzyak (st)	Penza	1.65±0.08	38.74±0.98	1.64±0.03	41.25±1.08
Kozyr	Penza	1.79±0.07	39.66±0.98	1.66±0.02	43.50±1.08
Baron	Penza	1.86±0.05	40.46±0.88	1.59±0.02	43.90±1.08
Dikii	Astrakhan	1.97±0.08	40.19±1.02	1.83±0.02	38.19±1.10
i.o.-4172	Sverdlovsk	1.57±0.07	38.53±1.01	1.54±0.03	36.71±1.08
i.o.-1357	France	1.69±0.08	38.69±0.98	1.74±0.03	38.03±1.09
i.o.-2219	Ukraine	1.81±0.09	37.42±0.88	1.57±0.03	35.61±0.98
i.o.-4155	Dagestan	1.76±0.07	38.41±0.88	1.66±0.02	37.79±0.99
i.o.-4164	Sweden	1.64±0.08	37.96±0.98	1.67±0.02	38.70±1.01
i.o.-4156	Mari El Republic	1.72±0.09	37.38±0.98	1.69±0.02	38.37±0.99
i.o.-4175	Czechoslovakia	1.61±0.09	37.79±0.98	1.56±0.03	36.95±1.08
i.o.-3290	Altai	1.85±0.09	39.58±0.88	1.73±0.02	38.29±1.08
i.o.-4165	Germany	1.72±0.08	38.83±0.88	1.67±0.02	38.27±1.08
LSD_{05}		0.12	1.03	0.04	1.12

N o t e. Panzyak variety is the standard (st), p = 0.05.

The seed oil content varied from 35.61 to 43.90%. The oiliest varieties were Baron and Kozyr, 43.90 and 43.60%, respectively. It can be said that they showed ecological plasticity when tested in various agroclimatic conditions. In the Penza region, it is worth noting i.o.-2219 (Ukraine), the yield of which was 1.81 t/ha, exceeding the standard variety Penzyak by 0.16 t/ha.

The realization of the productivity potential of varieties and lines of winter camelina both in the forest-steppe zone of the Middle Volga and in the steppe

zone of the Crimea was generally comparable and reached the values of 70.9-88.9 and 71.1-86.3%, respectively. The lines i.o.-3290 and Dikii used their capabilities most fully, 82.5-84.9 and 86.3-88.9%, respectively, which can be explained by their greater ability to withstand the action of abiotic stresses (Fig. 1).

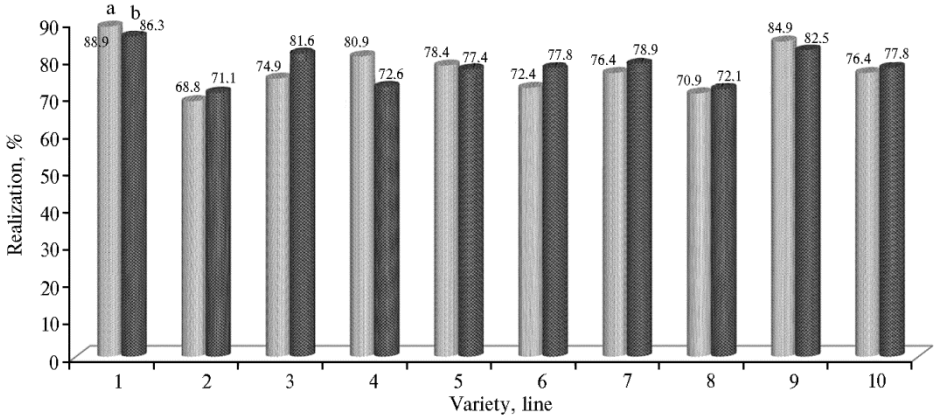

FIg. 1. Realization of potential productivity of camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) varieties and breeding lines cultivated in the Middle Volga (settlement Lunino, Penza Province) (a) **and the Steppe Crimea** (Klepinino village, Krasnogvardeiskii District) (b) **conditions:** 1 — Dikii, 2 — i.o.-4172, 3 — i.o.-1357, 4 — i.o.-2219, 5 — i.o.-4155, 6 — i.o.-4164, 7 — i.o.-4156, 8 — i.o.-4175, 9 — i.o.-3290, 10 — i.o.-4165 (2015-2017).

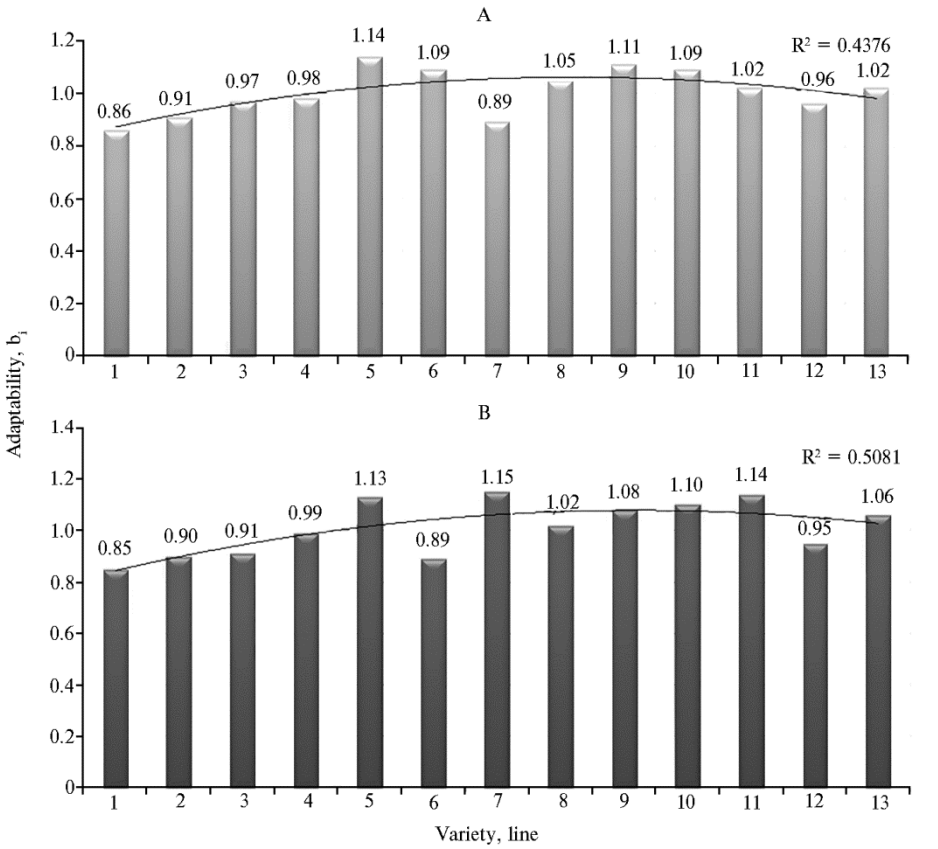

Fig. 2. Adaptability of camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) varieties and breeding lines cultivated in the Middle Volga (settlement Lunino, Penza Province) (A) **and the Steppe Crimea** (Klepinino village, Krasnogvardeiskii District) (B) **conditions:** 1 — variety Penzyk (standard, st), 2 — variety Kozyr, 3 — variety Baron, 4 — Dikii, 5 — i.o.-4172, 6 — i.o.-1357, 74 — i.o.-2219, 8 — i.o.-4155, 9 — i.o.-4164, 10 — i.o.-4156, 11 — i.o.-4175, 12 — i.o.-3290, 13 — i.o.-4165 (2015-2017).

The most stable and plastic both under the conditions of the Middle Volga region and the steppe Crimea were the breeding lines Dikii (b_i = 0.98-0.99) and i.o.-3290 (b_i = 0.96-0.95), which turned out to be more adapted to various, including unfavorable, growing conditions (Fig. 2). Lines with the regression coefficient $b_i > 1.0$ are of the intensive type, respond well to the improvement of agrotechnological conditions, but more often decrease their productivity under stressful agroclimatic conditions [22, 23].

The productivity of a crop depends on the components of the yield structure, the contribution of which to the final yield is determined by the influence of genotype and environmental factors [24] and is assessed by the criterion of adaptability (K_0) to the cultivation conditions [31]. In our experiments, for both regions on average, in the lines i.o.-3290 (85.4 g/m^2) and Dikii (88.9 g/m^2) the fitness criterion values were the highest (Table 3). Low K_0 values were noted in the lines i.o.-4172 (Sverdlovsk) and i.o.-4175 (Czechoslovakia), 56.3 and 59.6 g/m^2, respectively. An increase or decrease in the K_0 values in the camelina samples occurred as a result of changes in the ratio of the contributions of the main components to the final seed yield.

All breeding lines had positive K_0 values, which was mainly due to compensatory effects of an increase in the number of pods per plant, the number of seeds per pod, and the 1000-seed weight. Upon a negative value of one of the parameters, the positive criteria were overlapped by positive effects on other components of the seed yield. This explains different response of varieties to climatic factors during the growing season, as well as plant adaptability and fitness to the regions of cultivation.

3. Plant fitness criterion (K_0) **of camelina (*Camelina sylvestris* Waller ssp. *pilosa* Zing.) varieties and breeding lines cultivated in different regions** (2015-2017-year average)

Variety, line	Total fitness criterion, g/m^2	Contribution of yield structure component to K_0, %		
		pod number	seeds per pod	1000-seed weigh, g
Panzyak (st)	83.7	78.6	28.8	14.2
Kozyr	84.1	81.2	33.1	15.2
Baron	84.9	75.9	26.3	17.2
Dikii	88.9	119.6	–30.7	35.1
i.o.-4172	56.3	46.5	–21.6	32.8
i.o.-1357	79.5	79.5	96.8	–47.3
i.o.-2219	72.3	102.0	50.8	17.2
i.o.-4155	74.6	85.6	–43.1	61.8
i.o.-4164	69.8	78.6	55.6	–40.9
i.o.-4156	75.1	77.5	–27.7	50.2
i.o.-4175	59.6	51.3	–28.3	–33.9
i.o.-3290	85.4	75.9	29.4	45.3
i.o.-4165	71.9	66.3	50.3	–64.0

N o t e. Panzyak variety is the standard (st).

Currently, due to changes of climatic conditions, the assessment of the crop adaptability and stability is one of the most important in breeding. Among the first publications on assessing the stability and adaptability of winter camelina varieties is the work of Prakhova et al. [20], where the varieties Penzyak, Kozyr, Baron and Peredovik were tested in various agroecological conditions of Volgograd, Krasnodar, the Republic of Crimea, and the Penza region. We could not find other similar publications for winter camelina. However, a large number of similar works are known for other crops (also with regard to ecologically sustainable agriculture), including wheat [32], barley [33], corn [34], flax [24], rapeseed [18].

Korolev et al. [24] reported about the possibility of identifying adaptive and stable forms in fiber flax in the conditions of Belarus. Diederichsen et al. [35] carried out such studies at the All-Russian Research Institute of Flax (Torzhok). Based on the analysis of the works of other authors and the assessment of the breeding material

of winter camelina, we have identified a number of promising varieties that exceed the existing varieties in terms of adaptability and stability and can produce stable yields upon a deterioration in temperature conditions and water supply.

Thus, as a result of the agroecological assessment of winter camelina for the main parameters of productivity, stability and ecological plasticity in two contrasting agroecological regions, we identified varieties characterized by high productivity and resistance to various cultivation conditions. The most stable and plastic breeding lines are Dikii (b_i = 0.98-0.99) and i.o.-3290 (b_i = 0.96-0.95), which significantly exceeded the Penzyak variety in both regions. These samples have a high selection value and can be used to derive varieties with high stable productivity and adaptability to the contrasting conditions of the regions. Among the studied samples, i.o.-4155, i.o.-4156 and i.o.-4165 had low but stable yields over the years (1.66-1.76 t/ha) and b_i = 1.02-1.10, which shows their responsiveness to changes in cultivation conditions

REFERENCES

1. Zhuchenko A.A. *Adaptivnaya sistema selektsii rastenii (ekologo-geneticheskie osnovy)* [Adaptive plant breeding system (eco-genetic fundamentals)]. Moscow, 2001: 529, 539 (in Russ.).
2. Joshi S.K., Ahamada S., Meher L.C., Agarwal A., Nasim M. Growth and yield response of Camelina Sativa to inorganic fertilizers and farmyard manure in hot semi-arid climate of India. *Adv. Plants Agric. Res.*, 2017, 7(3): 1-6 (doi: 10.15406/apar.2017.07.00258).
3. Ghamkhar K., Croser J., Aryamanesh N., Campbell M., Kon'kova N., Francis C. Camelina (*Camelina sativa* (L.) Crantz) as an alternative oilseed: molecular and ecogeographic analyses. *Genome*, 2010, 53(7): 558-567 (doi: 10.1139/G10-034).
4. Imbrea F., Jurcoane S., Hălmăjan H.V., Duda M., Botoş L. *Camelina sativa*: a new source of vegetal oils. *Romanian Biotechnological Letters*, 2011, 16(3): 6263-6270.
5. Katar D., Arslan Y., Subaşı İ., Tekirdağ Z., Fakültesi D. Ankara ekolojik koşullarında farklı ekim zamanlarının ketencik (*Camelina sativa* (L.) Crantz) bitkisinin yağ oranı ve bileşimi üzerine olan etkisinin belirlenmesi. *Journal of Tekirdag Agricultural Faculty*, 2012, 3: 84-90.
6. Waraich E.A., Ahmed Z., Ahmad R., Ashraf M.Y., Saifullah, Naeem M.S., Rengel Z. *Camelina sativa*, a climate proof crop, has high nutritive value and multiple-uses: a review. *Australian Journal of Crop Science*, 2013, 7(10): 1551-1559.
7. Moser B.R. Camelina (*Camelina sativa* L.) oil as biofueis feedstock: golden opportunity or false hope. *Lipid technology*, 2010, 22(12): 270-273 (doi: 10.1002/lite.201000068).
8. Zazulya L.N., Nagornov S.A., Romantsova S.V., Malakhov K.S. *Dostizheniya nauki i tekhniki APK*, 2009, 12: 58-60.
9. Buyankin V.I. *Masla i zhiry*, 2008, 3: 19-22 (in Russ.).
10. Dobre P., Jurcoane Ş. *Camelina sativa* — an oilseed crop with unique agronomic characteristics. In: *Scientific Papers, Series A, Vol. LIV.* Bucharest, 2011: 425-430.
11. Sharma N. Assessment of biofuel potential in India. *International Journal of Recent Scientific Research*, 2017, 8: 17125-17127 (doi: 10.24327/ijrsr.2017.0805.0287).
12. Wu X., Leung D. Orthogonal array design for biodiesel production optimization — using ultrasonic-assisted transesterification of *Camelina sativa* L. Crantz oil. *Proc. of the World Renewable Energy Congress.* Linköping, Sweden, 2011, V. 57: 79-86 (doi: 10.3384/ecp1105779).
13. Sidorov E.A., Ukhanov A.P., Zelenina O.N. *Izvestiya Samarskoi gosudarstvennoi sel'skokhozyaistvennoi akademii*, 2013, 3: 49-54 (in Russ.).
14. Mohammed Y.A., Chen C., Lamb P.F., Afshar R.K. Agronomic evaluation of camelina (*Camelina sativa* (L.) Crantz) cultivars for biodiesel feedstock. *BioEnergy Research*, 2017, 10: 792-799 (doi: 10.1007/s12155-017-9840-9).
15. Vinovets V.G., Lisnyak A.D., Ivanov M.V. *Naukovo-tekhnichnii byuleten' Institutu oliinikh kul'tur NAAN*, 2014, 20: 86-91 (in Russ.).
16. Loshkomoinikov I.A., Kuznetsova G.N. *Kormoproizvodstvo*, 2009, 4: 24-27 (in Russ.).
17. Ryabtseva N.A. *Sel'skoe, lesnoe i vodnoe khozyaistvo*, 2015, 1(40): 14-17 (in Russ.).
18. Mostofa U.H., Nazrul I., Monjurul K., Noor H.M. Performance of rapeseed and mustard (*Brassica* sp.) varieties/lines in north-east region (Sylhet) of Bangladesh. *Agricultural Research & Technology*, 2016, 1(5): 001-006 (doi: 10.19080/ARTOAJ.2016.02.555576).
19. Bekuzarova S.A., Dulaev T.A. *Novye netraditsionnye rasteniya i perspektivy ikh ispol'zovaniya*, 2016, 12: 182-184 (in Russ.).
20. Prakhova T.YA., Smirnov A.A., Prakhov V.A., Turina E.L., Kulinich R.A. *Trudy Kubanskogo gosudarstvennogo agrarnogo universiteta*, 2017, 66: 203-207 (doi: 10.21515/1999-1703-66-203-207)

(in Russ.).
21. Campbell M. Camelina — an alternative oil crop. In: *Biokerosene*. M. Kaltschmitt, U. Neuling (eds.). Springer, Berlin, Heidelberg, 2018: 259-275 (doi: 10.1007/978-3-662-53065-8_12).
22. *FAO Data Dissemination*. Available: http://www.fao.org/statistics/databases/ru/. No date.
23. Galasso I., Manca A., Braglia L., Ponzoni E., Breviario D. Genomic fingerprinting of Camelina species using c TBP as molecular marker. *American Journal of Plant Sciences*, 2015, 6(8): 1184-1200 (doi: 10.4236/ajps.2015.68122).
24. Korolev K.P., Bome N.A. Evaluation of flax (*Linum usitatissimum* L.) genotypes on environmental adaptability and stability in the North-Eastern Belarus. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 3(52): 615-621 (doi: 10.15389/agrobiology.2017.3.615eng).
25. Kil'chevskii A.V. *Vestnik VOGiS*, 2005, 4(9): 518-526 (in Russ.).
26. Betancor M.B., Li K., Bucerzan V.S., Sprague M., Sayanova O.V., Usher S.L., Han L., Norambuena F., Torrissen O.J., Napier J.A., Tocher D.R., Olsen R.E. Oil from transgenic *Camelina sativa* containing over 25 % n-3 long-chain PUFA as the major lipid source in feed for Atlantic salmon (*Salmo salar*). *British Journal of Nutrition*, 2018, 119(12): 1378-1392 (doi: 10.1017/S0007114518001125).
27. *Metodika provedeniya polevykh agrotekhnicheskikh opytov s maslichnymi kul'turami* /Pod redaktsiei V.M. Lukomtsa [Methodology for field agrotechnical trials of oilseeds. V.M. Lukomts (ed.)]. Krasnodar, 2010 (in Russ.).
28. Dospekhov B.A. *Metodika polevogo opyta s osnovami statisticheskoi obrabotki rezul'tatov issledovanii* [Field trial methodology with the basic data statistical processing]. Moscow, 1985 (in Russ.).
29. Kil'chevskii A.V., Khotyleva L.V. *Geneticheskie osnovy selektsii rastenii. Obshchaya genetika rastenii* [Genetic basis of plant breeding. General plant genetics]. Minsk, 2008: 50-56 (in Russ.).
30. Nettevich E.D. *Doklady RASKHN,* 2001, 3: 50-55 (in Russ.).
31. Belenkevich O.A. Fiziologo-geneticheskie aspekty vzaimosvyazei mezhdu rasteniyami yarovogo yachmenya v monotsenoze [Physiological and genetic aspects of spring barley plant interaction in a monocenosis. *Sel'skokhozyaistvennaya biologiya*, 2002, 3: 57-61.
32. Voss-Fels K.P., Stahl A., Wittkop B., Lichthardt C., Nagler S., Rose T., Chen T.W., Zetzsche H., Seddig S., Majid Baig M., Ballvora A., Frisch M., Ross E., Hayes B.J., Hayden M.J., Ordon F., Leon J., Kage H., Friedt W., Stützel H., Snowdon R.J. Breeding improves wheat productivity under contrasting agrochemical input levels. *Nat. Plants,* 2019, 5(7): 706-714 (doi: 10.1038/s41477-019-0445-5).
33. Ceccarelli S., Grando S., Capettini F., Baum M. Barley breeding for sustainable production. In: *Breeding major food staples* /M.S. Kang, P. Priyadarshan (eds). Blackwell Publishing, Oxford, 2008: 193-225 (doi: 10.1002/9780470376447.ch7).
34. Reeves T.G., Thomas G., Ramsay G. *Save and grow in practice. Maize, rice, wheat. A guide to sustainable cereal production.* FAO, Rome, 2016.
35. Diederichsen A., Rozhmina L., Kudrjavceva A. Variation patterns within 153 flax (*Linum usitatissimum* L.) genebank accessions based on evaluation for resistance to fusarium wilt, anthracnose and pasmo. *Plant Genetic Resources*, 2008, 6: 22-32 (doi: 10.1017/S1479262108913897).

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PRODUCTIVITY POTENTIAL OF DRUPE FRUIT VARIETIES — BIOMORPHOLOGICAL FEATURES OF FORMATION AND REALIZATION UNDER THE CLIMATIC CONDITIONS OF SOUTH RUSSIA

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Abstract

Productivity, the main characteristic of a variety of fruit crops, which is due to a set of elements, determines the resistance of a crop to environmental stress factors and its suitability for industrial growing conditions. The impact of climatic stresses annually results in only 30-40% realization of productivity potential of fruit plants. In fruit plants, including sweet and ordinary cherries, fruit-bud differentiation takes a long time. In the conditions of the southern Russia (Prikubanskaya gardening zone of the Krasnodar Territory), it begins in July of the current year and ends in April-May of the next year. The climate in the Krasnodar Territory is quite mild, generally favorable for the cultivation of sweet and ordinary cherries. Nevertheless, even in the south of Russia, there is a high risk of annual abiotic stresses, negatively affecting fruit plants and impeding realization of their productivity potential. In the present work, a comprehensive biomorphological study was first undertaken to assess the regional productivity potential of sweet and ordinary cherries of various ecological and geographical origins. Our research aimed to establish the regional patterns of productivity element formation during morpho- and organogenesis in *Prunus* L. species and hybrids, to identify the most vulnerable stages in plant annual development cycles, and to distinguish the varieties with high and sustainable yielding under risky weather conditions. Three introduced *Cerasus avium* (L.) Moench varieties of different ecogeographic origins (Valerii Chkalov, Melitopolskaya Chernaya, and Polyanka), six domestic *C. avium* varieties (Kavkazskaya, Sashen'ka, Volshebnitsa, Dar izobiliya, Alaya, and Mak), four introduced *C. vulgaris* Mill. varieties (Kelleris, Nefris, Fanal, and Erdi Botermo), and seven interspecific hybrids *C. vulgaris* × *C. avium* (Kirina, Dombaziya, Duk Ivanovna, Duk Khodosa, Igrushka, and Shalun'ya) were studied during 2006-2019 (Experimental Production Center of the North-Caucasian Federal Scientific Center of Horticulture, Viticulture, Winemaking, Krasnodar; 6×4, 7×3, and 5×3 m plant spacing patterns). *Cerasus mahaleb* (L.) Mill. and *Cerasus avium* L. plants were rootstock plants, all experiments were arranged in three replications. Phenology of all stages and sub-stages of the annual (vegetative and generative) plant growth was described as per BBCH system (Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany). Morphogenesis of yield components was investigated in 15 generative buds two times in December and February, weekly in March-April, and weekly in July-November by light microscopy (Olympus BX41, Olympus Corporation, Japan). In spring, flowers, ovaries, and fruits were gradually counted on third-order skeletal branches, and the degree of their reduction were calculated to estimate biological productivity. The number of fruit buds and growth buds was estimated twice a week in August and September. To assess the winter hardiness, 90 vegetative buds and 180 fruit buds from different sides of the lower and upper branches of 3 trees of the same variety were collected in 3 replicates. It was found that the reduction of productivity elements in the stressful conditions of southern gardening occurs annually at the same (critical) stages of development of fruit plants. These stages are i) reproductive organ initiation; ii) archesporium formation in anthers; iii) pollen mother cells, iv) mono- and binuclear pollen grains, and v) macrosporogenesis. The critical periods of yield formation in the conditions of the Krasnodar Territory are II-III decades of March, and I-II decades of April, May and July, when special

agrotechnology should be applied to reduce the impact of stress and to increase yields. A direct correlation was revealed between temperature and air humidity during each critical period, the fruiting coefficient and the final yield ($r = +0.97$, $p < 0.001$). In our tests, the following samples are characterized by high fruiting rates and sustainable yields: domestic cherry varieties Kavkazskaya (62% and 31.3 t/ha, respectively), Alaya (46% and 30.0 t/ha), Sashen'ka (45% and 26.6 t/ha), and Volshebnitsa (42% and 23.3 t/ha), interspecific hybrids Duk Khodosa (46% and 23.3 t/ha), Kirina (44% and 23.3 t/ha), and Duk Ivanovna (44 % and 20.0 t/ha), and an ordinary cherry variety Kelleris (45% and 20.0 t/ha). These varieties and hybrids can be cultivated in commercial orchards and used as genetic donors in breeding for sustainable productivity.

Keywords: drupe fruit, sweet cherry, ordinary cherry, varieties, abiotic factors, adaptive potential, biological productivity, generative development, organogenesis, yielding

Stresses caused by current global climatic changes negatively affect the productivity of agricultural plants, including drupe fruit plants of the genus *Prunus* L. [1, 2]. According to the forecasts, in the future, an increase in the average air temperature by 1.5-2.7 °C is likely [1, 3], which can lead to a shift in the annual biorhythms of fruit crops and disrupt the production process [4]. Obviously, under the influence of stress factors, varieties will be unable to maximize their yield potential [5]. A deeper understanding of how the reproductive parts of a plant develop underlies measures to preserve future fruit harvests at critical, most vulnerable stages of plant ontogenesis.

Krasnodar Territory is a southern Russian region with quite favorable weather and climatic conditions for fruit plant cultivation [6]. Nevertheless, even in the south, the risk of annual abiotic stresses is high [7]. These are frosts during the dormant period, return spring frosts, precipitation, fog, dry winds and high temperatures during the flowering period, which cause damage to reproductive organs, partial or complete yield loss [8, 9]. About 50% of fruit yields depend on weather conditions [10], 40% depend on a varietal biological potential [11], and 10% on anthropogenic factors [7, 12]. Other researchers report a higher level of specific influence of the variety on the yield of drupe fruit plants [6, 8].

The fruit yield formation is preceded by a long period, including initiation and differentiation of fruit buds, which are the consecutive stages in the annual development cycle. At each stage, processes occur that determine the productivity of a particular crop and variety [10, 13]. In fruit plants, including sweet cherries and ordinary cherries, bud differentiation takes a long period [5, 14]. In the conditions of the southern region of Russia (Prikubanskaya zone of gardening in Krasnodar Territory), it begins in July of this year and ends in April-May of the next year [5]. In the apple tree, differentiation lasts on average 140-150 days [10, 12], in the sweet cherry it takes 117-130 days [6]. By the beginning of the dormant period (December), floral pistil primordia and stamen primordia appear in fruit buds of sweet cherry and ordinary cherry [15], which makes the flowers less frost-resistant and more vulnerable [8]. Unlike cherry plants, the fruit buds of apple plants are less differentiated, which determines their greater winter hardiness [12, 16].

It was found that frosts from –26.0 to –28.0 °C during dormancy lead to significant freezing or death of fruit buds in sweet cherry [17], and temperatures from –29.0 to –30.0 °C cause freezing or death of reproductive shoots in ordinary cherry [14]. Data are given that open flowers of sweet cherry and ordinary cherry die at temperatures from –2.0 to –2.2 °C, closed buds at –2.4 to –5.0 °C [17], and that pistils of flowers are especially sensitive to spring frosts [9, 18]. It has been proven that some drupe fruit crops (apricot, cherry plum, Russian plum, sweet cherry) are caused to early wake up if the sum of low negative

temperatures during dormancy is below the required values [19]. This biological feature increases the frost impact on generative organs [20, 21], leading to asynchronous development of flower organs [22, 23] and male generative dysfunctions with a decreased viability and quality of pollen and impaired fertilization [21, 24].

High temperature stress also adversely affects the generative sphere of fruit plants [25]. It has been established that differentiation of apple fruit buds is optimal at 23.0-30.0 °C [26] and stops at temperatures above 30.0 °C [27]. It is believed that maturation of sweet cherry and ordinary cherry pollen begins at 22-23.0 °C [24, 28], whereas the temperature of 25.0 °C is fatal for the cherry plum ova [29]. According to some studies, extremely high temperatures shorten the period of effective pollination [30], and when flower buds are set in such conditions, the morphogenesis and, hence, the future harvest are under threat [31, 32].

Thus, it is obvious that temperature stresses do not allow fruit plants to maximize their yield potential, since the plants generate a physiological stress response that allows cells to survive [33]. To summarize, the pattern of yield component formation and yield potential realization in fruit crops under temperature stress remain fragmentarily studied and insufficiently clarified, which prompted us to perform this research.

Here we present data on yield potential of sweet cherry and common cherry varieties of various ecogeographic origin assessed in the conditions of Krasnodar Territory by complex biomorphological parameters. Five critical stages of organogenesis and their timing under stresses have been identified. It has been found out that the observed yields depend strongly on the types of temperature stress during these periods and on the number of fruiting elements after reduction.

This research aimed to identify patterns of morpho- and organogenesis in the genus *Prunus* L., which determine the development of yield components under environmental stress, to reveal the most stress-sensitive stages of annual plant cycle based on the resultant yielding, and to recognize the most productive sweet cherry and ordinary cherry varieties for intensive gardening in the southern region of the Russian Federation.

Materials and methods. In the research we used 3 introduced varieties of sweet cherry *Cerasus avium* (L.) Moench of different ecological and geographical origin (Valery Chkalov, Melitopolskaya chernaya, Polyanka) (Ukraine), 6 varieties of domestic selection (Caucasian, Sasha, Volshebnitsa, Dar Izobiliya, Alaya, Mak) (Russia), 4 introduced varieties of ordinary cherry Cerasus vulgaris Mill., the Kelleris (Denmark), Nefris (Poland), Fanal (Germany), Erdi Botermo (Hungary), and 7 interspecific hybrids (sweet cherry × ordinary cherry), the Kirina (Russia), Dombazia (Central Asia), Duk Ivanovna (Ukraine), Duk Khodosa (Ukraine), Igrushka (Ukraine), Shalun'ya (Ukraine). The trials were performed at Tsentralnoe Experimental Production Center (the North Caucasian Federal Research Center of Horticulture, Viticulture, Winemaking, Krasnodar, Prikubanskaya zone of gardening) in 2006-2019. Agrotechnologies were common for the region. Tree planting patterns were 6×4, 7×3 and 5×3 m. Seedlings of antipka (Mahaleb cherry) *Cerasus mahaleb* (L.) Mill. and wild sweet cherry *Cerasiis avium* L. were used as the rootstocks (one cultivar — one variant according to the methodology of cultivar study), the experiments were arranged in three replications.

Phenological characterization of annual vegetative and generative growth

stages and substages was carried out as per BBCH international coding system (Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany) [34]. Biological productivity and the frost damage to fruit buds were assessed according to [35, 36]. During observations, the phenophases were documented (Canon EF-S camera, Japan), and the stages of organogenesis were examined using light microscopy (an Olympus BX41, Olympus Corporation, Japan).

On the tertiary skeleton branches, flowers, ovaries, and fruits were counted in spring, and their reduction was assessed. Fruit and growth buds were counted in August and September twice a week [5].

To study fruit morphogenesis by light microscopy, 15 generative buds were collected twice in December and February, and weekly in March-April and July-November.

For temporary slides, the fruit bud was cut with a razor blade from base to top, and transverse sections were fixed in distilled water on a glass slide. The slides were viewed under a microscope at a 50-100× zoom. The anthers were removed from fruit buds with a needle, pre-stained with acetocarmine on the microscope stage, pressed down with a cover glass and viewed at a 100-200× zoom (an Olympus BX41 microscope). The stages and substages of organogenesis and microsporogenesis were described as per Isaeva [37]. Specimens for anatomical study of fruit buds by light microscopy were prepared according to the improved method of Kiseleva [38].

Yield components appeared on short spurs with bud fascicles and on annual shoots with fruit buds were counted in August-September on 1-2 typical tertiary branches from the southern and eastern sides of a tree (a total of three trees per variety in a 3-fold repetition). The coefficient of productivity (Cp) was calculated as a fruit-to-flower percent ratio (the flowers per 1 m of fruiting branches were taken as 100%).

Winter hardiness was assessed in 90 vegetative and 180 fruit buds collected from three trees of each variety (from different sides of the lower and upper branches) in 3 replicates.

The arithmetic means (M), standard deviations (±SD) and LSD_{05} (a 95% probability level) were calculated using a standard Microsoft Excel 2013 software package. The coefficient of variation (Cv, %) and the relative standard error of the sample mean (sampling error) ($Sx = SD/\sqrt{M}$, %) were calculated [35]. Correlation analysis of damage to fruit buds depending on the cultivar and the year conditions was performed using the Statistica package (StatSoft, Inc., USA) (2019).

Results. The climate in the Prikubanskaya zone of the Krasnodar Territory is rather mild, generally favorable for the cultivation of sweet cherry and ordinary cherry. The average annual air temperature is 11.9-12.1 °C. The maximum temperatures in July-August reach 40.0-40.7 °C, the minimum temperatures in January-February can drop to –33.0 °C.

Weather conditions during study years showed dynamism and were indicative of a complex of stably recurring temperature stresses. So, in January 2006, abnormally low temperatures were observed (from –32.0 °C to –33.0 °C), which led to the death of all fruit crops [17]. In February 2014, freezing rain at –22, 2 °C caused the death of the harvest of all fruit plants. In January 2015 and December 2016, during the dormant period, after a long period of warm weather, the air temperature dropped to –21.0 °C and –17.0 °C, respectively, resulting in freezing of fruit buds in sweet cherry. During our observation, almost every two

1. **Reduction of yield components (fruit buds, flowers and ovaries) in sweet cherry *Cerasus avium* (L.) Moench and ordinary cherry *Cerasus vulgaris* Mill. varieties as influenced by weather stresses during critical periods of plant ontogenesis** (Tsentralnoe Experimental Production Center, the North Caucasian Federal Research Center of Horticulture, Viticulture, Winemaking, Krasnodar, 2006-2019)

Phenophase (BBCH stage)	Stage of organogenesis and microsporogenesis (timing)	Temperature and air humidity		Reduction, %
		optimal conditions	unfavorable conditions	
Initiation of flower organs in fruit buds	IV — emergence of generative parts (July decades I-II)	Below 30-33 °C at 40-47% air humidity	Above 35-37 °C at air humidity below 40%	20-30
Dormancy (00)	VI — archesporial tissue in anthers (December-January)	Above –24 °C at 65-75% air humidity	Below –28 °C at 75-80% air humidity	85-100
Bud scale elongation (bud burst) (51)	VII — pollen mother cells (March decades II-III)	Not lower than 9-10 °C at 60% air humidity	Below –10 °C at 65% air humidity	15-70
First flowers bloom—full flowering (60-65)	IX — mono- and bicellular pollen grains (April decades I-II)	Within 16-25 °C at 55-58% air humidity	From –3.0 to –6.0 °C; below 16 °C at 60% air humidity	20-95
Ovary emergence and growth (71-72)	X — macrosporogenesis (May decades I-II)	Within 25.0-28.0 °C at 54-60% air humidity	Above 28.0-30.0 °C at 75% air humidity	40-46

N o t e. For description of the varieties and hybrids, see section Materials and methods.

years (2009, 2010, 2014, 2015, 2018) in July-August, in the period of fruit set and differentiation, abnormally high temperatures (38.0-40.0 °C) combined with drought were noted for 4-5 days or more. During the flowering of sweet cherry and ordinary cherry plants, recurrent spring frosts (from –3.0 to –6.0 °C) occurred annually, leading to freezing and death of stamens and stigmas of pistils, and at temperatures below 16 °C and 60% air humidity the fertilization was disturbed (Table 1).

Of the 12 morphogenetic stages that we studied in sweet and ordinary cherry plants, five stage with a high rate of yield component mortality were the most vulnerable across study sites and years. We also determined the optimal and extreme conditions for each phenophase (organogenesis stage) (Table 1). Similar studies on apple trees identified only four critical stages [37].

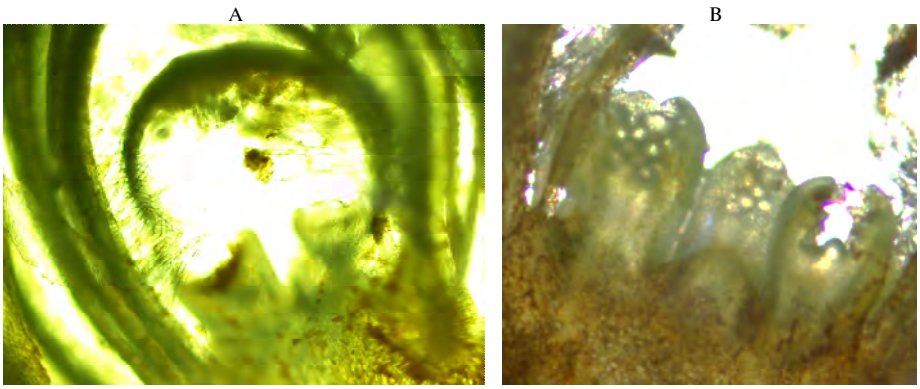

Fig. 1. Floral primordium in the fruit bud of sweet cherry *Cerasus avium* (L.) Moench variety Alaya at stage IV of organogenesis (A, July 23, 2017, unfavorable conditions of above 35-37 °C and air humidity below 40%) **and pistil primordium in the fruit bud of ordinary cherry *Cerasus vulgaris* Mill. variety Kirina at stage V of organogenesis** (B, November 10, 2018, above –6,5 °C at 65-75% air humidity are close to optimal conditions)**, the Prikubanskaya zone of gardening in Krasnodar Territory** (50-100× zoom, Olympus BX41, Olympus Corporation, Japan). For variety origin, see section Materials and methods.

During stage IV of organogenesis (July decades I-II), only 1-2 instead of 3-5 floral primordia emerged under unfavorable temperature conditions (see Table 1, Fig. 1, A), whereas, under those close to optimal, all the flower organs and archesporial anther tissue (three meristematic zones and more) were formed, which then reduced to floral primordia (see Table 1, Fig. 1, B). Under unfavorable temperature at stage IV, the reduction of flower organs averaged 20-30%.

Many researchers report that fruit buds have the highest winter hardiness at the state of primary archesporium, corresponding to stage VI [15, 29], which we noted in December-January (Fig. 2). We regard the stage VI as the second critical for organogenesis under the conditions of Krasnodar Territory (see Table 1) due to the high probability of frost and, thence, death of fruit buds. So, in 2006, the most abnormal in recent decades, temperature drop to –33.0 °C during the dormancy period killed 100% of flower buds in most sweet cherry varieties. We revealed a close correlation (r = +0.991, $p < 0.001$) between exposure to critical temperature and the death of fruit buds. In particular, 100% death of generative organs was characteristic of the introduced sweet cherry varieties Polyanka and Valery Chkalov. For the local varieties Alaya, Volshebnitsa and Dar izoboliya, with 90% death of fruit buds, the yields were 5.0-5.5 kg per tree, which indicates frost resistance. Interspecific hybrids for which a fruiting rate of 4.0-6.0 kg per tree upon 90-95% death of fruit buds

was indicative also showed high frost resistance. In the studied varieties of ordinary cherry, the rate of bud death was lower and averaged 85-90%, with a yield of 5.0-8.0 kg per tree.

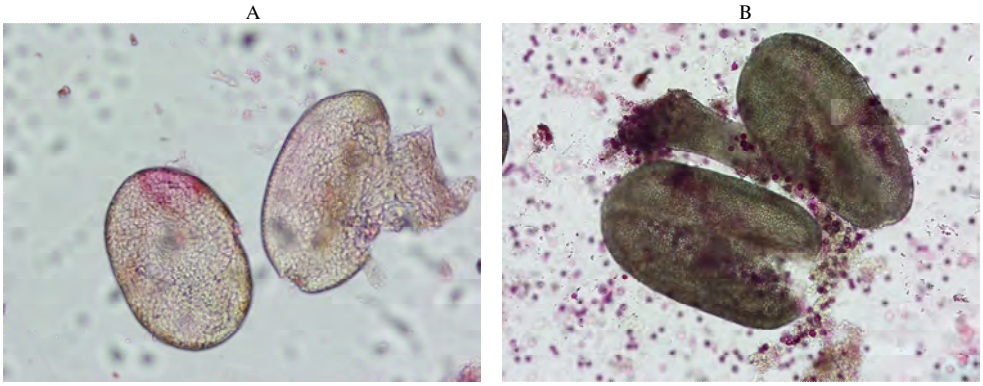

Fig. 2. Formation of archesporium tissue in anthers at stage VI of organogenesis (A, January 28, 2010, favorable temperature above –24 °C and 65-75% air humidity) **and mother cells of pollen at stage VII of organogenesis** (B, March 15, 2019 года, temperature not lower than 9-10 °C at 60% air humidity) **in sweet cherry *Cerasus avium* (L.) Moench variety Mak, the Prikubanskaya zone of gardening in Krasnodar Territory** (50-100× zoom, Olympus BX41, Olympus Corporation, Japan). For variety origin, see section Materials and methods.

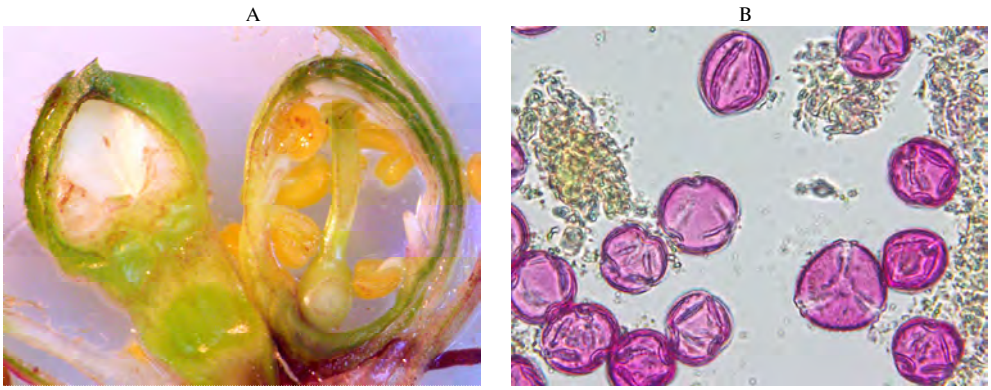

Fig. 3. Phenophases "first flowers bloom" in ordinary cherry *Cerasus vulgaris* Mill. variety Nefris at stage IX of organogenesis (A, April 5, 2018, favorable conditions) **and "full flowering" in ordinary cherry ordinary cherry *Cerasus vulgaris* Mill. variety Igrushka at stage IX of organogenesis** (B, 1-2-cell pollen, April 16, 2010, favorable temperature withing 16-25 °C at 55-58% air humidity)**, the Prikubanskaya zone of gardening in Krasnodar Territory** (50-100× zoom, Olympus BX41, Olympus Corporation, Japan). For variety origin, see section Materials and methods.

We regard the phenological phases "bud scale elongation" (stage VII of organogenesis) (see Fig. 2) and "first flowers bloom—full flowering" (stage IX) (Fig. 3) as the third and fourth critical stages that determine yield efficiency of sweet cherry plants. In the conditions of southern horticulture, stage VII of organogenesis falls on March-April, with recurrent spring frosts (see Table 1). At the beginning of the growing season, which coincided with the phenophase "bud scale elongation", the generative organs of sweet cherries are especially sensitive to temperature stresses. Some researchers believe that at air temperature from –3.0 to –6.0 °C at this stage, sweet and ordinary cherry varieties can almost completely lose their yield [11, 17], which is in line with our findings. Thus, frosts up to –6.2 °C on April 10, 2009, led to freezing and significant losses of yield components. In cherry varieties of local selection, the death of fruit buds was 80-85%, and in all introduced varieties it reached 90-95% with a reduced

yield of 3.0-7.0 kg per tree in both genetic groups. In interspecific hybrids, there was a 90-95% death of fruit buds with a low yield, 2.0-4.5 kg per tree. European varieties of ordinary cherry showed higher resistance to spring frosts (the fruit bud death rate of 75-78% and a yield of 7.0-8.0 kg per tree).

On March 25, 2013, the temperature several times dropped to –3.0 °C and even –4.0 °C, that caused freezing of 40-50% of fruit buds. However, these climatic factors caused only 40% death of generative organs in the domestic varieties Alaya and Kavkazskaya, with a yield of 18.0-20.0 kg per tree, giving, therefore, the reason to deem these varieties resistant to spring frosts. In interspecific hybrids and in in ordinary cherry varieties, 30-40% and 15-25% of yield components, respectively, were frozen, with yields of 10.0 and 22.0 kg per tree, respectively. Light microscopy of fruit buds showed that both in sweet cherry and ordinary cherry the anthers during the period of return frosts retained high-quality pollen without visible anomalies, but the pistils were damaged. That is, a pistil is less frost-resistant than other floral organs, which is consistent with the data obtained earlier [9, 24]. We revealed a higher resistance to low negative temperatures during the dormant period and at the beginning of the growing season in the ordinary cherry varieties as compared to the sweet cherry varieties. We also established varietal specificity and a close correlation between the critical temperature and the reduction of generative organs ($r = +0.97$, $p < 0.001$)

The phenophases “first flowers bloom—full flowering” (stage IX) (see Table 1, Fig. 3) turned out to be the most vulnerable to temperature stresses. For this stage, according to some reports, the optimal temperature ranges within 16.0-25.0 °C [17, 28], which is consistent with our observations. We found that air temperatures above 26.0 °C during flowering period (April decades I-II) accelerate flower senescence both in sweet cherry and ordinary cherry due to the drying out of the pistil and ovule, which led to impaired fertilization and a significant loss of yields.

Ovary emergence phenophase (stage X of organogenesis) was less susceptible to stress than others. At this stage (May decades I-II), in the conditions of southern horticulture, anomalously high temperatures are adverse factors causing a 40% loss of the ovary in sweet cherry varieties, 42% in interspecific hybrids and 46% in ordinary cherries (see Table 1).

Given the fact that the variety is the main factor providing stable high yields in fruit crops [12, 13], we evaluated the yield to distinguish among the studied varieties those that are the most productive under stress. Under favorable conditions, drupe fruit varieties form a significant number of vegetative and fruit buds the ratio of which differs significantly [8]. Our findings also confirm this statement ($Cv = 20\%$). In sweet cherry, depending on the variety, the number of fruit buds per 1 fruiting branch averaged from 68 (Kavkazskaya) up to 128 (Alaya), of vegetative buds from 26 (Dar izobiliya) up to 35 (Melitopolskaya chernaya). For the number of formed fruit buds, all sweet cherry varieties significantly exceeded the control ($LSD_{05} = 3.1$). In ordinary cherry varieties, the number of fruit buds per 1 fruiting branch varied from 68 (Fanal) up to 12 (Kelleris) and exceeded the control ($LSD_{05} = 3.5$). The number of vegetative buds in ordinary cherry varieties varied moderately ($Cv = 20\%$), from 25 (Fanal) up to 40 (Kelleris). In the ordinary cherry-sweet cherry hybrids, the fruit bud number was 64 for Dombazia and 118 for Kirina, and the vegetative bud number was 24 for Igrushka and 37 for Shalunya (Table 2). That is, the number of developing fruit buds was much higher than that of vegetative ones, which

indicates a high yield potential of sweet and ordinary cherry varieties.

2. Yield components in studied sweet cherry *Cerasus avium* (L.) Moench and ordinary cherry *Cerasus vulgaris* Mill. varieties of various ecogeographic origin (Tsentralnoe Experimental Production Center, the North Caucasian Federal Research Center of Horticulture, Viticulture, Winemaking, Krasnodar, 2006-2018)

Variety	Biological potential, number/m (M±SD)					Coefficient of productivity (Cp), %
	buds		flowers	ovaries	fruit	
	fruit	growth				
Sweet cherry *Cerasus avium* (L.) Moench						
Varieties of North-Caucasian Federal Scientific Center of Horticulture, Viticulture, Winemaking						
Kavkazskaya (St)	68±12	30±6	310±22	226±7	192±4	62
Sashen'ka	75±15	31±10	237±17	134±5	107±5	45
Volshebnitsa	98±12	29±5	384±28	222±10	160±3	42
Dar izobiliya	85±10	26±8	255±31	159±4	102±7	40
Alaya	128±14	33±5	430±20	266±5	196±5	46
Mak	76±11	32±7	220±19	129±6	75±6	35
Introduced sweet cherry varieties						
Valerii Chkalov	76±10	28±11	225±15	182±8	47±7	21
Melitopolskaya chernaya	94±18	35±6	405±23	173±10	98±3	23
Polyanka	80±15	29±15	325±20	186±9	125±5	35
LSD_{05}	3.1	2.8	7.4	5.5	5.6	3.1
SD, %	17.0	2.7	81.2	44.9	51.1	
$M_{average}$ for sweet cherry	89.0	30.0	310.1	186.3	122.4	
Cv, %	20.0	8.9	26.2	24.1	41.6	
Sx, %	5.6	0.9	27.1	14.9	17.0	
Ordinary cherry *Cerasus vulgaris* Mill.						
Introduced ordinary cherry varieties						
Kelleris	124±19	40±9	295±19	192±7	134±4	45
Nefris	103±16	29±5	322±25	164±11	120±2	37
Fanal	68±21	25±12	200±18	94±4	70±8	35
Erdi Botermo	88±18	30±10	228±16	110±5	87±5	38
Ordinary cherry-sweet cherry hybrids						
Dombaziya	64±15	31±14	160±22	82±8	52±9	33
Dyuk Ivanovna	95±10	37±8	250±20	161±11	110±4	44
Dyuk Khodosa	88±19	30±15	198±18	125±5	90±8	46
Vstrecha	102±12	33±11	306±11	173±8	119±3	39
Kirina (St)	118±7	36±9	280±10	168±10	123±5	44
Igrushka	97±17	24±17	286±17	130±7	105±2	37
Shalun'ya	108±11	37±13	247±15	153±4	95±7	38
LSD_{05}	3.5	2.7	3.4	2.8	2.2	
SD, %	18.5	5.1	51.3	35.3	24.6	
$M_{average}$ for ordinary cherry	95.9	32.0	252.0	141.1	100.5	
Cv, %	19.2	16.0	20.4	25.0	24.5	
Sx, %	5.6	1.5	15.5	10.6	7.5	

Note. St — standard variety; Cv — coefficient of variation, SD — standard deviation, M — arithmetic mean, Sx — relative standard error of the sample mean (sampling error). For the description of varieties and hybrids, see section Materials and methods. On average, the sample size per year was 2700-4500 fruit buds and 4500-6800 flowers for sweet cherry, and 1500-3200 fruit buds, 2500-4700 flowers for ordinary cherry.

Initiation of a large number of fruit buds is an important adaptive mechanism developed during evolution and used as an "insurance fund" in case of reduction of flowers, ovaries and fruits after exposure to stress factors [10, 37]. Coefficient of productivity is a significant parameter to characterize the yield potential of a variety [5, 13]. Out data show that the potential for flowers per 1 m of fruiting branches in sweet cherry varieties varied significantly (Cv = 26.2%), from 220 (Mak) to 430 (Alaya), in ordinary cherry varieties moderately (Cv = 20.4%), from 160 (Dombaziya) to 322 (Nefris) (see Table 2). There is an opinion that natural losses, or "cleansing", at the stages of ovary and fruit formation are quite high [5, 11]. According to our data, given the reduction of non-pollinated or lagging flowers at the stage IX of organogenesis, sweet cherry trees formed 129 ovaries in the Mak variety and 266 in the Alaya variety, which exceeded the control value in

the Kavkazskaya variety (226 ovaries on average) (LSD_{05} = 5.5). In ordinary cherry varieties, the number of ovaries (from 94 for Fanal variety up to 192 for Kelleris variety) was less than that in sweet cherry and significantly varying (Cv = 25.0%) (see Table 2).

On average, over the study years, the reduction at fruit set and fruit formation stages in sweet cherry varieties was 40%, in ordinary cherry 46%, in interspecific hybrids 42%.

Yield potential realization in sweet and ordinary cherries was assessed at XI-XII stages of organogenesis (i.e. at ripening). Sweet cherry varieties under favorable conditions generated 75-196 fruits, ordinary cherry 70-134 fruits, and interspecies hybrids 52-123 fruits per 1 m of fruiting branches.

At the final stages of development, the reduction of fruits significantly decreased, which made it possible to calculate the optimal coefficient of productivity. It averaged 43% in sweet cherry varieties, 40% in the hybrids, and 38% in ordinary cherry varieties. It should be noted that the varieties of local selection differed from a number of introduced ones by a higher rate of fruiting. So, it was 62% in the domestic variety Kavkazskaya, 46% in Alaya, 45% in Sashen'ka, and 42% in Volshebnitsa. The higher coefficient in sweet cherry varieties corresponds to a higher yield, averaging 45-50 kg per tree, or 29.9-33.3 t/ha, at the planting pattern of 5×3 m (see Tables 2, 3).

The varieties outstanding for a high percentage of fruits to flowers under the conditions of Prikubanskaya zone of gardening (the Krasnodar Territory) are Kelleris (45%) and Erdi Botermo (38%) of ordinary cherry, and Duk Khodoss (46%), Kirina (44%) and Duk Ivanovna (44%) of the interspecies hybrids. These varieties and hybrids also provide a fairly high yield (30-40 kg per tree, or 19.9-26.6 t/ha, at the planting pattern of 5×3 m) (see Tables 2, 3).

3. Yields of sweet cherry *Cerasus avium* (L.) Moench varieties and ordinary cherry *Cerasus vulgaris* Mill. varieties of various ecogeographic origin under climatic stresses of Prikubanskaya zone of gardening (the Krasnodar Territory) (Tsentralnoe Experimental Production Center, the North Caucasian Federal Research Center of Horticulture, Viticulture, Winemaking, Krasnodar, 2006-2018)

Variety	Productive performance							
	2006 (January, –33.0 °C)		2009 (April, –6.2 °C)		2013 (March, from –3.0 to –4.0 °C)		2018 (optimum)	
	R, %	kg/tree (*M*±SD)	R, %	kg/tree (*M*±SD)	R, %	kg/tree (*M*±SD)	R, %	kg/tree (*M*±SD)
Sweet cherry *Cerasus avium* (L.) Moench								
Varieties of North-Caucasian Federal Scientific Center of Horticulture, Viticulture, Winemaking								
Kavkazskaya (St)	90	5.5±1.0	80	7.0±2.0	40	18.0±2.5	0	35.0±5.0
Alaya	90	5.0±2.0	80	7.0±3.0	40	20.0±4.0	0	47.0±5.0
Dar izobiliya	92	4.5±1.5	82	5.0±2.5	45	17.0±3.5	0	40.0±3.5
Volshebnitsa	90	5.0±2.5	85	6.0±1.5	45	17.0±3.0	0	35.0±4.0
Mak	94	3.0±2.5	80	7.0±1.0	50	15.0±4.5	0	45.0±3.5
Sashen'ka	95	1.0±0.5	85	5.5±2.5	50	15.0±3.0	0	30.0±4.5
Introduced sweet cherry varieties								
Valerii Chkalov	100	1.0±0.5	95	0.0±0.0	52	12.5±4.0	0	15.0±5.0
Melitopolskaya chernaya	96	1.0±0.5	90	3.0±1.0	55	10.0±2.5	0	25.0±3.0
Polyanka	100	0.0±0.0	95	1.0±0.5	55	10.0±4.0	0	30.0±2.5
LSD_{05}	1.35	0.99	1.69	1.12	1.70	1.20	0	2.10
SD, %	4.0	2.2	6.2	2.7	5.8	3.5	0	10.0
$M_{average}$ for sweet cherry	94.1	2.8	85.7	4.6	48.0	14.9	0	33.6
Cv, %	4.3	75.8	7.2	58.0	12.0	23.0	0	29.0
Sx, %	1.3	30.7	2.1	0.9	1.9	1.2	0	3.3
Ordinary cherry *Cerasus vulgaris* Mill.								
Introduced ordinary cherry varieties								
Kelleris	85	8.0±2.0	75	8.0±1.5	20	22.0±4.5	0	30±4.5
Nefris	88	7.0±1.5	76	7.5±2.5	15	20.0±3.0	0	25±3.0
Fanal	85	7.5±2.5	75	7.5±1.5	20	18.0±3.5	0	20±3.5
Erdi Botermo	90	5.0±3.0	78	7.0±3.0	25	15.0±2.5	0	25±2.0

Continued Table 3

Ordinary cherry-sweet cherry hybrids								
Dombaziya	90	6.0±2.5	90	4.0±3.5	40	10.0±4.0	0	28±1.5
Dyuk Ivanovna	95	4.0±3.0	93	3.0±2.5	40	10.5±3.0	0	30±1.0
Dyuk Khodosa	90	5.0±2.5	90	4.5±1.5	30	15.0±2.0	0	35±2.0
Vstrecha	90	6.0±2.0	92	3.0±2.0	35	13.0±5.0	0	28±1.5
Kirina (St)	91	4.0±1.5	95	2.0±1.5	30	15.0±3.5	0	35±1.0
Igrushka	95	4.0±2.5	90	4.0±1.0	35	15.0±2.0	0	25±2.5
Shalun'ya	95	4.0±3.0	95	2.0±0.5	35	15.5±2.5	0	28±2.0
LSD_{05}	1.1	0.8	2.0	0.9	2.1	1.1	0	1.6
SD, %	3.6	1.5	8.4	2.3	8.5	3.6	0	4.4
$M_{average}$ for ordinary cherry	90.4	5.5	86.3	4.8	29.5	15.4	0	28.1
Cv, %	4.0	27.0	9.7	48.0	28.8	23.3	0	15.6
Sx, %	1.1	0.46	2.5	0.7	2.6	1.1	0	1.3

N o t e. St — standard variety; *Cv* — coefficient of variation, SD — standard deviation, *M* — arithmetic mean, Sx — relative standard error of the sample mean (sampling error). R — reduction (death) of fruit buds. For the description of varieties and hybrids, see section Materials and methods. On average, the sample size in stressful years for sweet cherry and ordinary cherry was 2500-2700 fruit buds.

It should be noted that high yielding of sweet cherry and ordinary cherry trees can be realized given that the impact of stress factors is minimal at all stages of generation of yield elements. Table 3 presents actual data on yielding of the varieties in extreme years. So, in 2006, during the dormant period, the temperature dropped to –33.0 °C, causing a 90-100% reduction of yield components. In this conditions, the sweet cherry varieties Alaya and Volshebnitsa stood out, with a yield of about 5.0 kg per tree. In ordinary cherry and sweet cherry-ordinary cherry hybrids, the rate of yield component reduction was lower than in sweet cherry, up to 85-95%. Thus, cherry varieties Vstrecha, Dombaziya, Duk Khodosa, Kelleris and Fanal were significantly superior to the control variety Kirin for actual yields (HCP_{05} = 0.8). In 2009, as a result of periodic frosts (–6,2 °C) during the flowering period, yield component reduction was high. In particular, in sweet cherry, it was 85-95%, with low variation between the varieties (*Cv* = 7.2%). In ordinary cherry and interspecies hybrids, 75-95% of fruit buds died, with insignificant variation between the varieties (*Cv* = 9.7%). n 2013, when the first flowers were blooming, the temperature dropped to –3.0 °C and –4.0 °C, which led to the death of 40-55% of the generative organs in sweet cherry and 15-40% in ordinary cherry and interspecific hybrids (see Table 3

Yield values of sweet cherry and ordinary cherry varieties indicate that these drupe fruit crops have a high yield potential, which is utmost realized under optimal weather conditions. So, in a favorable 2018, sweet cherry varieties had an average yield of 33.6 kg per tree. For yield values, sweet cherry varieties Dar izobiliya (40.0 kg per tree), Mak (45.0 kg per tree), and Alaya (47.0 kg per tree) outstood, significantly exceeding the control variety. Among ordinary cherry varieties which also had a high average yield in 2018 (28.1 kg per tree), Dyuk Ivanovna (30 kg per tree) and Kelleris (30 kg per tree) outstood.

So, under the conditions of Krasnodar Territory, in varieties and hybrids of sweet cherry and ordinary cherry, we have identified five critical stages of organogenesis, or phenological phases, when plants are most sensitive to temperature stresses, and the probability is very high of the significant rate of yield component reduction. These stages are initiation of flower organs in fruit buds, emergence of archesporium in the anthers (dormancy), generation of pollen mother cells (bud scale elongation), formation of mono- and bicellular pollen grains ("first flowers bloom—full flowering) and macrosporogenesis (ovary emergence and growth). The clarified average terms for these critical stages in the conditions of the Kuban zone of gardening are December-January, decades II-III of March, decades I-II of April, decades I-II of May,

and decades I-II of July, which allows us to detail recommendations ensuring sustainable yielding of drupe fruit crops. The realization of the yield potential of sweet cherry and ordinary cherry trees is closely dependent on the type of temperature stress and the number of yield components after their reduction at all vulnerable stages of organogenesis ($r = +0.97$, $p < 0.001$). The samples selected for productive performance, are domestic varieties of sweet cherry Kavkazskaya resistant to temperature stresses and the most productive in the conditions of southern horticulture (the fruits-to-flowers ratio of 62%, the yield of 31.3 t/ha), Sashen'ksa (45% and 26.6 t/ha, respectively), Volshebnitsa (42% and 23.3 t/ha), Alaya (46% and 30.0 t/ha), an ordinary cherry variety Kelleris (45% and 20.0 t/ha), and interspecies hybrids Dyuk Khodos (46% and 23.3 t/ha), Kirina (44% and 23.3 t/ha), and Dyuk Ivanovna (44% and 20.0 t/ha). They are recommended for commercial growing in the Krasnodar Territory and involved in breeding programs as genetic donors of high productivity and adaptability to temperature stresses.

REFERENCES

1. Bondarenko L.V., Maslova O.V., Belkina A. V., Sukhareva K.V. *Vestnik Rossiiskogo ekonomicheskogo universiteta im. G.V. Plekhanova*, 2018, 2(98): 84-93 (doi: 10.21686/2413-2829-2018-2-84-93) (in Russ.).
2. Campoy J., Ruiz D., Egea J. Dormancy in temperate fruit trees in a global warming context: a review. *Scientia Horticulturae*, 2011, 130(2): 357-372 (doi: 10.1016/j.scienta.2011.07.011).
3. Luedeling E. Climate change impacts on winter chill for temperate fruit and nut production: a review. *Scientia Horticulturae*, 2012, 144: 218-229 (doi: 10.1016/j.scienta.2012.07.011).
4. Else M., Atkinson C. Climate change impacts on UK top and soft fruit production *Outlook on Agriculture*, 2010, 39: 257-262 (doi: 10.5367/oa.2010.0014).
5. Alekhina E.M., Dolya Yu.A. *Plodovodstvo i vinogradarstvo Yuga Rossii*, 2013, 24(06): 10-17 (in Russ.).
6. Dolya Yu.A. *Formirovanie produktivnosti sortov chereshni v usloviyakh Severnogo Kavkaza. Kandidatskaya dissertatsiya* [The formation of sweet cherry productivity in the North Caucasus. PhD Thesis]. Krasnodar, 2011 (in Russ.).
7. Chumakov S.S. *Vozmozhnosti realizatsii biologicheskogo potentsiala plodovykh rastenii v raznovozrastnykh nasazhdeniyakh yuga Rossii* [Possibilities for realizing the biological potential of fruiting plants in different-aged stands of Southern Russia]. Krasnodar, 2011 (in Russ.).
8. Eremina O.V. V sbornike: *Metody i reglamenty optimizatsii strukturnykh elementov agrotsenozov i upravleniya realizatsiei produktsionnogo potentsiala rastenii* [In: Methods and regulations to optimize structural elements of agrocenoses and to manage realization of plants production potential]. Krasnodar, 2009: 185-189 (in Russ.).
9. Ozherel'eva Z.E., Gulyaeva A.A. *Sovremennoe sadovodstvo*, 2015, 3(15): 45-51 (in Russ.).
10. Buntsevich L.L. *Morfofiziologicheskie osobennosti formirovaniya urozhainosti yabloni domashnei* (*Malus domestica Borkh.*)*: monografiya* [Morphophysiological features of domestic apple try (*Malus domestica* Borkh.) yield formation: a monograph]. Krasnodar, 2012 (in Russ.).
11. Zhuchenko A.A. *Adaptivnoe rastenievodstvo: ekologo-geneticheskie osnovy* [Adaptive crop production: ecological and genetic aspects]. Kishinev, 1990 (in Russ.).
12. Doroshenko T.N., Klad' A.A., Gegechkori B.S. *Opredelenie produktivnosti plodovykh rastenii i priemy ee regulirovaniya* [Determination of fruit plant productivity and methods of its regulation]. Krasnodar, 1999 (in Russ.).
13. Dragavtsev V.A., Dragavtseva I.A., Mozhar N.V., Morenets A.S. *Trudy Kubanskogo gosudarstvennogo agrarnogo universiteta*, 2018, 72: 142-148 (doi: 10.21515/1999-1703-72-142-148) (in Russ.).
14. Spitsyn I.P. *Tsitoembriologicheskie, geneticheskie i ekologicheskie aspekty produktivnosti vishni. Avtoreferat doktorskoi dissertatsii* [Cytoembryological, genetic and environmental aspects of cherry productivity. DSc Thesis]. Michurinsk, 2003 (in Russ.).
15. Taranov A.A. *Plodovodstvo*, 2005, 17(1): 63-67 (in Russ.).
16. Buntsevich L.L., Kostyuk M.A., Paletskaya E.N. *Plodovodstvo i yagodovodstvo Rossii*, 2013, 31(1): 55-61 (in Russ.).
17. Egorov E.A., Il'ina I.A., Prichko T.G., Dragavtseva I.A., Popova V.P., Alekhina E.M., Alferov V.A., Artyukh S.N., Khvostova I.V., Yakuba G.V., Cherkezova S.R., Podgornaya M.E., Smolyakova V.M., Efimova I.L., Ul'yanovskaya E.V., Shafrostova N.K., Zaremuk R.Sh., Sergeev Yu.I., Lugovskoi A.P., Teren'ko G.N., Kuznetsova A.P., Bogatyreva S.V., Mozhar N.V., Govorushchenko S.V., Tyshchenko E.L., Shadrina Zh.A. *Adaptivnyi potentsial sadovykh kul'tur yuga Rossii v usloviyakh stressovykh temperatur zimnego perioda (metodicheskie rekomendatsii)*

[Adaptive potential of garden crops in Southern Russia under stressful temperatures of the winter period (methodical recommendations)]. Krasnodar, 2006 (in Russ.).
18. Salazar-Gutiérrez M.R., Chaves B., Anothai J., Whiting M., Hoogenboom G. Variation in cold hardiness of sweet cherry flower buds through different phenological stages. *Scientia Horticulturae,* 2014, 172: 161-167 (doi: 10.1016/j.scienta.2014.04.002).
19. Hedhly A. Hormaza J.I., Herrero M. Warm temperatures at bloom reduce fruit set in sweet cherry. *Journal of Applied Botany and Food Quality*, 2007, 81(2): 158-164.
20. Zhang L., Ampatzidis Y., Whiting M.D. Sweet cherry floral organ size varies with genotype and temperature. *Scientia Horticulturae*, 2015, 182: 156-164 (doi: 10.1016/j.scienta.2014.09.051)
21. Woznicki T.L., Heide O.M., Sønsteby A., Måge F., Remberg S.F. Climate warming enhances flower formation, earliness of blooming and fruit size in plum (*Prunus domestica* L.) in the cool Nordic environment. *Scientia Horticulturae*, 2019, 257: 108750 (doi: 10.1016/j.scienta.2019.108750).
22. Rodrigo J., Herrero M. Effects of pre-blossom temperatures on flower development and fruit set in apricot. *Scientia Horticulturae*, 2002, 92(2): 125-135 (doi: 10.1016/S0304-4238(01)00289-8).
23. Götz K.-P., Chmielewski F.-M., Homann T., Huschek G., Matzneller P., Rawel H.M. Seasonal changes of physiological parameters in sweet cherry (*Prunus avium* L.) buds. *Scientia Horticulturae*, 2014, 172: 183-190 (doi: 10.1016/j.scienta.2014.04.012).
24. Vitkovskii V.L. *Morfogenez plodovykh rastenii* [Fruit plant morphogenesis]. Leningrad, 1984 (in Russ.).
25. Zhang L., Ferguson L., Whiting M.D. Temperature effects on pistil viability and fruit set in sweet cherry. *Scientia Horticulturae*, 2018, 241: 8-17 (doi: 10.1016/j.scienta.2018.06.039).
26. Kolomiets I.A. *Preodolenie periodichnosti plodonosheniya yabloni* [Overcoming the periodicity of fruiting apple trees]. Kiev, 1961 (in Russ.).
27. Sergeeva N.N., Buntsevich L.L. Morphogenetic features of development and the feed regime of an apple-tree in the conditions of south of Russia. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2010, 5: 92-97 (in Russ.).
28. Hedhly A., Hormaza J.I., Herrero M. Effect of temperature on Pollen Tube Kinetics and Dynamics in sweet cherry, *Prunus avium* (*Rosaceae*). *American Journal of Botany*, 2004, 91(4): 558-564 (doi: 10.3732/ajb.91.4.558).
29. Dragavtseva I.A. *Razvitie tsvetkovykh pochek alychi v svyazi s zimostoikost'yu ee sortov v usloviyakh Kryma. Avtoreferat kandidatskoi dissertatsii* [Development of cherry plum flower buds in connection with the winter hardiness of varieties in Crimea. PhD Thesis]. Odessa, 1966 (in Russ.).
30. Sanzol J., Herrero M. The "effective pollination period" in fruit trees. *Scientia Horticulturae*, 2001, 90(1-2): 1-17 (doi: 10.1016/S0304-4238(00)00252-1).
31. Nen'ko N.I., Kiseleva G.K. V knige*: Sovremennye metodologiya, instrumentarii otsenki i otbora selektsionnogo materiala sadovykh kul'tur i vinograda* [In: Modern methodology, tools for assessing and selecting breeding material for garden crops and grapes]. Krasnodar, 2017: 66-78 (in Russ.).
32. Koutinas N., Pepelyankov G., Lichev V. Flower induction and flower bud development in apple and sweet cherry. *Biotechnology and Biotechnological Equipment*, 2010, 24(1): 1549-1558 (doi: 10.2478/V10133-010-0003-9).
33. Rabadanova K.K., Tyutereva E.V., Matskevich V.S., Deminchik V.V., Voitsekhovskaya O.V. Cellular and molecular mechanisms controlling autophagy: a perspective to improve plant stress resistance and crop productivity (review). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2018, 53(5): 881-896 (doi: 10.15389/agrobiology.2018.5.881eng).
34. Fadón E., Herrero M., Rodrigo J. Flower development in sweet cherry framed in the BBCH scale. *Scientia Horticulturae*, 2015, 192: 141-147 (doi: 10.1016/j.scienta.2015.05.027).
35. Dzhigadlo E.N., Kolesnikova A.F., Eremin G.V., Morozova T.V., Kan'shina M.V. V knige: *Programma i metodika sortoizucheniya plodovykh, yagodnykh i orekhoplodnykh kul'tur* /Pod redaktsiei E.N Sedova, T.P. Ogol'tsovoi [In: The program and methodology to study fruit, berry and nut-bearing crop varieties. E.N Sedov, T.P. Ogol'tsova (eds.)]. Orel, 1999: 300-350 (in Russ.).
36. Dzhigadlo E.N., Shchekotova L.A., Turovtsev N.I., Morozova T.V., Eremin G.V. V knige: *Programma i metodika selektsii plodovykh, yagodnykh i orekhoplodnykh kul'tur* /Pod redaktsiei E.N. Sedova [The program and method for fruit, berry and nut-bearing crop breeding. E.N. Sedov (ed.)]. Orel, 1995: 234-313 (in Russ.).
37. Isaeva I.S. *Morfofiziologiya plodovykh rastenii. Kurs lektsii* /Pod redaktsiei F.M. Kuperman [Morphophysiology of fruit plants. Lecture course. F.M. Kuperman (ed.)]. Moscow, 1974 (in Russ.).
38. Kiseleva G.K. V knige: *Sovremennye metodologiya, instrumentarii otsenki i otbora selektsionnogo materiala sadovykh kul'tur i vinograda* [Modern methodology, tools for assessing and selecting breeding material for garden crops and grapes]. Krasnodar, 2017: 51-60 (in Russ.).

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ACCUMULATION OF BIOACTIVE SUBSTANCES AND CHEMICAL ELEMENTS IN *Echinacea purpurea* (L.) Moench MEDICINAL HERB AS INFLUENCED BY SOIL APPLICATION OF COPPER, AN ESSENTIAL MICROELEMENT

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Abstract

Copper, an essential element in human metabolism, is a trace element for plants and animals. It plays a significant role in physiological processes (i.e. in photosynthesis, respiration, carbohydrate and protein metabolism), increases productivity, improves plant quality characteristics and increases resistance to adverse factors. A particular concern is the need to apply copper fertilizers in the biogeochemical provinces with Cu deficit in the soil. *Echinacea purpurea* (L.) Moench is one of the best-selling plant-based medicines in many developed countries around the world. It has been widely used in medicine and veterinary medicine for immunocorrection. However, publications on the effect of trace elements on the yield and content of biologically active substances in the medicinal raw materials of the *E. purpurea* are very few. Our study presents the results confirming the role of copper fertilizers in increasing the content of biologically active substances in the medicinal raw materials of *E. purpurea*, as well as in its enrichment with certain trace elements. The work aimed to assess the influence of essential microelement (Cu) on the accumulation of certain biologically active substances (tanning substances, carotene, vitamin C) and chemical elements (zinc and copper) in the medicinal raw material of *E. purpurea* variety Znakhar. The plot tests were run in the conditions of the southern forest-steppe of Western Siberia (an experimental field of Omsk Stolypin State Agrarian University, Omsk, May-September 2016-2018). The experiment design was as follows: absolute control (without fertilizers), N_{125} (N-based fertilizer), N_{125} + 0.25MAC Cu (the maximum allowed concentration) (2.3 kg a.m./ha), N_{125} + 0.5MAC Cu (4.7 kg a.m./ha), N_{125} + 0.75MAC Cu (7.0 kg a.m./ha), N_{125} + 1MAC Cu (9.4 kg a.m./ha). The soil of the test site was meadow chernozem, low-power, low humus, medium loamy with a 5.2 % humus content, 10.0 mg/kg nitrate nitrogen, 394 mg/kg mobile phosphorus, 749 mg/kg exchange potassium; pH 6.5-6.8. The mobile copper level in the soil was 0.3 mg/kg. The experiment was arranged in four replicates, plots were systematically located in several tiers, with a plot size of 10 m^2. Copper acetate $(CH_3COO)_2Cu$ (32 %) was used as Cu fertilizer, ammonium nitrate (34.4%) was an N-based fertilizer. The crop was planted at the end of May 2016, 24 plants per plot, with 70×60 cm spacing. Fertilizers were manually incorporated into the soil to a 10-15 cm depth before planting (at tilling), and uniformly distributed throughout the entire plot area. Plants were collected in September, during *E. purpurea* mass flowering phase. Tannings, carotene, and ascorbic acid were quantified in the medicinal raw material of *E. purpurea*. The concentration of trace elements (copper, zinc) in powdered herb samples was determined by atomic absorption spectroscopy. We have found out that a single application of copper fertilizers contributed to accumulation of tanning substances, ascorbic acid and carotene in the herb raw material. On average, over three years of our research, the level of bioactive substances in the raw material (grass) increased reliably ($p < 0.05$), to 16.6 mg% for tanning, to 5.8 mg% for ascorbic acid, and to 51.2 mg/kg for carotene. A copper acetate dose of 9.4 kg a.m./ha resulted in maximum concentration of the bioactive substances. Each 1 kg of Cu fertilizer increased the content of tannins by 0.51 mg%, ascorbic acid by 0.29 mg%, and carotene by 2.56 mg/kg ($p < 0.05$). Thus, the use of $(CH_3COO)_2Cu$ contributes to an improved medicinal value of *E. purpurea*. The copper fertilizer dosages correlated tightly with the level of mobile zinc

and copper in medicinal raw materials ($r = 0.98$, $p < 0.05$). Each 1 kg of copper added with N_{125} increased the content of mobile copper and zinc in *E. purpurea* raw material by 0.23 and 1.15 mg/kg, respectively. These values are b coefficients that evaluate the effects of copper fertilizers and, together with the regression equations we obtained, allow practitioners to draw up protocols for applying micronutrient fertilizers in meadow chernozem soils at an early stage of *E. purpurea* plant development in specific growing conditions.

Keywords: *Echinacea purpurea* (L.) Moench, meadow chernozem soil, essential microelements, copper, zinc, bioactive substances, southern forest-steppe, Western Siberia.

The World Health Organization recognizes herbal medicines as a significant part of the pharmaceutical industry market. More than 80% of the world's population is increasingly using medicinal plants as the main source of medical care. This is not only the growing population of China and India, but also many developed countries [1]. About 80% of all modern pharmaceuticals are obtained directly or indirectly from plant sources [2, 3]. According to forecasts, in the next 10 years, the use of herbal remedies in the world market will grow and reach 60% [4]. Herbal medicinal products have a number of advantages: no side effects, low toxicity, availability, complex effects on the body (anti-inflammatory, antimicrobial, antispasmodic, analgesic, antitoxic) [5].

In the main agricultural regions of the Russian Federation, the soil area with a low levels of mobile trace elements (copper, zinc, etc.) reaches 50-90% of the surveyed territory (6, 7). The critical amount of trace elements in soils leads to significant losses in crop production. Bioenrichment of agricultural plants [8, 9], including medicinal crops, with various bioelements, for example, copper, may be a convenient solution.

Copper is an essential chemical element along with iron, zinc, iodine, and selenium [10]. Cu acts as a cofactor for many enzymes [11]. Copper and its compounds play a significant role in physiological processes in plant cells, being involved into respiration, photosynthesis, carbohydrate and phosphorus metabolism, protein synthesis, nitrogen fixation and reduction [12, 13]. It has a positive effect on the water regime of plants, their drought and frost resistance, increases plant resistance to various diseases. Copper fertilizers increase the content of sugars, fat, ascorbic acid, vitamin A, and group B vitamins in crop production [14, 15].

Application of essential trace elements activates enzymatic processes in medicinal plants, which leads to biosynthesis and the accumulation of biologically active substances, improving the valuable qualities of medicinal raw materials [16]. Microelements in plants are in an accessible, organically bound form, which enable their better assimilation. In the plant organism, essential trace elements bind to biologically active substances, which enhances the pharmacological effects [17].

Echinacea purpurea (L.) Moench is one of the best-selling herbal medicines in many developed countries [18]. It is widely used in medicine and veterinary medicine as an immunocorrector. All plant organs contain biologically active substances — vitamins A and C, tannins, flavonoids, essential oils, anthocyanins and alcamides [19-21]. Fundamental and clinical studies have proven the antiviral, anti-inflammatory, antioxidant, antibacterial and antimycotic effects of *Echinacea purpurea* [22]. However, scant data are available on the effect of microelements on the yield and the content of biologically active substances in the medicinal raw material of *E. purpurea* [23, 24].

This paper provides the first confirmation of the role of copper fertilizers in increasing the content of biologically active substances in the medicinal raw material of *Echinacea purpurea* and its enrichment with microelements.

Our objective was to assess the effect of an essential trace element (Cu) on the accumulation of some biologically active substances (tannins, carotene,

vitamin C) and chemical elements (zinc and copper) in the medicinal raw material of *Echinacea purpurea*.

Materials and methods. *Echinacea purpurea* (L.) Moench variety Znakhar was grown in the conditions of the southern forest-steppe of Western Siberia was carried out in May-September 2016-2018 (Stolypin Omsk GAU, Omsk). The soil of the experimental site is meadow chernozem, shallow low-humus, medium loamy. Agrochemical analysis was performed by conventional methods [25], the 0-30 cm layer contained 5.2% humus (according to Tyurin), 10.0 mg/kg nitrate nitrogen (according to Kochergin), 394 mg/kg mobile phosphorus and 749 mg/kg exchangeable potassium (according to Chirikov), absorption capacity of 25.2-28.2 mg-eq/100 g, 0.3 mg/kg mobile copper; pH values of aqueous extracts ranged within 6.5-6.8.

The plots were put in test for the following treatments: control without fertilizers; N_{125}; N_{125} + 0.25MPC (maximum permissible concentration) Cu (2.3 kg Cu/ha); N_{125} + 0.5MPC Cu (4,7 kg Cu/ha); N_{125} + 0.75MPC Cu (7.0 kg Cu/ha); N_{125} + MPC Cu (9.4 kg Cu/ha). Ammonium nitrate (34.4%) was referred to as a background, and copper acetate $(CH_3COO)_2Cu$ (32%) was considered as fertilizer. The N_{125} dosage was chosen as a baseline due to the low $N\text{-}NO_3$ concentration in the soil, as only a balanced macronutrient composition allows the micronutrients to take full effect. Doses of copper fertilizers were calculated based on MPC Cu (3 mg/kg) and copper content before planting. The experiment was repeated four times, the sequence of variants was systematic, in several tiers, using 10 m^2 plots.

The approved zonal agrotechnology was applied. *E. purpurea* planting was carried out at the end of May 2016, 24 plants per plot with 70×60 cm plant spacing (70 cm between rows and 60 cm within the row) in order to create an optimal density of 24 thousand plants per hectare, providing a 0.4 m^2 space per plant. Fertilizers were manually incorporated to a depth of 10-15 cm when digging prior to planting and evenly distributed over each plot.

Plant samples were collected according to the variants in September in the phase of mass flowering of the culture. The medicinal raw material (grass) was dried in the shade in well-ventilated rooms. The contents of bioactive substances were measured and the values were converted to absolutely dry matter basis. Tannins were measured as per GOST 24027.2-80 [26], carotene as per GOST 13496.17-95 [27], ascorbic acid as per Murry [28[. Trace elements (copper, zinc) were measured according to GOST 30178-96 [29] by the atomic absorption method (a Varian AA-140 spectrometer, Akvilon, Russia) in the samples ground to a powder state.

For statistical processing, we used standard software packages Microsoft Office Excel 2007 and STATISTICA 6.0 (StatSoft, Inc., USA). Mean values (M*)* and standard errors of means (±SEM) were calculated, and regression and correlation were assessed. Differences were considered statistically significant at $p < 0.05$. To determine the relationship between the studied parameters, the correlation coefficients (*r*) were calculated. The values of the correlation coefficients were considered significant at $p < 0.05$

Results. Microelements can increase the content of vitamins and other biologically active substances in medicinal plants. In particular, there are evidences of their positive effect on the synthesis of vitamins C, A, and $B_{1\text{-}12}$ [30, 31]. In medicinal raw materials of *E. purpurea* enriched with copper, we determined the content of tannins, ascorbic acid, and carotene (Table 1).

Tannins (tannic acids) are important plant secondary metabolites in the

plant organism. They provide astringent, antibacterial, antienzymatic, anti-inflammatory effects, and also protect plants from mammals and insects [32]. According to Zaprometov [33], the leaves of *E. purpurea* contain up to 7.2-10.2% tannins. In our experiments, tannins increased from 13.07 to 16.58 mg% on average over the study years depending on the doses of copper acetate. The maximum concentration of tannins (16.58 mg%) occurred when applying 9.4 kg Cu/ha. Differences in the amount of tannins for all treatments were significant at $p < 0.05$ as compared to both the control and the N_{125} when applied separately.

1. Concentration of bioactive compounds in medicinal raw materials of *Echinacea purpurea* (L.) Moench at flowering as influenced by Cu fertilizer dosage ($n = 24$, M±SEM, Omsk, 2016-2018)

Treatments	Tannins, mg%	Ascorbic acid, mg%	Carotene, mg/kg
Control (without fertilizers)	10.34±1.82	2.11±1.12	25.69±6.48
Background (N_{125})	11.76±1.02	3.07±0.58	27.65±5.38
N_{125} + 0.25MPC Cu (2.3 kg Cu/haa)	13.07±0.28	3.85±0.14	32.31±2.74
N_{125} + 0.5 ПДК Cu (4,7 кг д.в/га)	14.23±0.38	4.54±0.25	40.65±1.97
N_{125} + 0.75ПДК Cu (7,0 кг д.в/га)	15.37±1.02	5.18±0.61	45.45±4.69
N_{125} + ПДК Cu (9,4 кг д.в/га)	16.58±1.71	5.84±0.99	51.21±7.95

N o t e. All treatments differ form control and N_{125} background at a statistically significant level ($p < 0.05$).

Andrade et al. [34] note the ability of tannins to chelate copper, which determines their antioxidant activity. The positive effect of copper is also due to its role of a key component of many biological compounds and involvement in biosynthesis of secondary metabolites, including tannins [35]. Our findings are consistent with reports about direct dependence of the concentration of tannins on the amount of nutrients, in particular Cu and Mn, which serve as cofactors of enzymes involved in phenol decomposition and lignin biosynthesis. Copper deficiency impairs lignification in plants [36].

2. Regression and correlation between concentration of bioactive compounds in medicinal raw materials of *Echinacea purpurea* (L.) Moench and the dosage fertilizers (Omsk, 2016-2018)

Content of compounds (x)	Regression	Correlation r
Tannins	$y = 0.51x + 11.82$	0.99 ($p < 0.05$)
Ascorbic acid	$y = 0.29x + 3.13$	0.99 ($p < 0.05$)
Carotene	$y = 2.56x + 27.45$	0.99 ($p < 0.05$)

We revealed a high relationship ($r = 0.99$ at $p < 0.05$) between the doses of copper fertilizers and the content of tannins (Table 2). The regression coefficient shows that copper acetate applied at 1 kg Cu/ha provides a 0.51 mg% increase in tannins.

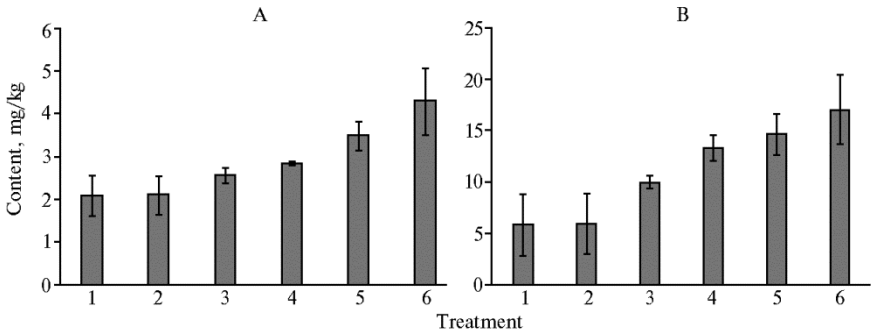
Ascorbic acid (vitamin C) is a micronutrient and an essential chemical compound that participates in many metabolic processes, performs antitoxic and antioxidant functions, increases resistance and protective properties of the body. To compensate for the deficiency of vitamin C, artificial enrichment of plant products and foodstuffs is currently widely used [37]. Carotene which plays photoprotective, light-harvesting, and structural role in plant photosynthesis, participates in the process of cell growth and division, promotes activation of immune system, helps the normal functioning of the gonads, affects growth, participates in the process of visual perception, and it also is an anticarcinogen. The intake of provitamin A into the body occurs mainly with plant foods, which are the main primary source of carotenoids [38].

Ascorbic acid was found in the leaves of *E. purpurea* [39]. In our research, the calculated doses of copper had a positive effect on the accumulation of vitamins in medicinal raw materials (see Table 1). The content of ascorbic acid and carotene were becoming significantly higher ($p < 0.05$) as the copper acetate dosage

increased. The amount of vitamin C varied upon copper application from 3.85 to 5.84 mg%, carotene from 32.31 to 51.21 mg/kg. The maximum content of vitamins was noted for N_{125} + MPC Cu. According to Zagumennikov et al. [40], *Echinacea purpurea* accumulates small amounts of ascorbic acid, while plant nutrition and age have an insignificant effect on the content of vitamin C.

Each 1 kg of the copper fertilizer increased the concentration of ascorbic acid by 0.29 mg%, carotene by 2.56 mg/kg (see Table 2). Correlation analysis indicated a very strong correlation between the content of vitamins and the copper doses ($r = 0.99$ at $p < 0.05$).

Copper interacts with various chemical elements, including zinc, iron, molybdenum, sulfur, and selenium [41]. Therefore, we compared the content of two microelements, zinc and copper, in the medicinal raw materials of *E. purpurea* (Fig.).



Content of mobile forms of Cu (A) **and Zi** (B) **in medicinal raw materials of *Echinacea purpurea* (L.) Moench at flowering as influenced by Cu fertilizer dosage:** 1 — control (without fertilizers), 2 — N_{125} (a background), 3 — N_{125} + 0,25MPC Cu (2.3 kg Cu/ha), 4 — N_{125} + 0.5MPCCu (4.7 kg Cu/ha), 5 — N_{125} + 0.75MPC Cu (7.0 kg Cu/ha), 5 — N_{125} + MPC Cu (9.4 kg Cu/ha) ($n = 24$, $M \pm$SEM, Omsk, 2016-2018).

Many chemical elements can inhibit or enhance the intake of other elements, that is, their action may be either synergistic or antagonistic [42]. Therefore, we investigated the effect of copper fertilizers in the soil—plant system.

The copper and zinc concentration in the aboveground mass of *E. purpurea* had a clearly pronounced linear dependence on the doses of copper fertilizers applied. The relationships between different doses of copper acetate and the amount of Cu and Zn in plants during the flowering period were characterized by the equations:

$$y = 0.23Cu + 1.98 \ (r = 0.98 \text{ at } p < 0.05),$$
$$y = 1.15Zn + 6.78 \ (r = 0.98 \text{ at } p < 0.05).$$

In 2016, the coefficient (b) characterizing the copper effect on the content of mobile Cu and Zn in the medicinal raw materials was 0.23 and 1.15 mg/kg, respectively, for each kilogram of copper fertilizers in doses of 2.3, 4.7, 7.0 and 9.4 kg/ha (with N_{125} as a background). To increase the Cu content in medicinal raw materials by 1 mg/kg, 4.3 kg of copper fertilizers are required ($r = 0.98$ at $p < 0.05$). The obtained normative b valued (0.23 and 1.15 mg/kg) can be used to predict how the amount of available nutrients in medicinal raw materials will increase when micronutrient fertilizers are applied. Many researchers also noted that the accumulation of copper in plants increased with an increase in the dose of applied copper fertilizers [43-45].

Thus, incorporation of copper acetate into the soil, together with macrofertilizers, allows for an increase in the content of biologically active substances (tannins, vitamins C and A) in the medicinal raw materials of *Echinacea purpurea* (L.) Moench. Our findings provide a significant increase ($p < 0.05$) in tannins to

16.58 mg%, in vitamin C up to 5.84 mg%, and in carotene up to 51.21 mg/kg. The Cu micro-fertilizer also contributes to the enrichment of raw materials with microelements (copper and zinc). The concentration of copper in the raw material increased by 2.19 mg/kg as compared to N_{125} applied separately (a background) and amounted to 4.27 mg/kg ($p < 0.05$). The highest observed zinc concentration was 17.06 mg/kg, which is 11.17 mg/kg higher than the value for N_{125} as a background. The obtained linear regression equations and coefficients b of the copper effect enable development of practical application of microelements on meadow chernozem soils in the conditions of the southern forest-steppe of Western Siberia. Our results also expand the databases on the biochemical composition of medicinal raw materials grown with the use of copper fertilizers.

REFERENCES

1. Jamshidi-Kia F., Lorigooini Z., Amini-Khoei H. Medicinal plants: Past history and future perspective. *Journal of Herbmed Pharmacology,* 2018, 7(1): 1-7 (doi: 10.15171/jhp.2018.01).
2. Hariharan P., Subburaju T. Medicinal plants and its standardization — a global and industrial overview. *Global Journal of Medicinal Plant Research*, 2012, 1(1): 10-13.
3. Nirmal S.A., Pal S.C., Otimenyin S.O., Aye T., Elachouri M., Kundu S.K., Thandavarayan R.A., Mandal S.C. Contribution of herbal products global market. *The Pharma Review*, 2013: 95-104.
4. Bogoyavlenskii A.P., Aleksyuk P.G., Turmagambetova A.S., Berezin V.E. *Farmatsevticheskie nauki*, 2013, 6(5): 1184-1187 (in Russ.).
5. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, 2013, 4: 177 (doi: 10.3389/fphar.2013.00177).
6. Kabata-Pendias A., Pendias H. *Trace elements in soils and plants.* CRC Press, Boca Raton, London, New York, Washington, D.C., 2001.
7. Aristarkhov A.N., Bushuev N.N., Safronova K.G. *Agrokhimiya*, 2012, 9: 26-40 (in Russ.).
8. Dimpka C.O., Bindraban P. Fortification on micronutrients for efficient agronomic production: a review. *Agronomy for Sustainable Development*, 2016, 36: 1-26 (doi: 10.1007/s13593-015-0346-6).
9. Valença A.W., Bake A., Brouwer I.D., Giller K.E. Agronomic biofortification of crops to fight hidden hunger in sub-Saharan Africa author links open overlay panel. *Global Food Security*, 2017, 12: 8-14 (doi: 10.1016/j.gfs.2016.12.001).
10. Vavoulidou E.E., Avramides E.J., Papadopoulos P., Dimirkou A.T., Charoulis A., Konstantinidou-Doltsinis S. Copper content in agricultural soils related to cropping systems in different regions of Greece. *Communications in Soil Science and Plant Analysis*, 2005, 36(4-6): 759-773 (doi: 10.1081/CSS-200043367).
11. Pavelkova M., Vysloužil J., Kubova K., Vetchy D. Biological role of copper as an essential trace element in the human organism. *Czech and Slovak Pharmacy*, 2018, 67(4): 143-153.
12. Broadley M., Brown P., Buerkert A., Camak I., Cooper J., Eichert T., Engels C., Fernandez V., Kirkby T., Eckhard G., Hawkesford M., Horst W., Huber D., Kichey T., Ludwi B., Jian Feng Ma., Marschner P., Neumann E., Neumann G., Schlecht H., Rengel Z., Romheld V., Schjoerring J., Moller I.S., Weinmann M., White P., Wiesler F., Zhao F. *Marschner's mineral nutrition of higher plants.* Elsevier Ltd., 2012 (doi: 10.1016/B978-0-12-384905-2.00025-X).
13. Braz J. Copper in plants. *Brazilian Journal of Plant Physiology*, 2005, 17(1): 145-156 (doi: 10.1590/S1677-04202005000100012).
14. Burkhead J.L., Gogolin Reynolds K.A., Abdel-Ghany S.E., Cohu C.M., Pilon M. Copper homeostasis. *New Phytologist,* 2009, 182(4): 799-816 (doi: 10.1111/j.1469-8137.2009.02846.x).
15. Printz B., Lutts S., Hausman J.-F., Sergeant K. Copper trafficking in plants and its implication on cell wall dynamics. *Frontiers in Plant Science*, 2016, 7: 601 (doi: 10.3389/fpls.2016.00601).
16. Makarova V.G., Laksaeva E.A., Martynov E.G. *Rossiiskii mediko-biologicheskii vestnik imeni akademika I.P. Pavlova*, 2006, 3: 29-35 (in Russ.).
17. Krasnov E.A., Savel'eva E.E., Ryzhakova N.K., Reshetov Ya.E., Gataullina A.R. *Khimiya rastitel'nogo syr'ya*, 2017, 4: 145-151 (doi: 10.14258/jcprm.2017041934) (in Russ.).
18. Nonvitamin and nonmineral nutritional supplements. S.M. Nabavi, A.S. Silva (eds.). *Academic Press*, 2019 (doi: 10.1016/C2016-0-03546-5).
19. Sharifi-Rad M., Mnayer D., Morais-Braga M.F.B., Carneiro J.N.P., Bezerra C.F., Coutinho H.D.M., Salehi B., Martorell M., del Mar Contreras M., Soltani-Nejad A., Uribe Y.A.H., Yousaf Z., Iriti M., Sharifi-Rad J. Echinacea plants as antioxidant and antibacterial agents: From traditional medicine to biotechnological applications. *Phytotherapy Research*, 2018, 32(9): 1653-1663 (doi: 10.1002/ptr.6101).
20. Kumar R.M., Ramaiah S. Pharmacological importance of *Echinacea purpurea. International Journal of Pharma and Bio Sciences*, 2011, 2(4): 304-314.
21. Shekarchi M., Hajimedipoor H., Khanavi M., Rustaie A. The effects of plant age and harvesting

time on chicoric and caftaric acids content of *E. purpurea* (L.) Moench. *Iranian Journal of Pharmaceutical Sciences,* 2012, 8(3): 203-208.
22. Khasina E.I. *Izvestiya Samarskogo nauchnogo tsentra Rossiiskoi akademii nauk*, 2014, 16(5-2): 1030-1032 (in Russ.).
23. Babaeva E.Yu. *Osobennosti mineral'nogo pitaniya ekhinatsei purpurnoi v usloviyakh Nechernozemnoi zony RF. Avtoreferat kandidatskoi dissertatsii* [Features of the *Echinacea purpurea* mineral nutrition in the Non-chernozem zone of the Russian Federation. PhD Thesis]. Moscow, 2000 (in Russ.).
24. Hajagha R., Kirici S., Tabrizi L., Asgharzadeh A., Hamidi A. Evaluation of growth and yield of purple coneflower (Echinacea purpurea l.) in response to biological and chemical fertilizers. *Journal of Agricultural Science*, 2017, 9(3): 160-171 (doi: 10.5539/jas.v9n3p160).
25. *Agrokhimicheskie metody issledovaniya pochv* /Pod redaktsiei A.V. Sokolova [Agrochemical methods in soil research. A.V. Sokolov (ed.)]. Moscow, 1975 (in Russ.).
26. *GOST 24027.2-80 Syr'e lekarstvennoe rastitel'noe. Metody opredeleniya vlazhnosti, soderzhaniya zoly, ekstraktivnykh i dubil'nykh veshchestv, efirnogo masla* [GOST 24027.2-80 Raw medicinal vegetable. Methods for determination of moisture, ash content, extractive compounds and tannins, essential oils]. Moscow, 1999 (in Russ.).
27. *GOST 13496.17-95 Korma. Metody opredeleniya karotina* [GOST 13496.17-95 Feed. Methods for determining carotene]. Moscow, 2011 (in Russ.).
28. Mineev V.G., Sychev V.G., Amel'yanchik O.A., Bolysheva T.N., Gomonova N.F., Durynina E.P., Egorov V.S., Edemskaya N.L., Karpova E.A., Prizhukova V.G. *Praktikum po agrokhimii* /Pod redaktsiei V.G. Mineeva [Workshop on agrochemistry. V.G. Mineev (ed.)]. Moscow, 2001 (in Russ.).
29. *GOST 30178-96 Syr'e i produkty pishchevye. Atomno-absorbtsionnyi metod opredeleniya toksichnykh elementov* [GOST 30178-96 Raw materials and food products. Atomic absorption method for determination of toxic elements]. Moscow, 1997 (in Russ.).
30. Collins J.F., Klevay L.M. Copper. *Advances in Nutrition*, 2011, 2(6): 520-522 (doi: 10.3945/an.111.001222).
31. Kaiser C., Wernery U., Kinne J., Marker L., Liesegang A. The role of copper and vitamin A deficiencies leading to neurological signs in captive cheetahs (*Acinonyx jubatus*) and lions (*Panthera leo*) in the United Arab Emirates. *Food and Nutrition Sciences*, 2014, 5(20): 1978-1990 (doi: 10.4236/fns.2014.520209).
32. Hassanpour S., Maheri-Sis N., Eshratkhah B., Mehmandar F.B. Plants and secondary metabolites (Tannins): a review. *International Journal of Forest, Soil and Erosion*, 2011, 1(1): 47-53.
33. Zaprometov M.N. *Osnovy biokhimii fenol'nykh soedinenii* [Fundamentals of biochemistry of phenolic compounds]. Moscow, 1974 (in Russ.).
34. Andrade R.G. Jr., Dalvi L.T., Silva J.M. Jr., Lopes G.K., Alonso A., Hermes-Lima M. The antioxidant effect of tannic acid on the in vitro copper-mediated formation of free radicals. *Archives of Biochemistry and Biophysics*, 2005, 437(1): 1-9 (doi: 10.1016/j.abb.2005.02.016).
35. Khathutshelo M.V., Mpumelelo N., Wonder N., Fhatuwani M.N. Effects of foliar spray application of selected micronutrients on the quality of bush tea. *Horticultural Science*, 2016, 51(7): 873-879 (doi: 10.21273/hortsci.51.7.873).
36. Santos R.M., Fortes G.A.C., Ferri P.H., Santos S.C. Influence of foliar nutrients on phenol levels in leaves of *Eugenia uniflora*. *Revista Brasileira de Farmacognosia*, 2011, 21(4): 581-586 (doi: 10.1590/S0102-695X2011005000112).
37. Araya M.M., Pizarro F., Olivares M., Arredondo M., González M., Méndez M. Understanding copper homeostasis in humans and copper effects on health. *Biological Research*, 2006, 39(1): 183-187 (doi: 10.4067/S0716-97602006000100020).
38. Napoli J.L. Vitamin A. Biochemistry and physiological role. In: *Encyclopedia of human nutrition (second edition)*. Elsevier, Berkeley, CA, 2005: 339-347 (doi: 10.1016/B0-12-226694-3/02280-8).
39. Mistríková I., Vaverková S. *Echinacea* — chemical composition, immunostimulatory activities and uses. *Thaiszia - Journal of Botany*, 2006, 16: 11-26.
40. Zagumennikov V.B., Molchanova A.V., Babaeva E.Yu., Petrova A.L. Accumulation of ascorbic acid in fresh *Echinacea purpurea* plants and their processing products. *Pharmaceutical Chemistry Journal*, 2015, 48(10): 671-674 (doi: 10.1007/s11094-015-1168-1).
41. Osredkar J., Sustar N. Copper and zinc, biological role and significance of copper/zinc imbalance. *Journal of Clinical Toxicology*, 2011, 3: 1-18 (doi: 10.4172/2161-0495.S3-001).
42. Wang Y., Ou Y.L., Liu Y.Q., Xie Q., Liu Q.F., Wu Q., Fan T.Q., Yan L.L., Wang J.Y. Correlations of trace element levels in the diet, blood, urine, and feces in the chinese male. *Biological Trace Element Research*, 2012, 145(2): 127-135 (doi: 10.1007/s12011-011-9177-8).
43. Kumar R., Mehrotra N.K., Nautiyal B.D., Kumar P., Singh P.K. Effect of copper on growth, yield and concentration of Fe, Mn, Zn and Cu in wheat plants (*Triticum aestivum* L.). *Journal of Environmental Biology*, 2009, 30(4): 485-488.
44. Kaewchangwat N., Dueansawang S., Tumcharern G., Suttisintong K. Synthesis of copper-chelates derived from amino acids and evaluation of their efficacy as copper source and growth stimulator for *Lactuca sativa* in nutrient solution culture. *Journal of Agricultural and Food Chemistry*, 2017, 65(45): 9828-9837 (doi: 10.1021/acs.jafc.7b03809).
45. Stepien A., Wojtkowiak K. Effect of foliar application of Cu, Zn, and Mn on yield and quality indicators of winter wheat grain. *Chilean Journal of Agricultural Research*, 2016, 76(2): 220-227 (doi: 10.4067/s0718-58392016000200012).

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THE BALANCE OF CALCIUM IN THE GRASS ECOSYSTEMS OF THE TEREK-KUMA LOWLAND

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Abstract

Terek-Kuma lowland occupies the North-Western part of the Precaspian lowland. The soil-plant cover of the territory is determined by the aridity of the climate with frequent winds, light granulometric and saline soils, high pasture load. In this study, for the first time in the Terek-Kuma semidesert conditions, the productivity of structural parts of grass ecosystems and calcium reserves during the most favorable (April) and arid (August) periods are determined for the main soil types. The work aimed to determine the accumulation, distribution and reserves of calcium in the structure of phytomass with regard to a soil type. The research was performed at the Kochubey Biosphere Station, Precaspian Institute of Biological Resources of the Dagestan Scientific Center RAS in 2011-2016 on grass phytocenoses of light chestnut and meadow-chestnut soils and saline typical automorphic soils. Assessment of plant matter and Ca accumulation in green mass, rags, steppe felt, and roots, and calculation of the Ca budget of the ecosystems were carried out according to A.A. Titlyanova et al. (1988). The content of Ca in plants was determined by capillary electrophoresis (a Drops-105M system, Lumex, Russia) with special software Elforan (Lumex, Russia). The greatest amount of phytomass accumulated on light-chestnut soil. On meadow-chestnut soil photosynthesizing parts, rags, steppe felt and roots accumulated 2.3, 1.5, 2.3 and 2.2 times less, respectively, and on typical saline soil 2.6, 1.7, 2.5 and 2.7 times less than on light-chestnut soil. This was likely caused by a decrease in projective coverage from 77.0 % on light chestnut soil to 48.5 % on meadow-chestnut soil and 43.5 % on typical saline soil. In the species patterns the dominants were *Poaceae* (51 % for light-chestnut soil) and *Asteraceae* (30 % and 17 % for meadow-chestnut soil and saline soil, respectively). The root weight for meadow-chestnut soil and typical saline soil was 2.2 and 2.9 times less than for light-chestnut soil. Their share in the total phytomass depending on soil types ranged from 85.0 to 87.2 %. In green parts, the concentration of Ca, depending on the season and the soil type, was in the range of 0.40-0.48 %, in rags it was 0.50-0.54 %, in felt 1.00-1.31 %, and in underground parts 1.14-1.38 %. By soil types, it decreased as light chestnut soil > meadow-chestnut soil > saline soil due to pH changes (8.6 > 8.2 > 8.0), an increase in the degree of salinity and the shift of salinity from the sulfate type to the sulfate-chloride type. Reserves of Ca in the above-ground parts during the growing season on light-chestnut soil amounted to 2.32 kg/ha per year and exceeded 2.7- and 3.1-fold, respectively, similar

indicators for meadow-chestnut soil and typical saline soil. Ca reserves in the root mass for all types of soils were 12.6 times more than in the aboveground parts. After plant matter decomposition, steppe felt and underground organs contribute to light- chestnut soil 42.0 and 58.0 % of the calcium consumed from the soil, to meadow-chestnut soil 36.0 and 64.0 %, respectively; for the typical saline soil these amounts are 1.1- and 2.3-fold, respectively. It was revealed that the difference in the dynamics of Ca accumulation in components of a semi-desert plant community (green mass, rags, steppe felt, and roots) depends on the plant species composition, soil type and season.

Keywords: phytocoenosis, phytomass accumulation, plant matter translocation, Ca accumulation, Ca reserves, calcium budget

The Terek-Kuma lowland (the northwestern part of the Caspian lowland) is characterized by light grain size and saline soils, arid climate, high pasture load, and degraded soil and vegetation cover. Soils with a low content of humus and the main nutrients of plants in combination with an unfavorable water-salt regime have low productivity [1]. However, these estimates were obtained only for the photosynthesizing part of the phytomass, while the structure of the phytocenosis also contains other elements, the rags, steppe felt, and roots.

The elemental composition of phytocenoses in the Terek-Kuma lowland was studied by many rsearchers. In particular, there are data on the relative content of calcium in the vegetating air-dry mass (hay) [2, 3], but there is no similar information regarding the structure of the vegetation cover (green mass, rags, felt, and roots). The lack of these data hinders estimation of the reserves of biophilic elements, including Ca, and its balance in herbal ecosystems, including differentiated by the main types of soils.

On the territory of the Terek-Kuma lowland, the content of Ca in plants is higher than in the adjacent Prisulakskaya lowland, 2.014±0.02 vs. 1.623±0.25 g/kg [4]. A gradual decrease in its accumulation is regularly noted from November to April next year. Gireev et al. [4] explain this by the transformation of meadow-wormwood communities into ephemeral-wormwood and wormwood ones. It can be assumed that such a decrease in the amount of Ca in phytocenoses has another reason and is associated with the translocation of substances between the elements of the phytocenosis structure across the stages of plant development.

Moisture is noted to have a decisive effect on the accumulation of chemical elements in plants, in particular, the Ca content increases with increasing precipitation [5, 6]. It was also revealed that favorable hydrothermal conditions during the growing season contribute to an increase in Ca content in soil [7].

In this study, productivity of herbal ecosystems and Ca reserves in the Terek-Kuma semi-desert was differentiated with regard to components of phytomass structure for the main soil types during climatically most favorable (April) and dry (August) seasons, and the Ca balance in ecosystems was calculated

The work aimed to study calcium accumulation in plant matter, taking into account its structure, and the prevalence of this element in herbal ecosystems on the main types of soils of the Terek-Kuma lowland.

Materials and methods. The study was carried out at the Kochubey Biosphere Station (the Precaspian Institute of Biological Resources of the Dagestan Federal Research Center RAS, 44.40720 N, 46.24771 E) in 2011-2016. Seasonal accumulation, reserves and budget of calcium (April, August) were analyzed in detail in 2013-2015. Herbaceous phytocenoses on light-chestnut and meadow-chestnut soils and a typical automorphic solonchak of the Terek-Kuma lowland were surveyed. In the 0-30 cm layer of light-chestnut soil contained 1.12% humus, 1.11 mg/100 g P_2O_5, 20.12 mg/100 g K_2O, the density was 1.14 g/cm^3, the lowest moisture capacity was 20.6%; for meadow-chestnut, the parameters were 0.54%, 0.7 mg/100 g, 18.2 mg/100 g, 1.42 g/cm^3, and 26.7%, respectively; for a typical automorphic solonchak these were 0.48%, 0.66 mg/100 g, 16.8 mg/100 g,

1.45 g/cm^3, and 30.3%. The type of salinity varied from chloride-sulfate to chloride depending on the hydrothermal conditions of the seasons, the salinity varied from weak to very strong.

Plant matter accumulation per time unit per area unit, Ca content in green mass, rags, steppe felt and roots, and calculation of Ca balance in ecosystems were carried out according to Titlyanova [7]. The Ca content in plant matter was determined by capillary electrophoresis (Kapel-105M system equipped with Elforan software, Lumex, Russia) in the mode of determination of cations and anions [8]. A total of 150 samples were analyzed in 3 biological and analytical replicates.

The Latin names of the species are given according to Murtazaliev [9].

Statistical processing was carried out using Microsoft Excel 2010 software. The mean values (*M*) and errors of the mean (±SEM), linear regression equations, correlation coefficient (*r*), and determination coefficient (*R*) were calculated.

Results. In the conditions of the plain, phytocenoses are most productive on light chestnut soil. On average, for 2011-2016, the accumulation of air-dry phytomass here was 2.2 times higher than for meadow-chestnut soil. On typical automorphic solonchak, the productivity of the phytocenosis decreased 2.6 times as compared to that for light-chestnut soil (Table).

On meadow-chestnut soil and typical saline, the level of accumulated rags (102.8 and 100.5%, respectively) was the same as for green mass, and on light-chestnut soil it was 33.7% less, which was associated with the species composition of phytocenoses.

Air-dry weigh of above ground parts and roots accumulated structural components of vegetation cover for different soil types of the Terek-Kuma lowland (Kochubey Biosphere Station of the Precaspian Institute of Biological Resources, the Dagestan Federal Research Center RAS, 2011-2016)

Component	Soil	Phytomass, t/ha per year (*M*±SEM)	Ca	
			concentration, % (*M*±SEM)	reserves, kg/ha
Green mass	Light-chestnut	4.84±0.02	0.48±0.02	2.32
	Meadow-chestnut	2.11±0.01	0.41±0.01	0.86
	Typical saline	1.89±0.03	0.40±0.03	0.76
Rags	Light-chestnut	3.21±0.02	0.54±0.03	1.73
	Meadow-chestnut	2.17±0.01	0.50±0.01	1.08
	Typical saline	1.91±0.04	0.50±0.02	0.96
Steppe felt	Light-chestnut	4.32±0.03	1.31±0.01	5.66
	Meadow-chestnut	1.89±0.02	1.30±0.02	2.46
	Typical saline	1.71±0.02	1.00±0.01	1.71
Roots	Light-chestnut	83.71±0.03	1.38±0.02	115.52
	Meadow-chestnut	38.19±0.03	1.38±0.03	52.70
	Typical saline	31.19±0.02	1.14±0.03	35.56

On light chestnut soil, the proportion of ephemera from the bluegrass family, which were represented by *Eragrostic minor* Host., *Poa bulbosa* L., and *Eremopyrum orientale* L., was 51.4% by the number and 19.6% by weight. On the meadow-chestnut soil and saline soil, the phytomass of *Artemisia taurica* Willd and *A. lercheana* Web. ex Stechm remained rather high, 37.7% by number and 83.7% by weight), since their rags for April-August did not have time to completely turn into steppe felt.

According to some publications, the contribution of underground organs to the total mass of herbaceous phytocenoses is 50-90% [10, 11]. In our studies, the weight of roots for meadow-chestnut soil and typical saline soil turned out to be 2.2 and 2.9 times less than for light-chestnut soil. The root weight comprised 85.0-87.2% from the total phytomass yield depending on the soil type. Such a high ratio of aboveground and underground mass (1:6.8-1:5.7) is typical for all arid regions worldwide [12, 13].

Our data show that the following regression equations describe the relationship between the accumulation of aboveground (x) and underground (y) mass in ecosystems in semi-desert conditions, depending on the type of soil:

for light chestnut soil $y = 0.6935x + 73.7823$ ($r = 0.97$, R = 0.96);

for meadow chestnut soil $y = 1.1804x + 30.7254$ ($r = 0.96$, R = 0.95);

for typical saline soil $y = 1.7925x + 21.0415$ ($r = 0.97$, R = 0.95).

The main reason for the decrease in the phytomass on the meadow- chestnut soil and saline soil is typically an increase in soil salinity and a change in its character towards an increase in the ratio of Cl^-:SO_4^{2-} ions [11]. It should be noted that the productivity of the grass ecosystems of the Terek-Kuma lowland was significantly lower than in other regions of the country, where it is 15-20 t/ha [10, 11]. Only on light-chestnut soil this indicator approached the lower of the indicated limits, and on meadow-chestnut soil and typical saline soil it turned out to be 2.2-2.6 times lower. Similar results were obtained by other authors in the territory of our study [5], in the Astrakhan region [14], in the dry and desertified steppe of Tuva [12, 13], in the arid regions of Iran [15], Panama [5], China [16], and in regions with arid climates.

In plant life, Ca plays an important role in the formation of cell walls, takes part in maintaining the structure of chromosomes, mitochondria, and ribosomes [17, 18], increases salt tolerance [19-21], plant resistance to heat stress [22] and negative effects of heavy metals [23], optimizes the growth and development of roots [24].

For 2013-2015 (April, August), the average values of Ca content in the green mass and other elements of vegetation cover in general corresponded to the accumulated yield of phytomass and amounted to 0.48% for light-chestnut soil, 0.41% for meadow-chestnut soil, and 0.40% for typical saline soil. Significant differences were noted in the content of the element among plant species. For light-chestnut soil, 51% of the total projective cover (77%) fell on cereals — *Anisantha tectorum* (L.) Nevski, *Bromus squarrosus* L., *Poa bulbosa* L., *Eragrostis minor* Host and *Eremopyrum triticeum* (Gaertn.) Nevski, 15% — on *Artemisia taurica* Willd and *A. lercheana* Web. ex Stechm, 5% — on saltwort, including *Ceratocarpus arenarius* L., *Salsola iberica* Sennen & Pau, *Petrosimonia brachiata* (Pall.) Bunge, 3% — on *Silene conica* L. and *Herniaria incana* Lam., and 3% — on *Alussum desertorum* Stapf. The calcium content was 1.03% for wormwoods, 0.93% for *Chenopodioideae*, 0.38% for *Caryophyllaceae*, 0.22% for *Cruciferae* and 0.58% for cereals.

On the meadow-chestnut soil, the projective cover of *Poaceae* plants decreased 5.1 times, of *Caryophyllaceae* 2.0 times, and *Cruciferae* 1.5 times, while wormwoods from the *Asteraceae* family increased 2.0 times. Despite the fact that on this type of soils the proportion of *Asteraceae* and *Chenopodioideae* soils with a high Ca accumulation increased, the average content of the element decreased to 0.47% in the *Poaceae* group compared to that for light-chestnut soil, and up to 0.90% in the *Chenopodioideae* with insignificant deviations in other families. On a typical saline soil, the total projective cover by phytocenosis (43.5%) approached the value on meadow-chestnut soil (48.5%), but the content of the element decreased only in saltwort (by 0.24%) with relatively similar values in other groups of plants.

The decrease in Ca content in the phytomass as light-chestnut soils > meadow-chestnut soils > typical saline soil, we associate with a slight decrease in the pH of the soil solution in the same row (8.6 > 8.2 > 8.0), which becomes the reason for more easy availability of Ca from soils, the same is confirmed by other authors [25]. In addition, the increase in salinity and the transition from its sulfate

type to the sulfate-chloride type affect [11]. These factors significantly reduce the availability of Ca for plants from meadow-chestnut soil and typical saline soil compared to the light-chestnut type.

It is known that the influx of ions of various metals from the soil into the root system and aboveground organs of plants is due to the dynamics of osmotic pressure with the participation of ion transporter encoded by different genes [26]. The following equation describes the role of soil type (x) and species composition of phytocenoses (y) in changes in Ca concentration (z):

$$z = 0{,}1057 - 0{,}4999x + 1{,}1898y.$$

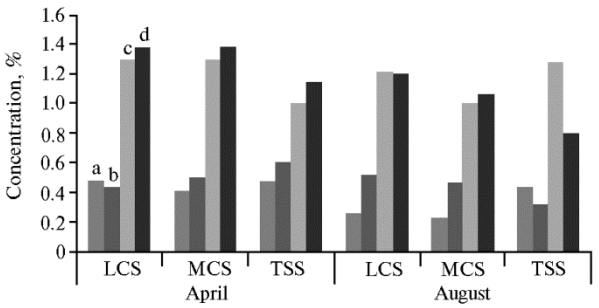

Fig. 1. Ca accumulation in phytocenoses of the Terek-Kuma Lowland depending on soil types and seasons: LCS — light-chestnut soil, MCS — meadow-chestnut soil, TSS — typical saline soil; a — green mass, b — rags, c — steppe felt, d — roots (Kochubey Biosphere Station of the Precaspian Institute of Biological Resources, the Dagestan Federal Research Center RAS, 2013-2015).

In the rags and felt, the Ca content leveled off, and in the underground organs it decreased from 1.38% for the first two types of soils to 1.14% for typical saline soil (Fig. 1). A sufficiently high amount of Ca in the phytomass under the conditions of a non-flush water regime promoted the maintenance of a neutral and slightly alkaline reaction in the soil solution [10].

The reserves of the element in the root mass for all soils were higher than in the aboveground one, by an average of 12.6 times (73.81 kg/ha vs. 5.85 kg ha). Given the above data, the Ca balance was compiled in biogeocenoses of the considered soil types (Fig. 2). In herbal ecosystems, calcium is consumed significantly more than in forest ones [16]. In our study, for light-chestnut soil, 42.0% of a total 125.23 kg/ha Ca, consumed by the phytocenosis per year, returned to the soil during the decomposition of steppe felt, the value for underground organs was 58.0%. For the meadow-chestnut soil, the share of Ca received during the felt decomposition decreased to 36.8%, while for a typical saline soil it increased to 48.4%.

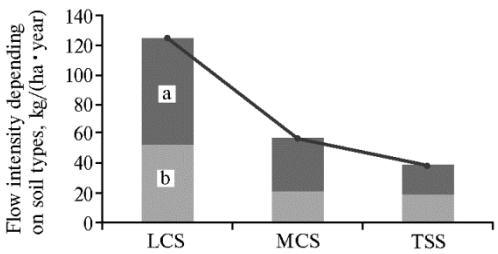

Fig. 2. Calcium budget in phytocenoses of the Terek-Kuma lowland depending on the soil type: LCS — light chestnut soil, MCS — meadow chestnut soil, TSS — typical saline soil; a — entered the soil during the decomposition of underground organs, b — entered the soil during the decomposition of felt; the graph shows amount of Ca taken up by growing plants (Kochubey Biosphere Station of the Caspian Institute of Biological Resources, the Dagestan Federal Research Center RAS, 2013-2015).

The relatively high productivity of phytocenoses on light-chestnut soil contributes to the enrichment of its upper horizons with Ca and other biophilic elements (Fe, Si, Al), which serve as the main factor in the biosynthesis of secondary clay minerals that form the absorbing soil complex. The high content of Ca in the phytomass of herbaceous plants (along with Mg and S) leads to the saturation of the absorbing complex of soils of the Terek-Kuma lowland with this strong coagulator, which ensures the formation of favorable agrophysical soil

properties and an improvement in its structure [1].

For all types of soils, a higher Ca content was observed in spring compared to the summer period (1st decade of August), which is explained by the favorable water regime of the soil during this period, promoting the entry of chemical elements into plants. A decrease in the amount of Ca in August is associated with an increase in soil salinity, an increase in the osmotic pressure of the soil solution, and a slowdown in the supply of soil moisture and nutrients to plants. These data confirm the validity of the conclusion that the intensity of the influx of chemical elements, including Ca, into plants is associated with favorable hydrothermal conditions [10], and not with the transformation of meadow-wormwood communities into ephemero-wormwood and wormwood [3, 4].

Another reason for the decrease in the accumulation of Ca in pasture phytocenoses by the end of the growing season or by the next spring is the outflow of some elements from aging and dying plant tissues into young or newly formed ones. According to Titlyanova [10], the process of re-location of nutrients is universal and inherent in all ecosystems. Consequently, on the main types of the studied soils, a sufficient content of Ca in the phytomass and its subsequent supply to the upper part of the soil profile, becomes the basis for the biological cycles of Ca in herbal cenoses.

Thus, in the conditions of Terek-Kuma lowland, the light-chestnut soil provides the maximum reserves of photosynthesizing phytomass (4.84 t/ha per year), rags (3.21 t/ha per year), steppe felt (4.32 t/ha per year) and roots (83.71 t/ha per year). These values decreased 2.3, 1.5, 2.3 and 2.2 times, respectively, for meadow-chestnut soil, and 2.6. 1.7. 2.5 and 2.7 times for a typical saline soil. The accumulation of Ca in the aboveground and underground parts corresponded to the yield of phytocenoses. Its content averaged 0.48-0.40% in the green mass, 1.0-1.3% in felt, and 1.14-1.38% in underground organs. The maximum reserves of the element in the aboveground parts during the growing seasons accumulated the light-chestnut soil (9.71 t/ha per year), exceeding similar indicators for meadow-chestnut soil and typical saline soil 2.7-fold and 3.1-fold, respectively. During decomposition of steppe felt, 42.0% of Ca consumed by plants was returned to the light-chestnut soil, 36.0% to the meadow-chestnut soil, and 48.4% to the typical saline soil. The same amount of the element moved to the above-ground organs. During the decomposition of underground organs, plants received, according to soil types, 58.0, 64.0 and 51.6% of the released Ca, respectively. It was revealed that the difference in the dynamics of Ca accumulation in the phytomass structural components (photosynthesizing parts, rags, steppe felt, and roots) of semi-desert phytocenoses depends on the plant species composition, soil type, and season.

REFERENCES

1. Balamirzoev M.A., Mirzoev E.M.-R., Adzhiev A.M., Mufaradzhev K.G. *Pochvy Dagestana. Ekologicheskie aspekty ikh ratsional'nogo ispol'zovaniya* [Soils of Dagestan — environmental aspects of rational use]. Makhachkala, 2008 (in Russ.).
2. Usmanov R.Z., Osipova S.V., Dzhalalova M.I., Babaeva M.A. *Yug Rossii: ekologiya, razvitie*, 2008, 3(3): 109-111 (in Russ.).
3. Zagidova R.M., Biibolatova Z.D., Asgerova D.B., Abdurashidova P.A. *Vestnik Dagestanskogo gosudarstvennogo universiteta*, 2015, 30(1): 60-65 (in Russ.).
4. Gireev G.I., Luganova S.G., Salikhov Sh.K. *Vestnik Tambovskogo universiteta. Seriya: Estestvennye i tekhnicheskie nauki*, 2013, 18(3): 767-770 (in Russ.).
5. Santiago LS., Schuur EA., Silvera K. Nutrient cycling and plant-soil feedbacks along a precipitation gradient in lowland Panama. *Journal of Tropical Ecology*, 2005, 21(4): 461-470 (doi:

10.1017/s0266467405002464).
6. Dijkstraa F.A., Heb M., Johansenc M.P., Harrisonc J.J., Keitel C. Plant and microbial uptake of nitrogen and phosphorus affected by drought using ^{15}N and ^{32}P tracers. *Soil Biology and Biochemistry*, 2015, 82: 135-142 (doi: 10.1016/j.soilbio.2014.12.021).
7. Titlyanova A.A., Bazilevich N.I., Snytko V.A., Dubynina S.S., Kopoteva T.A., Magomedova L.N., Mironycheva-Tokareva N.P., Nefed'eva L.G., Semenyuk N.V., Tishkov A.A., Ti Tran, Khakimzyanova F.I., Shatokhina N.G., Shmakova E.I. *Biologicheskaya produktivnost' travyanykh ekosistem. Geograficheskie zakonomernosti i ekologicheskie osobennosti* /Pod redaktsiei V.B. Il'inoi [Biological productivity of grassy ecosystems. Geographical patterns and environmental features. V.B. Il'ina (ed.)]. Novosibirsk, 1988: 109-127 (in Russ.).
8. Komarova N.V., Kamentsev Ya.S. *Prakticheskoe rukovodstvo po ispol'zovaniyu sistem kapillyarnogo elektroforeza «KAPEL'»* [Practical guide for using capillary electrophoresis systems «KAPEL'»]. St. Petersburg, 2006 (in Russ.).
9. Murtazaliev R.A. *Konspekt flory Dagestana* /Pod redaktsiei R.V. Kamelina [Outline of Dagestan flora. R.V. Kamelin (ed.)]. Makhachkala, 2009 (in Russ.).
10. Titlyanova A.A. *Pochvovedenie*, 2007, 12: 1422-1429 (in Russ.).
11. Gasanov G.N., Asvarova T.A., Gadzhiev K.M., Akhmedova Z.N., Abdulaeva A.S., Bashirov R.R., Arslanov M.A. *Izvestiya Samarskogo nauchnogo tsentra RAN*, 2016, 18(2): 59-64 (in Russ.).
12. Kurbatskaya S.S. *Novye issledovaniya Tuvy*, 2009, 4: 209-224 (in Russ.).
13. Titlyanova A.A., Sambuu A.D. *Izvestiya RAN. Seriya biologicheskaya,* 2014, 6: 621-630 (in Russ.).
14. Tyutyuma N.V., Bulakhtina G.K. *Trudy instituta geologii Dagestanskogo nauchnogo tsentra RAN*, 2016, 1: 68-72 (in Russ.).
15. Saffariha M., Azarnivand H., Tavili A. Effects of grazed exclosure on some of nutrient elements of aerial and underground organs of *Artemisia sieberi*, *Stipa hohenacheriana* and *Salsola rigida*. *International Journal of Agronomy and Agricultural Research*, 2014, 4(2): 62-70.
16. Zuo X.A., Knops J.M.H., Zhao X.Y., Zhao H.L., Li Y.Q., Guo Y.R. A positive correlation between plant diversity and productivity is indirectly caused by environmental factors driving spatial pattern of vegetation composition in semiarid sandy grassland. *Biogeosciences Discussions*, 2011, 8: 1795-1801.
17. *Marschner's mineral nutrition of higher plants.* 3rd edition. P. Marschner (ed.). Amsterdam, Netherlands, Elsevier/Academic Press, 2011 (doi: 10.1017/s001447971100130x).
18. Shen J., Yuan L., Zhang J., Bai Z., Chen X., Zhang W., Zhang F. Phosphorus dynamics: from soil to plant. *Plant Physiology*, 2011, 156(3): 997-1005 (doi: 10.1104/pp.111.175232).
19. Khan M.N., Siddiqui M.H., Mohammad F., Naeem M. Interactive role of nitric oxide and calcium chloride in enhancing tolerance to salt stress. *Nitric Oxide*, 2012, 27(4): 210-218 (doi: 10.1016/j.niox.2012.07.005).
20. Hadi M.R., Karimi N. The role of calcium in plants' salt tolerance. *Journal of Plant Nutrition*, 2012, 35(13): 2037-2054 (doi: 10.1080/01904167.2012.717158).
21. Manishankar P., Wang N., Köster P., Alatar A., Kudla J. Calcium signaling during salt stress and in the regulation of ion homeostasis. *Journal of Experimental Botany*, 69(17): 4215-4226 (doi: 10.1093/jxb/ery201).
22. Tan W., Meng Q.W., Brestic M., Olsovska K., Yang X. Photosynthesis is improved by exogenous calcium in heat-stressed tobacco plants. *Journal of Plant Physiology*, 2011, 168(17): 2063-2071 (doi: 10.1016/j.jplph.2011.06.009).
23. Huang D., Gong X., Liu Y., Zeng G., Lai C., Bashir H., Zhou L., Wang D., Xu P., Cheng M., Wan J. Effects of calcium at toxic concentrations of cadmium in plants. *Planta*, 2017, 245(5): 863-873 (doi: 10.1007/s00425-017-2664-1).
24. Zhang T., Yang J., Sun Y., Kang Y., Yang J., Qi Z. Calcium deprivation enhances non-selective fluid-phase endocytosis and modifies membrane lipid profiles in Arabidopsis roots. *Journal of Plant Physiology*, 2018, 226: 22-30 (doi: 10.1016/j.jplph.2018.04.002).
25. González-Fontes A., Navarro-Gochicoa M.T., Ceacero C.J., Herrera-Rodríguez M.B., Camacho-Cristybal J.J., Rexach J. Chapter 9 — Understanding calcium transport and signaling, and its use efficiency in vascular plants. In: *Plant macronutrient use efficiency. Molecular and genomic perspectives in crop plants.* M.A. Hossain, T. Kamiya, D.J. Burritt, L.-S.P. Tran, T. Fujiwara (eds.). Academic Press, 2017: 165-180 (doi: 10.1016/B978-0-12-811308-0.00009-0).
26. Krämer U., Talke I.N., Hanikenne M. Transition metal transport. *FEBS Letters*, 2007, 581(12): 2263-2272 (doi: 10.1016/j.febslet.2007.04.010).

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HOW THE BASIDIOMYCETES RESPOND TO BIOGENIC ASPARTATE-BOUND METALS(II) OF VARIABLE VALENCY IN GROWTH MEDIA

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Abstract

Current studies on artificial mushroom cultivation are aimed at optimizing mineral nutrition and the delivery of metals(II). Organically bound trace metals are superior to their inorganic precursors. Cu, Mn, Fe, Zn, and Co metal(II) complexation with essential amino acids seems to be a solution to the bioavailability problem, thus making amino acid chelates of biogenic metals relevant for study. In particular, aspartic acid salts potentially could improve cultivable mushroom growing due to use of bioavailable organic compounds of microelements. However, a comprehensive study on mineral nutrition of cultivated mushrooms using biogenic metal chelates has not been conducted previously. This paper is the first to discover and characterize the effect of metal(II) aspartates on growth, biochemical response, antibacterial activity of mycelium submerged cultures, and fruiting-body formation in basidiomycetes *Ganoderma lucidum* strain 1315, *Grifola umbellate* strain 1622, *Laetiporus sulphureus* strain 120707, *Lentinula edodes* strain F-249, and *Pleurotus ostreatus* strains 69, BK1702 and HK352. The work was aimed at elucidating and comparing action of the variable-valent metal(II) aspartates on the physiological and biochemical parameters of the basidiomycetes. Glucose- and wheat powder-based nutrient media supplemented with 1×10^{-4} mol/l Cu(II), Mn(II), Fe(II), Zn(II), and Co(II) aspartates were used to grow mycelia in submerged culture, comparing growth parameters and production of fruiting bodies. Media without any supplements or with 2×10^{-4} mol/l L-aspartic acid were control. Antimicrobial activity of the metal-containing biosamples against plant pathogenic bacteria *Micrococcus luteus* B-109, *Pectobacterium carotovorum* subsp. *carotovorum* (strains 603 and MI), *Pectobacterium atrosepticum* 1043, *Pseudomonas fluorescens* EL-2.1, *Xanthomonas campestris* B-610 was determined by agar well diffusion method. A pool of secondary metabolites was analyzed by high performance liquid chromatography/high resolution time-of-flight mass-spectrometry method. Metal levels in specimens were quantified by atomic absorption spectroscopy technique. The fruiting body formation was tested in the lab and upon commercial growing. In lab tests, it was established that amino acid chelates of biogenic metals(II) intensify mycelium growth in liquid-submerged culture and fruiting body formation. Chelates of copper, manganese, zinc, and to a lesser extent iron, exhibited the significant growth-promoting effect on the basidiomycetes' mycelium under the submerged culture conditions, especially in respect to lacquered polypore, umbrella polypore, and sulfur-yellow polypore. The additives of $Cu(Asp)_2$, $Mn(Asp)_2$, $Zn(Asp)_2$ showed only slight stimulation or even inhibition of *P. ostreatus* 69 growth. Aspartic acid caused a suppressing impact on mycelia accumulation, regardless of the basidiomycetes' taxonomic characteristics. At the oyster mushroom fermentation in the presence of biogenic metal aspartates, the interstrain distinctions occurred among rapidly and slower growing cultures in relation to the metal chelates' exogenic

action. Thus, in assays with $Cu(Asp)_2$ and $Zn(Asp)_2$, the strain *P. ostreatus* BK1702 had an advantage over others in accumulating biomass. Manganese chelate exerted the most profound positive effect on the fast-growing strain *P. ostreatus* HK352. The latter, however, was suppressed in its development to the greatest extent compared with BK1702 or 69, when the cobalt organic salt appeared in the liquid medium. Earlier we discussed in detail the items related to these substances' increased level resulted from the exogenic action of some compounds. As a biochemical response of cultures to the above aspartates occurrence in the starting nutrient media, the organic substances with double bond, which were not detected in the absence of the same additives, appeared in the growth liquid. These substances were aromatic alcohol 2-phenylethanol, as well as *para*-hydroxyphenylacetic acid, the latter's maximal extracellular concentration evaluated by the analytical signal being observed at $Mn(Asp)_2$ introduction. According to the data we gathered by physicochemical research, the metal(II) aspartates, notably $Mn(Asp)_2$, $Cu(Asp)_2$ in the growth liquid induced the increased level of 5-hydroxymethyl-2-furaldehyde, dihydropyrone (structurally similar to kojic acid), *para*-hydroxyphenylacetic acid, which antioxidative properties are important for mushroom culture. Positive impact of the certain combinations of Mn(II), Cu(II), Fe(II), Zn(II) chelate compounds on *P. ostreatus* vital functions could be efficiently used for elaborating upon scientific foundations and developing the technology of mushroom mineral nutrition, including wide-scale growing. Biogenic metal aspartates could serve as the active ingredient in biopreparations for commercial mushroom culture. Oyster mushroom fruit bodies and mycelium parameters provided by the aspartates implementation allowed us to propose manganese(II) chelate for put into practice.

Keywords: basidiomycetes, physiological and biochemical features, biometals, amino acid chelates, aspartates

Interest in studying the influence of microelements on the physiological, cultural, biochemical properties of edible and medicinal higher mushrooms is due, on the one hand, to wide practical use, on the other, to the uniqueness of basidiomycetes as objects of microbiological and biochemical studies [1, 2]. The cultivation of xylotrophic macromycetes is, in fact, a biotechnological utilization of lignocellulosic waste, effectively converted into human food or feed with high nutritional value and improved bioavailability [3, 4].

Significant efforts to improve production of functional food ingredients and natural nutritional supplements aim at production of microbial biomass enriched with biometals [5-7]. The enrichment of mushroom cultures with substances containing essential elements can be very effective [8]. Therefore, the optimization of mineral nutrition and delivery of metals(II), such as copper, manganese, iron, zinc, cobalt, is essential for artificial cultivation of mushrooms.

The organic form of trace elements has significant advantages over inorganic precursors [9-11], in addition, when used in the form of inorganic salts, metals are unavailable for utilization by organisms [12-14]. Chemical complexation of metals(II) (in particular Cu, Mn, Fe, Zn, Co) with essential amino acids seems to be a solution to the problem of bioavailability [15, 16].

Interaction of metal ions with amino acids at a molar rate of 1:(1-3) leads to chelation through the formation of covalent-coordination bonds [17]. Amino acids and products of enzymatic degradation of proteins. i.e. small peptides are ideal ligands, since they possess at least two functional groups necessary for the formation of a ring structure with a metal [18]. Metal ions bind to carboxyl groups, and in aspartic acid complexes, some metal ions are able to form a chelate bond with amino groups [19, 20]. Therefore, stability constants of a number of metal complexes of aspartic acid are mainly determined by the metal ion affinity for the amino group [21] followed by selective binding of metal ions and their transfer through building complexes with aspartate [22, 23]. Coordination complexes of trace elements increase the absorption of minerals by the body [24]. Bioconjugation of metals with amino acids is a valuable tool for the functionalization of natural proteins and peptides [25, 26].

Our previous work studied the biological activity of fungal substations when the nutrient medium contained inorganic salts of biometals(II) [27]. Min-

eral salts of biometals as an exogenous source of microelements in deep fungal cultures did not provide a positive biological effect. So far, systemic studies to optimize mineral nutrition of cultivated mushrooms using aspartates of biogenic metals have not been carried out.

This paper is the first to discover and characterize the effect of aspartates of metals(II) with variable valency on growth, biochemical response in submerged cultures, antibacterial activity, and fruiting body formation in basidiomycetes of genera *Ganoderma*, *Grifola*, *Laetiporus*, *Lentinula*, and Pleurotus.

Our work aimed to reveal and compare the action of aspartates of metals(II) with variable valency on physiological and biochemical parameters of basidiomycetes.

Materials and methods. Basidiomycetes used in the experiments were zizhi (*Ganoderma lucidum* 1315), umbrella polypore (*Grifola umbellata* 1622), chicken-of-the-woods (*Laetiporus sulphureus* 120707), shiitake mushroom (*Lentinula edodes* F-249), and tree oyster mushroom (*Pleurotus ostreatus*). Strains *P. ostreatus* BK1702 and HK352 were obtained from the collection of basidiomycetes of the Institute of Biochemistry and Physiology of Plants and Microorganisms RAS (IBPRM RAS), *P. ostreatus* 69 was provided by the Institute of Microbiology of the National Academy of Sciences of Belarus. Mushroom cultures were maintained on wort agar (4 degrees Balling) in the dark at 4 °C.

Bacterial test systems for antimicrobial activity assay were selected based on the World Federation for Culture Collection — WFCC, #975, World Data Center of Microorganisms — WDCM, #1021. Plant pathogenic bacteria *Micrococcus luteus* B-109, *Pectobacterium carotovorum* subsp. *carotovorum* (strains 603 and MI), *Pectobacterium atrosepticum* 1043, *Pseudomonas fluorescens* EL-2.1, *Xanthomonas campestris* B-610 were used to measure antibacterial activity of submerged cultures of mushroom mycelium grown in the presence of chelates.

Glucose-asparagine culture medium contained 9.0 g/l D-glucose (300 mM carbon concentration) and 1.5 g/l L-asparagine (20 mM nitrogen concentration); yeast extract culture medium contained 10 g/l D-glucose and 1 g/l yeast extract. For solid media, 1.8-2.0% (w/v) agar was added.

Oyster mushroom liquid inoculum was cultured in wheat flour medium based on decoction of wheat flour at 26 °C. For culture medium, 100 ml of cold water was added to 20 g of premium wheat flour, stirred until homogeneous. The suspension was poured into 900 ml of boiling water in a thin stream, boiled for 2-3 minutes, and autoclaved at a 1 atm extra pressure.

Micrococcus luteus, *Pectobacterium carotovorum* subsp. c*arotovorum*, *Pectobacterium atrosepticum*, and *Pseudomonas fluorescens* were grown on the medium containing 10.0 g/l meat extract, 10.0g/l peptone, and 5.0 g/l NaCl. The medium for *Xanthomonas campestris* had the following composition: 20.0 g/l glucose, 10.0 g/l yeast extract, and 20.0 g/l $CaCO_3$. For solid media, 18 g/l Bacto® Agar (Difco Laboratories Inc., USA) was added, pH was adjusted to 7.2-7.4. All bacteria were cultured at 28 °C.

Cu(II), Mn(II), Fe(II), Zn(II), and Co(II) aspartates added to glucose- or wheat decoction-based media had the general formula $M(Asp)_2$, where Asp is aspartic acid, and were used at a 1×10^{-4} mol/l metal concentration in the medium. A 2×10^{-4} mol/l aspartic acid was another additive. Aspartates were non-hygroscopic free flowing fine powders of lilac color for cobalt, blue for copper, beige for iron, slightly beige for manganese, and white for zinc. Aspartates of metals(II) were derived from direct interaction of metal sulfates with a stoichiometric amount of aspartic acid (Asp) via complexation in neutral conditions,

followed by thermal spraying to dry [28].

For mycelial culture, 14-day-old mycelium of *G. lucidum*, *G. umbellata*, *L. sulphureus*, *L. edodes*, and *P. ostreatus* grown on beer wort agar (4 degrees Balling) was used as an inoculum. A 5 mm wort agar disc taken with a sterile metal punch from one zone of mycelium in a Petri dish was the dose of inoculum. Three disks per 50 ml of liquid medium were put into flasks to grow liquid culture mycelium, and 1 disk was put in the center of a Petri dish to grow mycelium in solid medium. The flour medium was inoculated with mycelium grown on solid beer wort in a Petri dish for 7-10 days.

Concentrated stock solutions of organic and inorganic salts in 50% (v/v) aqueous ethanol were added, with an automatic pipette, into each flask or molten agar cooled to a temperature of ~ 40 °C after autoclaving, prior to pouring Petri dishes. Calculation of the resultant concentration of M^{2+} cations in nutrient media considered the specified dilution. Growth media without aspartates or aspartic acid served as a control for mushroom cultures, including oyster mushroom strains.

P. ostreatus inoculum for growing fruiting bodies was cultured according to the standard technology on durum wheat grain substrate [29]. The grain was exposed to hot water (90 °C) for 20 min and twice (with a 24 h interval) autoclaved at 1 atm for 30 min in 500 ml containers. The wheat grain substrate was inoculated with 14-day old mycelium from submerged cultures grown in flour medium with or without (control) of metal aspartates. Mycelial inoculum was also grown in flour medium with pairs of amino acid chelates added to sterile wheat flour decoction. On days 3, 5, 7, 9, and 14 of growth, the grain substrate was shaken to provide better colonization and inspect contamination by competing microflora. Cultivation lasted 2 weeks at 24-26 °C.

Sunflower husk, the most available and therefore popular lignocellulosic substrate for industrial mushroom farming in Russia, was used in the experiments. Fruiting bodies were grown in lab tests according to a standard technique with pasteurized substrate. After 2 weeks in the dark, containers with colonized substrate were exposed to light in a humid chamber. On day 15 after inoculation, all the studied strains produced primordia. Further, the growth of fruiting bodies was monitored daily. The intensity of the substate colonization by *P. ostreatus* when using myceliated grain (grain spawn) from inoculation with liquid cultures grown with and without $M(Asp)_2$ additive was estimated to assess the effect of metal(II) aspartates. Metal aspartates were also applied to sunflower seed husk lignocellulosic substrate. The production of fruiting bodies was first assessed in the lab tests and then in a mushroom farm.

Mycelium grown in submerged culture was separated using filters previously weighed on an analytical balance, then dried to constant weight, and weighed again. The increase in biomass compared to 3-hour culture was measured in the control (without aspartates or Asp) and in test samples. The growth rate in submerged culture was expressed as dry biomass accumulation per unit time during culturing. The effect of exogenous aspartates or aspartic acid on mycelium liquid culture was expressed as biomass accumulation in the presence and absence of L-aspartates or Asp.

Metal-containing biosamples of fungal origin were obtained as described for inorganic salts [27]. The sensitivity of plant pathogens to fungal bioagents was measured by agar well diffusion method as the radius of the zones of bacterial growth inhibition around the well minus the diameter of the well itself. If the zones were oval in shape, the largest and smallest radius of the zone was measured to calculate the average value. The estimates were deemed indicators

of bactericidal activity.

Metal levels in biosamples were quantified by atomic absorption spectroscopy on an iCE 3000 C093500037 v1.30 spectrometer (Thermo Fisher Scientific, USA) at the Symbiosis IBPRM RAS Center for Collective Use (CCU) of scientific equipment in physical and chemical biology and nanobiotechnology.

Effects of the chelates on the fungal secondary metabolic profile were assessed by comparing control growth media extracts and those with 10^{-4} mol/l metal(II) aspartate (an UltiMate 3000 liquid chromatograph, Thermo Fisher Scientific, USA, coupled with a maXis Impact quadrupole time-of-flight mass spectrometric detector, maXis 4G, Bruker Daltonics, Germany). Separation was carried out on a column (150×2.1 mm) Acclaim ™ 120 C18 (2.2 μm) (Thermo Fisher Scientific, USA) in the mobile phase gradient elution mode.

The mobile phases contained 0.1% formic acid in water with the addition of 5 mM ammonium formate (A) and 0.1% formic acid in acetonitrile (B). Gradient elution mode was as follows: 0 min — 98% A + 2% B, 15 min — 100% B, 20 min — 100% B, 30 min — 98% A + 2% B, with the flow rate of 0.3 ml · min^{-1}, the optimal temperature of the chromatographic column of is 35 °C, the injection volumes of 20 μl. An ionBooster device (Bruker Daltonics, Germany) was used for electrospray ionization. Selection of the optimal parameter values was described in previous works [30, 31].

The registered ion masses ranged within 200-500 Da. Sodium formate (10 mM) in aqueous isopropanol solution of (1:1) was a calibrant. Calibration was performed automatically when recording a chromatogram. The error in determining the ion masses did not exceed ±5 ppm (n = 3).

TargetAnalysis-1.3. software (Bruker Daltonics, Germany) was used for identification. Chromatograms of the total ionic current and chromatograms of extracted ion masses were processed using DataAnalysis-4.1 software (Bruker Daltonics, Germany), the isotope distribution of analytes was drawn up with IsotopePattern software (Bruker Daltonics, Germany).

Microsoft Excel software package was used for data statistical processing. The arithmetic mean values (M) and standard deviations (±SD) are given. The values of the parametric Student's t-test were found for the 95% significance level.

Results. Biogenic metal chelates of amino acida, in particular aspartates, allow the use of microelements in a bioavailable organic form for artificial cultivation of mushrooms. Aspartic acid is used as a substance that forms a compound with metals, while the molar ratio of aspartic acid:metal is 2:1.

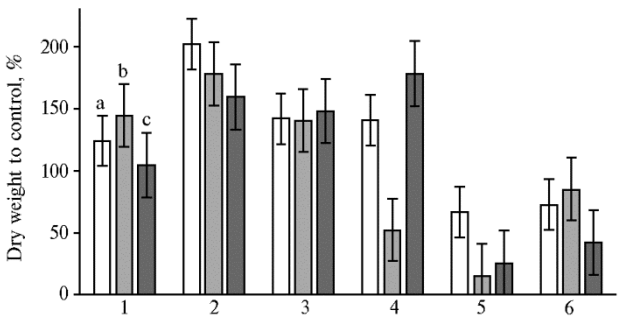


Fig. 1. Biomass accumulation in 21-day old submerged cultures of *Ganoderma lucidum* 1315 (a)**, *Grifola umbellata* 1622** (b)**, and *Laetiporus sulphureus* 120707** (c) **grown in a yeast extract-glucose nutrient medium with additives:** 1 — $Cu(Asp)_2$, 2 — $Mn(Asp)_2$, 3 — $Fe(Asp)_2$, 4 — $Zn(Asp)_2$, 5 — $Co(Asp)_2$, 6 — Asp (M±SD).

In contrast to inorganic salts of biometals(II) in our previous study [27], aspartates which have low toxicity and high biological activity [28] showed a high potential to mycelial biomass production and fruiting body production in lab tests with 1×10^{-4} mol/l trace element level in the nutrient media.

In submerged cultures, most of the additives showed a significant growth-stimulating effect, especially for *Ganoderma lucidum* 1315, *Grifola umbellata* 1622,

Laetiporus sulphureus 120707 (Fig. 1). The dry biomass statistically significantly ($p < 0.05$) exceeded the control values without aspartates.

Stimulation of basidiomycetes by metal cations can be due to their ability to bind and stabilize key compounds for mycelium growth which molecules have various chemical structures, dimensional and charge characteristics, solubility, lipophilic properties, and reactivity. The distribution of charges plays a certain role. The negative charge on the mycelium surface, which promotes complexation during metal-ligand interactions, is provided by chitin, a component of the fungal cell wall [32], and carboxyl, amine, thiol, amide, imine, thioether, and phosphate functional groups [33]. There is not just a chemical reaction between the metal-containing compounds and the mycelium surface, in which the biomass passively binds metal ions according to known physicochemical mechanisms. The interaction with aspartates is metabolically dependent, which undoubtedly contributes to diverse responses of different fungal species to the same metal compound (see Fig. 1).

However, organic salts $Cu(Asp)_2$, $Mn(Asp)_2$, $Zn(Asp)_2$ showed very weak stimulation or even inhibited *P. ostreatus* 69 despite a significant positive effect on the growth other mushroom species (Fig. 2).

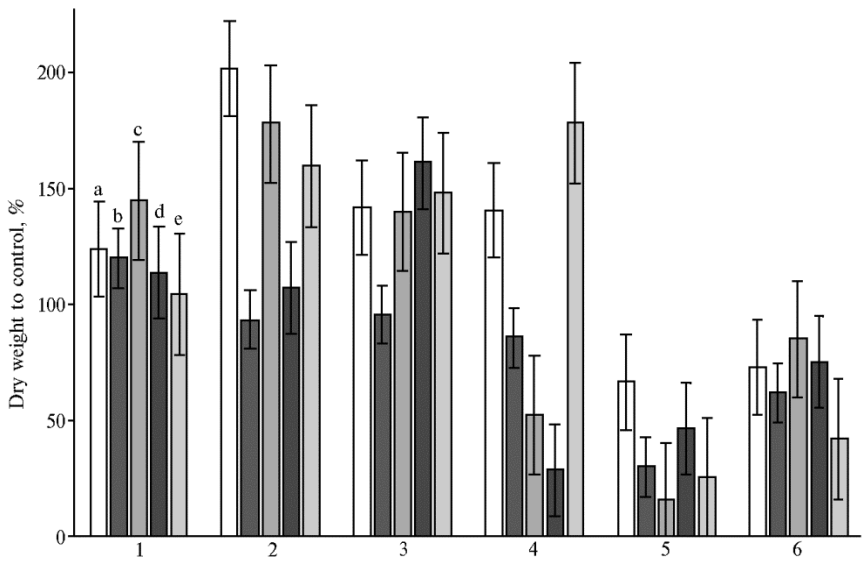


Рис. 2. Biomass accumulation in 21-day old submerged cultures of *Ganoderma lucidum* 1315 (a)**, *Lentinula edodes* F-249** (b)**, *Grifola umbellata* 1622** (c)**, *Pleurotus ostreatus* 69** (d)**, and *Laetiporus sulphureus* 120707** (e) **grown in a yeast extract-glucose nutrient medium with additives:** 1 — $Cu(Asp)_2$, 2 — $Mn(Asp)_2$, 3 — $Fe(Asp)_2$, 4 — $Zn(Asp)_2$, 5 — $Co(Asp)_2$, 6 — Asp (M±SD).

The pronounced dependence on the taxonomic characteristics of basidiomycetes during their artificial cultivation with inorganic metal salts has been experimentally confirmed. On the example of copper and zinc, the species-specificity of the effects of metals was shown for tens of cultures of edible mushrooms of different taxonomic positions [6, 34]. Eleven xylotrophic strains of *Trametes* fungi in submerged cultures significantly differed in the activity of lignin-modifying enzymes in the presence of copper, iron, and manganese salts [35]. Cu(II), Fe(II), and Zn(II) sulfates had different effects on the yield of biomass and polysaccharide metabolites of the fungus *Antrodia cinnamomea* [36].

Thence, the problem arose of selecting a *P. ostreatus* strain for more efficient use of amino acid chelates for spawn and fruiting bode production.

Fungiculture of *P. ostreatus* is promising primarily due to its high productivity, valuable nutritional composition, significant protein content [37, 38], and utilization of substrates unsuitable for any other purposes, namely the non-food waste of agriculture and industry [39-41]. Current studies of *P. ostreatus* growth in liquid media aimed at developing a technology for submerged mycelium culture to produce biomass for feed and food purposes, and as a source of various physiologically active drugs [42, 43] and biotechnologically valuable products [44-46]. Submerged culture is known as a fast and efficient method of producing liquid spawn in the cultivation of this mushroom [47, 48].

To reveal the effects of exogenous aspartates and aspartic acid on slow- and fast-growing strains of *P. ostreatus*, we compared growth of *P. ostreatus* 69, *P. ostreatus* BK1702 and *P. ostreatus* HK352 in a liquid synthetic nutrient medium with glucose and yeast extract supplemented or not supplemented with metal(II) (Cu, Mn, Fe, Zn, Co) L-aspartates and Asp. The dry weight of mycelium characterized the growth rate of *P. ostreatus* strains in the submerged culture. In 28 days after inoculation, the biomass in the absence of additives was 49.00±8.00, 124.86±6.38, and 244.7±15.77 mg/100 ml for the strains 69, BK1702 and HK352, respectively. Dry biomass accumulation in *P. ostreatus* HK352 was the highest.

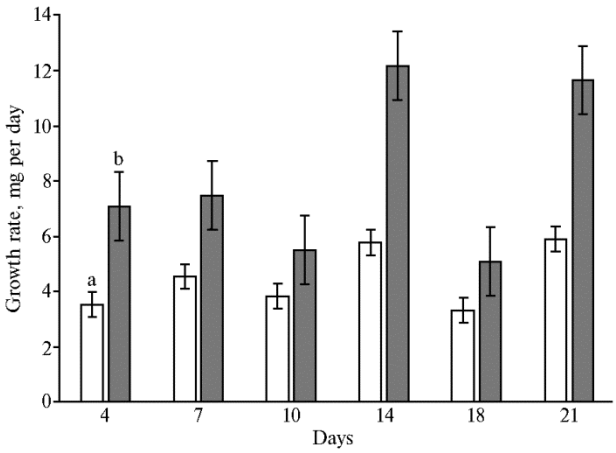


Fig. 3. Growth rate of *Pleurotus ostreatus* 69 (a) **and *P. ostreatus* HK352** (b) **in liquid yeast extract-glucose nutrient medium** (*M*±SD).

The high rate of mycelium growth in liquid culture is associated with advantages over contaminating species, significant competitiveness with extraneous microflora, the ability to utilize various carbon-containing compounds, including those hardly available, of plant waste from agriculture and timber processing industry. *P. ostreatus* HK352 grew up the fastest compared to other oyster mushroom strains, while *P. ostreatus* 69 grew slower than others (Fig. 3).

It turned out that growth characteristics significantly contribute to the sensitivity of *P. ostreatus* submerged cultures to the presence of metal(II) L-aspartates in the nutrient medium (Fig. 4). Aspartic acid showed a weak effect, and interstrain differences were practically not notable in the experiment.

Organic cobalt salt in the medium of the fastest growing strain HK352 suppressed the biomass accumulation to the greatest extent (see Fig. 4). Exogenous $Mn(Asp)_2$ and $Fe(Asp)_2$ caused changes in this parameter of the same or opposite direction compared to the controls. The sensitivity of slow- and fast-growing oyster mushroom strains to $Cu(Asp)_2$ and $Zn(Asp)_2$ was not the same. These treatments were favorable for *P. ostreatus* BK1702 (see Fig. 4). The revealed cultural characteristics should be taken into account when selecting strains for artificial culture.

Trace elements at physiological levels can be incorporated into the active site or act as active modulators of fungal enzymes [49]. It was shown that

the activity of *P. ostreatus* laccase, endo-1,4-β-glucanase, and 1,4-β-glucosidase increases in the presence of zinc and copper [50]. Zinc, copper and iron in the culture medium can have a strong effect on the composition of the fungal cell wall, as well as on the content of polyphenols and polysaccharides, which are involved in the antioxidant, antitumor, immunomodulatory, and other biological activities of basidiomycetes [51, 52].

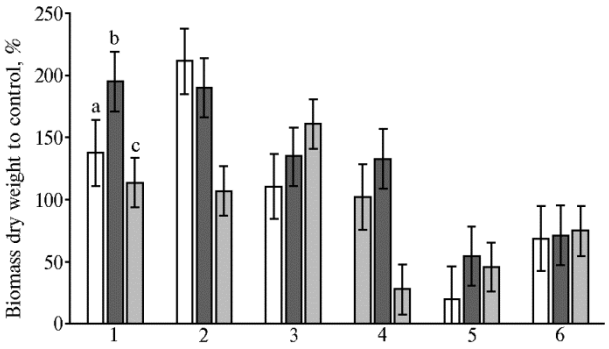


Fig. 4. Biomass accumulation in 21-day old submerged cultures of *Pleurotus ostreatus* HK352 (a), ***P. ostreatus* BK1702** (b), **and *P. ostreatus* 69** (c) **grown in a yeast extract-glucose nutrient medium with additives:** 1 — $Cu(Asp)_2$, 2 — $Mn(Asp)_2$, 3 — $Fe(Asp)_2$, 4 — $Zn(Asp)_2$, 5 — $Co(Asp)_2$, 6 — Asp (M±SD).

However, it should be borne in mind that only relatively low concentrations of trace metals are necessary for the growth and development of fungal cultures and the activity of various enzymes [6]. Cobalt has a rather high toxicity for fungi, and biomineralization of Co sulfate compounds is poorly studied even for metal-tolerant species [53], which explains the higher sensitivity to $Co(Asp)_2$ in all the strains we studied. Possibly, the synergistic effects of the fungal metallothionein compositions, which are responsible for binding metal cations of different toxicity [54], lead to an enhanced bioaccumulation of cobalt and, consequently, a slowed down accumulation of fungal biomass.

We have characterized the biochemical response of submerged cultures of basidiomycetes to the exogenous metal chelates. Analysis of the extracellular levels of metals in the chelates by atomic absorption spectroscopy reveled that during a 2-week submerged growth the amount of metal in the culture liquid decreased several times vs. the initial concentration in the nutrient medium (Table 1). Aspartates were involved in the growth and development of fungal mycelium.

1. General characterization of samples and metal detection in filtrates of fungal culture liquid at different culture ages (M±SD)

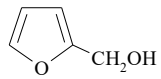
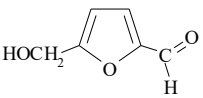
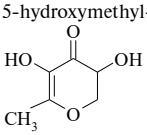
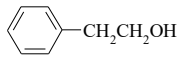
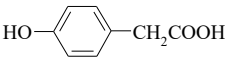
Samples	M^{2+} concentration, μg/ml	
	day 0	day 14
Lentinula edodes Cu 10^{-4} mol/l	25.6	3.78±0.05
Ganoderma applanatum Cu 10^{-4} mol/l	25.6	1.49±0.06
Ganoderma lucidum Cu 10^{-4} mol/l	25.6	3.58±0.07
Grifola umbellata Cu 10^{-4} mol/l	25.6	2.54±0.02
Pleurotus ostreatus Cu 10^{-4} mol/l	25.6	3.83±0.01
Laetiporus sulphureus Cu 10^{-4} mol/l	25.6	2.51±0.03
Lentinula edodes Fe 10^{-4} mol/l	22.4	8.14±0.02
Ganoderma applanatum Fe 10^{-4} mol/l	22.4	2.90±0.01
Ganoderma lucidum Fe 10^{-4} mol/l	22.4	2.50±0.02
Grifola umbellata Fe 10^{-4} mol/l	22.4	3.44±0.01
Pleurotus ostreatus Fe 10^{-4} mol/l	22.4	3.61±0.01
Laetiporus sulphureus Fe 10^{-4} mol/l	22.4	1.93±0.01
Lentinula edodes Mn 10^{-4} mol/l	22.0	7.45±0.07
Ganoderma applanatum Mn 10^{-4} mol/l	22.0	0.33±0.01
Ganoderma lucidum Mn 10^{-4} mol/l	22.0	4.30±0.01
Grifola umbellata Mn 10^{-4} mol/l	22.0	6.39±0.19
Pleurotus ostreatus Mn 10^{-4} mol/l	22.0	6.98±0.02
Laetiporus sulphureus Mn 10^{-4} mol/l	22.0	6.26±0.02
Lentinula edodes Zn 10^{-4} mol/l	26.0	3.82±0.03
Ganoderma applanatum Zn 10^{-4} mol/l	26.0	3.12±0.01

Continued Table 1

Ganoderma lucidum Zn 10^{-4} mol/l	26.0	4.04±0.02
Grifola umbellata Zn 10^{-4} mol/l	26.0	3.35±0.01
Pleurotus ostreatus medium1 Zn 10^{-4} mol/l	26.0	2.66±0.07
Laetiporus sulphureus Zn 10^{-4} mol/l	26.0	2.95±0.01
Lentinula edodes Co 10^{-4} mol/l	23.6	8.48±0.01
Ganoderma applanatum Co 10^{-4} mol/l	23.6	5.17±0.01
Ganoderma lucidum Co 10^{-4} mol/l	23.6	7.86±0.01
Grifola umbellata Co 10^{-4} mol/l	23.6	5.56±0.03
Pleurotus ostreatus Co 10^{-4} mol/l	23.6	8.89±0.01
Laetiporus sulphureus Co 10^{-4} mol/l	23.6	7.87±0.03

The effect of the added metal(II) chelates on the pool of secondary metabolites of fungal cultures was assessed by high-performance liquid chromatography/high-resolution time-of-flight mass spectrometry. The method combines simple and fast sample preparation with the identification and sensitive determination of low molecular weight secondary metabolites in biological samples.

2. Main characteristics of extracellular compounds in *Lentinula edodes* culture medium with 10^{-4} mol/l metal(II) aspartate (high-resolution mass spectrometry of positive ions $[M+H]^+$)

Analite	Empirical formula	$[M+H]^+$, m/z	Signals for samples with M(Asp)2					
			Cu	Mn	Fe	Zn	Co	C
 2-hydroxymethylfuran	$C_5H_6O_2$	99.0441	500	nf	500	6000	nf	nf
 5-hydroxymethyl-2-furaldehyde	$C_6H_6O_3$	127.0389	3000	4000	3000	nf	3000	н.о.
 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one	$C_6H_8O_4$	145.0495	6000	600	8000	nf	nf	nf
 2-phenylethanol	$C_8H_{10}O$	123.0804	1000	nf	1000	nf	nf	nf
 4-hydroxyphenylacetic acid)	$C_8H_8O_3$	153.0546	2500	5000	nf	nf	nf	nf

N o t e. C —no additives, nf — not found.

The extracts from the nutrient medium supplemented with 10^{-4} mol/l metal(II) aspartates were compared with the control (Table 2). The results showed that aspartates of some metals(II) increased the production of 5-hydroxymethyl-2-furaldehyde and dihydropyrone (see Table 2) in the culture. Our previous works discussed in sufficient detail the data on the increased content of these substances as a result of exogenous action of some compounds [55, 56]. Thus, the medium for submerged culture of basidiomycete *Lentinula edodes* in the presence of 1,5-diphenyl-3-selenpentanedione-1,5 $C_6H_5COCH_2SeCH_2COC_6H_5$, the diacetophenonyl selenide, bis(benzoylmethyl)selenide, DAFS-25 [57], which leads to an increase in the growth rate of the fungus and the activity of its extracellular lectins, and also serves as an antioxidant, contains 2-hydroxymethylfuran, 5-hydroxymethyl-2-furaldehyde, 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one [55].

In the presence of transition metals (Cu, Mn, Fe, Co) [58] in the form of

aspartates, the conversion of carbohydrates into 5-hydroximethylfurfural [59] seems to occur via catalytic hydrolysis and dehydration of hexose-containing components of the nutrient medium leading to the formation of 5-hydroxymethyl-2-furaldehyde. The latter inhibits tyrosinase which is responsible for the synthesis of the fungal pigment melanin, that is, it serves as an inhibitor of melanogenesis [60]. The recognized importance should be mentioned for an innovative way of chemical utilization of hexose-containing raw materials to produce 5-hydroxymethyl-2-furaldehyde, a promising intermediate product for chemical industry (production of food additives, pharmaceuticals, polymeric materials, additives to motor oils and biofuel precursors) [55, 59, 60].

Some samples of culture liquid contained 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (see Table 2). Note the similarity of the structure of the discovered dihydropyranone and 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one, also called kojic acid. Kojic acid is a known inhibitor of melaninogenesis in fungi, which is mediated by an increase in tyrosinase activity and occurs under conditions of oxidative stress [61, 62]. Enhanced synthesis of this substance as compared to the culture without additives indicates an increased antioxidant activity of submerged culture mycelium grown in the presence of aspartates we studied in the work. This is another evidence of an increase in the adaptive potential of a mushroom culture grown in the presence of aspartates, especially chelates of copper, iron, and manganese.

Organic substances with double bonds, including phenolic ones, not registered in the control, appeared in the culture medium as a biochemical response of fungal strains to aspartates. $Mn(Asp)_2$ caused the maximum extracellular concentration of para-hydroxyphenylacetic acid, as it followed from the intensity of the analytical signal (see Table 2). This phenolic acid was detected in the mycelium of some higher fungi, e.g. *Chroogomphus rutilus* [63], *Suillus granulatus* [64], and *Clitocybe nuda* [65].

It is believed that the antioxidant activity of mushroom extracts correlates with the total content of phenolic substances [66]. It was 4-hydroxyphenylacetic acid that contributed to the accumulation of phenols in the mycelium to the greatest extent than other exogenous para-hydroxy-substituted phenolic compounds [56].

The increase in the number of compounds with antioxidant properties that we found in the presence of a number of aspartates in submerged basidiomycete cultures is consistent with the data available for other organisms. It is known that the antioxidant properties of compounds can be enhanced when they are used in various synergistic compositions with other antioxidants [67] and with substances that do not in themselves exhibit this biological activity [68, 69]. The aspartates of Cu, Mn, Zn, and Mg were characterized by inhibitory activity against xanthine oxidase and NADPH oxidase, reducing the production of oxygen radicals by these enzymes. The most active inhibitors of oxidative stress were aspartates of the transition metals copper [70, 71] and manganese [72]. The effect of zinc [73] and magnesium [74] aspartates can be associated with the influence on the rate of spontaneous superoxide ion dismutation. It is reasonable to assume that $Cu(Asp)_2$, $Mn(Asp)_2$ and $Zn(Asp)_2$ act as biomimetics of Cu-, Zn-, or Mn-dependent superoxide dismutases. The antioxidant properties of zinc aspartate as an effective inhibitor of production of the most reactive hydroxyl radicals [75] were also demonstrated in experiments with laboratory animals [76].

The obtained results suggest that amino acid chelates of copper, iron, and manganese can affect the production of compounds important for mushroom culture adaptation.

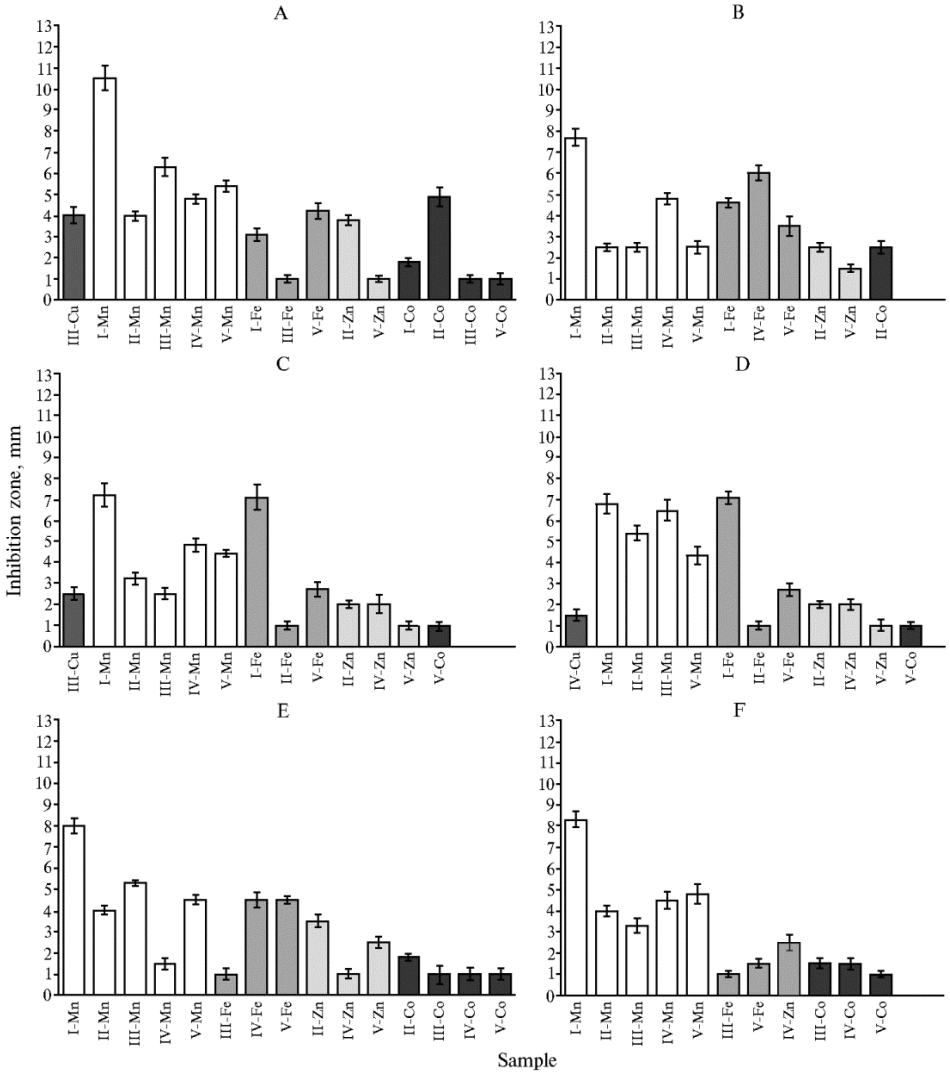


Рис. 5. Inhibitory activity of Cu-, Mn-, Fe-, Zn-, and Co-containing biocomposites based on *Pleurotus ostreatus* HK352 (I), ***Ganoderma lucidum* 1315** (II), ***Lentinula edodes* F-249** (III), ***Grifola umbellata* 1622** (IV), ***Laetiporus sulphureus* 120707** (V) **against bacterial cultures:** A — *Pectobacterium atrosepticum* 1043, B — *Pectobacterium carotovorum* subsp. *carotovorum* MI, C — *Xanthomonas campestris* B-610, D — *Pectobacterium carotovorum* subsp. *carotovorum* 603, E — *Micrococcus luteus* B-109, F — *Pseudomonas fluorescens* EL-2.1 (*M*±SD, agar diffusion method).

To better characterize the biochemical response of basidiomycetes to metal chelates with aspartic acid, we measured the antibacterial activity of products from submerged mushroom cultures grown in the presence of chelates against plant pathogenic bacteria *Micrococcus luteus*, *Pectobacterium carotovorum* subsp. *carotovorum* (two strains), *Pectobacterium atrosepticum*, *Pseudomonas fluorescens*, *Xanthomonas campestris*. A total pool of extracellular metabolites was extracted from submerged cultures of *G. lucidum*, *G. umbellata*, *L. sulphureus*, *L. edodes*, and *P. ostreatus*. The extracts contained products of biotransformation of the introduced organic metal(II) complexes by fungi. The bactericidal effect of the studied metal-containing biocomposites was assessed with plant pathogenic bacteria as a test culture by agar diffusion methods (Fig. 5).

At first glance, the discussed above property of cobalt(II) to inhibit the growth of microorganisms does not correlate with the low bactericidal activity of

its biocomposites of fungal origin. Only in 50% of cases, Co-containing biosamples showed at least minimal toxicity towards plant pathogens, and only *G. lucidum*-derived composites had a zone of inhibition of more than 2 mm in tests with *P. atrosepticum* (Fig. 5, A) and *P. carotovorum* MI (see Fig. 5, C). The M(II) concentration in potentially antibacterial samples with cobalt and other metals was the same. It can be assumed that the production of extracellular metabolites, reduced due to a slow increase in biomass in the presence of cobalt aspartate, does not make a sufficient contribution to the formation of Co-composites. In our opinion, production of fungal substances with low content of extracellular antimicrobial metabolites leads to a sharply reduced ability to suppress the studied plant pathogenic bacteria. Additional indirect confirmation of the importance of the chemical environment of the metal should be considered when using its compounds to combat plant pathogens.

Aspartates of other metals which stimulated growth of the basidiomycetes (see Fig. 1) differed quite significantly in the effect of metal-containing biosubstances on bacterial plant pathogens. Virtually no inhibition of bacterial growth occurred in the presence of copper biocomposites. Only from the culture liquid of *Lentinula edodes* it was possible to obtain an antibacterial sample characterized by a noticeable zone of growth inhibition for *P. atrosepticum* (see Fig. 5, A) and *X. campestris* (see Fig. 5, C). The biocomposite from *Grifola umbellata* was ineffective (see Fig. 5, D). Probably, the induction of copper-containing lytic enzymes in the presence of this cation, noted in a large number of microorganisms [44, 46] and necessary for assimilation of nutrient substrate by bacteria, favors the survival of plant pathogens. This is also facilitated by bioproduction of bacterial copper reductases [32] which catalyze the transformation of metal into less toxic chemical forms.

The manganese is believed to be responsible for the induction of manganese peroxidase in microorganisms, primarily higher fungi. However, studies [50] have shown a negative correlation between the maximum of Mn-peroxidase activity and the exogenous Mn concentration. It was found that this enzymatic activity in the presence of Mn is lower than in its absence. Possibly, this was partly due to the high antimicrobial activity of fungal substances that contain manganese in a bioavailable organic form as observed in our experiment (see Fig. 5). When they act on test bacteria, the indicated peroxidase activity may decrease. Simultaneously, upon stimulation with Mn^{2+} ions, the activity of laccases (and lignin peroxidases) may not be detected, as is the case in microorganisms with different taxonomic affiliation and ecological strategy [77]. Indeed, the highest quantitative characteristics of bactericidal action were found in the variant with $Mn(Asp)_2$. The effect of this manganese chelate as a component of the submerged culture medium used to produce potentially bactericidal samples was much more pronounced than that of $Zn(Asp)_2$ and especially of $Fe(Asp)_2$, and manifested itself against test strains of plant pathogens (see Fig. 5). The *Pectobacterium carotovorum* subsp. *carotovorum* 603 insensitive to the manganese-containing inhibitor from *Grifola umbellate* was the only exception (see Fig. 5, D).

In our tests, the highest antibacterial effect with the absolute maximum size of zone of *P. atrosepticum* growth inhibition was characteristic of the bioagents based on extracellular metabolites of *Pleurotus ostreatus* grown in the culture media with $Mn(Asp)_2$ (see Fig. 5, A). The same producer of the organic part of the biocomposite containing Zn(II) is in second place. However, this Zn-containing bioagent acted exclusively on the plant pathogenic *P. carotovorum* 603 (see Fig. 5, D).

Other Zn(II) fungal-based composites showed moderate bactericidal activity and were effective in less than half of the tests with $Zn(Asp)_2$. We failed to produce a Zn-containing antibacterial agent containing extracellular metabolites of one of the basidiomycetes, *Lentinula edodes*. That is, along with the selective growth inhibitor of *P. carotovorum* 603, all fungal metal-containing biosamples for which the size of bacterial growth inhibition zone was at least 8 mm were derived using $Mn(Asp)_2$ (see Fig. 5, E, F).

The iron aspartate-based bioagents in some tests were slightly inferior in bactericidal action (see Fig. 5, C) or even showed slightly greater efficiency (see Fig. 5, D) compared to $Mn(Asp)_2$, but these results were obtained using *Pleurotus ostreatus* only. Iron biocomposites of fungal origin had almost no effect on *Ps. fluorescence* (see Fig. 5, F).

Soil microorganisms, including members of the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Azotobacter*, can be used to increase the phytoavailability of minerals, to improve plant health and for biocontrol [24, 32]. Such lab tests were successful. However, the practical use of the bacterial biocontrol method requires a comprehensive consideration of the originality and uniqueness of the plant protection strategy depending on the cultivar, the chemical properties of the soil, and the external environment. The ability of potentially biocontrol preparations to compete with resident rhizobacteria during root colonization in natural ecosystems varies greatly and is not always predictable. Nevertheless, the problem of the interaction of drugs based on the biocomposites we proposed with rhizobacteria-based formulations is relevant. The fact that in our experiments the bactericidal activity against *Pseudomonas fluorescens* turned out to be the least pronounced (see Fig. 5) is of interest to address the problem of negative impact of biocontrol drugs on resident rhizobacteria. In particular, biologicals based on biocomposites to which the *P. fluorescens* is tolerant or those compatible with useful rhizobacteria can be used. Thence further studies are promising to identify the fungicidal properties of metal-containing agents of fungal origin.

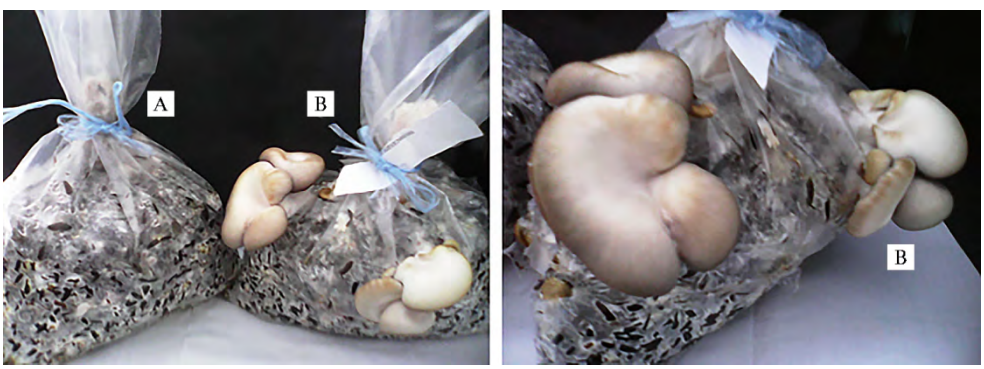


Рис. 6. Growth of *Pleurotus ostreatus* NK352 mycelium on sunflower husk substrate. The grain spawn was produced in liquid cultures without additives (A) and with 1.0×10^{-4} mol/l $Mn(Asp)_2$ (B).

Our experiments assessed the possibility of using metals(II) L-aspartates as an active ingredient of biopreparations with complex (growth-stimulating and adaptogenic) action for industrial cultivation of *P. ostreatus*. The accumulation of biomass of submerged mycelium of *P. ostreatus* strains under the influence of exogenous $Mn(Asp)_2$ (see Fig. 2) changed in the same mode as that in control, so an assumption can be made about the most pronounced positive effect of manganese chelate on the fast-growing *P. ostreatus* HK352 strain. The

liquid inoculum was obtained in the medium with a decoction of wheat flour, as well as in the media supplemented with Cu, Mn, Fe, or Zn aspartates. The colonization of grain substrate by *P. ostreatus* mycelium upon inoculation with liquid culture grown in the presence of the listed organic salts was more intensive than in the control.

The mycelium obtained on a wheat grain substrate was used as an inoculum for growing fruiting bodies on a sunflower seed husk substrate. Given the intensity of colonization of this lignocellulosic substrate by *P. ostreatus* mycelium grown in the presence of $Mn(Asp)_2$ (Fig. 6), the manganese(II) chelate had the most favorable effect on the formation of oyster mushroom fruiting bodies.

Oyster mushroom growth is peculiar depending on trace metal chelates of amino acids as additives, which is significant to select proper *P. ostreatus* strains for biotechnological cultivation. E.g., the growth index under the influence of exogenous $Fe(Asp)_2$ (see Fig. 2) changes in the opposite way to the growth parameters of the control strains (see Fig. 2), that is, the greatest tolerance to iron chelate, including its mixtures with other aspartates, may be expected in the slow-growing strain *P. ostreatus* 69. At the same time, the patterns of *P. ostreatus* sensitivity to $Cu(Asp)_2$ and $Zn(Asp)_2$ (see Fig. 2) shows a clear advantage of moderately growing strain BK1702.

The impact of mixtures of aspartates on the growth and development of cultures of three *P. ostreatus* strains was assessed based on combination of growth parameters of the cultures and the strain tolerance to the exogenous effect of the metal chelates.

We added metal aspartates to the liquid medium to produce liquid spawn, then to treat the grain substrate for the production of grain spawn, and finally to treat the lignocellulosic substrate to grow *P. ostreatus* fruiting bodies. It turned out that $Mn(Asp)_2 + Zn(Asp)_2$ and $Cu(Asp)_2 + Zn(Asp)_2$ have the greatest stimulating effect on the *P. ostreatus* submerged mycelium growth and the rate of grain substrate colonization, in addition, the fruiting occurs faster and is more intensive.

Our findings allow us to propose some principles for application of trace metal organic salts in growing oyster mushroom mycelium. Combination of manganese and zinc aspartates in an equimolar ratio provides the fastest growth of *P. ostreatus* cultures. In *P. ostreatus* strains with a moderate growth rate, combination of copper and zinc aspartates at a 1:2 molar ratio enhances submerged mycelium production in all nutrient media. In slow-growing *P. ostreatus* strains, treatment of the dense substrate with iron and zinc aspartates in a molar ratio of 1:2 leads to higher fruiting body formation. Chelated compounds of biogenic metals in the above combinations had a positive effect on the *P. ostreatus* vital activity.

Thus, amino acid chelated biogenic metals(II) are the factors for the intensification of spawn and fruiting body lab production of macrobasidiomycetes. Our findings reveal a significant growth-stimulating effect of chelates of copper, manganese, zinc and, to a lesser extent, iron on the submerged culture of basidiomycetes, especially *Ganoderma lucidum*, *Grifola umbelata*, and *Laetiporus sulphureus*. $Cu(Asp)_2$, $Mn(Asp)_2$ and $Zn(Asp)_2$ weakly stimulate or even inhibit the growth of *Pleurotus ostreatus* 69 though have a pronounced positive effect on the other fungal cultures. Aspartic acid exhibits an inhibitory effect regardless of the taxonomic characteristics of basidiomycetes. The oyster mushroom growing in the presence of biogenic metal aspartates revealed differences between fast- and slow-growing strains in the response to the exogenous metal chelates. $Cu(Asp)_2$ and $Zn(Asp)_2$ are favorable for the *P. ostreatus* BK1702 strain while $Mn(Asp)_2$ has

the most pronounced positive effect on the fast-growing *P. ostreatus* HK352 strain. The physicochemical results indicate that metal(II) aspartates, especially $Mn(Asp)_2$ and $Cu(Asp)_2$, affect the biosynthesis of 5-hydroxymethylfurfural, dihydropyrone (structural analogue of kojic acid) and para-hydroxyphenylacetic acid, i.e. the compounds with antioxidant properties which are important for mushroom culture adaptiveness. The positive effect of combinations of chelated Mn(II), Cu(II), Fe(II), and Zn(II) compounds on the vital activity of *P. ostreatus* should be studied to reveal fundamentals of mineral nutrition of higher mushrooms and can be used in practical mushroom farming. This work suggests a biotechnological scheme for the application of organic salts of microelements to culture oyster mushroom mycelium. The obtained results indicate high potential of mixtures of metal(II) chelates (aspartates) when used for production of mycelial biomass and mushroom fruiting bodies in lab conditions, as well as the prospects of commercial biologicals based on aspartates of biogenic metals. The patterns of oyster mushroom mycelial growth and formation of fruiting bodies in the presence of $Mn(Asp)_2$ allow us to recommend the manganese(II) chelate for practical use.

REFERENCES

1. Ogidi C.O., Oyetayo V.O., Akinyele B.J., De Carvalho C.A., Kasuya M.C.M. Food value and safety status of raw (unfermented) and fermented higher basidiomycetes, *Lenzites quercina* (L.) P. Karsten. *Preventive Nutrition and Food Science*, 2018, 23(3): 228-234 (doi: 10.3746/pnf.2018.23.3.228).
2. Rathore H., Prasad S., Kapri M., Tiwari A., Sharma S. Medicinal importance of mushroom mycelium: mechanisms and applications. *Journal of Functional Foods*, 2019, 56: 182-193 (doi: 10.1016/j.jff.2019.03.016).
3. Guo L.-Q., Lin J.-Y., Lin J.-F. Non-volatile components of several novel species of edible fungi in China. *Food Chemistry*, 2007, 100(2): 643-649 (doi: 10.1016/j.foodchem.2005.09.087).
4. Akyüz M., Kirbağ S. Effect of various agro-residues on nutritive value of *Pleurotus eryngii* (DC. ex Fr.) Quel. var. *ferulae* Lanzi. *Journal of Agricultural Sciences*, 2010, 16: 83-88.
5. Figlas D., Oddera M., Curvetto N. Bioaccumulation and bioavailability of copper and zinc on mineral-enriched mycelium of *Grifola frondosa*. *Journal of Medicinal Food*, 2010, 13(2): 469-475 (doi: 10.1089/jmf.2008.0284).
6. Matute R.G., Serra A., Figlas D., Curvetto N. Copper and zinc bioaccumulation and bioavailability of *Ganoderma lucidum*. *Journal of Medicinal Food*, 2011, 14(10): 1273-1279 (doi: 10.1089/jmf.2010.0206).
7. Poursaeid N., Azadbakht A., Balali G.R. Improvement of zinc bioaccumulation and biomass yield in the mycelia and fruiting bodies of *Pleurotus florida* cultured on liquid media. *Applied Biochemistry and Biotechnology*, 2015, 175(7): 3387-3396 (doi: 10.1007/s12010-015-1510-9).
8. Liu J., Bao W., Jiang M., Zhang Y., Zhang X., Liu L. Chromium, selenium, and zinc multimineral enriched yeast supplementation ameliorates diabetes symptom in streptozocin-induced mice. *Biological Trace Element Research*, 2012, 146(2): 236-245 (doi: 10.1007/s12011-011-9248-x).
9. Zafar M.H., Fatima M. Efficiency comparison of organic and inorganic minerals in poultry nutrition: a review. *PSM Veterinary Research*, 2018, 3(2): 53-59.
10. Gharibzahedi S.M.T., Jafari S.M. The importance of minerals in human nutrition: bioavailability, food fortification, processing effects and nanoencapsulation. *Trends in Food Science & Technology*, 2017, 62: 119-132 (doi: 10.1016/j.tifs.2017.02.017).
11. Egorova K.S., Ananikov V.P. Toxicity of metal compounds: knowledge and myths. *Organometallics*, 2017, 36(21): 4071-4090 (doi: 10.1021/acs.organomet.7b00605).
12. Tuhy Ł., Samoraj M., Michalak I., Chojnacka K. The application of biosorption for production of micronutrient fertilizers based on waste biomass. *Applied Biochemistry and Biotechnology*, 2014, 174(4): 1376-1392 (doi: 10.1007/s12010-014-1074-0).
13. Witkowska Z., Rusek P., Witek-Krowiak A., Chojnacka K., Tuhy Ł., Samoraj M. Production of dietary feed supplements enriched in microelements in a pilot plant biosorption system. *International Journal of Environmental Science and Technology*, 2016, 13(4): 1089-1098 (doi: 10.1007/s13762-016-0945-z).
14. Gins M., Gins V., Motyleva S., Kulikov I., Medvedev S., Kononkov P., Pivovarov V. Mineral composition of amaranth (*Amaranthus* L.) seeds of vegetable and grain usage by ARHIVBSP se-

lection. *Potravinarstvo Slovak Journal of Food Sciences*, 2018, 12(1): 330-336 (doi: 10.5219/863).
15. Beshkenadze I., Chagelishvili A., Begheluri G., Zhorzholiani N., Gogaladze M., Urotadze S., Klarjeishvili N. New generation premixes for rabbit nutrition. *Annals of Agrarian Science*, 2016, 14(4): 288-291 (doi: 10.1016/j.aasci.2016.06.001).
16. De Marco M., Zoon M.V., Margetyal C., Picart C., Ionescu C. Dietary administration of glycine complexed trace minerals can improve performance and slaughter yield in broilers and reduces mineral excretion. *Animal Feed Science and Technology*, 2017, 232: 182-189 (doi: 10.1016/j.anifeedsci.2017.08.016).
17. Canaj A.B., Kakaroni F.E., Collet A., Milios C.J. α-Amino acids: natural and artificial building blocks for discrete polymetallic clusters. *Polyhedron*, 2018, 151: 1-32 (doi: 10.1016/j.poly.2018.05.005).
18. Yu X., Chau M.C., Tang W.K., Siu C.K., Yao Z.P. Self-assembled binuclear Cu(II)-histidine complex for absolute configuration and enantiomeric excess determination of naproxen by tandem mass spectrometry. *Analytical Chemistry*, 2018, 90(6): 4089-4097 (doi: 10.1021/acs.analchem.7b05407).
19. Sajadi S.A.A. Metal ion-binding properties of the L-aspartic acid and tartaric acid, a coparative investigation. How can be increased the dosage of mineral absorption in the body. *Advances in Bioscience and Biotechnology*, 2010, 1(4): 354-360 (doi: 10.4236/abb.2010.14047).
20. Boles G.C., Hightower R.L., Coates R.A., McNary C.P., Berden G., Oomens J., Armentrout P.B. Experimental and theoretical investigations of infrared multiple photon dissociation spectra of aspartic acid complexes with Zn^{2+} and Cd^{2+}. *The Journal of Physical Chemistry B*, 2018, 122(14): 3836-3853 (doi: 10.1021/acs.jpcb.8b00801).
21. Murphy J.M., Gaertner A.A., Williams T., McMillen C.D., Powell B.A., Brumaghim J.L. Stability constant determination of sulfur and selenium amino acids with Cu(II) and Fe(II). *Journal of Inorganic Biochemistry*, 2019, 195: 20-30 (doi: 10.1016/j.jinorgbio.2019.03.001).
22. Künzle M., Eckert T., Beck T. Metal-assisted assembly of protein containers loaded with inorganic nanoparticles. *Inorganic Chemistry*, 2018, 57(21): 13431-13436 (doi: 10.1021/acs.inorgchem.8b01995).
23. Hamley I.W. Protein assemblies: nature-inspired and designed nanostructures. *Biomacromolecules*, 2019, 20(5): 1829-1848 (doi: 10.1021/acs.biomac.9b00228).
24. Garg M., Sharma N., Sharma S., Kapoor P., Kumar A., Chunduri V., Arora P. Biofortified crops generated by breeding, agronomy, and transgenic approaches are improving lives of millions of people around the world. *Frontiers in Nutrition*, 2018, 5: 12 (doi: 10.3389/fnut.2018.00012).
25. Biswal B.K., El Sadany M., Kumari D., Sagar P., Singhal N.K., Sharma S., Stobdan T., Shanmugam V. Twin function of zein−zinc coordination complex: wheat nutrient enrichment and nanoshield against pathogenic infection. *ACS Sustainable Chemistry & Engineering*, 2018, 6(5): 5877-5887 (doi: 10.1021/acssuschemeng.7b04174).
26. Ohata J., Martin S.C., Ball Z.T. Metal-mediated functionalization of natural peptides and proteins: panning for bioconjugation gold. *Angewandte Chemie International Edition*, 2019, 58(19): 6176-6199 (doi: 10.1002/anie.201807536).
27. Tsivileva O.M., Perfil'eva A.I., Ivanova A.A., Pavlova A.G. *Biomika*, 2018, 10(2): 210-213 (doi: 10.31301/2221-6197.bmcs.2018-30) (in Russ.).
28. Voronin S.P., Golubov I.I., Gumenyuk A.P., Sinolitskii M.K. *Biodostupnaya forma mikroelementnykh dobavok v kormovye smesi dlya zhivotnykh i ptits. Pat. 2411747 (RF). Zayavl. 25.12.2008. Opubl. 20.02.2011. Byul. № 5* [A bioavailable form of trace element-based additives to feed for animals and birds. Patent 2411747 (RF). Appl. 25.12.2008. Publ. 20.02.2011. Bull. № 5] (in Russ.).
29. Stamets P. *Growing gourmet and medicinal mushrooms*. Berkeley, 1993.
30. Amelin V.G., Korotkov A.I., Volkova N.M. High-resolution time-of-flight mass spectrometry combined with high-performance liquid chromatography for detection and determination of quinolones and sulfonamides in foods. *Journal of Analytical Chemistry*, 2015, 70(14): 1629-1634 (doi: 10.1134/S1061934815140026).
31. Amelin V.G., Andoralov A.M., Volkova N.M., Korotkov A.I., Nikeshina T.B., Sidorov I.I., Timofeev A.A. *Analitika i kontrol'*, 2015, 19(2): 189-207 (doi: 10.15826/analitika.2015.19.2.010) (in Russ.).
32. Hansda A., Kumar V., Anshumali. A comparative review towards potential of microbial cells for heavy metal removal with emphasis on biosorption and bioaccumulation. *World Journal of Microbiology and Biotechnology*, 2016, 32(10): 170 (doi: 10.1007/s11274-016-2117-1).
33. Javanbakht V., Alavi S.A., Zilouei H. Mechanisms of heavy metal removal using microorganisms as biosorbent. *Water Science and Technology*, 2014, 69(9): 1775-1787 (doi: 10.2166/wst.2013.718).
34. Rabinovich M., Figlas D., Delmastro S., Curvetto N. Copper-and zinc-enriched mycelium of *Agaricus blazei* Murrill: bioaccumulation and bioavailability. *Journal of Medicinal Food*, 2007, 10(1): 175-183 (doi: 10.1089/jmf.2005.064).
35. Kachlishvili E., Asatiani M.D., Kobakhidze A., Elisashvili V. Evaluation of lignin-modifying

enzyme activity of *Trametes* spp. (Agaricomycetes) isolated from Georgian forests with an emphasis on *T. multicolor* biosynthetic potential. *International Journal of Medicinal Mushrooms*, 2018, 20(10): 971-987 (doi: 10.1615/IntJMedMushrooms.2018028186).
36. Lin T.Y., Tseng A.J., Chao C.H., Lu M.K. Microelements induce changes in characterization of sulfated polysaccharides from *Antrodia cinnamomea*. *International Journal of Biological Macromolecules*, 2018, 120(A): 952-958 (doi: 10.1016/j.ijbiomac.2018.08.112).
37. Majesty D., Ijeoma E., Winner K., Prince O. Nutritional, anti-nutritional and biochemical studies on the oyster mushroom, *Pleurotus ostreatus*. *EC Nutrition*, 2019, 14(1): 36-59.
38. Mutukwa I.B., Hall III C.A., Cihacek L., Lee C.W. Evaluation of drying method and pretreatment effects on the nutritional and antioxidant properties of oyster mushroom (*Pleurotus ostreatus*). *Journal of Food Processing and Preservation*, 2019, 43(4): e13910 (doi: 10.1111/jfpp.13910).
39. Nam W.L., Phang X.Y., Su M.H., Liew R.K., Ma N.L., Rosli M.H.N.B., Lam S.S. Production of bio-fertilizer from microwave vacuum pyrolysis of palm kernel shell for cultivation of Oyster mushroom (*Pleurotus ostreatus*). *Science of The Total Environment*, 2018, 624: 9-16 (doi: 10.1016/j.scitotenv.2017.12.108).
40. Phan C.W., Wang J.K., Tan E.Y.Y., Tan Y.S., Sathiya Seelan J.S., Cheah S.C., Vikineswary S. Giant oyster mushroom, *Pleurotus giganteus* (Agaricomycetes): current status of the cultivation methods, chemical composition, biological, and health-promoting properties. *Food Reviews International*, 2019, 35(4): 324-341 (doi: 10.1080/87559129.2018.1542710).
41. Sangeetha K., Senthilkumar G., Panneerselvam A., Sathammaipriya N. Cultivation of oyster mushroom (*Pleurotus* sp.) using different substrates and evaluate their potentials of antibacterial and phytochemicals. *International Journal of Research in Pharmaceutical Sciences*, 2019, 10(2), 997-1001 (doi: 10.26452/ijrps.v10i2.371).
42. Vetvicka V., Gover O., Karpovsky M., Hayby H., Danay O., Ezov N., Hadar Y., Schwartz B. Immune-modulating activities of glucans extracted from *Pleurotus ostreatus* and *Pleurotus eryngii*. *Journal of Functional Foods*, 2019, 54: 81-91 (doi: 10.1016/j.jff.2018.12.034).
43. Zhu B., Li Y., Hu T., Zhang Y. The hepatoprotective effect of polysaccharides from *Pleurotus ostreatus* on carbon tetrachloride-induced acute liver injury rats. *International Journal of Biological Macromolecules*, 2019, 131: 1-9 (doi: 10.1016/j.ijbiomac.2019.03.043).
44. Pozdnyakova N., Dubrovskaya E., Chernyshova M., Makarov O., Golubev S., Balandina S., Turkovskaya O. The degradation of three-ringed polycyclic aromatic hydrocarbons by wood-inhabiting fungus *Pleurotus ostreatus* and soil-inhabiting fungus *Agaricus bisporus*. *Fungal Biology*, 2018, 122(5): 363-372 (doi: 10.1016/j.funbio.2018.02.007).
45. Karthikeyan V., Ragunathan R., Johney J., Kabesh K. Production, optimization and purification of laccase produced by *Pleurotus ostreatus* MH591763. *Research & Reviews: A Journal of Microbiology and Virology*, 2019, 9(1): 56-64.
46. Noman E., Al-Gheethi A., Mohamed R.M.S.R., Talip B.A. Myco-remediation of xenobiotic organic compounds for a sustainable environment: a critical review. *Topics in Current Chemistry*, 2019, 377(3): 17 (doi: 10.1007/s41061-019-0241-8).
47. Bamigboye C.O., Oloke J.K., Burton M., Dames J.F., Lateef A. Optimization of the process for producing biomass and exopolysaccharide from the king tuber oyster mushroom, *Pleurotus tuber-regium* (Agaricomycetes), for biotechnological applications. *International Journal of Medicinal Mushrooms*, 2019, 21(4): 311-322 (doi: 10.1615/IntJMedMushrooms.2019030357).
48. Zhang W.R., Liu S.R., Kuang Y.B., Zheng S.Z. Development of a novel spawn (block spawn) of an edible mushroom, *Pleurotus ostreatus*, in liquid culture and its cultivation evaluation. *Mycobiology*, 2019, 47(1): 97-104 (doi: 10.1080/12298093.2018.1552648).
49. Stajić M., Vukojević J., Knežević A., Milovanović I. Influence of trace elements on ligninolytic enzyme activity of *Pleurotus ostreatus* and *P. pulmonarius*. *BioResources*, 2013, 8(2): 3027-3037 (doi: 10.15376/biores.8.2.3027-3037).
50. Baldrian P., Valášková V., Merhautová V., Gabriel J. Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead and zinc. *Research in Microbiology*, 2005, 156(5-6): 670-676 (doi: 10.1016/j.resmic.2005.03.007).
51. Yan H., Chang H. Antioxidant and antitumor activities of selenium-and zinc-enriched oyster mushroom in mice. *Biological Trace Element Research*, 2012, 150(1-3): 236-241 (doi: 10.1007/s12011-012-9454-1).
52. Ma X.K., Zhang H., Fam H. Influence of rutin, $FeSO_4$, Tween 80, aspartate and complex vitamins on synthesis of fungal exopolysaccharide. *Carbohydrate Polymers*, 2013, 92(2): 1188-1196 (doi: 10.1016/j.carbpol.2012.10.027).
53. Glukhova L.B., Frank Y.A., Danilova E.V., Avakyan M.R., Banks D., Tuovinen O.H., Karnachuk O.V. Isolation, characterization, and metal response of novel, acid-tolerant *Penicillium* spp. from extremely metal-rich waters at a mining site in Transbaikal (Siberia, Russia). *Microbial Ecology*, 2018, 76(4): 911-924 (doi: 10.1007/s00248-018-1186-0).
54. Ramesh G., Podila G.K., Gay G., Marmeisse R., Reddy M.S. Different patterns of regulation for the copper and cadmium metallothioneins of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *Applied and Environmental Microbiology*, 2009, 75(8): 2266-2274 (doi:

10.1128/AEM.02142-08).
55. Pankratov A.N., Tsivileva O.M., Tsymbal O.A., Yurasov N.A. *Izvestiya Saratovskogo universiteta. Novaya seriya. Seriya: Khimiya. Biologiya. Ekologiya*, 2015, 15(3): 16-25 (doi: 10.18500/1816-9775-2015-15-3-16-25) (in Russ.).
56. Tsivileva O.M., Pankratov A.N., Misin V.M., Zavyalov A.Yu., Volkov V.A., Tsymbal O.A., Yurasov N.A., Nikitina V.E. Antioxidant properties of the Artist's Conk medicinal mushroom, *Ganoderma applanatum* (Agaricomycetes), upon cultivation with *para*-substituted phenolic compounds and tea leaf extracts. *International Journal of Medicinal Mushrooms*, 2018, 20(6): 549-560 (doi: 10.1615/IntJMedMushrooms.2018026329).
57. Drevko B.I., Drevko R.I., Antipov V.A., Chernukha B.A., Yakovlev A.N. *Sredstvo dlya lecheniya i profilaktiki infektsionnykh zabolevanii i otravlenii zhivotnykh i ptits, povyshayushchee ikh produktivnost' i sokhrannost'. Pat. 2171110 (RF) MPK 7 A 61 K 33/04. Zayavl. 26.05.1999. Opubl. 27.07.2001. Byul. № 21* [A composition for the treatment and prevention of infectious diseases and poisoning of animals and birds, increasing their productivity and safety. Patent 2171110 (RF) MPK 7 A 61 K 33/04. Appl. 26.05.1999. Publ. 27.07.2001. Bull. № 21] (in Russ.).
58. Sharofova M.U., Sagdieva Sh.S., Abdullaev S.F., Maslov V.A., Rakhimi F., Mirshakhi M. *Doklady Akademii nauk Respubliki Tadzhikistan*, 2018, 61(4): 350-359 (in Russ.).
59. Khokhlova E.A., Kachala V.V., Ananikov V.P. Conversion of carbohydrates to 5-hydroxymethylfurfural: the nature of the observed selectivity decrease and microwave radiation effect. *Russian Chemical Bulletin*, 2013, 62(3): 830-835 (doi: 10.1007/s11172-013-0112-3).
60. Sharma V.K., Choi J., Sharma N., Choi M., Seo S.-Y. *In vitro* anti-tyrosinase activity of 5-(hydroxymethyl)-2-furfural isolated from *Dictyophora indusiata*. *Phytotherapy Research*, 2004, 18(10): 841-844 (doi: 10.1002/ptr.1428).
61. Kim Y.M., Yun J., Lee C.-K., Lee H., Min K.R., Kim Y. Oxyresveratrol and hydroxystilbene compounds inhibitory effect on tyrosinase and mechanism of action. *Journal of Biological Chemistry*, 2002, 277(18): 16340-16344 (doi: 10.1074/jbc.M200678200).
62. Razak D.L.A., Fadzil N.H.M., Jamaluddin A., Rashid N.Y.A., Sani N.A., Manan M.A. Effects of different extracting conditions on anti-tyrosinase and antioxidant activities of *Schizophyllum commune* fruit bodies. *Biocatalysis and Agricultural Biotechnology*, 2019, 19: 101116 (doi: 10.1016/j.bcab.2019.101116).
63. Çayan F., Tel G., Duru M.E., Öztürk M., Türkoğlu A., Harmandar M. Application of GC, GC-MSD, ICP-MS and spectrophotometric methods for the determination of chemical composition and *in vitro* bioactivities of *Chroogomphus rutilus*: the edible mushroom species. *Food Analytical Methods*, 2014, 7(2): 449-458 (doi: 10.1007/s12161-013-9644-2).
64. Tel G., Deveci E., Küçükaydın S., Özler M.A., Duru M.E., Harmandar M. Evaluation of antioxidant activity of *Armillaria tabescens*, *Leucopaxillus gentianeus* and *Suillus granulatus*: the mushroom species from Anatolia. *Eurasian Journal of Analytical Chemistry*, 2013, 8(3): 136-147.
65. Chen J.-T., Su H.-J., Huang J.-W. Isolation and identification of secondary metabolites of *Clitocybe nuda* responsible for inhibition of zoospore germination of *Phytophthora capsici*. *Journal of Agricultural and Food Chemistry*, 2012, 60(30): 7341-7344 (doi: 10.1021/jf301570y).
66. Cheung L.M., Cheung P.C.K., Ooi V.E.C. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, 2003, 81(2): 249-255 (doi: 10.1016/S0308-8146(02)00419-3).
67. Karpukhina G.V., Emanuel N.M. *Doklady Akademii Nauk SSSR*, 1984, 276(5): 1163-1167 (in Russ.).
68. Burlakova E.B., Mazaletskaya L.I., Sheludchenko N.I., Shishkina L.N. Inhibitory effect of the mixtures of phenol antioxidants and phosphatidylcholine. *Russian Chemical Bulletin*, 1995, 44(6): 1014-1020 (doi: 10.1007/BF00707045).
69. Mazaletskaya L.I., Sheludchenko N.I., Shishkina L.N. Role of the nitrogen-containing moiety of phosphatidylcholines in the mechanism of inhibiting action of their mixtures with natural and synthetic antioxidants. *Petroleum Chemistry*, 2008, 48(2): 105-111 (doi: 10.1134/S0965544108020059).
70. Lanfranco L., Novero M., Bonfante P. The mycorrhizal fungus *Gigaspora margarita* possesses a CuZn superoxide dismutase that is up-regulated during symbiosis with legume hosts. *Plant Physiology*, 2005, 137(4): 1319-1330 (doi: 10.1104/pp.104.050435).
71. Sharma S.S., Dietz K.J. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany*, 2006, 57(4): 711-726 (doi: 10.1093/jxb/erj073).
72. Aguilar F. Manganese ascorbate, manganese aspartate, manganese bisglycinate and manganese pidolate as sources of manganese added for nutritional purposes to food supplements. *European Food Safety Authority Journal*, 2009, 7(6): 1114.
73. Mohamed E.T., Said A.I., El-Sayed S.A. Protective effect of zinc aspartate against acetaminophen induced hepato-renal toxicity in albino rats. *Journal of Radiation Research and Applied Sciences*, 2011, 4(2(B)): 709-723.
74. Le Prell C.G., Ojano-Dirain C., Rudnick E.W., Nelson M.A., DeRemer S.J., Prieskorn D.M., Miller J.M. Assessment of nutrient supplement to reduce gentamicin-induced ototoxicity. *Journal of the Association for Research in Otolaryngology*, 2014, 15(3): 375-393 (doi: 10.1007/s10162-014-0448-x).

75. Afanas'ev I.B., Suslova T.B., Cheremisina Z.P., Abramova N.E., Korkina L.G. Study of antioxidant properties of metal aspartates. *Analyst*, 1995, 120(3): 859-862 (doi: 10.1039/AN9952000859).
76. Özkan K.U., Boran C., Kilinc M., Garipardic M., Kuruta E.B. The effect of zinc aspartate pretreatment on ischemia-reperfusion injury and early changes of blood and tissue antioxidant enzyme activities after unilateral testicular torsion-detorsion. *Journal of Pediatric Surgery*, 2004, 39(1): 91-95 (doi: 10.1016/j.jpedsurg.2003.09.013).
77. Turkovskaya O.V., Dubrovskaya E.V., Grinev V.S., Balandina S.A., Pozdnyakova N.N. Degradative activity and production of the extracellular peroxidases by micromycetes with different ecological strategy, *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(1): 65-75 (doi: 10.15389/agrobiology.2019.1.65eng).

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PRODUCTION OF ALTERNARIOL IN THE POPULATIONS OF GRAIN FEED-ASSOCIATED SMALL SPORE *Alternaria* SPECIES

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Abstract

Modern science has strong evidence that *Alternaria* fungi pose a serious toxic hazard. *Alternaria* species can grow well on various substrates and in a wide range of temperatures and humidity, occupying different ecological niches in this way, and can produce several types of especially dangerous secondary metabolites (S.M. Tralamazza et al., 2018). The most well-studied *Alternaria* mycotoxin alternariol (AOH), a dibenzo-α-pyron derivative, exhibits high cytotoxicity, genotoxic and mutagenic effects (Z. Mao et al., 2014). However, the ability of *Alternaria* fungi to produce this toxin still remains poorly studied. In Russia, a significant prevalence of small spore *Alternaria* species on grain crops (Ph.B. Gannibal, 2004, 2006; T.Yu. Gagkaeva et al., 2012), and an increase in the frequency of *Alternaria* occurrence and AOH accumulation in grain and feed mixtures (G.P. Kononenko et al., 2019, 2020) have been recently reported. In this article, we first showed that the species *A. tenuissima*, *A. alternata*, and *A. arborescens* can cause AOH contamination of grain feeds. The work aims to investigate AOH production by the grain feed-associated *Alternaria* species. *Alternaria* fungi were isolated from 57 feed samples of different types (wheat, barley, corn and oats, sunflower seeds, wheat bran and mixed feeds). Monoconidial isolates identified by morpho-cultural features as *A. tenuissima* (Nees et T. Nees: Fries) Wiltshire, *A. alternata* (Fr.) Keissl, and *A. arborescens* E.G. Simmons, and another 14 isolates assigned to *A. infectoria* species group were cultured for 7 days at 25 °C on a panel of 4 mycological media, the potato-carrot agar (PCA), hay infusion agar (HAY), malt extract agar (MEA), and vegetable juice agar (V-8). AOH was detected in extracts using a certified commercial kit for indirect competitive enzyme-linked immunosorbent assay (ELISA). Among the isolates belonging to *A. infectoria* species group, 13 were devoid of producing ability while for one of them the accumulation of AOH was observed on all growth media in quantities 2.0±0.2, 14±3, 18±4 and 220±30 μg/g, respectively. Evaluation of the biosynthetic potential of *A. tenuissima* and *A. alternata* showed the highest degree of its realization on MEA growth medium in terms of the total number of producers and the share of highly active and superactive isolates. The total amount of AOH accumulation in these conditions for both species was almost the same and amounted to 73 and 71 μg/g, respectively. *A. arborescens* isolates provided the highest AOH production on V-8, HAY, and MEA media in amounts equal to 106, 64, and 31 μg/g, respectively. The peculiarities of metabolic response of *A. tenuissima*, *A. alternata* and *A. arborescens* species to environmental changes and a rapid method to assess toxigenicity of *Alternaria* fungi during taxonomic identification are discussed.

Keywords: alternariol, *Alternaria tenuissima*, *Alternaria alternata*, *Alternaria arborescens*, *Alternaria infectoria* species group, grain feeds, ELISA

Modern research convincingly evidence that fungi of the genus *Alternaria* pose a serious toxicological hazard [1]. Owing to the possibility of active growth on different substrates in a wide range of temperatures and humidity, these fungi occupy a vast ecological niche and are capable of producing secondary metabolites of several structural types with especially dangerous negative action [2-5]. In the group of dibenzo-α-pyrones, the most known is alternariol (AOL) possessing high cytotoxicity, genotoxic and mutagenic effects [6].

Assessment of the risk of mycotoxin contamination of natural objects

susceptible to infection by microscopic fungi implies a phased implementation of a complex of mycological and mycotoxicological investigations. First, in a representative set of samples that most fully characterizes the object of the survey, the main species are identified, and the data on the toxin-forming ability of the set of isolates are extrapolated to the entire population. Similar projects for grain infected with *Alternaria* fungi has already been undertaken several times for agroecosystems in Latin America [7-9], Europe [10, 11] and Asia [12, 13], but comparison of the results often failed because of the ambiguity of approaches to fungal species discrimination and differences in the experimental testing schemes. In Russia, the potential for the AOL formation by *Alternaria* fungi that infect grain products remains practically unexplored, and only one of the works of German researchers provides data for 24 isolates from grain of one field in the Novosibirsk region [14].

It should be admitted that the difficulties associated with the variety of methods for determining species of *Alternaria* fungi are objective. The taxonomic system of this genus is currently improving [15, 16]. Morphological identification is recognized as not always unambiguous, since some traits can overlap between different species. The development of molecular methods, as well as a polyphase approach using metabolic profile data, is still at the stage of information accumulation [17-19]. In this regard, when assessing biological objects, researchers are increasingly inclined to choose the traditional approach based on a set of morphological characteristics of reproductive structures and sporulation under controlled conditions [20].

The procedure for testing fungi toxin formation in vitro undoubtedly needs to be unified. For this purpose, it is preferable to use homogeneous agar media but not grain substrates, on which the required precision of determination is not always achieved. Short-term 7-day incubation at 23-25 °C on such matrices followed by analysis of metabolites in blocks of mycelial-spore biomass is widely used in chemotaxonomy of the genera *Penicillium* and *Aspergillus* [21, 22].

Here, we for the first time evaluated the ability of *Alternaria* fungi to produce AOL on a panel of four growth media recommended for the identification of the members of this genus. Our findings revealed the species-specific metabolic response to the type of growth medium and, in addition, showed for the first time that three morphological species, the *Alternaria tenuissima*, *A. alternata*, and *A. arborescens* may be involved into grain feed contamination with this toxin.

Our goal was to study the potential for the production of alternariol in populations of small-spore species of the genus *Alternaria* fungi associated with grain feed.

Materials and methods. Primary cultures of *Alternaria* fungi were isolated from 57 samples of seven types of grain feeds, the wheat grain (29 samples), mixed feed (12 samples), barley grain (7 samples), sunflower seeds (6 samples), and wheat bran, corn grain and oats (one sample each). The fungal species was identified by cultural and morphological characteristics as described [23]. Monoconidial cultures of *Alternaria tenuissima* (Nees et T. Nees:Fries) Wiltshire, *A. alternata* (Fr.) Keissl and *A. arborescens* E.G. Simmons derived from conidial suspensions in 0.1% sterile Tween 80. Controlling the number of conidia in one drop with a diameter of 0.4 cm, 3-4 drops were put into sterile Petri dishes filled with a thin layer of melted and cooled Chapek-Dox agar and cultured for 1 day at 23-25 °C. The agar fragments with single germinated conidia, after being examined under a microscope, were put onto a Chapek-Dox agar slant.

A scheme of 1 isolate—1 sample was applied for testing each *Alternaria* species. In total, there were 14 polyconidial isolates of the *A. infectoria* group of species and monoconidial isolates of *A. tenuissima* (23 isolates), *A. alternata* (20

isolates) and *A. arborescens* (8 isolates, including 3 strains isolated from barley, Nos. 157011, 158011, and 529051 of the Yachevsky Laboratory of Mycology and Phytopathology mycological herbarium, All-Russian Research Institute of Plant Protection, St. Petersburg, Russia).

For morpho-cultural characterization of isolates assigned to the group of *A. infectoria* species, yeast extract sucrose agar (YES) was used. Ten-day old cultures in Chapek-Dox agar served as inocula. The growth media were potato-carrot agar (PCA), hay infusion agar (HAY) prepared as per [24], malt extract agar (MEA) (Liofilchem S.r.l., Italy) and vegetable juice agar (V-8) prepared from vegetable juice (LLC South Juice Company, Krasnodar Territory, Belorechensk, Russia) as per [23]. Each inoculum (in 3 replicates) was put into a 10 ml flask (a bottom diameter of about 18 mm) with 1.5 ml of one of the above media. The flasks were closed with cotton-gauze plugs, additionally wrapped with Parafilm M (Merck KGaA, Germany) and incubated in the dark for 7 days at 25 °C; then each flask was added with 1.5 ml of a mixture of acetonitrile and water, a volume ratio of 84:16, and vigorously shaken at the beginning and end of stationary 14-hour extraction. The AOL levels in the extracts were quantified using ELISA test for alternariol [25] with 0.01 μg/g detection limit of the toxin.

The data were processed using descriptive statistics in Microsoft Excel 2013, the results were expressed as arithmetic means of the values (*M*) with an error of the sample mean (±SEM).

Results. The recent data of an extensive study of the *Alternaria* species composition in the European Russia indicate that *A. tenuissima* and *A. infectoria* species complex are most common in cereal seeds, while *A. alternata* and *A. arborescens* are much less frequent [26-28]. The results of the first survey of grain fodder definitely indicated that *A. tenuissima* predominates in the mycobiota of wheat and barley grains [29].

In this work, using more than 150 samples of grain feeds (mainly wheat, barley, sunflower seeds and mixed feeds with a high proportion of grain components), we managed to form sets of enough size only for isolates of *A. tenuissima*, *A. alternata* and *A. infectoria* group of species. Isolates identified as *A. arborescens* were found only in 5 samples, that is why the accessions of similar origin were additionally taken. We thought it expedient to test isolates on a panel of media recommended for the species identification procedure, since this approach could be used in the future for the rapid detection of toxin production in these fungi already at the stage of mycological analysis. Each of the 66 strains were grown under identical conditions on PCA, MEA, HAY and V-8 agars. This work is the world's first comparative analysis of toxin production in *A. tenuissima*, *A. alternata*, *A. arborescens* and *A. infectoria* group on PCA, HAY and V-8 growth media.

All *A. tenuissima* isolates produced AOL on MEA, HAY, and V-8, while no toxin was found on PCA in two isolates (Table 1). On all media, fluctuations in the amount of the toxin in isolates were three orders of magnitude. We noted this feature earlier, and it was even more pronounced. E.g. the AOL accumulation in 15 *A. tenuissima* isolates (wort agar, 7 days, 25 °C) ranged from 0.8 to 710 μg/g [29]. In 17 strains isolated from grain in the Novosibirsk region (rice grain, 14 days, 25 °C), the variation was more significant, from 0.405 to 26900 μg/g [14]. The *A. tenuissima* isolates showed a group response to the type of nutrient medium. On MEA the predominant accumulation occurred in 12 isolates of those tested, V-8 agar was preferable for 5 isolates, and another 5 isolates produced comparable amounts of AOL on all three substrates. On average, over the entire set of isolates, the level of AOL accumulation decreased as MEA > V-8 > HAY. The toxin concentration of 10 μg/g or more was detected in

17 cultures on MEA, in 16 cultures on V-8, and in 10 cultures on HAY. Overactive production (above 100 μg/g) was noted on MEA and V-8, respectively, in 7 and 2 isolates, but it was not observed on HAY. In general, as followed from the mean values for the entire set, the greatest metabolic response in *A. tenuissima* was achieved on MEA, while on HAY and V-8 it was slightly lower.

1. Alternariol (AOL) **production by monoconidial isolates of *Alternaria tenuissima* from grain feeds on different agar media (**7 days, 25 °C) (*M*±SEM)

Isolate No., n = 23	AOL, μg/g substrate			
	PCA	MEA	HAY	V-8
204/1	0.04±0.01	105±20	26±3	38±4
215/1	0.50±0.10	22±4	16±3	23±2
221/1	0.07±0.00	253±35	37±7	12±1
222	0.80±0.20	138±18	20±4	72±12
225	2.90±0.40	73±15	37±6	12±2
228	–	30±7	4.8±0.6	0.3±0.0
233/1	0.04±0.00	10±2	23±6	80±14
234/2	–	33±3	7.3±1.1	117±23
236/1	0.02±0.00	26±6	59±8	19±3
241/1	0.20±0.00	200±43	53±8	37±7
242/1	0.90±0.20	39±7	1.5±0.3	3.9±0.2
255	0.04±0.00	5.5±0.7	0.3±0.0	12±2
337/1	0.04±0.00	0.1±0.0	0.1±0.0	0.2±0.0
342/1	0.03±0.00	58±8	87±15	120±25
357/1	0.06±0.01	21±4	4.7±0.9	6.1±0.9
359/1	2.30±0.40	100±18	1.7±0.2	19±2
368/1	50.00±8.00	2.0±0.1	0.2±0.0	0.2±0.0
372/1	1.10±0.20	31±5	0.3±0.0	2.7±0.5
381/1	0.20±0.00	23±6	9.2±1.4	12±2
384/1	0.05±0.00	5.8±0.9	5.9±0.9	3.6±0.7
392	0.10±0.00	402±43	69±13	25±6
395/1	0.10±0.00	3.7±0.6	0.1±0.0	13±2
397	0.20±0.00	106±18	0.5±0.1	24±4
n^+ (n^{10}/n^{100})	21 (0/0)	23 (17/7)	23 (10/0)	23 (16/2)
Range	0.02-2.90	0.1-402	0.08-87	0.2-120
Average, n^+	0.5	73	20	28

N o t e. n — the number of tested isolates; n^+ — the number of AOL producing isolates; n^{10} — the number of isolates with AOL production of > 10 μg/g; n^{100} — the number of isolates with AOL production of > 100 μg/g; a dash means that mycotoxin was not detected (detection limit of 0.01 μg/g); PCA — potato-carrot agar, MEA — malt extract agar, HAY — hay infusion agar, V-8 — vegetable juice agar.

Table 2 shows that the range of fluctuations in the AOL concertation on MEA and V-8 for *A. alternata* was 4 and 5 orders of magnitude. It was just as wide as in several *A. alternata* strains (rice grain, 14 days, 25 °C) isolated from grain in the Novosibirsk region [14]. The toxin was not detected in four isolates on PCA (Nos. 223/2, 380/1, 385/1 and 388/1), in three isolates on HAY (Nos. 216/2, 388/1 and 418/4) and in two on V-8 (Nos. 216/2 and 418/4). The average value for the set of isolates (1.3 μg/g, with 0.03 to 5.3 μg/g in range) on PCA was significantly lower than on other nutrient media.

2. Alternariol (AOL) **production by monoconidial isolates of *Alternaria alternata* from grain feeds on different agar media** (7 days, 25 °C) (*M*±SEM)

Isolate No., n = 20	AOL, μg/g substrate			
	PCA	MEA	HAY	V-8
210	0.3±0.0	155±24	100±20	267±38
216/2	2.2±0.3	2.8±0.4	–	–
219	0.6±0.1	3.1±0.5	2.8±0.5	2.8±0.4
220	0.1±0.0	125±25	23±4	89±10
223/2	–	272±38	1.9±0.3	3.5±0.5
227/1	3.3±0.7	133±30	10±2	19±3
233/2	0.4±0.1	114±20	25±4	13±2
235/2	0.1±0.0	130±30	70±12	70±10
238/1	0.3±0.0	6.5±1.2	27±6	10±2
342/2	0.03±0.01	3.3±0.6	8.9±1.3	2.0±0.2
358/1	0.5±0.1	20±3	11±2	38±3
377/1	0.9±0.2	10±2	14±3	14±3
380/1	–	0.2±0.0	0.3±0.1	0.4±0.1

Continued Table 2

383/1	0.1±0.0	43±7	35±7	50±9
385/1	–	246±39	71±15	365±43
388/1	–	0.04±0.01	–	0.07±0.01
390/1	0.6±0.2	80±15	57±10	461±70
396/1	2.2±0.4	72±10	48±8	19±3
399	3.6±0.8	7.0±1.3	53±8	40±8
418/4	5.3±1.2	0.1±0.0	–	–
n^+ (n^{10}/n^{100})	16 (0/0)	20 (12/7)	17 (13/0)	18 (12/3)
Range	0.03-5.3	0.04-272	0.3-100	0.07-267
Average, n^+	1.3	71	33	81

N o t e. n — the number of tested isolates; n^+ — the number of AOL producing isolates; n^{10} — the number of isolates with AOL production of > 10 μg/g; n^{100} — the number of isolates with AOL production of > 100 μg/g; a dash means that mycotoxin was not detected (detection limit of 0.01 μg/g); PCA — potato-carrot agar, MEA — malt extract agar, HAY — hay infusion agar, V-8 — vegetable juice agar.

The toxin production was comparable for V-8 and MEA (81 and 71 μg/g, respectively) and slightly less for HAY (33 μg/g). On PCA, producers with high activity were not detected at all, while on the other three media, the AOL accumulation of more than 10 μg/g occurred in 12-13 isolates. Ultra-high activity of 100 μg/g and more not observed on HAY, was established for 3 isolates on V-8 and for 7 isolates on MEA.

A. alternata and *A. tenuissima* could be grouped according to the response to the medium composition, with predominant accumulation on MEA (6 isolates), on HAY (2 isolates with amounts comparable to those on MEA) and V-8 (3 isolates), as well as on all three media (5 isolates). There were only few isolates producing the largest amounts of toxin on V-8 (No. 390/1) and with approximately equal AOL accumulation on PCA and MEA (No. 216/2). The isolate No. 418/4 from barley grain showed the abnormal response with the highest accumulation on PCA. In general, the highest metabolic response in *A. alternata* occurred on MEA and V-8 media. A more pronounced synthesis of AOL on these two media compared to wort agar and YES was previously shown by us for some strains identified as *A. tenuissima* and *A. alternata* [30].

Despite the obvious differences in response to the type of growth medium, substrate profiles of toxin production, and wide range of variation of AOL amounts in *A. tenuissima* and *A. alternata* (see Tables 1, 2), an estimate based on the total number of producers and the proportion of highly active and overactive isolates shows the highest level of realization of AOL biosynthetic potential of *A. tenuissima* and *A. alternata* on MEA. The total amount of AOL accumulated under these conditions for both species was practically the same, up to 73 and 71 μg/g, respectively.

A. arborescens isolates produced AOL in all media (Table 3), but in the amounts which varied significantly less than in the two species described above (see Tables 1, 2), being 1-2 orders of magnitude. However, the available set size was noticeably inferior to that for *A. tenuissima* and *A. alternata*; therefore, it is possible to state this fact, but a direct comparison is hardly correct. Indeed, for *A. arborescens*, significant fluctuations have been described; for example, for single strains from grain in the Novosibirsk region, the range of fluctuations in the amount of AOL was extremely wide [14]. However, in previous testing three strains of this species isolated from corn and sunflower seeds, the intensity of AOL production on MEA (3.3-36 μg/g) was quite comparable with fluctuations within the same order of magnitude [31].

Among the isolates, there were those with the highest AOL production on HAY and V-8, as well as on HAY and MEA. We would like to note that abnormal response to the type of medium is also possible, e.g., for the *A. arborescens* No. 100041 isolated in 2007 from wheat leaves in Syria the AOL level in the series PCA > MEA > HAY, V-8 decreased from 15±3 to 0.03±0.01 μg/g (unpublished

authors' data). The same type of metabolic response was characteristic of the *A. alternata* strain No. 418/4, which we isolated from barley grain (see Table 2). In the set as a whole, the highest AOL production occurred on V-8, HAY, and MEA media. The total AOL accumulation under these conditions was 106, 64, and 31 μg/g, respectively.

3. Alternariol (AOL) **production by monoconidial isolates of *Alternaria arborescens* from grain feeds on different agar media** (7 days, 25 °C) (M±SEM)

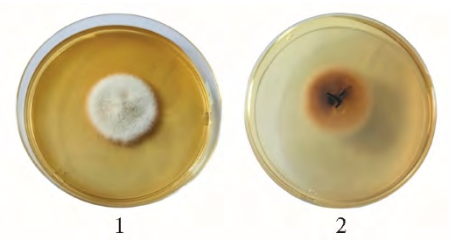
Isolate No., n = 8	AOL, μg/g substrate			
	PCA	MEA	HAY	V-8
3/3	1.9±0.3	79±14	117±22	173±30
15/6	1.5±0.3	10±2	11±3	20±6
19/4	0.8±0.1	4.4±0.5	47±11	8.8±1.6
39/4	0.9±0.1	4.0±0.5	6.8±1.2	13±4
338/1	1.5±0.2	77±13	133±20	12±2
157011	1.9±0.3	69±14	113±17	129±25
158011	1.3±0.2	2.0±0.2	69±13	488±38
529051	0.9±0.2	6.7±1.3	11±2	4.3±0.8
n^+ (n^{10}/n^{100})	8 (0/0)	8 (3/0)	8 (7/3)	8 (6/3)
Range	0.8-1.9	2.0-79	6.8-133	4.3-488
Average, n^+	1.3	31	64	106

N o t e. n — the number of tested isolates; n^+ — the number of AOL producing isolates; n^{10} — the number of isolates with AOL production of > 10 μg/g; n^{100} — the number of isolates with AOL production of > 100 μg/g; a dash means that mycotoxin was not detected (detection limit of 0.01 μg/g); PCA — potato-carrot agar, MEA — malt extract agar, HAY — hay infusion agar, V-8 — vegetable juice agar.

Differently directed shifts in the intensity of AOL biosynthesis on the growth media panel, which we observed in isolates of all three species, are possibly associated with intraspecific individual or group peculiarities of genome functional activity with the participation of regulators of metabolic processes. A more detailed study of this issue is of particular value for the development of the chemotaxonomy of *Alternaria* fungi, which has received increasing attention in recent years [32-34]. Our findings on the advantage of the commercial substrate MEA, as well as two other mycological media, V-8 and HAY are important to improve lab techniques for studying biosynthetic capabilities of these microscopic fungi, which was started in 1990th [35, 36] and is still discussed in the scientific papers [14, 37].

All fungal isolates which, on day 10 of growth at 25 °C on YES medium, formed a weakly colored aerial mycelium and colonies of different structures and densities, were assigned to the *A. infectoria* species group. For 13 of them, there was a distinct similarity in cultural and morphological characteristics (with small differences). The diameter of the colony was 45-60 mm, the structure of the aerial mycelium was predominantly cotton-like (from sparse to more dense), in its color a white-pink tone dominated which interspersed with gray, beige or olive-gray tones interspersed, the reverse side of the colony was light brown or dark brown, mainly with radial grooves. In the cultures possessing such characteristics AOL was not detected on the entire panel of growth media, and this result was consistent with that previously obtained on single isolates [25]. However, one of the cultures, *A. infectoria* No. 6/10 significantly differed from the others in growth rate (colony of 80 mm in diameter), low, relatively dense, slightly heavy aerial mycelium of light beige color with a pink sector (20 mm), the reverse side of the colony had a light brown color with radial grooves, with light orange in the sector area (Fig.). This isolate turned out to be an active producer of AOL with a sharp increase in activity on V-8 (220±30 μg/g) vs. 2.0±0.2; 14±3 and 18±4 μg/g on PCA, MEA and HAY, respectively. We also noted a similar response to a change in the growth medium in some members of *A. alternata* and *A. tenuissima* (see Tables 1, 2). According to German researchers, two isolates of *A. infectoria* from grain in the Novosibirsk region also produced AOL in contrasting amounts, the extremely low and ultrahigh [14]. Interestingly, recently, when studying a group of *A. infectoria* species on a

modified YES medium, two morphological types differing in pigmentation were revealed [38]; however, unfortunately, their ability to form toxins was not assessed.



Macroscopic characteristics of the *Alternaria* infectoria group isolate No. 6/10 (yeast extract sucrose agar, YES, 10 days, 25 °C): 1 — top view, 2 — back view.

The ability of *A. infectoria* to produce AOL is still open [1]. Most publications report that its synthesis is not characteristic of this group of species [18]; however, in several studies, toxinogenic producers were found among isolates [14, 16, 34]. According to the latest data, this group is characterized by production of toxins of another structural series, the perylenequinones [14, 16].

This work submits data on a cumulative assessment of *A. tenuissima*, *A. alternata* and *A. arborescens* from grain feeds based on a series of indicators, which experimentally confirms the pronounced and almost identical ability of the isolates to produce AOL. These results generally correspond to those obtained earlier for some representatives of these species [29-31], and also agree with the data of foreign researchers for species found in grain and grain products in Argentina, Brazil [7-9], Mediterranean countries, Slovakia, Italy [10, 11, 39] and Korea [13].

The principle of a set formation we used (1 isolate—1 sample) allows us for the first time to conclude about the high toxigenic potential of populations of three small-spore species, the *A. tenuissima*, *A. alternata*, and *A. arborescens* and their involvement in the AOL contamination of grain feed. According to recent data, AOL contamination in Russia in recent years has acquired particular relevance for corn feed grain [40] and grain-based mixed feeds [41].

Thus, our research revealed that among the *Alternaria* fungi colonizing grain fodder, populations of three morphological species, *A. tenuissima*, *A. alternata* and *A. arborescens* are capable of active alternariol production (more than 10 μg/g growth medium for most isolates) and may be involved in feed contamination, while the contribution of the *A. infectoria* group is unlikely. Further development of the population approach used in the presented work is important for improving methods of assessing the risks of feed contamination with toxicants of mycogenic origin. The successful use of typical mycological media for *in vitro* express testing of isolates confirms that it is possible to combine the control of toxigenic potential with species identification. The correspondence between morpho-cultural features and the phylogenetic position of these fungi, on the one hand, and the profile of their toxic secondary metabolites, on the other, remains a key component of the database, which has been actively constructed in recent years to clarify systematics of the genus *Alternaria*.

REFERENCES

1. Tralamazza S.M., Piacentini K.C., Iwase C.H.T., De Oliveira Rocha L. Toxigenic *Alternaria* species: impact in cereals worldwide. *Current Opinion in Food Science*, 2018, 23: 57-63 (doi: 10.1016/j.cofs.2018.05.002).
2. Ostry V. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal*, 2008, 1(2): 175-188 (doi: 10.3920/WMJ2008.x013).
3. EFSA on Contaminants in the Food Chain (CONTAM). Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal*, 2011, 9(10): 2407 (doi: 10.2903/j.efsa.2011.2407).
4. Lou J., Fu L., Peng Y., Zhou L. Metabolites from *Alternaria* fungi and their bioactivities. *Molecules*, 2013, 18(5): 5891-5935 (doi: 10.3390/molecules18055891).
5. Lee H.B., Patriarca A., Magan N. *Alternaria* in food: ecophysiology, mycotoxin production and

toxicology. *Mycobiology*, 2015, 43(2): 93-106 (doi: 10.5941/MYCO.2015.43.2.93).
6. Mao Z., Sun W., Fu L., Luo H., Lai D., Zhou L. Natural dibenzo-α-pyrones and their bioactivities. *Molecules*, 2014, 19(4): 5088-5108 (doi: 10.3390/molecules19045088).
7. Patriarca A., Azcarate M.P., Terminiello L., Fernández Pinto V. Mycotoxin production by *Alternaria* strains isolated from Argentinian wheat. *International Journal of Food Microbiology*, 2007, 119(3): 219-222 (doi: 10.1016/j.ijfoodmicro.2007.07.055).
8. Mašková Z., Tančinová D., Barboráková Z., Felšöciová S., Cisarová M. Comparison of occurrence and toxinogenity of *Alternaria* spp. isolated from samples of conventional and new crossbred wheat of Slovak origin. *Journal of Microbiology, Biotechnology and Food Sciences*, 2012, 1(4): 552-562 (doi: 10.15414 /jmbfs.2016.5.6.552-556).
9. Oviedo M.S., Sturm M.E., Reynoso M.M., Chulze S.N., Ramirez M.L. Toxigenic profile and AFLP variability of *Alternaria alternata* and *Alternaria infectoria* occurring on wheat. *Brazilian Journal of Microbiology*, 2013, 44(2): 447-455 (doi: 10.1590/S1517-83822013000200017).
10. Andersen B., Nielsen K.F., Fernández Pinto V., Patriarca A. Characterization of *Alternaria* strains from Argentinean blueberry, tomato, walnut and wheat. *International Journal of Food Microbiology*, 2015, 196: 1-10 (doi: 10.1016/j.ijfoodmicro.2014.11.029).
11. Ramires F.A., Masiello M., Somma S., Villani A., Susca A., Logrieco A.F., Luz C., Meca G., Moretti A. Phylogeny and mycotoxin characterization of *Alternaria* species isolated from wheat grown in Tuscany, Italy. *Toxins*, 2018, 10(11): 472 (doi: 10.3390/toxins10110472).
12. Li F., Toyazaki N., Yoshizawa T. Production of *Alternaria* mycotoxins by *Alternaria alternata* isolated from weather-damaged wheat. *Journal of Food Protection*, 2001, 64(4): 567-571 (doi: 10.4315/0362-028x-64.4.567).
13. Nguyen T.T.T., Kim J., Jeon S.J., Lee C.W., Magan N., Lee H.B. Mycotoxin production of *Alternaria* strains isolated from Korean barley grains determined by LC-MS/MS. *International Journal of Food Microbiology*, 2018, 268: 44-52 (doi: 10.1016/j.ijfoodmicro.2018.01.003).
14. Zwickel T., Kahr S.M., Rychlik M., Müller E.H. Chemotaxonomy of mycotoxigenic small-spored *Alternaria* fungi — do multitoxin mixtures act as an indicator for species differentiation? *Frontiers in Microbiology*, 2018, 9: 1368 (doi: 10.3389/fmicb.2018.01368).
15. Andersen B., Thrane U. Secondary metabolites produced by *Alternaria infectoria* and their use as chemotaxonomic markers. *Mycotoxin Research*, 1996, 12(2): 54-60 (doi: 10.1007/BF03192262).
16. Andersen B., Thrane U. Differentiation of *Alternaria infectoria* and *Alternaria alternata* based on morphology, metabolite profiles, and cultural characteristics. *Canadian Journal of Microbiology*, 1996, 42(7): 685-689 (doi: 10.1139/m96-093).
17. Andersen B., Krøger E., Roberts R.G. Chemical and morphological segregation of *Alternaria alternata*, *A. gaisen* and *A. longipes*. *Mycological Research*, 2001, 105(3): 291-299 (doi: 10.1017/S0953756201003446).
18. Pinto V.F., Patriarca A. *Alternaria* species and their associated mycotoxins. In: *Mycotoxigenic Fungi: Methods in Molecular Biology*, *vol. 1542*. A. Moretei, A. Susca (eds.). Humana Press, New York, NY, 2017: 13-32 (doi: 10.1007/978-1-4939-6707-0_2).
19. Lawrence D.P., Rotondo F., Gannibal P.B. Biodiversity and taxonomy of the pleomorphic genus *Alternaria*. *Mycological Progress*, 2016, 15(1): article 3 (doi: 10.1007/s11557-015-1144-x).
20. Gannibal Ph.B., Yli-Mattila T. *Mikologiya i fitopatologiya*, 2005, 39(4): 13-23 (in Russ.).
21. Frisvad J.C., Smedsgaard J., Larsen T.O., Samson R.A. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Studies in Mycology*, 2004, 49: 201-241.
22. Frisvad J.C., Hubka V., Ezekiel C.N., Hong S.-B., Nováková A., Chen A.J., Arzanlou M., Larsen T.O., Sklenár F., Mahakarnchanakul W., Samson R.A., Houbraken J. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Studies in Mycology*, 2019, 93: 1-63 (doi: 10.1016/j.simyco.2018.06.001).
23. Simmons E.G. *Alternaria. An identification manual*. CBS, Utrecht, 2007.
24. *Introduction to food- and airborne fungi*. R.A. Samson, E.S. Hoekstra, J.S. Frisvad, O. Filtenborg (eds.). CBS, Utrecht, 2000.
25. Burkin A.A., Kononenko G.P. *Immunopatologiya, allergologiya, infektologiya*, 2009, 2: 7-8 (in Russ.).
26. Gannibal F.B. *Mikologiya i fitopatologiya*, 2004, 38(3): 19-28 (in Russ.).
27. Gannibal F.B. *Mikologiya i fitopatologiya*, 2008, 42(4): 359-368 (in Russ.).
28. Gagkaeva T.Yu., Gannibal F.B., Gavrilova O.P. *Zashchita i karantin rastenii*, 2012, 1: 37-42 (in Russ.).
29. Piryazeva E.A., Kononenko G.P. *Materialy chetvertogo s"ezda mikologov Rossii «Sovremennaya mikologiya v Rossii», vol. 1* [Proc. Fourth Congress of Russian mycologists «Modern mycology in Russia»]. Moscow, 2017: 175-177 (in Russ.).
30. Ustyuzhanina M.I., Burkin A.A., Kononenko G.P., Piryazeva E.A., Zotova E.V. Alternative assay media for alternariol production by *Alternaria* species. *Proc. of the VIII International Conference on Environmental, Industrial and Applied Microbiology — BioMicroWorld2018 «Global progress in applied microbiology: a multidisciplinary approach»*. A. Méndez-Vilas (ed.). Formatex Research Center, Badajoz, Spain, 2018: 1-5.
31. Kononenko G.P., Ustyuzhanina M.I., Orina A.S. Multi-substrate screening the ability to produce

alternariol among *Alternaria arborescens* strains. *Journal of Veterinary Science & Technology*, 2019, 10: 41-42.
32. Andersen B., Krøger E., Roberts G. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research*, 2002, 106(2): 170-182 (doi: 10.1017/S0953756201005263).
33. Andersen B., Sørensen J.L., Nielsen K.F., van den Ende B.G., de Hoog S. A polyphasic approach to the taxonomy of the *Alternaria infectoria* species-group. *Fungal Genetics and Biology*, 2009, 46: 642-656 (doi: 10.1016/j.fgb.2009.05.005).
34. Patriarca A., da Cruz Cabral L., Pavicich M.A., Nielsen K.F., Andersen B. Secondary metabolite profiles of small-spored *Alternaria* support the new phylogenetic organization of the genus. *International Journal of Food Microbiology*, 2019, 291: 135-143 (doi: 10.1016/j.ijfoodmicro.2018.11.022).
35. Kostecki M., Grabarkiewicz-Szczęsna J., Chelkowski J. Biosynthesis and preparation of five *Alternaria* metabolites. *Mycotoxin Research*, 1991, 7(1): 3-7 (doi: 10.1007/BF03192156).
36. Häggblom P., Hiltunen M. Regulation of mycotoxin biosynthesis in *Alternaria*. *Mycotoxin Research*, 1991, 7(1): 8-10 (doi: 10.1007/BF03192157).
37. Oviedo M.S., Ramirez M.L., Barros G.G., Chulze S.N. Impact of water activity and temperature on growth and alternariol and alternariol monomethyl ether production of *Alternaria alternata* isolated from soybean. *Journal of Food Protection*, 2010, 73(2): 336-343 (doi: 10.4315/0362-028X-73.2.336).
38. Kosiak B., Torp M., Skjerve E., Andersen B. *Alternaria* and *Fusarium* in Norwegian grains of reduced quality — a matched pair sample study. *International Journal of Food Microbiology*, 2004, 93(1): 51-62 (doi: 10.1016/j.ijfoodmicro.2003.10.006).
39. Logrieco A., Bottalico A., Solfrizzo M., Mulé G. Incidence of *Alternaria* species in grains from Mediterranean countries and their ability to produce mycotoxins. *Mycologia*, 1990, 82(4): 501-505 (doi: 10.2307/3760022).
40. Kononenko G.P., Burkin A.A., Zotova E.V., Smirnov A.M. *Rossiiskaya sel'skokhozyaistvennaya nauka*, 2019, 3: 28-31 (doi: 10.31857/S2500-26272019328-31) (in Russ.).
41. Kononenko G.P., Burkin A.A., Zotova E.V. *Veterinariya segodnya*, 2020, 1(32): 60-65 (doi: 10.29326/2304-196X-2020-1-32-60-65) (in Russ.).