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BACTERIAL MICROORGANISMS ASSOCIATED WITH THE PLANT TISSUE CULTURE: IDENTIFICATION AND POSSIBLE ROLE
(review)

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Abstract

Effective sterilization of plant explants and antiseptics rules compliance do not exclude the presence of so-called covert (endophytic) bacteria in in vitro cultures. But the role of these bacteria in tissues cultures has been not enough studied whereas it was related to the explants regeneration capacity and the possibility of animal and human cells transformation under in vitro cultivation. Bacterial strains pathogenic to humans can be stably maintained in cultivated tissues and ex vitro plants. The broadening of bacterial environments creates ecological and genetic risks leading to necessity of careful monitoring of endophytic communities in plants used as raw food and at use of in vitro technologies in practical plant growing and food production. Identification of bacterial microorganisms colonizing in vitro plant cultures allows studying the bacteria effect on the host, realizing special chemotherapy and developing the microorganisms' databases. Two methods of identification are the most widespread: more available traditional one that does not allow detecting non-cultured forms (its base is the use of cultural and morphological characteristics as well as chemical and biochemical reactions) and molecular-genetic one. At the second approach different 16S rRNA sequences are studied using metagenomic DNA and appropriate specific primers; these sequences have conserved sites identical for all prokaryotes and variable ones suitable for species specific regions identification. Internal transcribed spacers (ITS) are being mainly used to distinguish the microorganisms at the species level and even at strains one. Taxonomy of in vitro cultures' bacterial endophytes indicates to their diversity and absence of specific composition as for cultures of plants belonging to different taxa as for different plant organs explants. Among identified endophytic bacteria potentially useful for intact plants *Streptomycete*, *Pantoea agglomerans* and others were found as well as those pathogenic for humans, e.g. *Ralstonia mannitolytica*, *Staphylococcus epidermidis*, *Corynebacterium amycolatum*, *Bacillus neonatiensis, Salmonella* and *Nocaridia* spp. At in vitro plant cultivation durable symptomless bacterial presence is caused on the one hand by bacterial growth repression with factors accompanying plant explants cultivation (pH, temperature below bacterial optimum, activation of the defense mechanisms), and on the other hand by simultaneous bacteria support due to exudates secreted by plant explants. The rapid bacterial cells proliferation can begin even at small changes in initial conditions, at increase in plant exudates concentrations and per se in consequence of in vitro cultivation as a stress at the absence of whole organism regulatory role. As the number of subcultivations increases a portion of plant cultures with latent bacterial contamination increases too; no-cultured endophytes have been reported to acquire the status of cultured ones. Covert bacterial contamination could depress regeneration, micropropagation, cause death of in vitro cultivated objects, restrict the protocols repeatability and concern induction of epigenetic somaclonal variability. For instance *Acinetobacter* and *Lactobacillus plantarum* filtrates extracted from degrading calluses strongly reduced shoot regeneration at inoculation in explants or addition into a medium; bacteria *Mycobacterium obuense* and *M. aichiense* repressed seeds development in in vitro cultures. The article accents the problem of gnotobiological plant cultures (specifically in in vitro collections of plants genetic banks) development caused by difficulties in identification and elimination of bacterial microorganisms.

Keywords: plant tissue culture, bacterial microorganisms, antibacterial therapy.

When working with in vitro plant tissue culture, the presence of bacterial contamination is largely determined by the quality of sterility [1, 2]. However, an effective sterilization of plant explants and compliance with the antiseptics

rules do not exclude the presence of covert bacteria in in vitro cultures (without visual growth and specific symptoms) [3-5]. Bacterial organisms, the native habitat of which is air, soil, plants and human, are detected and identified using microbiological, molecular and genetic and biochemical methods both in the long-term passaged plant cultures and plant cultures initiated in vitro [6-14]. Latent bacterial infections, defined by many researchers as internal or endophytic, are detected in calli and microplants cultivated in vitro, as well as in various explants such as shoot apices, buds, and meristems [15-22]. Bacterial endophytes performing a number of functions that are important for plants have always been and continue to be the subject of numerous studies [23]. At the same time, the role of endophytic bacteria in tissue cultures is less well studied, but it is of utmost interest both in fundamental and applied aspects. Specifically, bacterial endophytes are considered as a key factor that defines the regenerative capacity of explants along with the genotype and cultivation conditions [18]. They are studied as a possible promising source of new components for the use in the microbiology and medicine practices [24]. Moreover, attention is drawn to bacterial endophytes due to the accumulation of data indicating the conventionality of historical division of microorganisms into phytopathogenic, pathogenic for animals (human) and non-pathogenic [25]. It was shown that human pathogenic bacterial strains can be steadily preserved in passaged cultures and ex vitro plants [14], and bacteria *Agrobacterium tumefaciens* can transform in vitro cultured human cells [26] and sea urchin embryos [27]. The enhanced bacteria habitat creates environmental and genetic risks that necessitate careful monitoring of endophytic communities, especially in plants used as raw food [14, 28]. This problem is relevant for the plant tissue culture as well, since in vitro techniques are widely used in plant growing practices and food production.

The purpose of this survey was to collect and organize data related to detection, identification, structure, dynamics, possible role, and elimination of latent bacterial contaminations in the plant tissue culture.

In literature, bacterial microorganisms, the presence of which in the in vitro cultivated plant objects is not accompanied by visual displays and specific symptoms, are referred to as latent, covert, endogenous, internal, and endophytic, and often these terms are used as synonyms. Most often, these bacterial microorganisms in the plant tissue culture are called latent. One of the papers [29] emphasizes that the term «latent» is borrowed from the plant pathology, where it is used to describe asymptomatic pathogens, while bacterial microorganisms in the plant tissue culture are not necessarily pathogens (they can exert either negative, positive or no impact). The author of the cited paper [29], along with other researchers [30, 31], believes that it is more appropriate to use the term «covert» for these bacterial microorganisms. Many researchers call covert bacterial microorganisms «endophytes» due to their presence in the culture of plant objects that underwent surface sterilization. We will use the term «endophytic bacteria» as it is used by the authors of the cited papers.

According to the widely used definition, endophytes are microorganisms that live inside the plant during the whole or part of the life-cycle and do not cause symptoms of diseases [32]. In nature, they enter the plant through the stomata, wounds, and root system. A significant role in the formation of endophytic microflora is played by transfer of microorganisms through seeds, as well as their introduction by vector organisms, the invertebrates and fungi [28, 32]. Introduced microorganisms may be included in the plant microflora at the point of entry and/or distributed throughout the plant [32], and obligation is not a prerequisite [33].

Endophytic bacteria have been found in cell cytoplasm, intercellular

space [34] and vascular system [35] of plants. In numerous papers the presence of endophytic microorganisms in in vitro cultivated plant explants was documented by light and electronic microscopy, and using in situ hybridization [15, 16, 21, 36-38].

Sources of bacterial microflora. Endophytic bacteria are derived from epiphytic associations of plant rhyzosphere and phytosphere. The initial explants mostly are the causal factors of endophitic infection during in vitro cultivation. Aseptic explants are hardly prepared from rosette, woody and perennial plants [12, 38], in case of wet habitats or sampling when the weather was wet and warm, and also from the diseased plants [21, 39]. Infection can occur when specific explants are used, in particular, the underground organs (root, rhizome, corm) [40, 41], the buds which are tightly covered with multilayer scales, the fragments of epidermis, especially hairy one [42, 43]. Some bacterial epiphytes can remain inaccessible to disinfecting agents, particularly in the closed stomata, in folds on the surface of the root cuttings, or in the epidermal intercellular space [5, 9].

Systemic infection of in vitro plant culture can also be due to bacterial contamination of the operator's position or the operator himself, glassware and instruments used [2, 44]. Spores of some bacterial species remain viable after autoclaving [36] and in ethanol [37].

Approach to detection and identification. There are different ways to reveal a latent bacterial contamination. In particular, selective media, physiological tests, bacteriophages, specific fatty acid and protein assay are commonly used. Besides, recently improved MALDI TOF (Matrix assisted laser desorption/ion-ization time of flight) mass spectrometry and molecular markers (i.e. RAPD-PCR — random amplified polymorphic DNA polymerase chain reaction, REP-PCR — repetitive extragenic palindromic polymerase chain reaction, AFLP — amplified fragment length polymorphism, ARDRA — amplified ribosomal DNA restriction analysis, 16S rRNA) are successful in bacterial typing. All they are specific at different taxonomic levels, being mostly suitable for the estimation at family, genus and species levels. For subspecies, biovars and strain attributing, current biochemical and molecular genetic techniques are preferable [45).

A conventional approach to bacteria detection and identification is based on their cultural and morphological properties, as well as the biochemical tests [46] carried out with no expensive equipment. However, the methods of classical microbiology are more available but thrivelles in case of non-cultivated forms unable to metabolize the nutrient substrate. Molecular identification of the genotypes is based on the analysis of conservative rRNA genes which present in all bacterial cells and are genus-specific in most microorganisms [23]. For identification, the genes of 23S rRNA of ~3000 bp, 16S rRNA of ~1500 bp and Internal Transcribed Spacers (ITS) should be sequenced [47]. In the 16S rRNA genes there are both conservative regions characteristic for all prokaryotes and species-specific sites suitable for identification [48, 49]. The sequences of 16S—23S rRNA ribosomal spacers are even more informative due to their high variability in size and structure compared to the genes themselves. Thus, the ITS are preferably used to attribute the microbial species and strains [50]. The ITS and 16S rRNA gene fragments are amplified in PCR with metagenomic DNA and specific primers [13, 51, 52]. After sequencing PCR products their homology to DNA sequences deposited in GenBank database should be estimated [53] for taxonomic identification. According to A.V. Pinevich [54], genome sequencing has been reported for 60 bacterial species while their total number is 5007.

Identification of bacteria in in vitro culture. Identifi-

cation of bacterial colonization of in vitro plant tissue culture allows us to study effects of microorganism on the host cells, to apply specific chemotherapy, and to create databases with regard to microorganisms associated with plant tissue cultures. In early papers there were data mostly obtained by classical methods including study of growth on different media, Gram staining, morphology and color of the colonies [4, 6, 9, 10, 46]. Due to advances in studying taxonomic diversity among bacteria associated with plant tissue cultures by means of molecular methods, the database of these microorganism progressively increases. In the Table there is a taxonomic composition of bacterial endophytes from in vitro plant culture for a relatively limited range of the samples tested which indicates a diversity of bacterial form able to colonize plant tissue cultures as a very specific niche quite different from the natural one. It also should be noted the absence of specific bacterial composition in case the plants were from different systematic groups and the explants derived from different organs. The data on bacterial identification reported earlier for plant tissue cultures allow us to make the same conclusion [4, 6, 9, 10].

Among identified endophytes there are those potentially useful for intact plants, namely *Streptomycete*, *Pantoea agglomerans*, etc., as well as pathogenic for humans, in particular, *Ralstonia mannitolytica*, *Staphylococcus epidermidis*, *Corynebacterium amycolatum*, *Bacillus neonatiensis*, *Salmonella* and *Nocaridia* spp. [13].

Dynamics of bacterial expression. Long symptomless presence of endophytic bacteria in in vitro plant cultures is due to two opposite processes, the growth limitation and the support of viability. In the course of cultivation the bacteria growth and reproduction are suppressed by some factors, such as acidification, suboptimal temperature (25 °C), and probable activation of defense mechanisms against microorganism in the tissue culture [55]. At the same time, the exudates secreted by explants support the viability of bacteria, since most bacteria, despite the heterotrophy, are not sustainable in the absence of plant [12, 55]. As a result, the number of bacteria associated with the plant tissue culture is at a medium level leading to symptomless and long persistence of infectious agents which are difficult to remove.

Rapid proliferation of bacterial cell can occur under slight modification of the conditions, such as an increased temperature, changes in acidity or nutrient contents (in particular, due to additional N from destroyed explant tissues) [55, 56] or at high cytokinin levels in the media for subcultivation of old cultures [8, 57]. Proliferation is induced by activated secretion of exudates by explants which, in turn, can be stimulated by temperature, growth of the culture, or transfer to the rooting medium [55]. Intensified bacterial growth can lead to visible symptoms in in vitro culture and/or observed growth on the media used for explant cultivation [3]. When bacteria migrate from the cultivated tissues into the medium, they usually form a turbid halo observed by many researchers [7, 10, 11, 58, 59]. In vitro cultivation could be a stress factor stimulating growth of endophytes which under these conditions are not controlled in the same way as in an intact plant [16].

In numerous papers it is noted that the more is passage number, the higher rate of latent contamination can observed, while the rate of pronounced infection decreases. Besides, the composition of microbial community can also change, i.e. the number of Gram-positive microorganisms increases as well as the rate of those capable to growth on a nutrient media [4, 13, 21]. Thus, in micropropagated banana plants the endophytes were nonculturable for three passages and detected only by sequencing 16S rRNA gene (viable but nonculturable bacteria — VBNC). Nevertheless, from 4^{th} to 18^{th} passage the culturable bacteria were

found in the same microplant [13, 21]. The authors of the cited papers suggested that in the course of plant cultivation the VBNC endophytes can change their status.

Impact on colonized in vitro plant cultures. Bacteria associated with plant tissue culture can adversely affect the regeneration of callus, cell suspension and protoplasts [38, 56], depress microclonal propagation, shoot growth and rooting, cause death of samples cultivated in vitro and ex vitro [3, 11, 13, 21, 29, 36, 37, 57, 60]. Latent contamination may negatively affect the reproducibility of protocols [29] and be associated with the emergence of epigenetic somaclonal variants [61]. A probable reason for the negative effect of latent bacterial microorganisms is their increased number. Some researchers consider that fact in a connection with plant tissue culture death after second or third subcultivation [18, 62]. In our investigation, the second and third passages turned out to be critical for in vitro micropropagation of raspberry explants [59]. A depressive effect of bacteria can also be due to changes in medium pH or nutrient level (particularly, because of consumption of saccharose) or synthesis of herbicidal substances [63].

Study of the impact of axenic bacteria on in vitro plant culture is of special interest. It was shown that *Acinetobacter* and *Lactobacillus plantarum* filtrates from degrading callus decreased sharply the shoot regeneration when adding to medium or inoculating explants [56, 63]. *Mycobacterium obuense* and *M. aichiense* depressed seed development in vitro [38].

As far as the helpful effect of endophytes on in vitro plant cultures the researchers began to study later, the data obtained are less numerous. In some papers a positive influence of *Methylobacterium* on induction of organogenesis and embryogenesis was reported [15, 17, 64-68]. Presumably the *Mycobacterium* sp., *Methylobacterium* spp., *Pseudomonas* spp., *Rhodotorula minuta* endophytes detected in pine tissue culture by in situ hybridization can affect positively the in vitro morphogenesis [17] similar to that observed in animal cell culture [69]. The stimulation of somatic morphogenesis by *Bacillus circulans* was reported in *Pelargonium* × *hortorum* Bailey [70].

Thus, bacterial microorganisms associated with plant tissues in in vitro cultures, on one side, are the factors depressing explant growth, development and viability, and on the other side, they can effect them positively.

In gene banks the plants are not preliminarily checked for endophyte contamination before cryoconservation. Gnotobiotic state of certified plant material allows us to avoid transfer of infection under micropropagation and is a criterion of safe storage of the genotypes in the controlled conditions. In the course of certification the main viruses, mycoplasmas and bacterial microflora are analyzed and eliminated from the plant material [39]. Diversity, significant number and changes in bacteria of in vitro plant cultures necessitate its regular checking for contamination by various methods that complicates the procedure in big collections of plant gene banks.

Endophytes of in vitro plant cultures

Plant genus, species (variety)	Culture type (time of in vitro cultivation)	Genus, species of bacteria (frequency, %)	Reference
Chrysanthemum (Arka Swarna)	Microplants (1-7 passages)	Morphtypes of *Curtobacterium citreum*	[8]
Pinus sylvestris	Callus culture	*Hormonema dematioides* (isolates L, M), *Methylobacterium extorquens* (isolate F), *Pseudomonas synxantha* (isolates G, H, J), *Pseudomonas* sp. (isolates K, N), *Rhodotorula minuta* (isolate T)	[16]
Pinus sylvestris	Callus and suspension cultures	*Methylobacterium extorquens*	[18]
Prunus cerasus (Montmorency)	Microplants	*Pseudomonas aeruginosa*	[19]

Table (continued)

Bactris gasipaes	Microplants	*Brevibacillus* sp., *Moraxella* sp.	[20]
Musa sp.	Microplants (long microcloning)	*Alcaligenes*, *Bacillus* spp., *Brachybacterium*, *Brevibacterium*, *Brevundimonas*, *Corynebacterium*, *Enterobacter, Klebsiella*, *Kocuri*, *Methylobacterium*, *Microbacterium*, *Oceanobacillus*, *Ochrobactrum, Pantoea*, *Pseudomonas*, *Ralstonia*, *Staphylococcus*, *Tetrasphaer* spp.	[21]
Musa sapientum (Chini champa)	Shoot tips (1-2 week cultivation)	Gram-positive: *Bacillus megaterium*, *Cellulomona uda*, *C. flavigena*, *Corynebacterium paurometabolum* Gram-negative: *Erwinia cypripedii*, *Klebsiell* sp., *Pseudomonas* sp.	[22]
Larix, Picea	Suspension culture (6-8 week cultivation)	*Acinetobacter*	[56]
Chrysanthemum (Arka Ravi)	Microplants	*Enterobacter*, *Methylobacterium* spp., *Ralstonia*	[57]
Rubus idaeus, *Fragaria ananassa*, *Cerasus vulgaris*, *Ribes nigrum*	Microplants from in vitro collection	*Arthrobacter* (23.5 %), *Bacillus* (51.5 %) More rare are *Agrobacterium*, *Bacterium*, *Brevibacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Pseudomonas*	[58]
Jatropha curcas	Leaf explants	*Enterobacter ludwigii*	[62]
Fragaria ananassa (Camarosa, Sweet Charlie, Oso-Grande)	Meristem	17 bacterial strains of *Bacillus*, *Sphingopyxis*, *Virgibacillus*	[64]
Musa sp.	Shoot tips	*Bacillus*, *Brevibacillus*, *Paenibacillus*, *Staphylococcus* spp., *Virgibacillus*; *Actinobacteria* (*Cellulomonas*, *Micrococcus*, *Corynebacterium*, *Kocuria* spp.); α-*proteobacteria* (*Paracoccus* sp.); Y-*proteobacteria* (*Acinetobacter* spp., *Pseudomonas*)	[51]
Ilex dumosa	Segments of shoot nodes	*Achromobacter*, *Stenotrophomonas maltophilia*	[71]
Echinacea	Microplants	*Acinetobacter*, *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Wautersia* (*Ralstonia*)	[72]
Carica papaya	Shoot tips	*Agrobacterium* (*A. tumefaciens*), *Bacillus* (*B. benzoevorans*), *Brevundimonas* (*B. aurantiaca*), *Enterobacter* (*E. cloacae*), *Methylobacterium* (*M. rhodesianum*), *Microbacterium* (*M. esteraromaticum*), *Pantoea* (*P. ananatis*) (70 %), *Sphingomonas*, *Wautersia* (*Ralstonia*)	[73]
Potato	Microplants	*Bacillus pumilus*	[74]
Carica papaya	Shoot tips (1 month cultivation)	*Lysinibacillus fusiformis*, *Paenibacillus* sp., *Pantoea* sp., *Ralstonia mannitolilytica*, *Sphingomonas* sp.	[75]
Limonium simuatum	Microplants	*Alcaligenes* sp., *Pasteurella multocida*, *Stenotrophomonas maltophilia*	[76]
Ananas comosus	Microplants (5 year cultivation)	*Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*	[77]
Piper nigrum, *Piper colubrinum*, *Taxus baccata* subsp. *wallichiana*, *Withania somnifera*	Callus culture (primary explants)	*Aminobacter*, *Flavobacterium*, *Morococcus*, *Paracoccus Pseudomonas*, *Psychrobacter*, *Rhizobacter*	[78]

Antibacterial treatment. Antibiotics are used to eliminate bacterial microorgamisms [79]. Some antibiotics used for plant chemotherapy are described in a review of G. Seckinger et al. [80]. In order to eliminate bacterial contamination from plant culture the antibiotics should possess bactericide effect, being also inexpensive, non-toxic to humans, soluble in the medium with no influence on pH [9, 61]. The choice of most active antibiotics of wide spectrum (or effective in specific combinations) is more successful if the target bacteria are identified. In case of combination, particularly for synergistic antibiotics, the risk of resistant bacteria emergence decreases, nevertheless, some antibiotics

are the incompatible chemicals neutralizing each other [71, 72]. Most bacteria identified in plant culture are Gram-negative, and they are most hard to eliminate because of, in fact, two layer cell membrane preventing antibiotics input. After the chemotherapy the plant material should be checked for the presence of bacterial contamination for 2-3 passages [71].

Usage of antibiotics is complicated by different reasons. The specific concentration should be optimized, and its effect on plant tissue culture should be taken into account. Antibiotic-resistant strains inevitably occur. Some antibiotics destroy chloroplasts and mitochondria resulting in chlorosis and morphological changes in explants [6, 9]. The advances in antibacterial therapy with regard to plant tissue culture will be largely determined by progress in investigations and development of new generation of antibacterial preparations.

So, diversity of latent bacterial microflora, the endophytes, of in vitro plant cultures is significant and includes forms which can influence on the colonized plant culture both negatively and positively. As the number of plant culture passages increases, the bacteria titer may increase, too, and composition of bacterial association as well as bacterial culturability can change. Complications in obtaining gnotobiotic cultures, particularly in vitro collections of plant gene banks, are caused by difficulties in detection and elimination of bacterial microflora.

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SNOW MOLDS: HISTORY OF THE STUDY AND CONTROL
(review)

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Abstract

Snow mold is caused by pathogenic low-temperature fungi and fungi-like pathogens which can attack grassy winter and perennial plants and even woody plants. Pathogens infect crops in autumn and develop under snow and early in spring at low temperatures. History of the emergence of the terminology for pathogenic low-temperature fungi, the appearance of the «snow mold» terms and domestic «vyprevaniye» (eng. «dumping-off») are represented, and various snow molds and their pathogens in Russia are described. Recent advances in agrochemical, chemical, biological and breeding technologies used to provide snow mold control are under consideration in detail, particularly data obtained in North America (USA, Canada), North Europe (Sweden, Norway, Finland), Asia (Japan) with special attention to the investigations in Russia. Crop rotation using crops being not the host plants of these pathogens and thus resistant to them is considered as rather effective agrotechnology decreasing plant damage from snow molds, and also deep tillage, early or late sowing, show thawing by its covering with black materials, monthly use of some composts are also discussed. Seed sterilization against *Microdochium nivale* infection is widely used in North Europe. In Russia the Baytan, Benlat, Granoza and Pentiuram are used on winter crops against *Typhula incarnata* and *T. idahoensis* (syn. *T. ishikariensis*). Fungicides are economically effective in the years of strong damage from snow molds, at that, pathogenic species differ in sensitiveness to fungicides. Characteristics of definite fungicides used are discussed. Biological suppression also is a method for anti-snow mold protection. For the purposes, the antagonistic agents effective in summer when snow molds are dormant, as well as low temperature agents active in the period of snow mold development can be used. Natural suppressors such as composts and antagonistic organisms were successful against *Typhula spp*. No special breeding for plant resistance to snow molds was carried out in Russia until recent time, nevertheless, in numerous investigations some grain crop species resistant to snow mold have been revealed. Particularly, by immunological assessment of 500 specimens from the VIR World Collection (N.I. Vavilov All-Russian Institute of Plant Industry, St. Petersburg) and domestic varieties the resistant forms are described as follows: Shatilovskaya tetra, Populyatsiya I-82 tetrs, Sibirskaya krupnozernaya, Taezhnaya, Kirovskaya 89, Vyatka 2, Dymka, Rosinka, Ilim, Falenskaya 4, Purga, F4-92, Chulpan 3, Korotkostebel'naya 6, Khar'kovskaya 88, Tatarskaya 1, Bezenchukskaya 88, Volkhova, Takovskaya 29; LAD-287 St-2614, Antonnisnie, Leelondzkie Kartowe № 1, Leelondzkie Krotnoslomix × Baltycnie (Poland), Epos, Rerus (DDR), Inzucht 74/2, Inzucht 108/8 (Sweden), к-10953 (Finland), Feniks (Belgium), к-11385 (Yugoslavia), к-11150, к-11389 (Portugal), к-11306 (Argentina), к-11179, к-11180 (USA), к-11388 (Tajikistan), к-11398 (Georgia), к-11131 (Azerbaijan), Belta tetra (Belarus), Beve (Ukraine).

Keywords: low temperature fungi, snow molds, anti-snow mold agrotechnologies, chemical fungicides, biomethod, breeding for plant resistance to snow molds.

The snow mold is caused by pathogenic low-temperature fungal and fungi-like pathogens which attack overwintering plants [1, 2]. The low-temperature fungi have been previously called psychrophilic (effectively grow at low temperatures) and psychrotrophic (affect plants at low temperatures, but can grow in mesophilic conditions). These definitions were suggested by R.Y. Morita [3] with regard to low-temperature bacteria. T. Hoshino and N. Matsumoto [4] have recently proposed a new term, «cryophilic fungi», because fungi are more complex organisms than bacteria, and their development cycle often comprises both mesophilic and psychrophilic stages. Show mold pathogens can attack not only grassy winter and perennial plants, but also woody plants, such as first-year pine seedlings [5] or the lower parts of tree branches under snow [6].

In the USSR, the term «damping-out» was used in snow mold studies [7-9]. This term was introduced in the 19th century, before the studies of low-temperature fungi began [10], and it reflects physiological changes in plants, rather than the infectious nature of the disease [11, 12]. In the Soviet Union and Russia, only the disease caused by the fungus *Fusarium nivale* (Fr.) Ces. was called «snow mold» [13]. There are many names of the snow mold in the world scientific literature: pink snow mold caused by the fungus *Microdochium nivale* (Fr.) Samuels & Hallett [syn. *Fusarium nivale* (Fr.) Ces.] [14-16]; gray snow mold, caused by *Typhula incarnata* Lasch. ex Fr. [17, 18]; speckled (or gray) snow mold caused by the fungus *T. ishikariensis* S. Imai [17, 19, 20]; sclerotial snow mold, caused by fungus *Sclerotinia borealis* Bubak & Vleugel [syn. *Myriosclerotinia borealis* (Bubak & Vleugel) Kohn, *S. grami-nearum* Elenev et Solkina], often called «snow scald» or «snow blight» [21, 22]; as-yet-unnamed, relatively recently described snow mold caused by the fungus *S. nivalis* I. Saito (23]; Pythium snow mold, caused by fungi-like Oomycetes *Pythium* spp. (*P. iwayamai* S. Ito, *P. okanoganense* P.E. Lipps and *P. paddicum* L.) [24-26], etc.

It is hard to control the snow mold because pathogens usually infect crops in autumn and develop under snow and early in spring at low temperatures. Plant spraying with fungicides is difficult or impossible in this period. Various snow mold forms are favored by the thick snow cover and prolonged spring. Plant freezing provokes the growth of the sclerotial snow mold caused by the necrotroph *S. borealis.* Conversely, non-frozen soil promotes the damage by the biotroph *T. ishikariensis*. Therefore, the sclerotial snow mold prevails in the areas where plants are subject to freezing (Volga Region, Urals, Siberia) [27]. However, Novosibirsk populations of the fungus *T. ishikariensis* are much more adapted to freezing than Moscow strains [28]. In addition, it is difficult to predict winter conditions; explosive development of the snow mold is a quite rare phenomenon, and the cost of protective measures is high.

The snow mold is controlled using agrotechnical, chemical and biological methods. Another way to reduce damage by the snow mold is selection of snow mold-resistant plants.

Agrotechnical method. In rye cultivation, the crop rotation and deep ploughing reduce the *S. borealis* inoculum quantity within the field [5, 29] but do not provide efficient control of the fungus because airborne ascospores are mainly transferred from uncultivated lands, such as meadows.

Placement of solar-absorbing black materials on snow promotes thawing and makes it possible to reduce damage from the snow mold. Thus, it was V.V. Gulyayev who recommended to deliberately get rid of snow in late spring with prolonged thawing (especially in depressed areas) by dispersing fragmented peat or ash over the surface [5]. Many authors [30-32] have studied the influence of graphite and carbon powder on acceleration of snow thawing. Mechanical removal of snow is impractical [33] and may increase the damage of plants due to their high sensitivity to frosts in early spring after snow removal [34].

Crop rotation and deep ploughing are key measures to reduce the number of sclerotia in soils. Such agronomic practice contributes to minimization of the forage legume damage caused by *S. trifoliorum* and *S. sativa* on overwintering dicotyledonous crops [35, 36].

Due to early sowing of the winter wheat in autumn, stronger plants can better sustain an attack by the speckled snow mold than small ones in case of later planting [22, 34, 37]. The fields sown very late sometimes avoid getting infected, but once infected, plants perish [34]. Early planting leads to destruction of leaves by the snow mold, however, in general, the plant grows again from the tillering node and recovers. Field trials and experiments in climatic chambers [38, 39] confirm that larger plants survive better such snow molds as speckled

and pink ones, but the larger the plant, the greater is the number of sclerotia on it, which leads to greater infection load on soil.

Although snow mold growth is significantly influenced by the seeding time, it does not depend on other agricultural practices (tillage, equipment, seeding depth) in this period [34, 37, 39].

Nitrogen fertilization has the greatest effect on the pathogenic process. Nitrogen application on lawn grasses leads to increased growth of the snow mold [40]. Additional fertilization with some composts on a monthly basis is efficient with regard to containment of a wide range of diseases, including the snow mold caused by the fungi *Typhula* spp. and *Microdochium nivale* [41].

In case of rotation with the crops that are not referred to host plants for snow mold pathogens, snow mold growth decreases. The winter wheat cultivated in the field where spring wheat has been under cultivation for several years is affected by the fungus *T. idahoensis* in a lesser extent than in case if seeding after winter wheat [31]. After the medick, the number of *T. idahoensis* sclerotia was greater than after winter wheat [42]. Whereas the wheat seeded after leguminous plants (medick, white sweet-clover or pea) demonstrated insignificant damage by the snow mold, the extent of such damage increased with every subsequent winter wheat seeding [37].

C h e m i c a l m e t h o d . Grain sterilization against the snow mold [43, 44] has been tested and is widely used to control the *Fusarium* seed infection caused by *M. nivale* in North Europe [45]. In Russia, it was recommended to treat winter grain crops [*T. incarnata* and *T. idahoensis* (syn. *T. ishikariensis*)] with Baitan Universal, Baitan, Benlate (Fundazol), Granozan and Pentiuram [46] against the Typhula blight, although the recommendation on sterilization using benzimidazole chemicals, such as Benomyl, Fundazol, etc., against the *Typhula* spp. causes doubt. Efficiency of fungicides against various species of snow mold pathogens is different, and, in some cases, such treatment may even stimulate the snow mold growth. For example, the extent of affection by the fungi of the genus Typhula increases in case of treatment with Benomyl [47, 48], Cercobin M, Bavistin [49]. Stronger progression of the *Typhula* blight in case of Benomyl is associated with mycelium growth stimulation [50] and suppression of antagonistic mycobiota [51]. *Typhula* blight affection may also increase in case of treatment with other pesticides, such as insecticide Dimetalan [52].

In the middle of the 20th century, mercurial and many other fungicides, which are now prohibited for environmental reasons, were used for snow mold control [53, 54]. A negative aspect is that mercurial pesticides are not decomposed into non-toxic components; instead, they persist in soil, for example, on golf courses [55], and may contaminate adjacent aquatic ecosystems [56]. Use of mercury compounds in fungicides is prohibited in Russia [57].

There is an opinion that field crop spraying against the snow mold is not cost-effective in general [33]. Although fungicide can efficiently protect grain crops [44, 47, 58], epiphytoties are sporadic, and it is impossible to determine in advance if treatment is needed. Spraying with fungicides is economically feasible in years with severe damage, when substantial benefits can be obtained (for example, increase in yield, product quality improvement or stable income). As a result, the majority of papers on fungicide application discuss studies on grass plots, especially on golf courses, due to their high cost [59, 60].

In northern Japan, as reported by I. Saito et al. [61], fungicide spraying on leaves early in winter is a mandatory practice in wheat cultivation because the snow mold reduces wheat yield. On wheat fields, *S. borealis* is usually encountered in combination with one or more pathogens causing the snow mold, for example, *T. ishikariensis* or *M. nivale*. Therefore, a need arises for a fungicide which is effective against all these pathogens, or a mixture of fungicides.

On Hokkaido, where the winter wheat is cultivated more extensively than elsewhere in Japan, the following active substances of fungicides are recommended for snow mold control: Fluazinam against *S. borealis*, *T. ishikariensis*, *T. incarnata* and *M. nivale*, Benomyl against *S. borealis*, Thiophanate-methyl against *S. borealis*, Iminoctadine Triacetate against *S. borealis* and *M. nivale*.

In Canada, before formation of snow cover, the snow mold is controlled by spraying of fungicides with the following active substances: Chloroneb, Chlorotalonyl, Iprodione, Propiconazole and Quintozene [62].

In the USA, a list of allowed chemicals (by active ingredients) against the snow mold is wider: Azoxystrobin, Chloroneb, Chloroneb + Thiophanate-methyl, Chlorotalonyl + Fenarimol, Chlorotalonyl + Thiophanate-Methyl, Cyproconazole, Fenarimol, Flutolanyl, Iprodione, PCNB (penta-chloronitrobenzene), Propiconazole, Tiram, Triadimefon, Vinclozolin [63].

At present, in the Russian Federation, in accordance with the list of fungicides allowed for use in 2014 [57], fungicides with the following active substances may be applied: Azoxystrobin, Iprodione, Propiconazole, Tiram, Triadimefon, Fenarimol, Chlorotalonyl, Cyproconazole (it should be noted that there are no recommendations on their use on lawn grasses and winter grain crops against the snow mold). With regard to the snow mold (including the *Typhula* one), it is advised to perform preplanting treatment of winter rye grains with preparations with the following active substances: Carbendazim, the preparations Kolfugo Super, SC (suspension concentrate), Kolfugo Super Color, SC (Agro-Chemie Kft., Hungary); Ferazim, SC (LLC Agro Expert Group, Russia); Karbonar, SC (LLC Agrobyuro RUS, Russia); Carbendazim + Carboxin, the preparation Kolfugo Duplet, SC (Agro-Chemie Kft., Hungary); complex mixture: Klotianidin + Fluoxastrobin + Prothioconazole + Tebuconazole, the preparation Scenic Combi, SC (Bayer CropScience AG, Germany).

It is reported about the Terminator preparation with the same active substances as in Kolfugo Duplet [64], however, it is not included in the list of pesticides allowed for use in the Russian Federation [57].

There are no registered fungicides against the sclerotial snow mold of the rye (*S. borealis*) in Russia. According the data of All-Russian Research Institute of Plant Protection (St. Petersburg) [64], this disease is especially common in the Central and Volga-Vyatka Regions where crop losses achieve 20–25%.

Fungicide testing has shown that the most efficient protection of grasses and winter grain crops against the gray snow mold (*T. ishikariensis*) is provided by spraying the plant leaf surface, before formation of perennial snow cover, with preparations Alto (0.2 l/ha), Alto Super (0.4 l/ha) (Syngenta AG, Switzerland), or combination of the above treatments with preplanting treatment of seeds with preparations Dividend Star (2 l/t) and Dividend Total (2 l/t) (Syngenta AG, Switzerland) [65]. Sterilization of winter grain crop seeds against the gray snow mold alone is not efficient, but, taking into account the protective action of these treaters against root rots, it is reasonable to combine seed sterilization with ground treatment using proved fungicides [65]. In field conditions, the best protection of winter grain crops against the snow mold (*M. nivale* and *T. ishikariensis*) was provided by crop spraying with Alto Super and Tilt at rate of 0.5 l/ha before formation of snow cover. Biological efficiency of application of these preparations was 96.1% and 93.1%, respectively. It has been found [66] that plant treatment with growth regulators Obereg (LLC Orton, Russia) and Silk (CJSC Elkha-Silk and Sayany-Elkha, Russia) delays plant infection by the fungus *T. ishikariensis* by 30 days; in case of fungicide Alto Super, the delay is within 75-90 days, depending on snow cover thickness and longevity. The protective action of Alto Super was observed within 75-90 days under weather conditions favorable for Typhula blight development and 90-105 days under less fa-

vorable conditions. When seed treatment with preparation Maxim was combined with autumn spraying with Alto Super, wheat plants were better than the control during the vegetation period with regard to physiological parameters (chlorophyll content, water-retaining capacity, dry weight of plants) and, ultimately, with regard to the yield which was higher by 70-80 % than that of the control plants. Biological efficiency of autumn treatment of winter crops with Alto Super (0.5 l/ha) was 80–90% and did not depend on the seed treaters applied. It is notable that even the half-rate (0.25 l/ha) was efficient in conditions of moderate development of the Typhula blight. In a study on 10 species of lawn grasses [67], eight species of the genus *Fusarium* Link, including *M. nivale*, have been considered as pink snow mold pathogens. It has been found that fungicide Bravo, SC (active substance—Chlorotalonyl, 500 g/l) was efficient in case of autumn spraying at rate of 2.0 l/ha for grass stands of the English ryegrass and red fescue. In 2010-2011, this preparation reduced pink snow mold development by a factor of 2–4 as compared with reference preparation Quadris, SC (Azoxystrobin, 250 g/l, as an active substance).

Because it is difficult to forecast weather conditions, multiple treatments are often required instead of one. New active substances and formulas are brought to the market from time to time; however, protection against the snow mold using fungicides requires an alternative [68].

Biological method. In development of a biological method, it is necessary to begin with the study of relationships between organisms [69]. In case of low-temperature forms, antagonists may be represented by both mesophylls during the aestivation of snow mold pathogens (sclerotia), and low-temperature bioagents active in the course of pathogen development [70].

Both natural suppressors, such as composts, and antagonistic organisms were successfully used against the snow mold caused by *Typhula* spp. in a number of studies.

Monthly application of relatively small amounts of suppressive composts (5 kg/100 m^2) during the vegetation period can suppress many lawn grass diseases, including the snow mold caused by the fungi of genus *Typhula* [71, 72]. Also, shocking doses (100 kg/100 m^2) of some composts are efficient when applied on golf courses in late autumn. The main problem associated with the use of suppressive composts is their variable efficiency by years and plots [73].

The influence of 164 bacterial isolates in seed treatment against the root rot caused by *Fusarium culmorum* and the snow mold pathogen *M. nivale* has been investigated in Sweden. The performance of three *Pseudomonas fluorescens* and one isolate of the genus *Pantoea* (isolate MF 626) has been noted: they were as efficient as fungicide Guazatine [74]. The experiments aimed at comparing seed treatment and spaying with the bacterium *Pseudomonas brassicacearum* (strain MA_{250}) during planting have demonstrated some efficiency, with a less noticeable effect in case of spraying [75].

Biofungicide Elena Zh applied on winter wheat Bezenchukskaya is biologically efficient against the snow mold at the level of chemical fungicide Ferazim, SC; also, it exhibits growth-stimulating properties, which allows it to ensure high yield of grain crops even in severe weather conditions (drought) [76].

Some species of the genus *Trichoderma* are antagonists of summer sclerotia of *T. incarnata* and can reduce the inoculum potential of the gray snow mold pathogen. The viability of sclerotia significantly decreased after incubation with *Trichoderma* cultures within 6 days [77].

It has been found [78] that in summer in field conditions over 90 % of the sclerotia of *T. incarnata* are naturally killed by mycoparasites, whereas the sclerotia of *T. ishikariensis* of biotype A mainly survive. The mycoparasites *Co-*

niothyrium minitans Campbell, *Gliocladium roseum* Bain. and *Trichoderma* spp. were extracted from the sclerotia of *T. incarnata*. All of them also parasitized *T. ishikariensis* of biotype A in laboratory conditions. However, a practical result was not achieved because even several survived sclerotia in autumn may lead to spread of the pathogen with basidiospores, and field treatment of the sclerotia of *T. ishikariensis* of biotype A with mycoparasites is very difficult during the plant vegetation period.

Attempts to use bacteria as antagonistic organisms have not led to noteworthy results, although the pseudomonas fluorescens isolates antagonistic to *Typhula incarnata* and *T. ishikariensis* of biotypes A, B, and C have been revealed [78-80]. In addition, two strains of *Bacillus* sp. with similar properties have been isolated [80].

At first, *Typhula phacorrhiza* was considered as a pathogen previously not observed on lawns, but it also turned out that this species was not pathogenic on the creeping bent in field trials with inoculation. Conversely, the fungus suppressed the gray snow mold development [81-83]. In Japan, a similar fungus, *T. phacorrhiza,* suppressed the snow mold on the English ryegrass [84]. In Canada, the *T. phacorrhiza* isolates extracted from wheat residues had different capability of suppressing the gray snow mold in field trials within more than 3 years [85, 86]. The efficiency of more than 29 isolates of *T. phacorrhiza* has been screened on 14 plant species against *T. ishikariensis* and *T. incarnata* [87, 88], and the most active strain TP94671 has been identified. No strong correlation between laboratory and field trial data was noted in *T. phacorrhiza* isolate tests [89], therefore the laboratory results cannot serve as preliminary data for selection of antagonistic strains [89]. Gray snow mold forcing-out from fields is obviously promoted by high capacity for utilization of structural and reserve carbohydrates in combination with a wider optimum temperature range in case of *T. phacorrhiza* as compared with pathogenic *T. ishikariensis* and *T. incarnata* [90].

One possible problem associated with the use of *T. phacorrhiza* as a bioagent is its potential pathogenicity. Some isolates of *T. phacorrhiza* were pathogenic on wheat under controlled environment conditions and field conditions [91, 92]. In other field trials, the isolates of *T. phacorrhiza* were not pathogenic with regard to a number of lawn grass species [89].

In Russia, *T. phacorrhiza* was first applied for control of the most aggressive speckled snow mold (caused by *T. ishikariensis*) by S.V. Tazina [93]. The autumn application of the fungus *T. phacorrhiza* on winter wheat crops with the *T. ishikariensis* infection background led to less damage by the speckled snow mold in spring. Biological efficiency of winter grain crop protection using the fungus *T. phacorrhiza* (200 g/m^2) was 75.2 %, which is by 30.3 % and 17.6 % higher than after treatment with Fundazol and Bayleton, respectively.

A psychrotolerant hyperparasite, *Trichoderma atroviride* P. Karst, has been found in the subarctic areas of Alaska. It has been revealed that it suppressed the development of the wide range of snow mold pathogens: *Coprinus psychromorbidus*, *Microdochium nivale*, *Myriosclerotinia* (*Sclerotinia*) *borealis*, *Pythium* spp., *Typhula incarnata*, *T. idahoensis* and *T. ishikariensis* (biological species 1 according to N. Matsumoto et al.) [94-98]. *T. atroviride* is a mesophyll which is well adapted to cold conditions. Its temperature range is from 4 °C (or lower) to 33 °C, which makes it possible to use it for control of the phytopathogens causing damage to the roots, stems and other organs of plants in cold conditions when plant tissues are vulnerable. *T. atroviride* rapidly grows and produces large quantity of spores. Isolate CHS 861 of *T. atroviride* is naturally resistant to Metalaxyl (Ridomil), Captan and PCNB (Terrachlor) [95]. *T. atroviride*

can use snow molds as a feed source. The hyphae of the fungus easily penetrate cell walls and interweave with the hyphae of the snow mold pathogen. The cells disintegrate and are rapidly lysed. The chitin-lytic enzymes produced by *T. atroviride* obviously play an important role in mycoparasitism on snow mold pathogens [96].

Other organisms that can suppress the snow mold pathogen growth, such as the fungus *Actemonium boreale* described by J.D Smith & J.G.N. Davidson [99] in Canada, have been identified as well. It is also antagonistic with regard to other snow mold pathogens and exhibits slight parasitic properties on two grass species, however, it is unable to suppress *M. nivale* and low temperature basidiomycete (LTB) in trials under controlled conditions [100].

The studies of the winter wheat nematofauna in pink snow mold niduses [101-104] have resulted in identification of several species of low-temperature mycotrophic nematodes (*Aphelenchoides saprophillus* Franklin, *Paraphelenchus tritici* Baranovskaya, *Aphelenchus avenae* Bastian) consuming pink snow mold pathogen *M. nivale*. Among the mycohelminth species added into test tubes with the mycelium of the fungus *M. nivale*, the most intensive development was observed for *A. saprophillus*. At 5 °C this species destroyed the fungus mycelium within 60-70 days after adding into the test tube, its number being 1,208 specimens per test tube. In the presence of *P. tritici* or *A. avenae,* after 60-70 days, the fungus mycelium was only on 40-50 % of the surface of the growth medium. The number of nematodes was significantly less than in test tubes with *A. saprophillus*. A microplot field trial with application of the mycohelminth *A. saprophillus* (160,000 specimens, 80,000 specimens and 38,000 specimens) on the winter wheat crops infected by the pink snow has shown that biological efficiency was 62.7%, 52.7% and 43.1%, respectively. In the first case, the efficiency was by 6.7 % and 45.3 % higher than in the second and third ones, respectively. Nematodes had no influence on economic efficiency (yield), but they substantially reduced the degree of disease development and improved the productive qualities of plants. Thus, mycohelminths capable of multiplication at low temperatures may be used as potential bioagents in order to control the pink snow mold of the winter wheat, and it can be conceived that the most efficient bioagent against the pink snow mold pathogen is the mycohelminth *A. saprophillus* which significantly limits the population of the low-temperature fungus in the autumn-winter-spring period.

Selection method. Resistance to snow molds has been studied, first of all, for economically significant cultivated plants, such as winter grain crops. The history of selection works aimed at breeding the snow mold resistant varieties of these crops in North America, North-European countries and Japan has been already presented in details [105]. In Russia, targeted studies related to selection by resistance to snow molds have not been carried out, obviously, because it is impossible to use the climatic chambers capable of creating near-winter conditions, as was done in the USA [106, 107] and Japan [108], and also by the reason that epiphytoties of the most harmful snow mold pathogen in Russia, *Typhula ishikariensis*, are quite seldom.

Nevertheless, a number of investigations aimed at identifying grain crop species resistant to various snow mold species have been carried out in Russia. Particularly, against the infection background, an immunological assessment of 500 variety specimens from the VIR World Collection (N.I. Vavilov All-Russian Institute of Plant Industry, St. Petersburg) and domestic varieties has been made by damping-out percentage and intensity of leaf surface damage, and the following resistant specimens have been selected: the domestic varieties such as Shatilovskaya tetra, Populyatsiya I-82 tetra, Sibirskaya krupnozernistaya, Taezhnaya, Kirovskaya 89, Vyatka 2, Dymka, Rosinka, Ilim, Falenskaya 4, Purga, F4-92,

Chulpan 3, Korotkostebelnaya 6, Kharkovskaya 88, Tatarskaya 1, Bezenchukskaya 88, Volkhova, Talovskaya 29; the variety specimens from the VIR World Collection such as LAD-287 St-2614, Antonnisnie, Leelondzkie Kartowe № 1, Leelondzkie Krotnoslomix Baltycnie (Poland), Epos, Rerus (DDR), Inzucht 74/2, Inzucht 108/8 (Sweden), k-10953 (Finland), Feniks (Belgium), k-11385 (Yugoslavia), k-11150, k-11389 (Portugal), k-11306 (Argentina), k-11179, k-11180 (USA), k-11388 (Tajikistan), k-11398 (Georgia), k-11131 (Azerbaijan), Belta tetra (Belarus), Beve (Ukraine) [109].

There is an opinion that, besides *Triticum aestivum*, closely-related genera, such as *Secale*, *Aegilops*, *Haynaldia* and *Agropyron*, may have the germ plasm sources for improving wheat survival in winter, and it is assumed that *Ae. cylindrica* can be a new source of heritable resistance to the snow mold [110, 111]. This idea has been confirmed [93]. Among proved winter wheat varieties and hybrids against the infection background of *T. ishikariensis* and *M. nivale* [93], the best resistance to pathogens has been demonstrated by hybrids PPG-224 (wheat-agropyron hybrid) and PEG-149 (wheat-elymus hybrid). Based on chemical mutagenesis, a promising winter wheat variety, Imeni Rappoporta, has been obtained from PPG-186 [112, 113]. This variety surpassed reference varieties Mironovskaya 808, Zarya and Moskovskaya 39 in many parameters, including snow mold resistance.

It should be noted that Russian varieties and lines often turned out to be more resistant than foreign specimens in spite of the lack of targeted selection against snow molds. For example, a specimen from Russia, which is called Dormie (Sonya) by authors [114], has demonstrated much more resistance to the cottony snow mold pathogen (LTB, not encountered in Russia) in Canada, and variety Valuyevskaya is a standard of resistance to low temperatures (often associated with snow mold lesion) in Western countries.

Thus, it is difficult to control the snow mold because the pathogens usually infect crops in autumn and develop under snow and early in spring at low temperatures. There are different ways to control snow molds: agrochemical, chemical, biological and breeding methods. Corresponding developments are underway in all directions; however, selection for resistance continues to be a key strategy. In this regard, the progress is associated with identification of the genetic factors controlling the resistance, as well as with improvement of techniques for acceleration of stable line selection. The biological method is being actively developed, in particular, in Russia, where, besides fungal and bacterial organisms, a new bioagent, mycotrophic nematode, has been used for the first time.

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IDENTIFICATION OF THE *Lanr1* GENE OF RESISTANCE TO ANTHRACNOSE OF NARROW-LEAFED LUPINE (*Lupinus angustifolius* L.) USING DNA-MARKERS AnSeq3 AND AnSeq4

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Abstract

Anthracnose is one of the fungal diseases of the narrow-leafed lupine (*Lupinus angustifolius* L.) caused by *Colletotrichum lupini*. Resistance to anthracnose is not absolute in character, as the plants with high resistance can be affected by the pathogen but in less extent than those non-resistant. Recent suggestions of the total number of genes involved in control of anthracnose tolerance are discrepant. Current approach in breeding anthracnose-tolerant lupine is based on combination of non-allele genes of resistance in a single genotype. Specific DNA markers are being developed which are linked to the genes of resistance and can be used for rapid and effective selection of resistant plants, but prior to their application the efficacy of DNA marker should be specifically tested with the breeding material of interest to avoid false positive responces. The AnSeq3 and AnSeq4 DAN markers flanking *Lanr1* gene at 0.9 cM distance are considered the closest to it (H. Yang et al., 2012). In this article we report the possibility of using DNA markers AnSeq3 and AnSeq4, the single nucleotide polymorphisms (SNPs), in selecting forms resistant to anthracnose among varieties of narrow-leafed lupine. A total of 50 Russian, Belarusian, Polish and Australian varieties and samples were tested to detect the allele DNA markers of susceptibility or resistance to anthracnose. DNA was individually isolated from seeds in three replicates. Polymerase chain reaction (PCR) was used to detect alleles of DNA markers linked to the *Lanr1* gene. The list of tested plants, PCR mix composition and protocol are specified. PCR enzymes and reagents of the SibEnzyme company (Russia) were used. The praimers for AnSeq3 and AnSeq4 markers were site-specific and synthesized by the Syntol company (Russia). Polyacrylamide gel electrophoresis was used for visualization of the allele markers. The Australian varieties resistant and susceptible to anthracnose were used as a control for AnSeq3 and AnSeq4 alleles. DNA fragments of 92 and 87 base pairs corresponding to the markers AnSeq3 and AnSeq4, respectively, were obtained for all 50 breeding samples included in the study. For 13 Russian and 10 Polish varieties the marker alleles of susceptibility to anthracnose were detected. For BGB-6 Belarusian sample the resistance alleles were identified by AnSeq3 and AnSeq4 markers, and for Myrtan variety only the AnSeq3-specific pattern was shown. The rest of 21 Belarusian samples possessed the alleles of susceptibility to anthracnose. Earlier by means of other DNA markers, AntjM1 and AntjM2, we showed the absence of alleles linked to *Lanr1* in currently registered varieties originated from All-Russian Research Institute of Lupine. Thus, the DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* may be useful in breeding Russian and Belarusian anthracnose-resistant lupine varieties.

Keywords: *Lupinus angustifolius* L., anthracnose, polymerase chain reaction (PCR), DNA markers AnSeq3, AnSeq4.

Anthracnose is an infectious disease of many agricultural crops, including the blue lupine, *Lupinus angustifolius* L. This disease of lupine plants is caused by the fungus *Colletotrichum lupini* [1]. In the years of anthracnose epiphytoty development, significant reduction of yields is observed for the varieties susceptible to the pathogen. It is not recommended to use heavily affected lupine crops for seeding, grain forage and ensiling purposes [2]. It is reported that it

was the blue lupine where anthracnose was found for the first time in the USSR; it was damaged to the greatest extent as compared to the other species [3]. However, due to many years of breeding for resistance to anthracnose, some present-day varieties of *L. angustifolius* L. are tolerant to the disease. At the same time, it should be noted that this resistance is not absolute, but relative, because plant specimens with high resistance can be affected by the pathogen, but in less extent than those considered to be non-resistant.

Among researchers, yet there is no consensus about the total number of genes associated with resistance to anthracnose [4]. American varieties of the blue lupine, Rancher and Frost, and Australian varieties Marri, Illyarrie, Yandee and Danja carry dominant anthracnose resistance gene *An* [5]. Australian variety Kalya has dominant gene *Lanr2* providing medium resistance to anthracnose [6]. High anthracnose resistance of Australian varieties Wonga and Tanjil is controlled by dominant gene *Lanr1* [7]. Based on the results of hybridological analysis, the genotype of Belarusian varieties Mirtan and Pershatsvet, which exhibit resistance to anthracnose at the level of Wonga and Tanjil, contains blocks of three nonallelic dominant genes *Rcl1, Rcl2,* and *Rcl3*.

It should be emphasized that, at present, breeders from various countries carry out studies for creation of more anthracnose-resistant varieties by combining nonallelic genes of resistance to the disease in a single genotype [4]. DNA markers linked to disease-resistance genes are developed in order to rapidly and efficiently select anthracnose-resistant plants. Thus, Australian researchers have proposed DNA markers AntjM1 [7], AntjM2 [8], AnSeq3, and AnSeq4 [9] linked to gene *Lanr1*. With help of primers for the corresponding anthracnose resistance markers of the blue lupine, it is possible to determine if gene *Lanr1* is present in the specimens under study using the polymerase chain reaction (PCR) with subsequent analysis of DNA fragments.

Screening of breeding material with use of DNA markers makes it possible to identify anthracnose-resistant plants and recommend them for inclusion into the selection process. At the same time, according to the results obtained by Australian researches, DNA markers may be used in selection only after preliminary analysis of their efficiency for particular breeding specimens [10]. Earlier, in studies of anthracnose resistance controlled by gene *Lanr1*, we applied markers AntjM1 and AntjM2 for DNA analysis of 14 Russian and Belarusian varieties of the blue lupine [11]. At this point, DNA markers AnSeq3 and AnSeq4 seem to be the closest to *Lanr1*; they flank the gene at a distance of 0.9 cM [9].

The purpose of this work was to investigate a capability of using DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* in breeding of Russian and Belarusian specimens of the blue lupine. In this regard, we have assessed the presence of anthracnose resistance gene *Lanr1* in varieties and specimens of various selection origin using the two mentioned molecular markers.

Technoque. The test material was represented by 50 Russian, Belarusian, Polish and Australian varieties and specimens of the blue lupine (*Lupinus angustifolius* L.).

DNA was individually isolated from seeds in three replicates by a previously used method [11]. PCR was carried out in a Tertsik amplifier (DNA-Technology, Russia). 10 rl reaction mixture was composed of genomic DNA (10-20 ng), forward and reverse primers (0.3 μM), Taq polymerase E 338 (0.25 U), $MgCl_2$ (1.5 mM), dNTPs (0.15 mM), B321 AS buffer (1 μl). Enzymes and reagents of SibEnzyme (Russia) were used. Amplification conditions were es follows: initial denaturation for 1 min at 95 °C; then 30 cycles including denaturation for 30 s at 95 °C, annealing for 20 s at 51 °C for primers in case of marker AnSeq3 and at 53 °C for primers in case of AnSeq4, elongation for 10 s at 72 °C; final elongation for 2 min at 72 °C. The primers used for markers An-

Seq3 and AnSeq4 are site-specific [9] and have been synthesized by Sintol (Russia). DNA fragments were separated in 11-13 % polyacrylamide gels using a VE-20 vertical electrophoresis chamber (Helicon, Russia). PCR products were separated within 5-6 h at 270-300 V. The gel was placed into ethidium bromide solution (0.5 mg/l) for 20-30 min. After staining, it was visualized using a Gel-DocXR gel scanner (Bio-Rad, USA). The control for marker allele length determination comprised PCR products with DNA of the plants representing Australian varieties Tanjil, Wonga, Kalya.

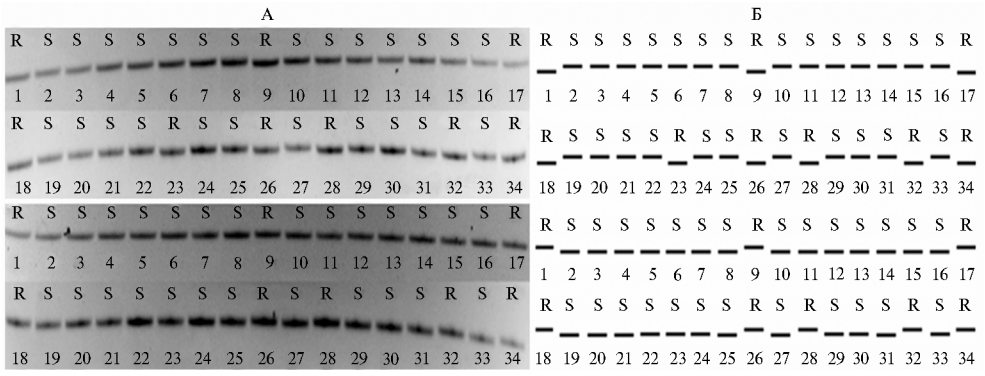
Results. We have investigated 50 blue lupine specimens of various origin (see table).

Blue lupine specimens included in the study for identifying the alleles of DNA markers AnSeq3 and AnSeq4 of anthracnose resistance gene *Lanr1*

Provided by Russia	Provided by Belarus
Russian varieties and specimens (total 13): Raduzhny, Siderat 38, Kristall, Snezhet, Belozerny 110, Smena, Nadezhda, Bryansky 123, Vityaz, Uzkolistny 53-02, Vektor, Belozerny 121, SN 78-07	Belarusian varieties and specimens (total 23): Privabny, Pershatsvet, Mitan, Gelena, Mirtan, Khvalko, Yan, BGB-6, Gerkules, Zhodinsky, Ranny, BGB-3, Mirtan 312, MK-VDS-Beis, PEL-MRL, Ramonok, 4B-129, BGB-1, BGB-2, BGB-4, Vada 40, Vasilek, Divny
Polish varieties and specimens (total 10): Baron, Boruta, Boyar, Graf, Zevs, Kalif, Neptun, Regent, Tsezar, Elf	
Australian varieties and specimens (total 4): Tanjil, Wonga, Kalya, W2248	

N o t e: The Russian and Belarusian breeding material was provided by All-Russian Research Institute of Lupine (Bryansk) and Scientific and Practical Center for Agriculture of NAS of Belarus (Zhodino), respectively.

DNA fragments of 92 and 87 base pairs corresponding to markers AnSeq3 and AnSeq4, respectively, were obtained for all 50 breeding specimens included in the study. The alleles of susceptibility (S) and resistance (R) to anthracnose have been determined in the course of comparison of the marker alleles identified for Australian control varieties and other breeding specimens. Alleles of AnSeq3 and AnSeq4 are single nucleotide polymorphisms (SNP) with the same length for each marker. In electrophoretic separation in polyacrylamide gel, the fragments corresponding to the marker alleles of resistance for AnSeq3, due to presence of one C→T base substitution, were positioned below the amplicons of the alleles of susceptibility, whereas inverted distribution was observed for AnSeq4 due to a single G→A substitution in the base sequence [7]. Thus, in case of varieties Tanjil and Wonga (positive controls of resistance for gene *Lanr1*), DNA fragments for the alleles of AnSeq3 and AnSeq4 located, respectively, below and above the amplified ones for variety Kalya (negative control of resistance for gene *Lanr1*). The PCR products obtained for DNA of each of the blue lupine variety specimens included in the study were reproduced in all three replicates. The marker alleles of AnSeq3 and AnSeq4 for different Russian, Belarusian, Polish and Australian varieties and specimens are shown in the figure below.



Results of electrophoretic separation of PCR products obtained for alleles of DNA markers AnSeq3

(1-34, top) **and AnSeq4** (1-34, bottom) **of susceptibility and resistance to anthracnose in the studied varieties and breeding specimens of various origin:** A — photo documentation, B — schematic representation; 1, 17, 18, 34 — Tanjil, 2 — Raduzhny, 3 — Siderat 38, 4 — Kristall, 5 — Snezhet, 6 — Belozerny 110, 7 — Smena, 8 — Nadezhda, 9, 26 — Wonga, 10, 27 — Kalya, 11 — Bryansky 123, 12 — Vityaz, 13 — Uzkolistny 53-02, 14 — Vektor, 15 — Belozerny 121, 16 — SN 78-07, 19 — Privabny, 20 — Pershatsvet, 21 — Mitan, 22 — Gelena, 23 — Mirtan, 24 — Khvalko, 25 — Yan, 28 — BGB-6, 29 — Gerkules, 30 — Zhodinsky, 31 — Ranny, 32 — W 2248, 33 — BGB-3; S and R — marker alleles of susceptibility and resistance, respectively, linked to gene *Lanr1* (12 % polyacrylamide gel; please refer to the body of the article for the description of the varieties and breeding specimens).

For the Russian specimens, the PCR analysis has not resulted in identification of R-alleles of DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* which controls the anthracnose resistance of Australian varieties Wonga and Tanjil. Earlier, using other DNA markers, in particular, AntjM1 and AntjM2 [11], we also obtained the information that the varieties of All-Russian Research Institute of Lupine which are currently included in the State Register of Selection Achievements of the Russian Federation do not contain resistant marker R-alleles linked to gene *Lanr1*. It is known that the genetic material that provided a basis for anthracnose resistance of Australian varieties Wonga and Tanjil was not used in breeding of the Russian varieties studied herein, which was confirmed by the results of the molecular genetic analysis of gene *Lanr1* DNA marker alleles. As with the Russian specimens, the Polish varieties were matched by anthracnose susceptibility marker alleles for gene *Lanr1*. With regard to phenotypic manifestations for the Polish varieties included in this study, there was no pronounced anthracnose resistance similar to that of Australian varieties Tanjil and Wonga, which is consistent with the data on markers AnSeq3 and AnSeq4.

Anthracnose susceptibility alleles were characteristic for most of the Belarusian specimens. Resistance alleles for two markers, AnSeq3 and AnSeq4, were only found in component BGB-6 of the blue lupine biological gene bank. This breeding specimen is closely related to varieties Wonga and Tanjil and also demonstrates high anthracnose resistance in field and laboratory trials [4].

For variety Mirtan, resistance marker alleles were only identified for AnSeq3. In a previous study of this variety with markers AntjM1 and AntjM2, we found susceptible alleles, and primers for marker AntjM2 allowed us to amplify the allele that was not identified for the other specimens, which characterizes the distinctiveness of the mentioned variety [11]. The variation of the anthracnose resistance/susceptibility alleles for markers An-Seq3 and AnSeq4 can be attributable to differences in the genetic make-up of Belarusian variety Mirtan and Australian variety Tanjil. In spite of the fact that Mirtan demonstrates relatively high anthracnose resistance, it was bred without the use of the Australian material resistant to the disease. Thus, it seems possible that, in case of variety Mirtan, marker AnSeq3 gives «false-positive» results as compared to marker AnSeq4, and the resistance allele identified is not associated with gene *Lanr1*.

In addition to variety Mirtan and specimen BGB-6, relatively high anthracnose resistance has been observed for variety Pershatsvet due to the presence of the block of several nonallelic genes *Rcl* in plant genotypes. It seems that the anthracnose resistance genes specific for this variety are not allelic to *Lanr1*, which is consistent with the results of these studies where anthracnose resistance for marker alleles linked to gene *Lanr1* was not observed for variety Pershatsvet. It should be noted that susceptibility alleles have been identified in the other Belarusian varieties of the blue lupine, which were not closely related to Australian varieties Wonga and Tanjil. R-alleles of markers AnSeq3 and AnSeq4 have also been found in Australian breeding line Walan 2248. This fact reflects the results of the anthracnose resistance breeding program implemented in Australia as part of creation of blue lupine varieties.

In general, it turned out that DNA markers AnSeq3 and AnSeq4 allows quite efficient identification of anthracnose resistant and susceptible genotypes by gene *Lanr1*. Thus, for 50 varieties and breeding specimens of the blue lupine, discrepant information about the alleles of the studied markers has been obtained only in case of variety Mirtan. These results have confirmed the need for preliminary analysis of DNA marker applicability in large-scale crossings because the use of «false-positive» marker alleles may lead to errors in selection of anthracnose resistant plants.

The stably high resistance of varieties Tanjil and Wonga carrying gene *Lanr1* has been confirmed in various test conditions [4, 5, 12]. Therefore, inclusion of gene *Lanr1* in the genotypes of new varieties obtained after crossing of Belarusian or Russian breeding material and anthracnose-resistant Australian specimens can improve total resistance to this disease. Appropriate DNA markers can be applied for individual, high-precision and rapid selection of the genotypes containing gene *Lanr1*.

So, the study of blue lupine (*Lupinus angustifolius* L.) specimens of various origin has not revealed anthracnose resistance alleles for markers AnSeq3 and AnSeq4 linked to gene *Lanr1* in case of Russian and Polish varieties. For Belarusian breeding specimen BGB-6, resistance alleles have been identified for both markers AnSeq3 and AnSeq4 studied, for variety Mirtan they were found only for marker AnSeq3. The controls for corresponding alleles of AnSeq3 and AnSeq4 were represented by the Australian breeding material resistant and susceptible to anthracnose. The undertaken studies have demonstrated that the use of DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* may be useful in breeding of the Russian and Belarusian varieties carrying anthracnose resistance gene *Lanr1*. At the same time, in case of large-scale crossings, it is necessary to preliminarily assess if the selected DNA markers are applicable or not because the use of «false-positive» marker alleles may lead to errors in selection of anthracnose resistant plants.

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GENETIC DIVERSITY STUDY OF MODERN RUSSIAN APPLE (*Malus* × *domestica* Borkh.) CULTIVARS BY THE SSR LOCI ANALYSIS

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Abstract

SSRs are one of the most suitable DNA-markers for assessment of genetic diversity of plant genetic resources. Microsatellites were used for development of saturated genetic maps of apple (*Malus* × *domestica* Borkh.) as well as for wide range of genetic diversity studies. Our study was aimed on the investigation of the genetic relationship within subcollection of modern Russian apple cultivars. Polymorphism of 12 microsatellite loci was estimated for 31 apple cultivars from the SKZNIISiV collection of genetic resources. These cultivars have been bred in North Caucasian Regional Research Institute of Horticulture and Viticulture (SKZNIISiV) and All-Russian Research Institute of Fruit Crop Breeding (VNIISPK). SSR-markers CH01f03b, CH01h01, CH01h10, CH02c06, CH02d08, CH04e05, CH05f06, CH01f02, CH02c11, Hi02c07, CH02c09 and CH03d07, which are recommended by Fruitbreedomics, the European consortium, were used in the study. According to the data of SSR-analysis from 5 to 10 alleles per locus were detected, with an average value of 7.75 alleles per locus. A total of 93 alleles were detected for all 12 loci. All apple cultivars showed individual, distinct SSR-profiles. Comparison with the data on the genetic diversity of the world apple tree gene pool suggests that the SSR-loci polymorphism in studied set of the apple cultivars is relatively high. Expected (H_e) and observed (H_o) heterozygosity varied within the ranges of 0.548-0.897 and 0.602-0.827 for H_o and H_e, respectively. The average values of these indexes are H_o = 0.786 and H_e = 0.755. PIC value ranged from 0.571 to 0.806, and 9 loci showed PIC value higher than 0.712. Results of UPGMA-analysis are consistent with the level of genetic heterogeneity of the studied cultivar set. Five clusters were determined. Distribution of cultivars into clusters in most cases is consistent with their genealogy. For example, Svezhest' cultivar, formed a distinct cluster № 1, as well as cultivars Imrus and Zimnee utro which formed cluster № 5 are originated from the cultivars, which are not presented as the parental forms of any studied cultivars. Cultivars of VNIISPK breeding such as Solnishko, Stroevskoe, Yubilei Moskvy, Afrodita and Start, which formed distinct cluster № 3 have one common parental cultivar. The structure of dendrite obtained when performing clustering on the results of SSR-analysis may be due to large number of unique alleles studied in genotypes that in turn is due to the high genetic diversity within the studied set of cultivars. At the same time, the fact of incorporation of cultivars with the same genealogy in the same clusters confirms the high significant genetic similarity within groups of such varieties. Results of the study allows to assess the level of the genetic diversity within the set of modern apple cultivars as well as can be used for confirmation of genealogy of apple cultivars and hybrids in the case of disputes, as well as for identification of varieties.

Keywords: apple tree, SSR-markers, genotyping, polymorphism, genetic diversity.

The use of DNA markers in analysis of plant genetic resources allows us to significantly extend the field of study, from assessment of genetic diversity and issues of variety certification to protection of breeders' copyright and determination of breeding material genetic purity.

DNA markers shall have certain properties and meet a number of requirements, including, in particular, a high level of polymorphism, a codominant nature of inheritance, optimum frequency of occurrence in genome, uniform distribution in chromosomes, easy assessment of marker parameters, high repro-

ducibility, capability of automated assessment and easy data exchange between laboratories [1]. Microsatellite DNA markers based on Simple Sequence Repeat (SSR) analysis fully comply with these requirements and are currently acknowledged to be one of the most efficient DNA marker systems used in breeding and genetics of cultivated plants, including the apple tree [2-4].

SSR-markers were used for construction of the first most complete genetic maps of the apple tree For example, C. Maliepaard et al. [5] developed one of the first apple tree genome maps with 17 linkage groups with the use of multiallelic markers, including SSR, based on the hybrid population created by crossing of varieties Prima and Fiesta. Later on, identification of new SSR loci in the apple tree genome made it possible to create more comprehensive genetic maps [6-9]. Information about the identified SSR loci and their genome localization provides a valuable scientific base for genetic studies of the apple tree, in particular, for mapping the genes responsible for economic characters, analyzing the genetic pattern of genetic resource collections, certifying the gene pool and investigating genetic diversity within the species *Malus* × *domestica* Borkh.

A wide range of studies aimed at assessing genetic polymorphism in collections of genetic resources (both modern varieties and native gene pool) have been performed by now. Work has been performed to clarify the questions regarding the genealogy of local varieties, formation of autochthonous geneplasm of the apple tree, its genetic interrelation with the global gene pool [10-15], as well as DNA certification of tree stocks [16, 17]. Uniqueness of the aboriginal gene pool has been revealed in a number of cases. The results obtained by A. Gharghani et al. [13] have shown that native Iranian varieties fall in between European apple tree varieties and the species *Malus orientalis* Uglitz. A. Patocchi et al. [18] have carried out a large-scale study where 88 microsatellite DNA markers were used to assess polymorphism for a total of about 2,000 specimens from European collections of genetic resources (varieties, hybrid populations). These data were used as a basis for recommendations on the most promising SSR markers for analysis of apple tree genomic polymorphism [18].

When genetic diversity of large genetic collections of the apple tree is studied, including wild forms and interspecies hybrids along with varieties, SSR markers also make it possible to objectively assess phylogenetic relationships at intra- and interspecies level [19, 20].

Analysis of SSR loci polymorphism in studies of genetic diversity allows making DNA certificates of genotypes. SSR fingerprints can be efficiently used for identification of specimens of collections, identification of doubles, etc.

Taking into account that the study of apple tree gene pool diversity is highly topical and the use of microsatellite markers in solution of such tasks is very promising, we have carried SSR genotyping of modern varieties for the genetic resource collection of North Caucasian Regional Research Institute of Horticulture and Viticulture (SKZNIISiV) and assessed genetic polymorphism in the modern domestic gene pool of the apple tree.

Technique. The material for the studies was the samples of modern apple tree varieties (SKZNIISiV and All-Russian Research Institute of Fruit Crop Breeding — VNIISPK, Orel, as the originators) stored in SKZNIISiV's collection of genetic resources ($n = 31$).

A total of 12 SSR markers were involved in the work, namely CH01f03b, CH01h01, CH01h10, CH02c06, CH02d08, CH04e05, CH05f06, CH01f02, CH02c11, Hi02c07, CH02c09 and CH03d07. The above listed SSR markers are recommended by European Consortium FruitBreedomics for study of apple tree genetic diversity (a primer sequence is available in a da-

tabase at http://www.hidras.unimi.it).

DNA was extracted using the CTAB method [21]. PCR was carried out using standard methods with preliminary optimization of parameters. The PCR mix contained 50-70 μg of DNA, 0.05 mM of dNTPs, by 0.3 μM of each primer, 0.5 μl of 10× reaction buffer, 2.5 mM of $MgCl_2$, 1 U of Taq polymerase; the final volume of 25 μl. Amplification conditions were as follows: initial denaturation for 5 min at 95 °C; denaturation for 10 s at 95 °C, primer annealing for 30 s at 58 °C, elongation for 30 s at 72 °C (30 cycles); final elongation for 3 min at 72 °C. The reaction was carried out in an Eppendorf Mastercycler Gradient amplifier (Germany), and the fragment analysis of SSR loci was performed using an ABI Prism 3130 automatic genetic analyzer (USA). The results of the fragment analysis were processed in the GeneMapper v. 4.1 software program.

In assessment of microsatellite analysis data, a genetic distance matrix was constructed using similarity coefficients (indices) according to M. Nei и W. Li [22]. The cluster analysis was performed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with the use of FreeTree Application v. 0.9.1.50 (ZDAT v.o.s.). The dendrogram construction was performed using the TreeView (Win32) v. 1.6.6 software program. The actual (H_o) and expected (H_e) heterozygosity was calculated using GenAlEx v. 6.3 software program. The PIC (Polymorphism Information Content) value was determined using the PICcalculator software program in the online mode (http://w3.georgikon.hu/pic/english/kodom.aspx).

Results. The selected microsatellite markers were tested using electrophoresis in 8 % nondenaturing polyacrylamide gel. After PCR parameter optimization, microsatellite DNA markers were grouped into multiplex kits (4 markers per kit) for simultaneous analysis by several loci. In this case, the sizes of the amplified fragments did not overlap, and each of the markers contained a specific fluorescent tag (FAM, TAMRA, R6G, ROX).

The fragment analysis gave clear reproducible results. As an example, Figure 1 (visualization of results in a working window of the GeneMapper v. 4.1 software program) shows data for variety Krasny Yantar. It can be seen in the electrophoretogram that there were two peaks for the markers indicative of two products, i.e. the corresponding loci are heterozygous. In the discussed case, these are markers CH01h01, CH01f03b, and CH02c06. One peak for locus CH01h10 on the electrophoretogram gave evidence of its homozygosity.

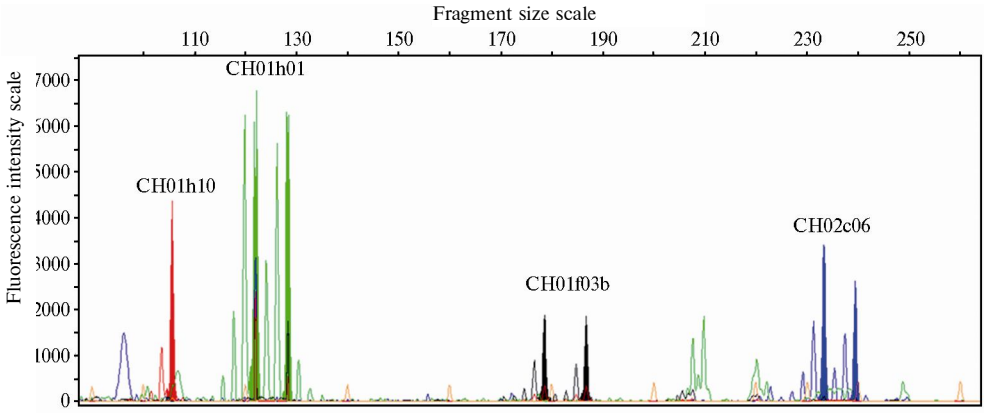

Fig. 1. Electrophoretic DNA fragment analysis for apple tree variety Krasny Yantar using a multiplex kit including SSR markers CH01h10, CH01h01, CH01f03b and CH02c06 (collection of North Caucasian Regional Research Institute of Horticulture and Viticulture, Krasnodar)

From the 12 markers used in the study, maximum and minimum polymorphism was revealed for locus CH02c11 (10 alleles) and locus CH01f03b (5 alleles), respectively. A total of 93 alleles were detected for all 12 loci. All the varieties studied had a unique set of alleles (the obtained SSR fingerprints are given in the database which is being registered now as an item of intellectual property).

Polymorphism characteristics for the microsatellite SSR markers used in assessment of genetic diversity of modern domestic apple tree varieties (*Malus* × *domestica* Borkh.) from the collection (n = 31, collection of North Caucasian Regional Research Institute of Horticulture and Viticulture, Krasnodar)

Marker	Range of amplified fragment sizes, base pairs	Number of identified alleles	PIC	H_o	H_e
CH01f03b	141-182	5	0.690	0.710	0.736
CH01h01	118-137	6	0.712	0.742	0.754
CH01h10	94-120	7	0.572	0.548	0.602
CH02c06	204-256	9	0.806	0.897	0.827
CH02d08	215-258	9	0.745	0.800	0.776
CH04e05	178-230	9	0.772	0.867	0.798
CH05f06	171-189	7	0.678	0.871	0.723
CH01f02	173-210	8	0.726	0.839	0.763
CH02c11	219-241	10	0.727	0.806	0.752
Hi02c07	104-166	9	0.752	0.897	0.781
CH02c09	235-259	7	0.737	0.774	0.772
CH03d07	188-228	7	0.747	0.679	0.776

N o t e: PIC — polymorphism information content, H_o — observed heterozygosity, H_e — expected heterozygosity.

The comparison of the total number of the alleles identified in our study with the results of the genetic diversity analysis for the apple tree from the global gene pool allows us to state that SSR loci polymorphism is quite high in the studied sample of domestic apple tree varieties. S. Pereira-Lorenzo et al. [14] have identified a total of 122 alleles as a result of analysis of 66 Spanish apple tree varieties by 10 SSR loci. At the same time, this figure for 27 native varieties was equal to 75 [14]. In the Finnish and Swedish apple tree gene pool (a total of 101 genotypes), 105 alleles have been identified by 9 loci. In this case, expected (H_e) and observed (H_o) heterozygosity values varied within 0.31–0.88 and 0.41–0.88, respectively [23], whereas in our study they ranged within 0.548-0.897 for H_o and 0.602-0.827 for H_e (see table). The average values of H_o and H_e in our study were equal to 0.786 and 0.755, respectively, which exceeded the figures obtained as a result of analysis of Scandinavian apple tree geneplasm (H_e = 0.72; H_o = 0.74) where the average number of alleles per locus was 11.6 (23). Based on the results of our study, this parameter in a sample of 31 domestic varieties was equal to 7.75. Studying the genetic diversity of native apple tree gene pool in north-eastern Spain (Aragon region), A. Pina et al. [24] have obtained the average value of 12.4 alleles from the analysis of 20 SSR loci in a sample of 130 genotypes. In their study, the authors compared the results with data on SSR loci polymorphism for 21 global varieties from the various regions of the world and determined that the corresponding average number of alleles per locus was equal to 8.2 [24].

The PIC values ranging from 0.572 to 0.806 (see table) indicate that most of the studied SSR markers have a high level of informativeness. In case of SSR locus CH01h10 with 7 alleles identified, the least PIC (0.572) value among the used markers was largely dependent on the prevalence of one of the alleles (with the size of 100 base pairs) over the other (it was found in 60% of the studied genotypes), as well as on the presence of two rare alleles (118 and 120 base pairs), either of which was identified only in one specimen.

Based on the SSR genotyping data, the extent of genetic similarity between the studied varieties has been assessed using the Unweighted Pair Group

Method with Arithmetic Mean (UPGMA) (Fig. 2).

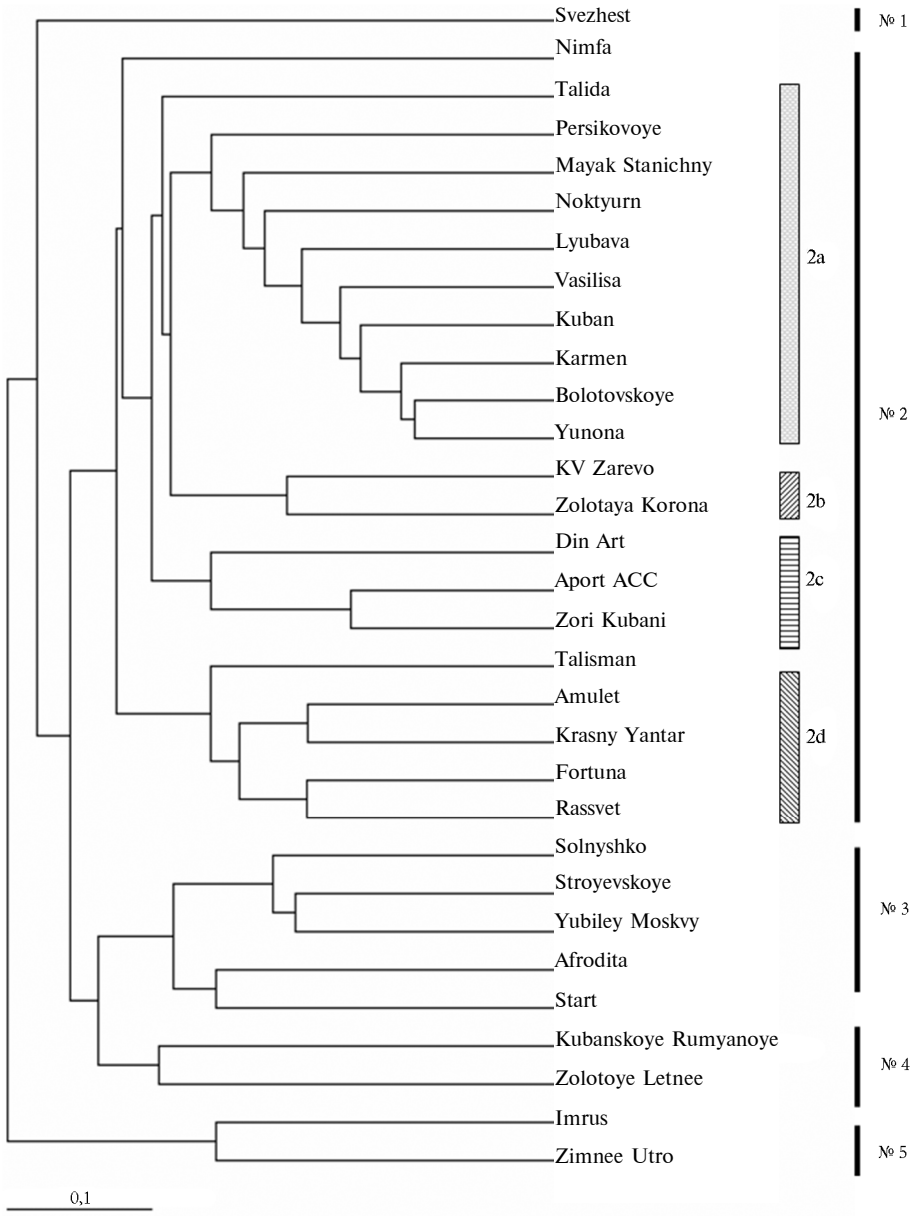

Fig. 2. The dendrogram of genetic similarity between the studied modern domestic varieties of the apple tree (*Malus* × *domestica* Borkh.), which was constructed by the UPGMA method: №№ 1-5 — clusters, 2a, 2b, 2c, 2d — subclusters (collection of North Caucasian Regional Research Institute of Horticulture and Viticulture, Krasnodar).

Five main clusters have been distinguished according to the extent of genetic similarity, i.e. № 1 — variety Svezhest; № 2 — varieties Nimfa, Talida, Persikovoye, Mayak Stanichny, Noktyurn, Lyubava, Vasilisa, Kuban, Karmen, Bolotovskoye, Yunona, KV Zarevo, Zolotaya Korona, Din Art, Aport ACC, Zori Kubani, Talisman, Amulet, Krasny Yantar, Fortuna, Rassvet; № 3 — Solnyshko, Stroyevskoye, Yubiley Moskvy, Afrodita, Start; № 4 — Kubanskoye Rumyanoye, Zolotoye Letnee; № 5 — Imrus, Zimnee Utro. In this case, cluster № 2 included four subclusters (2a-2d) and varieties Talida and Nimfa.

The results of the UPGMA analysis generally reflect genetic heterogeneity of the varieties from the studied sample. It is expressed, on the one hand, in the presence of the clusters combining several varieties at a single level or including only two (or even one in case of variety Svezhest) genotypes and, in the other hand, in formation of clusters with a complex structure (for example, № 2).

Clustering of the varieties is consistent with their origin to a large extent. For example, variety Svezhest [Antonovka Krasnobochka × PR12T67 (Uelsi × F_2 *M. floribunda*)] assigned to cluster № 1 and varieties Imrus (Antonovka Obyknovennaya × OR18T13) and Zimnee Utro (Liberty × Scarlet Staymared) forming cluster № 5 are originated from the varieties not represented in parent forms of any of the other varieties in the studied sample.

The reliability of the data obtained as a result of dendrogram construction is also confirmed by incorporation of varieties Afrodita, Start, Solnyshko, Yubiley Moskvy and Stroyevskoye having a common parent, the form 814 [F_2 (*M. floribunda 821* × *M. domestica)*] × Golden Delicious [25, 26], into a single cluster, which is the most probable cause of their apartness from most of the studied genotypes based on the clustering results. Varieties Talisman, Amulet, Krasny Yantar, Fortuna and Rassvet (subcluster 2d) are originated from hybrid combination Redfri × Papirovka Tetraploidnaya.

The complex dendrite structure obtained in the course of clustering, based on the results of the SSR analysis, may be due to a large number of unique and rare alleles in the studied genotypes, which, in turn, is caused by a high genetic diversity level within the sample [27].

Thus the results of the undertaken study confirm the efficiency of SSR markers for assessment of genetic diversity of the apple tree gene pool. Based on the data obtained from analysis of SSR loci polymorphism in 31 modern domestic varieties of the apple tree, we can say that the studied sample of genotypes is characterized by a high polymorphism level. At the same time, the fact of incorporation of varieties with similar genealogy in the same clusters confirms significant genetic similarity within the groups of such varieties. In this regard we do not recommend using the varieties included into one cluster as parent pairs in hybridization if it is necessary to obtain maximum genetic variability in hybrid populations. The results of the performed SSR genotyping can be considered in confirmation of variety and hybrid genealogy if one or both of their parent forms are included in the studied sample of genotypes.

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MICROSATELLITE LOCI POLYMORPHISM IN RUSSIAN BLACK CURRANT (*Ribes nigrum* L.) VARIETIES FROM COLLECTION OF ALL-RUSSIAN RESEARCH INSTITUTE OF BREEDING FRUIT CROPS

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Abstract

Black currant is the main berry crop in Russia. The need to improve its assortment requires new effective methods to be involved in breeding. Application of DNA markers as a modern approach in dealing with germplazm is intensively used abroad in works on black currant plants. We are the first in Russia who used SSR DNA markers in studying black currant gene pool. In this paper we report genotyping 27 black currant accessions from the collection of the All-Russian Research Institute of Breeding Fruit Crops, including 16 varieties originated from this institute, on 14 microsatellite loci. Electrophoresis in 6 % denaturing PAAG followed by staining with silver nitrate was used for separation of PCR products. All tested SSR loci, except MS06g03, have been found to be polymorphic. On average 4.9 alleles were amplified per locus. Three fragments have been amplified on DNA of some accessions at one SSR locus that is probably due to the duplication of these microsatellite loci in genome of these accessions. A total of 66 % of all amplified fragments were rare alleles with a frequency of occurrence equal to or less than 0.2. Eight unique alleles have been found. The observed heterozygosity ranged from 0.259 (g2-H21) to 1 (e4-D03) and averaged 0.608. Combinations of alleles at one locus allowed to distinguish from 3 (g2-H21, e1-O21) to 15 (g2-G12) genotypes. The minimum set of four loci (e4-D03, g1-M07, g1-E03, g2-B20) allowed to distinguish all the tested accessions, i.e. unique multilocus profile has been found for each sample. Pairwise genetic similarity coefficients ranged from 0.11 (between Odzhebin and Sharovidnaya varieties) to 0.95 (between Govtva and Rtishchevskaya varieties) and averaged 0.346. Cluster analysis of genetic similarity has been done. The dendrogram shows a few clusters with high bootstrap support. Distribution of alleles between related varieties was consistent with the pedigrees in the most cases, but discrepancies between pedigrees and SSR data also were found. Thus, the varieties Ocharovanie (№ 1168 × Ekzotika variety) and Ekzotika had no common alleles in three loci (e4-D03, g1-E03, g2-B20). It probably is due to pollination by other pollen or error in reproduction and transplantation or mutations in these loci. The varieties Odzhebin and Binar (Odzhebin × Naryadnaya) had no common alleles in two loci (g1-M07, g1-E03). Our results allow to recommend SSR markers for evaluation of domestic gene pool of black currant on genetic diversity, and to develop methods for the identification and certification of varieties of black currant.

Keywords: black currant, DNA markers, microsatellites, polymorphism, identification, gene pool, *Ribes nigrum* L.

The black currant occupies half the areas under berries in Russia [1]. As of 2013, the State Register of Selection Achievements Permitted for Use included 170 varieties on this key berry crop [2]. About 20 scientific institutions in Russia are involved in enhancing the assortment of its varieties. Breeding studies are aimed at improving the biochemical composition of berries, creating productive, stably fruit-bearing varieties which are disease- and insect-resistant, suitable for machine harvesting, etc. [1]. In the All-Russian Research Institute of Breeding Fruit Crops (VNIISPK), 26 varieties (12 of them are included in the State Register of Varieties Permitted for Use) have been submitted for state variety testing by now. An extensive collection of variety specimens, including over 100

introduced varieties and more than 700 home-bred forms, has been made.

The success of breeding studies greatly depends on investigation, selection and systematization of parent material [3]. The black currant has a diploid chromosome set (n = 7), which, to a certain extent, facilitates the study of its genome as compared to genomes of polyploid species. Scientists from the Scottish Crop Research Institute (Invergowrie, Dundee, Scotland, Great Britain) became pioneers in the use of DNA markers for genetic polymorphism analysis in the work with genetic resources of the black currant and other representatives of the genus *Ribes* L. They used RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter-Simple Sequence Repeat), SNP (Single Nucleotide Polymorphism) and other markers [4, 5]. As a result, based on microsatellite SNP and AFLP markers, the first genetic map of the black currant has been created [6], and a method for marker-mediated selection of genotypes with gall mite resistance gene *Ce* has been developed and is used [7]. In Russia, DNA marking for assessment of genetic collection of genus *Ribes* representatives has only been used relatively recently [8].

At present, we observe a tendency towards the use of highly reproducible codominant SSR and SNP markers. SSR (microsatellite) markers are used both in fundamental studies aimed at assessing genetic polymorphism, investigating phylogenetic relationships and creating genetic maps for linkage groups, and for application-oriented purposes, such as genealogy checking, development of identification and certification systems, search for the markers associated with economic characters, and marker-mediated selection of targeted genotypes at early stages of plant development [9]. SSR markers have been used for assessing the diversity of genetic collections of *Ribes* in Italy and North Europe [10, 11].

We were the first who used microsatellite markers for assessing intervarietal polymorphism, determining genetic similarity between variety specimens and checking the genealogy for black currant forms from the Russian collection of VNIISPK, which allowed us to discuss the prospects of using the polymorphism of microsatellite loci in work with the genetic resources of this berry crop.

The presented study was aimed at investigating the polymorphism of microsatellite loci for black currant varieties from VNIISPK's collection.

Technique. There were 27 varieties of the black currant from the collection of the All-Russian Research Institute of Breeding Fruit Crops, including 16 varieties bred by VNIISPK (Azhurnaya, Gratsiya, Gamma, Dachnitsa, Orlovsky Vals, Monisto, Orloviya, Iskusheniye, Chudnoye Mgnoveniye, Ocharovaniye, Slastena, Chernaya Vual, Ekzotika, Blakeston, Zusha, Kipiana), as well as varieties from other originators (Odzhebin, Titaniya, Sharovidnaya, Selechenskaya, Minai Shmyrev, Rtishchevskaya, Konsort, Binar, Govtva, Kanakhama, Yadrenaya) involved in the investigation. In addition, varieties Lentyai and Orlovskaya Serenada were analyzed by a part of the loci used. The results obtained for these varieties were taken into account only in the correspondence analysis of allele distribution between related varieties and analysis of genealogy data (allele distribution between related varieties was checked with all polymorphic loci, for which such data were available).

DNA was extracted from juvenile leaves using the CTAB method [12].

We have analyzed a total of 14 microsatellite loci (http://www.fruitbreeding.co.uk/RibesGenomicsSSRs.asp), i.e. e1-O01, e1-O21, e3-B02, e4-D03, g1-B02, g1-E03, g1-K04, g1-M07, g2-B20, g2-H21, g2-J08, g2-L17, g2-G12, MS06g03 [13]. The PCR analysis was carried out in 20 μl of reaction mixture containing 1× PCR buffer, 200 μM of nucleotides, 2 μM of forward primer, 2 μM of reverse primer, 0.3 U of Taq DNA polymerase and 10 ng of DNA in a

GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). The amplification reaction was carried out under the following conditions: preliminary denaturation for 5 min at 94 °C; denaturation for 30 s at 94 °C, primer annealing (at the temperature selected for each marker; refer to the «Results» section), DNA synthesis for 30 s at 72 °C (total 35 cycles); final elongation for 10 min at 72 °C.

The polymorphism of microsatellite loci was analyzed using electrophoretic separation of amplificates in 6 % denaturing polyacrylamide gel (PAAG) in a Sequi-Gen GT System chamber (38×50 cm) (Bio-Rad, USA) with subsequent staining with silver nitrate [14]. The sizes of fragments were determined by comparison with molecular weight marker 10 bp DNA Ladder (Invitrogen™, USA).

The allele frequency was calculated as a sum of homozygous genotype frequencies and a half-sum of frequencies of heterozygous genotypes containing this allele [15]. The expected heterozygosity was calculated as $H_e = 1 - p_i^2$, where p_i is a frequency of the *i*-th allele. In order to estimate the observed heterozygosity (H_o) in the corresponding locus, the number of heterozygous specimens was divided by the total number of specimens. Alleles with the frequency of 0.2 and less were considered as rare. The allele frequency, probability of identity in accordance with the Hardy-Weinburg law, HW P(ID), and probability of identity in sib analysis, Sib P(ID), were determined using the GENECAP software program [16]. The null allele frequency (r) was calculated by the formula $r = (H_e - H_{H.})/(1 + H_{o.})$. The Jaccard similarity coefficient calculation [17] and dendrogram construction were carried out using the PAST software program [18]. The dendrogram was constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [19] using a Jaccard coefficient; number of replications is 1,000.

Results. The analysis of 14 microsatellite loci (Table 1) for 27 currant variety specimens has revealed loci having from 1 (MS06g03) to 8 (e4-D03) alleles, which was equal to 4.9 alleles per locus on average. In this case, all loci but MS06g03 were polymorphic.

1. The designations and amplification conditions of the microsatellite loci used for the analysis of black currant (*Ribes nigrum* L.) variety specimens from the collection of All-Russian Research Institute of Breeding Fruit Crops

Locus	Linkage group[a]	Forward primer	Reverse primer	T, °C
e1-O01	6	CCT TTC CAG AGA AAA CTC AAA CA	AAG TAT GGG AAC AAC GGC AG	52
e1-O21	4	TCT CTC CAA CTG AGA AGG AAA A	GAT TTG TTC TTG TGC AGC GA	50
e3-B02	5	AAG ACG AAG ACG ACG ACG AT	CTG ATC TTT GCC GAA TGG TT	52
e4-D03	3	CCC AAA AGC AAA TTT AGG GT	GTG AGG CAT GGA ACC ACT TT	56
g1-B02	2	CGA CTT CAT CGC TCT CCT CT	CCA TTG ATT TGG TGA GGG T	50
g1-E03	1	TAA CTG CGG GGT TCC TAC AG	CCA CCG CTA CCA ATA ACC AT	56
g1-K04	1	TGT TCC CTG TTT CCT TCA AAA	GGA CGT GGA CGA TGA GAG TT	52
g1-M07	1	TCC CGT TAC TGG AGT GGT GT	CCA TGG TTT TCC GAT TTG TT	52
g2-B20	3	CTC CAT CAA ATC CCT CGT TT	TCT TGC TTC CCA AAC AGT ATC A	52
g2-H21	4	TGC CCT TTT TGG TCA TTT TC	CAA TCG TCG ATG AAG GTC TG	50
g2-J08	2	CGC CGA GCT CTA ATC ACT GT	ATA GCC CAT GCC CAT ATT CA	54
g2-L17	4	TTT GGA AAA CCT CCC CTT TT	GAG CTG TTG CTG TTG CCA TA	50
g2-G12	7	GTG ACC CAC CTA AAC CGT CC	GGA GTG GAG GGT TGG AAA AT	52
MS06g03	[b]	CGG AGG GTG TGC CGA AG	GCC CAG CCC ATA TCT GCT	52

N o t e: T — annealing temperature; a — on the genetic map of *Ribes nigrum* [6], b — black currant locus is not mapped.

For all black currant loci but MS06g03, we know the position of linkage groups on the genetic map (in particular, the analyzed loci are located in 7 different linkage groups) [6]. Primers for locus MS06g03 were initially developed based on an apple tree DNA sequence and mapped in linkage group 10 in a family obtained by of crossing of varieties Fiesta × and Discovery. MS06g03 is considered as a multilocus marker: in case of the apple tree, its amplification re-

sults in DNA fragments of 154-190 base pairs [13], and a fragment with the length of 140 base pairs is observed for all currant variety specimens. It is likely that this locus is located in the quite conservative part of the black currant genome.

In the majority of cases, maximum two fragments were identified for a specific locus for each genotype; however, for a number of specimens, three fragments were amplified in some loci (g2-B20 for variety Yadrenaya, e1-O01 for variety Selechenskaya, and in e3-B02 for Gamma, Gratsiya, Orloviya, Chernaya Vual, Sharovidnaya, Zusha, Kipiana). It is reported about amplification of more than two alleles in some microsatellite loci of diploid forms, which is associated with duplication of microsatellite loci either on the same chromosome or different chromosomes [20].

Based on the microsatellite profiles of 27 variety specimens, we have analyzed allele distribution for 13 of the polymorphic loci used (Table 2).

2. The characteristics of 13 polymorphic microsatellite loci identified for 27 black currant (*Ribes nigrum* L.) variety specimens from the collection of All-Russian Research Institute of Breeding Fruit Crops

Locus	*n*	Allele size, base pairs (frequency)								H_e	H_o	*r*	A, B
e1-O01	6	138[a]	140[ab]	142	145[a]	147	149[a]			0.737	0.769	–0.019	0.402,
		(0.093)	(0.023)	(0.419)	(0.163)	(0.209)	(0.093)						0.101
e1-O21	3	288[ab]	292	294						0.423	0.407	0.011	0.639,
		(0.029)	(0.714)	(0.257)									0.402
e3-B02	4	188	191	193[a]	194[a]					0.655	0.800	–0.088	0.467,
		(0.486)	(0.257)	(0.200)	(0.057)								0.177
e4-D03	8	197[a]	201[a]	205[a]	207[ab]	210[a]	212[a]	217[a]	224[a]	0.840	1.000	–0.087	0.342,
		(0.180)	(0.100)	(0.160)	(0.020)	(0.200)	(0.200)	(0.100)	(0.040)				0.046
g1-B02	3	203	205	207						0.610	0.590	0.016	0.495,
		(0.231)	(0.513)	(0.256)									0.216
g1-E03	7	232[ab]	235	237	239[a]	245[a]	249[ab]	270[a]		0.726	0.593	0.077	0.416,
		(0.025)	(0.400)	(0.275)	(0.175)	(0.075)	(0.025)	(0.025)					0.118
g1-K04	4	285[ab]	290	295	298[a]					0.560	0.556	0.003	0.530,
		(0.026)	(0.605)	(0.237)	(0.132)								0.250
g1-M07	7	200[a]	204[a]	207[a]	209	212[a]	217[a]	222[ab]		0.739	0.704	0.020	0.405,
		(0.140)	(0.140)	(0.047)	(0.442)	(0.140)	(0.070)	(0.023)					0.097
g2-B20	5	147[a]	168[a]	178	181[a]	185[a]				0.720	0.556	0.096	0.420,
		0.189	0.135	0.432	0.189	0.054							0.119
g2-H21	3	265[a]	267	273[a]						0.361	0.259	0.075	0.680,
		(0.156)	(0.781)	(0.063)									0.443
g2-J08	4	158[a]	160	162[a]	164[a]					0.514	0.444	0.046	0.562,
		0.083	0.667	0.167	0.083								0.274
g2-L17	6	150[a]	152[a]	154[a]	161	168[a]	171[a]			0.580	0.296	0.180	0.510,
		(0.063)	(0.063)	(0.094)	(0.625)	(0.094)	(0.063)						0.200
g2-G12	7	190[ab]	195[a]	203[a]	207[a]	209[a]	212	215		0.997	0.926	0.035	0.355,
		(0.022)	(0.178)	(0.089)	(0.089)	(0.156)	(0.244)	(0.222)					0.058
Average										0.652	0.608		0.192,
													0.479

N o t e: A — Sib P(ID) (probability of identity in sib analysis), B — HW P(ID) (probability of identity in accordance with the Hardy-Weinburg law); *n* — number of alleles, a — rare alleles, b — unique alleles amplified in one of 27 variety specimens studied.

The expected heterozygosity varied from 0.361 in locus g2-H21 to 0.997 in locus g2-G12 (0.652 on average), whereas the observed heterozygosity varied from 0.259 in locus g2-H21 to 1 in locus e4-D03 (0.608 on average). Thus, the average values of the observed and expected heterozygosity did not differ much, which was due to the nature of the object under study. As is known, allogamous plants, including the black currant, are characterized by greater heterozygosity than autophilous ones [15]. In addition, vegetative reproduction of fruit and berry crops makes it possible to fix the heterozygous state of the genotype regardless of how stable the recurrence of characters in the first generation might be. Therefore, distinctive features of such crops include high heterozygosity which is only strengthened by the use of a remote hybridization method and involvement of different species in the breeding process.

3. The variety-specific fragments amplified in the microsatellite loci of the black currant (*Ribes nigrum* L.) from the collection of All-Russian Research Institute of Breeding Fruit Crops

Variety specimen	Locus/size of fragment
Orloviya	g1-K04/285 base pairs, g1-E03/232 base pairs
Odzhebin	g1-M07/222 base pairs
Monisto	e4-D03/207 base pairs
Minai Shmyrev	e1-O21/288 base pairs
Binar	e1-O01/140 base pairs
Titaniya	g1-E03/249 base pairs
Sharovidnaya	g2-G12/190 base pairs

The observed heterozygosity was larger than the expected one in three loci (e1-O01, e3-B02, and e4-D03). Conversely, in the other nine loci, the expected heterozygosity was higher than the observed one, which is indicative of the presence of homozygous genotypes or a non-amplified null allele. The theoretical null allele frequency turned out to be a positive value for all loci, but e1-O01, e3-B02, and e4-D03, and ranged from –0.088 to 0.180.

The probability of identity of two genotypes, HW P(ID), varied from 0.046 for locus e4-D03 to 0.443 for locus g2-H21 (0.203 on average), and the probability of identity of two sibs, Sib P(ID), varied from 0.342 for locus e4-D03 to 0.680 for locus g2-H21 (0.489 on average).

A total of 45 of 68 alleles amplified in 14 loci (66 %) corresponded to rare alleles with the frequency equal to or smaller than 0.2. Also, eight unique fragments amplified only on DNA of one of the tested specimen varieties have been identified (Table 3).

Combinations of alleles in one locus allowed us to distinguish from 3 (g2-H21, e1-O21) to 15 (g2-G12) genotypes. For example, five combinations of alleles (genotypes) were observed in locus g1-K04 with 4 amplified alleles, and variety Orloviya had a unique allele with the size of about 285 base pairs (Fig. 1).

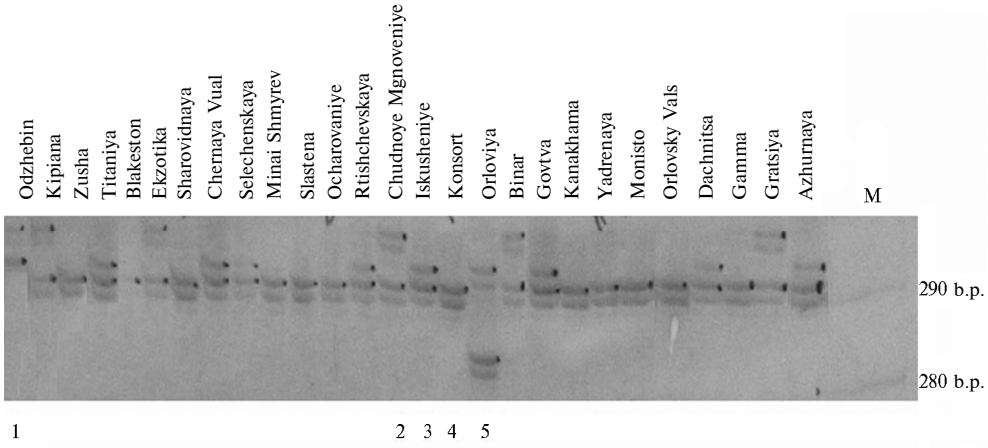

Fig. 1. The electrophoretogram of the fragments of microsatellite locus g1-K04 amplification for 27 studied specimen varieties of the black currant (*Ribes nigrum* L.) from the collection of All-Russian Research Institute of Breeding Fruit Crops: 1-5 — conditional genotypes according to locus; M — molecular weight marker (10 bp DNA Ladder, Invitrogen™, USA; fragment sizes are indicated on the right).

The minimum set of four loci (e4-D03, g1-M07, g1-E03, g2-B20) allowed us to distinguish all the tested variety specimens, i.e. a unique multilocus profile was obtained for each specimen.

Based on SSR spectra, we have constructed a similarity dendrogram for 27 variety specimens of the black currant (Fig. 2). The obtained pairwise genetic similarity coefficients ranged from 0.11 (between varieties Odzhebin and Sharovidnaya) to 0.95 (between varieties Govtva and Rtishchevskaya) with the

average value of 0.346.

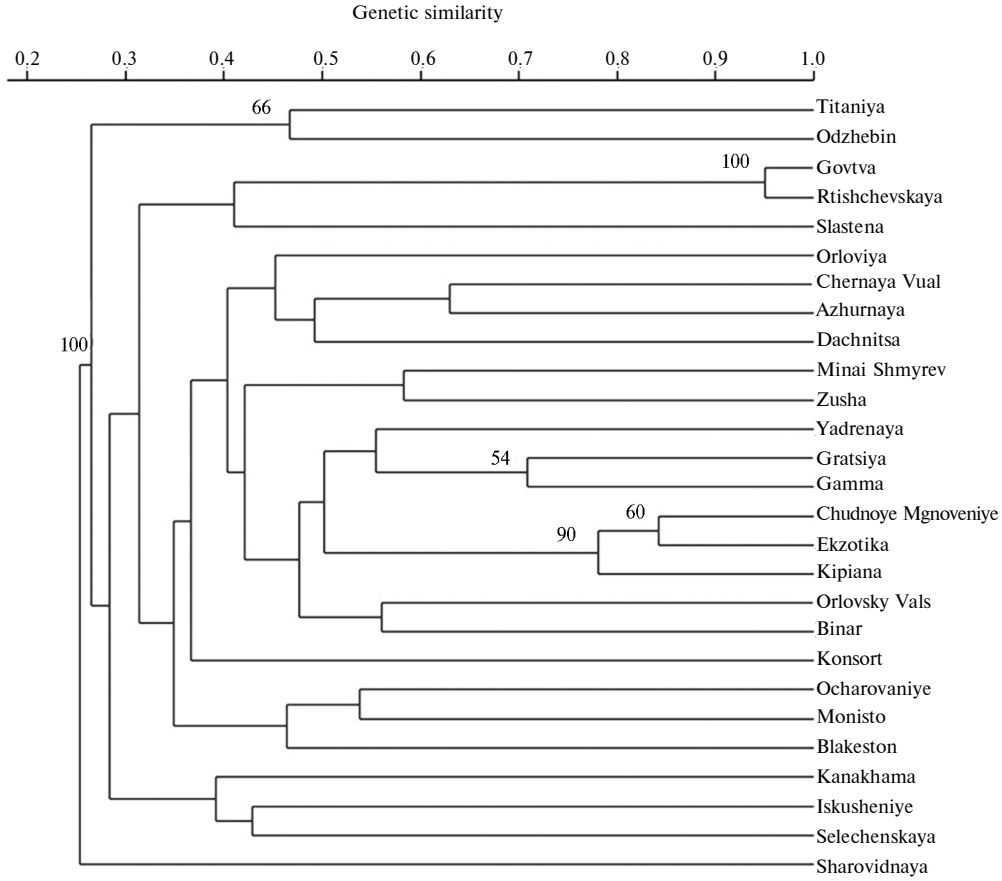

Fig. 2. The similarity dendrogram for 27 variety specimens of the black currant (*Ribes nigrum* L.) from the collection of All-Russian Research Institute of Breeding Fruit Crops, which was constructed based on the analysis of polymorphism of 13 microsatellite loci (the bootstrap values shown).

The dendrogram showed several distinct clusters with high bootstrap support (BS). At a short genetic distance, varieties Govtva and Rtishchevskaya (BS 100%) were collectively clustered. Varieties Chudnoye Mgnoveniye and Ekzotika merged at a somewhat greater genetic distance (BS 60 %); their parent forms are seedlings from one family. Then, variety Kipiana joined them (BS 90 %); its male parent was variety Ekzotika. Varieties Gamma and Gratsiya derived by selection from the same hybrid family as variety Kipiana were clustered separately from varieties Ekzotika and Kipiana. According to the data of RAPD analysis, clustering of varieties Gamma and Gratsiya with a high BS value was also observed, whereas variety Kipiana was not a part of this cluster [8]. This may indicate that varieties Gamma and Gratsiya are genetically more similar to each other than to varieties Kipiana and Ekzotika. An individual cluster was also formed by Swedish varieties Titaniya and Odzhebin (BS 66 %) having a Scandinavian subspecies of the black currant in their genealogy.

The analysis of allele flow through the genealogies of the related varieties has shown that, in the majority of cases, allele distribution between them was not in conflict with the genealogies. Thus, parent variety Lentyai and its derivatives in the first generation (varieties Azhurnaya, Orlovsky Vals, Slastena) had at least one common allele for all tested microsatellite loci. The same occurred

with variety Minai Shmyrev and the varieties derived from it the first generation (Zusha, Lentyai, Orlovskaya Serenada), as well as with variety Ekzotika and its descendants in the first generation (varieties Kipiana, Gamma, Gratsiya). However, variety Ocharovaniye, for which variety Ekzotika was a male parent according to the genealogy, had no common alleles in three loci (e4-D03, g1-E03, g2-B20). It is probably due to pollination by other pollen, or an error in reproduction and transplantation, or mutations in these loci. Varieties Odzhebin and Binar (derived by crossing of Odzhebin × Naryadnaya) had no common alleles in two microsatellite loci (g1-M07 and g1-E03).

In some cases, during genotyping of microsatellite loci of collected black currant varieties from North Europe, it was impossible to confirm genetic identity of same-name specimens from various collections, for example, one of 10 specimens of variety Odzhebin turned out to be non-identical to the other specimens [11]. S. Reim et al. used 11 microsatellite loci in order to clarify the origin of the varieties bred in Dresden-Pillnitz [21]. As a result, in a number of cases, it became evident which variety was involved in hybridization with pollination with mixed pollen of different varieties, and for some varieties, unreliability of genealogies was established both for female and male parent forms.

So, we were the first who carried out genotyping of black currant (*Ribes nigrum* L.) microsatellite loci in Russia. It has been established that the microsatellite loci have high polymorphism, and also rare and unique alleles have been identified, which made it possible to obtain a unique multilocus profile for each tested specimen. It is shown that we may check the relationship of specimens based on the analysis of microsatellite allele distribution. The obtained data indicate that there are good prospects for using the tested markers in the assessment of genetic diversity of the domestic black currant gene pool, as well as in the development of methods for identification and certification of varieties, which may be used for breeders' copyright protection.

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PRODUCTIVENESS AND NODULE ABILITY OF DIFFERENT VARIETIES OF COMMON BEAN (*Phaseolus vulgaris* L.) IN URALS CONDITIONS

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Abstract

Common bean (*Phaseolus vulgaris* L.) has a high nutritional value, its seeds contain a significant amount of protein and essential amino acids and vitamins. Furthermore, it can accumulate organic nitrogen in the soil due to nitrogen fixation. On the territory of the forest-steppe zone of Urals (Bashkortostan) in small plot experiments in homogeneous soil conditions during five years the elements of yield structure of four cultivars of common bean (Gornal, Ufimskaya, Zolotistaya and Elsa) were studied. In different soil and climatic conditions the distribution character (skewness) of seed production and nodule activity were compared. Correlations between agro-meteorological factors, nodules number and productivity were determined. In one of these experiments there were watering and using of start doses of fertilizers $(PK)_{45}$. The remaining experiments were carried out in a natural agricultural background without irrigation. The seeds yield of local cultivars (Gornal, Ufimskaya, Zolotiataya) in homogeneous soil conditions on leached chernozem (Ufa district) ranged from 18 to 22 kg/ha. High seed productivity of a new cultivar Elsa was mentioned. In the experiments without irrigation (Ufa, Iglinskiy, Chishminskiy, Bakalinskiy areas) the seed weight of different cultivars changed depending on the agro-meteorological conditions from 4 to 17 g/plant, being doubled at watering. An average rang of seed production variation for all years was 50 % for Zolotistaya cultivar, 53 % for Elsa, 57 % for Ufimskaya and 76 % for Gornal cultivars. The close relationship (r = 0.61) between the productivity of beans and the sum of active temperatures above 15 °C was revealed. Greater flexibility to the amount of heat was shown in the locally bred varieties Zolotistaya and Ufimskaya at r = 0.46 and r = 0.59, respectively, greater dependence on abiotic factors was demonstrated by Gornal and Elsa varieties at r = 0.78 and r = 0.84, respectively. Similar relations were revealed when the analysis of correlative links was made between the duration of the period of above 15 °C temperatures and the seed productivity. Thus, correlation was detected at r = 0.42 and r = 0.20 for Zolotistaya and Ufimskaya cultivars, and at r = 0.51 and r = 0.69, for Gornal and Elsa, respectively. In different conditions there were from 5 to 29 nodules formed at a plant. The average variation of nodule activity between all cultivars during all years was 15.3 %. However, for each cultivar under different soil and climatic conditions the individual average coefficients of variation differed, being 44 % for Gornal, 59 % for Zolotistaya, 65 % for Ufimskaya and 18 % for Elza varieties. In most experiments the positive skewness prevailed in both the number of nodules and productivity. Normal distribution and the negative skewness for all cultivars were observed in the experiment with watering, and occurred more frequently in plants of Zolotistaya cultivar. Number of nodules per plant and seed production correlated (r = 0.75-0.87) only under optimal water regime and mineral nutrition. On the gray forest soil the plants formed a symbiosis with local rhizobia, and on the chernozem soil the symbiotrophic nitrogen nutrition prevailed over mineral nitrogen one. The revealed high intravarietal variability of common bean plants and the asymmetric distribution of symbiotic activity and productivity suggest the possibility for selecting lines to improve the efficiency of symbiosis. A randomly formed supernodulating hybrid L20 of cultivar Elza was discovered to be promising for further breeding.

Keywords: bean cultivars, seed production, nodulation, agroclimatic factors, inside and between cultivar variability, skewness, South Urals.

The common bean (*Phaseolus vulgaris* L.) is distinguished from the other grain legume crops by its nutritional value and wide scope of application. Its food value is determined by significant (17-32 %) protein content in seeds, as well as by the presence of essential amino acids and vitamins. The bean has a

high potential as a soil-amendment crop. As known, it can accumulate more than 60 kg of biological nitrogen/ha per year due to nitrogen fixation. The bean is also used as a medical plant [1, 2]. In Russia, this crop is not referred to traditional ones, but it is successfully cultivated on individual farms.

By now, in the Republic of Bashkortostan (RB), new bean varieties Ufimskaya [3] and Zolotistaya [4] have been included in the State Register of Selection Achievements Permitted for Use along with previously released variety Gornal. Optimum seeding rates, seeding time and other agricultural methods of cultivation in the conditions of the Cis-Ural region have been developed for these varieties [5]; however, their symbiotic activity has not been practically studied yet. As is known [6], high intra- and intervarietal diversity with regard to symbiosis characters, which is typical for many cultivated forms of legumes, predetermines the directional selection of genotypes in order to improve symbiotic activity. It is one of the most important conditions for creation of systems of adaptive plant growing and environmentally sustainable crop farming [7].

Our work was aimed at investigating the productivity of different bean varieties and their nodule-forming capacity in symbiosis with local rhizobium races in conditions of the forest-steppe zone of the Cis-Ural region.

Technique. Four bush varieties of the common bean have been studied in field trials (Gornal, Ufimskaya, Zolotistaya and Elza). Variety Gornal bred at the Lgovskaya breeding station and in the All-Russia Research Institute of Legumes and Groat Crops was used as a standard. Ufimskaya and Zolotistaya are local varieties included in the State Register for the RB in 1998 and 2000, respectively [3, 4]. Variety Elza was obtained in France (k-14693 according to the catalogue of VIR, N.I. Vavilov Research Institute of Plant Industry, St. Petersburg). Field trials in the territory of the RB were carried out within five years (2003-2007) in various agrometeorological conditions.

On plot № 5 located in the Ufa area on leached chernozem, the following productivity characters were assessed in 2003-2007 (5 trials): grain weight, yielding capacity, number of seeds per plant and weight of 1,000 seeds for varieties Gornal, Ufimskaya, Zolotistaya and Elza. In this case, a total account of all plants on 1 m^2 was applied. Within the same years, nodular activity and seed productivity of bean plants were compared on four plots (№ 1, № 2, № 3, № 4; 8 trials).

In 2004, in the Ufa area, all four varieties were studied on gray forest, heavy clay-loam soil (plot № 2) with irrigation and application of starter doses of PK_{45} fertilizers. In the other cases, studies were carried out on the natural agricultural background without irrigation.

In 2003-2004, in the Iglino area, only variety Ufimskaya was cultivated; in 2005, in the Bakaly area there were only varieties Ufimskaya, Zolotistaya and Gornal. In 2006, the same varieties were assessed in the Iglino area, and also they were compared with tests of two varieties, Ufimskaya and Gornal, in the Ufa area. In 2006, varieties Zolotistaya and Elza were studied in the Chishmy area, and in 2007, they were cultivated in the Iglino area. Nodules (medium and large) and seed weight were taken into account for each plant at the end of the vegetation period at the bean maturation stage by analyzing 40-60 plants and determining a pattern of character distribution by asymmetry coefficients and variation coefficients.

In all trials, the bean was seeded by hand in wide rows (inter-row spacing of 45 cm) at the seeding rate of 15 viable seeds per linear meter, which corresponds to 333,000 pcs/ha. Seeding time was the 1st decade of June, except for seeding in the Chishmy area in 2006, when this term was carried over to the end of May; harvesting time was 2nd decade of September. The plot size was from 3

to 8 m^2. The trials were carried out in 4 replicates.

The data were processed statistically, using the standard programs of the Microsoft Excel software. The tables show arithmetical average values and standard errors.

Results. The bean is a warm-weather crop. Even germination of its seeds occurs at 12-15 °C; optimum temperature for plant growth and development is 20-25 °C, but successful bean setting is possible at 15 °C. In the years when the experiments were carried out, the dates when temperature crossed 15 °C fell within the period from mid-May to mid-June (Table 1). By this reason and in connection with the possibility of late-spring frosts in the conditions of the Southern Urals, we mainly adhered to the recommended seeding time [5] in the first decade of June. The sum of active temperatures is above 15 °C, and the normal duration of this period in the territory of the RB, according to data from the Bashkir Administration for Hydrometeorology and Environmental Monitoring, is within 70-100 days, and 72-101 days are required for complete bean ripeness for released varieties, which corresponds to the conditions of the region.

1. The characteristics of meteorological conditions in the period of the studies (Republic of Bashkortostan)

Year	Area	Ground frosts, date	T_{air} crossing 15 °C, date	$T_{ef.} > 15$, days	$T_{ef.} > 15$, °C	Precipitation (summer), mm	HTC
2003	Iglino	9.06	23.06-09.09	78 (84)	377 (237)	158 (200)	1.6
	Ufa	29.04	22.06-09.09	79 (90)	389 (265)	193 (172)	2.6
2004	Iglino	19.05	25.05-04.09	102 (84)	407 (237)	273 (200)	2.7
	Ufa	20.05	24.05-04.09	103 (90)	465 (265)	177 (172)	1.7
2005	Bakaly	n/a	11.05-20.08	102 (89)	353 (226)	204 (184)	1.1
	Ufa	17.04	11.05-20.08	99 (90)	386 (265)	167 (172)	n/a
2006	Iglino	20.05	01.06-12.09	103 (90)	391 (237)	155 (200)	1.0
	Chishmy	n/a	31.05-13.09	104 (94)	315 (267)	318 (167)	1.2
	Ufa	20.05	02.06-11.09	101 (90)	310 (265)	313 (172)	1.0
2007	Iglino	07.06	14.06-11.09	89 (84)	358 (237)	186 (200)	1.6
	Ufa	07.06	14.06-12.09	90 (90)	419 (265)	142 (172)	1.2

N o t e: n/a — not available, T_{air} — air temperature, $T_{ef.}$ — effective temperature, HTC — hydrothermal coefficient. Figures in brackets indicate normal values, *i.e.* long-term average annual values of the corresponding characteristics.

Refer to Table 2 for agrochemical soil parameters on the experimental plots.

2. The agrochemical characteristics of the plots where the field experiments were carried out (Republic of Bashkortostan, 2003–2007)

Plot №	Area	Soil	Humus content, %	Content of N_{total}, %	pH
1	Iglino	Gray forest, heavy clay-loam	3.9	0.25	6.1
2	Ufa	Gray forest, heavy clay-loam	3.8	0.26	6.1
3	Bakaly	Typical chernozem, medium clay-loam	8.5	0.46	6.8
4	Chishmy	Dark-gray forest, medium clay-loam	4.4	0.28	6.4
5	Ufa	Leached chernozem	6.7	0.37	6.4

3. Yielding capacity and productivity indices for different varieties of the common bean (*Phaseolus vulgaris* L.) cultivated on leached chernozem ($X \pm x$; Ufa area, Republic of Bashkortostan; 2003–2007)

Variety	Seed weight, g/plant	Number of seeds, pcs/plant	Weight of 1,000 seeds, g	Yielding capacity, centner/ha
Gornal	7.2±0.9	22.3±2.0	314±17.0	18.7±2.2
Ufimskaya	8.4±0.9	24.8±2.0	337±15.9	22.0±2.5
Zolotistaya	7.6±1.0	23.2±1.8	322±21.8	19.7±2.5
Elza	7.6±2.0	43.7±5.3	174±13.6	19.9±5.0

N o t e: *Cv* of seed weight — 30.1 %, *Cv* of number of seeds — 20.4 %, *Cv* of weight of 1,000 seeds — 14.1 %; LSD_{05} (least significant difference) for yielding capacity — 1.1 centner/ha. Data for variety Elza are given for 2003-2005.

The yielding capacity of released varieties Gornal, Ufimskaya and

Zolotistaya in trials on leached chernozem (plot № 5) for five years of studies was 18.7-22.0 centner/ha (Table 3) The highest productivity was observed for variety Ufimskaya. Relatively late-maturing variety Elza was distinguished by producing more seeds per plant; however, it had low yielding capacity due to poor bean filling and low germinating ability.

In the tests performed on other plots (Table 4), bean productivity was higher, which could be explained by specific features of the accounting process and trial conditions. In 2004, irrigation was used on plot № 2, which promoted the formation of seed weight from 18.5 to 27.0 g/plant in complete ripeness. Due to optimum moisture content, seed productivity doubled as compared to that of variety Ufimskaya cultivated without irrigation in the same year on plot № 1 in similar agrometeorological conditions. In trials of 2005-2007 without irrigation, seed weight was from 5 to 15 g/plant (8.8 on average) for variety Ufimskaya, from 4 to 17 g/plant (9.2 on average) for variety Gornal, from 6 to 14 g/plant (10.3 on average) for variety Zolotistaya, and from 11 to 16 g/plant (13.3 on average) for variety Elza. Narrower range of productivity variation on average for all years was observed for varieties Zolotistaya (Cv_{av} = 50 %) and Elza (Cv_{av} = 53 %); variation for Ufimskaya and Gornal was 57 % and 76 %, respectively.

4. The intervarietal diversity of nodule-forming and seed productivity characters for common bean (*Phaseolus vulgaris* L.) plants of different varieties ($X \pm x$, Republic of Bashkortostan)

Trial (year, plot)	Variety	Number of nodules			Seed weight		
		pcs/plant	*As*	*Cv*	g/plant	*As*	*Cv*
2003, № 1	Ufimskaya	10.8±0.7	161	39	8.8±0.6	59*	51
2004, № 1	Ufimskaya	24.5±0.7	135	23	10.8±0.7	188	48
2004, № 2	Ufimskaya	13.0±1.5	35*	61	18.5±1.0	210	31
	Zolotistaya	13.4±0.8	−134	36	19.3±0.8	39*	24
	Gornal	12.4±1.4	–31*	58	27.2±1.7	103	30
	Elza	15.3±1.1	82*	32	26.7±2.5	51*	43
2005, № 3	Ufimskaya	6.2±1.1	129	108	15.4±1.4	131	58
	Zolotistaya	4.7±1.0	288	131	14.2±1.3	105	58
	Gornal	5.2±1.1	297	122	17.4±2.4	727	86
2006, № 1	Ufimskaya	27.3±1.8	163	55	5.0±0.5	430	75
	Zolotistaya	21.9±2.8	250	76	6.1±0.7	202	67
	Gornal	18.4±2.2	237	78	6.2±0.6	190	65
2006, № 2	Ufimskaya	4.9±0.8	279	94	5.9±0.7	292	80
	Gornal	13.0±1.4	193	66	3.9±0.8	556	121
2006, № 4	Zolotistaya	29.4±1.8	86*	53	12.7±0.6	218	43
	Elza	21.4±2.5	1,000 (276)[a]	92	15.6±1.3	222	66
2007, № 1	Zolotistaya	11.5±1.2	98*	64	8.0±1.0	73*	57
	Elza	16.4±1.5	312	70	10.9±0.7	312	50

N o t e: № 1 — Iglino area, № 2 — Ufa area, № 3 — Bakaly area, № 4 — Chishmy area; *As* — asymmetry coefficient, % of critical value, *Cv* — variation coefficient, %; a — high asymmetry coefficient was due to the presence of a spontaneous creeping hybrid with 130 nodules in the sample, the figure in brackets indicates the asymmetry coefficient of the character in the population without this plant.
* Values corresponding to normal distribution ($p < 0.05$).

Based on the comparison of our data on productivity with the results of the experiments carried out in 2007-2008 on 11 common bean varieties in the south forest-steppe conditions of Western Siberia [8], it may be noted that the varieties tested in Siberia produced larger seed weight than varieties in the Cis-Ural region (on average of 5.6-35.0 and 12.0-20.0 g/plant, respectively).

With the regional climatic parameters being similar, experiments in the Omsk Region were distinguished by earlier time of bean seeding (2nd and 3rd decades of May). If frosts are not expected in Cis-Ural conditions according to a long-term forecast, it could be recommended to reschedule seeding dates from June to the last decade of May. We carried out such an experiment in 2006 on plot No. 4 in the Chishmy area. This lead to doubling of bean seed productivity as compared to plots №№ 1 and 2, not only due to better climatic conditions

(hydrothermal coefficient, HTC, was equal to 1.2 in the Chishmy area, and 1.0 in the Ufa and Iglino areas), but also due to a longer vegetation period and additional use of soil moisture in the early periods of plant development.

The analysis of correlation between the bean seed productivity and sum of active temperatures above 15 °C (r = 0.61 for all varieties) has revealed that the degree of this correlation is dissimilar for different varieties. Greater adaptability (independence from heat) was observed for varieties Zolotistaya and Ufimskaya (r = 0.46 and 0.59, respectively); varieties Gornal and Elza were less adaptive (r = 0.78 and 0.84, respectively). Similar regularities were found as a result of the analysis of correlative relationships between the duration of the period with temperatures above 15 °C and seed productivity: at r = 0.42 and r = 0.20 for varieties Zolotistaya and Ufimskaya, respectively; and at r = 0.51 and r = 0.69 for varieties Gornal and Elza, respectively.

An important reserve for increasing the adaptive potential of bean varieties is breeding of the varieties having high productivity due to symbiotrophic nutrition [9, 10]. Thus, the bean lines selected according to increase in their nodule formation rate surpassed the parent varieties in seed weight (by 74 %) and protein content (by 27 %) [11]. In our experiments aimed at studying the nodular potential of the varieties, the number of nodules in a bean maturation phase was 5-29 pcs/plant (see Table 4). The intervarietal variation of average nodule numbers per plant for the years of the studies was 15.3 %. It should be noted that the best bean variety specimens from VIR's collection had by 40-115 nodules per plant [12]. Based on the results of the trials carried out in the Omsk Region in 2006, high nodule-forming capacity (over 40 nodules) was observed for bean varieties Lika, Sekunda, Rashel and Zolushka [13]. We may suppose that the potential of breeding of the bean varieties and lines adapted to the conditions of the RB for high symbiotic activity is far from being exhausted.

In order to assess prospects for segregation of bean genotypes with the highest symbiotic activity within the variety, we considered the distribution pattern and intervarietal diversity of nodule formation rate and productivity characters by year within each plot (see Table 4). In most of the experiments, right-hand asymmetry ($As > 0$) prevailed with regard to both the number of nodules and productivity for different varieties. This coincides with the observations of other researchers [6, 14] and may be associated with the absence of previous plant selection for a symbiotrophic type of nutrition, the lack of highly active microsymbionts complementary to the host's genotype among local rhizobia, as well as with the influence of environmental factors limiting symbiotic efficiency (available nitrogen content or moisture deficit that reduces the exudation of attractants for nodule bacteria in the rhizosphere and outflow of fixed nitrogen to the above-ground organs of plants). It seems that moisture content conditions in our experiments have made a decisive contribution to the pattern of nodule-forming character distribution, because, in case of artificial irrigation with starter doses of potassium-phosphorus fertilizers in the Ufa area (plot № 2) in 2004, for all varieties, either a normal distribution pattern or uncommon negative asymmetry (mainly for variety Zolotistaya) was identified for the nodule-forming character. It should be noted that variety Zolotistaya demonstrated normal distribution by the number of nodules more often than the other varieties. The study of this variety is of interest with regard to identification of the genotypic properties which provide efficient attraction and/or selection of active bacterial partners by the macrosymbiont.

In order to breed the legume varieties and lines which are able to actively use symbiotically consumed nitrogen, it is important to determine correlation between the size of the nodule-forming apparatus and seed productivity. We

revealed close correlative relationship between the number of nodules in the maturation phase and the weight of seeds from one plant (r = 0.75 for variety Ufimskaya, r = 0.87 for varieties Zolotistaya and Elza) only in a trial with optimum nutrition and moistening conditions (plot № 2, 2004). On chernozem, (plot № 3, 2005), nodule formation activity of plants was low and did not correlate with productivity. On gray forest and dark-gray forest soils, nodule formation was in general better, but the correlative relationship between it and seed productivity was not found as well.

The comparison of nodule formation activity for each variety in different soil and climate conditions has allowed us to identify the variation range for this character (44.0 % in Gornal, 59.3 % in Zolotistaya, 65.0 % in Ufimskaya, and 18.4 % in Elza). It is likely that such diversity is connected with high flexibility of local adaptive varieties with regard to a capability of changing the nitrogen nutrition type from symbiotrophic to autotrophic under varying environmental conditions. The range of intervarietal diversity with regard to symbiotic activity (Cv_{av} = 63-81% for different varieties) offers opportunities for the directional selection of the high-efficiency intervarietal lines that actively use the potential of symbiotic nitrogen fixation.

As noted by N.S. Tsyganok [16], if principles of environmental and geographical remoteness and parent form contrast are met, hybridization is the most successful approach that allows us to obtain varieties with the required characters. The pea breeding pattern aimed at improving nitrogen fixation, which has been presented by K.K. Sidorova et al. [17], combines the use of hybridization with supernodular mutants, segregation of individual lines in F_2, recurrent selection of successful lines with supernodulation in F_3-F_7, and selection of individual and individual-group lines and plants. In our experiments on plot № 4 in 2006, we revealed a plant specimen of variety Elza that had formed a 5 m long stem (with the average value being 0.7 m) and produced more than 130 nodules. Taking this plant into account, a character distribution asymmetry coefficient was equal to 1,000 % of the critical value, or 276 % without taking it into account (see Table 4). It is likely that this plant turned out to be a spontaneously cross-pollinated hybrid with a creeping bean form. Its seeds (line L20) were selected for further study of the heritability of the character associated with high nodule-forming capacity and nondeterministic growth. Three generations of this and two other lines, L42 and L44, selected by high productivity have been field-tested [18].

So, all the bean varieties studied by us are good candidates for cultivation in the Cis-Ural region. It has been demonstrated that a close correlation exists between the bean productivity and the sum of active temperatures above, as well as the duration of the period of these active temperatures, which substantiates the account of adaptive characters, such as early ripeness and flexibility under changing environmental factors, as well in moisture deficit conditions observed in the Cis-Ural region, in the variety breeding process. Varietal features have been noted with regard to variation of these characters. It has been established that using artificial irrigation or rescheduling seeding dates from June to the end of May can double the seed yield. On gray forest soil, the plants of the tested varieties formed a symbiosis with local rhizobia, but the nodule formation rate did not correlate with the yielding capacity. High coefficients of correlation between the nodule-forming capacity of plants and seed productivity have been revealed only in a trial with the moisture regime and mineral nutrition conditions which are optimal for symbiosis. Under the same conditions, uncommon negative asymmetry with regard to the number of plant nodules and seed productivity was noted along with normal distribution of these characters for all varieties. On chernozem, the sym-

biotrophic type of nutrition was inferior to the autotrophic one. The high intravarietal plant diversity with regard to symbiotic activity of all varieties indicates that it is possible to select lines for improving symbiosis efficiency. We have found a spontaneous supernodulating hybrid L20 of variety Elza, which is promising for further breeding.

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CORRELATION OF BREEDING TRAITS THAT DETERMINE PRODUCTIVITY OF CHICKPEA (*Cicer arietinum* L.) ACCESSIONS FROM THE VIR COLLECTION IN THE CONDITIONS OF TAMBOV REGION

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Abstract

Increasing global temperature recently leads to climatic changes towards more drought conditions over large areas, so drought resistant plants should be wider cultivated. Chickpea is a drought resistant crop commercially cultivated in 2013 at 800 000 ha in some region of the Russian Federation with periodic droughts, except the Tambov region, though its geographic and climatic conditions could be appropriate for chickpea growing. For the first time in the Tambov region we investigated the formation of breeding traits that determine seed productivity in 629 chickpea accessions of different origin, including 44 countries, from the VIR (All-Russian Research Institute of Plant Industry, St. Petersburg) World Collection which were selected due to prior wide geographic testing in other Russian regions and abroad. Using statistical methods, i.e. factor, dispersion analysis and clustering, we defined breeding and noticeable traits, the influence of sowing norm, and the countries which were the most perspective as originators of chickpea. It is shown that the number of branches of the 2nd order, the number of pods per plant, plant height, the length of growing period mainly contributed into determining productivity of chickpea plants in the environmental conditions of the Tambov region. The weight of 1000 seeds had a positive relationship with the period from flowering to ripening. A significant association between the weight of seeds per plot and the number of plants was observed with the maximum rate found at 70-80 plants per plot. The variability of accessions in plant dray weight was the greatest and reached the value of Cv = 98.3 %. A total of 73 % of the variability of the investigated traits were influenced by three factors. The first one causing 38 % of variability comprised of a block of correlated traits, namely the number of branches of the 1st and 2nd order, the number of pods per plant and plant dry weight, which were associated with the number of seeds per plant. The second one of a 25 % influence included the periods from germination to flowering and from flowering to ripening, the height of the lower bean attachment and the plant height, and the third one with a 10 % effect on variability was the weight of 1000 seeds. In 330 studied forms the bean cracking rate was 10 % that met the standards of Volgogradskii 10 variety, in 202 accessions it exceeded 10 %, and 96 accessions were resistant to bean cracking being valuable for breeding. A total of 147 accessions were not affected by fusarium wilt, and another 120 accessions were very weakly attacked and damaged. By clustering we identified three groups of countries where the forms with higher seed weight per plant and weight of 1000 seeds as the most economically valuable traits were originated from. Accessions from the United States were characterized by larger weight of 1000 seeds and weight of seeds per plant, while in accessions from the former Soviet Union, Bulgaria and Afghanistan the weight of 1000 seeds and the weight of seeds per plant were low. For the rest countries both these parameters were close to average values. So in the Tambov region the k-3720, k-3721, k-3740, k-3771, k-3783, k-3785 from Syria; k-604, k-2340 from Turkey; k-431, k-437, k- 2176 from Mexico; k-1188, k-1335, k-1480, k-2197, k-2397 from Russia; k-2144 from Afghanistan; k-1491, k-1724, k-1727 from Uzbekistan and k-2597, k-2949 from the U.S. are the most perspective form in breeding for seed productivity. A total of 178 most prospective accessions were further tested in 2011-2013.

Keywords: chickpea, collection accessions, valuable breeding traits.

With regard to cultivated areas and grain production, the chickpea is in the third place in the world among legume crops after the soya and haricot bean: every year, the area under this crop is equal to about 12 million ha, and annual grain production is up to 9-10 million tons [1, 2]. The chickpea is cultivated in more than 55 countries with arid climate; it is a basic legume crop in South Asia, the Middle East, East Africa, the Western Mediterranean, Australia and

Mexico [3]. In countries with the rapid growth of the population (India, Pakistan, Mexico, Ethiopia), the chickpea holds a leading position among food products. Its seeds contain a large amount of protein (up to 30 %) with all amino acids and vitamins necessary for humans [4-6]. At the same time, chickpea seeds (unlike other legume crops) are characterized by reduced antinutrient content [7-9]. Due to symbiosis with nodule bacteria, the chickpea, like the other legumes, is a nitrogen-fixing plant and is considered to be the best predecessor for other crops in cultivation zones [10, 11].

In many agricultural regions of the Russian Federation which are prone to periodic droughts, chickpea cultivation areas have increased over the last years, because the chickpea is one of the most drought- and heat-resistant crops among grain legumes [12, 13]. In such territories, the chickpea remains the only crop which can be profitably cultivated in the structure of planted areas, and its cultivation contributes to soil amendment and improves the productivity of the crops following it (the winter wheat rotating the chickpea has the same yielding capacity as after black fallow, or even higher in some cases). The rapid growth of areas under the chickpea in Russia is associated with increase in demand for its grain both in the domestic and external market [14]. In 2001, they were equal to about 25,000 ha; in 2008 and 2011, they exceeded 100,000 ha; and in 2013, they achieved 800,000 ha. The chickpea is cultivated in the North Caucasian, Middle and Lower Volga, Ural and West Siberian Regions of the Russian Federation. The cultivated areas under the chickpea have also increased in the Central Black Soil region, particularly, in the Voronezh and Belgorod Regions.

The new millennium is characterized by global climate change towards warming. Increasingly larger territories are periodically prone to droughts. In this regard, in crop farming, a need arises to expand the zone where drought-resistant crops, including the chickpea, are cultivated [15-20].

Preliminary 2-year studies of the limited number of specimens confirmed that, in principle, it is possible to cultivate this crop in the Tambov Region [21], and, by this reason, we have investigated the world chickpea collection of VIR (N.I. Vavilov Research Institute of Plant Industry).

Genotype selection by one property often does not produce the desired result. In order to improve the efficiency of obtaining plants with high seed productivity, early ripeness and other properties which are significant in terms of breeding, we should know what interrelationships between their determinant characters are.

Under the conditions of the central part of European Russia, we have studied correlative relationships between yielding capacity elements for the chickpea specimens of various origin which are stored in VIR's collection.

Technique. Chickpea specimens (629 from 44 countries) were compared at the Yekaterininskaya experimental station of VIR (Tambov Region). Varieties Krasnokutsky 36 and Volgogradsky 10 released in the Russian Federation were used as standards. Seeding was carried out on April 26, 2011. Standards were seeded after every 10 plots; plot area in the trial was 1 m^2. Chickpea specimens from the collection were studied and assessed in compliance with the procedural guidelines and classifier of VIR [22-24]. Bean shattering before harvesting was described according to the classifier of the International Board for Plant Genetic Resources (IBPGR), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and International Center for Agricultural Research in the Dry Areas (ICARDA) [25], using the scale where 0 points indicates no shattering, 1 point corresponds to less than 10 % shattering, and 2 points indicate that shattering rate is more than 10 %. Within the vegetation period, specimens were assessed for damage by fusarium disease in compliance

with the procedural guidelines of VIR [24].

After harvesting, plants were subjected to structural analysis by the valuable breeding characters determining seed productivity and adaptation to mechanized cultivation. The following parameters were measured: plant height from soil to its highest point (cm), height of lower bean attachment (cm), number of primary branches at the stem base, number of primary branches in the apical part of the stem, weight of one plant with beans and root residues (g), number of beans on one plant, number of seeds on one plant, weight of seeds from one plant, and weight of 1000 seeds (g). Three plants were selected for analysis for each specimen.

Statistical analysis was performed using the StatSoft Statistica v. 6.0. Quantitative characteristics of specimens with different bean shattering rates were compared by the analysis-of-variance method. The characters that provided the highest differentiation of collection specimens were revealed using the factor analysis method. Distinctive features of specimen groups from various countries were studied using the single-factor analysis-of-variance and cluster analysis method. The dendrogram was constructed using the Euclidean distance and Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) [26]. The significance level of 5 % was used.

Results. Weather conditions in 2011 were consistent with the biological features of the chickpea, and grain harvest was obtained for all the specimens studied. Monthly average air temperatures in the vegetation period turned out to be higher than long-time annual average values and were equal to 7.4 °C in April (against 4.9 °C), 19.0 °C in May (against 13.9 °C), 23.5 °C in June (against 17.8 °C), and 28.0 °C in July (against 20.0 °C). Monthly total precipitation was also higher than long-time annual average values: 40.9 mm in April (against 32 mm), 47.3 mm in May (against 43 mm), 68.5 mm in June (against 57 mm), and 84.5 mm in July (against 63 mm).

Initial sprouts were noted on May 10-11 with full sprouts indicated on May 12-13; initial blossom was observed on June 1 with full blossom noted on June 3-18. Seed ripening for various chickpea specimens occurred on July 19-30. For standard varieties Volgogradsky 10 and Krasnokutsky 36, vegetation period was 69 and 73 days, respectively, and in case of collection varieties, it ranged from 67 to 78 days (70 days on average) with the range being 67-70 days for 535 of 629 specimens studied. The earliest ripeness (67 days) was noted for specimen k-3264 ILC-1289 (Turkey).

The majority (615) of the studied collection chickpea specimens had a standing bush form (sprawling at the top) (5 points); 6 of them had a creeping form (1 point); 2 specimens were characterized by a wide-branching bush form (3 points), and 9 specimens were distinguished by a compact bush form with high attachment of lower beans (7 points). In connection with the prevalence of one of the bush forms (standing, sprawling at the top), this character was not statistically analyzed.

The average plant height was 43 cm and 49 cm for the standards (variety Volgogradsky 10 and variety Krasnokutsky 36, respectively) and 36 cm (ranging from 22 to 65 cm) for collection specimens.

The plant dry weight was the most variable character (Cv = 98.3 %), which is indicative of pronounced differentiation of the collection under study, in particular, with regard to this character. The average dry weight of one plant was equal to 17 g with fluctuations from 3 to 186 g for different specimens. Significant differences between specimens were associated with increase of differentiation in a series of the characters determining the dry weight: number of lateral primary branches varied from 1 to 5 (Cv = 25.2 %), number of secondary

branches ranged from 1 to 9 (Cv = 57.4 %), number of beans from one plant was from 5 to 259 (Cv = 85.0%), number of seeds from one plant fluctuated from 5 to 292 (Cv = 88.8 %).

The number of seeds from one plant for standard varieties Volgogradsky 10 and Krasnokutsky 36 was 25 and 28, respectively. Specimens k-910 (Czechoslovakia); k-3718 Flip 85-1C, k-3764 (Syria); k-1810 № 29 (Moldova); k-399 (Bulgaria) and k-217 (Afghanistan) were distinguished by the number of seeds per plant, which ranged from 97 to 292.

The weight of 1000 seeds was 278 g for both standards and 394 g on overage for collection specimens (minimum and maximum were equal to 150 g and 695 g, respectively). The following specimens were distinguished by this character: k-3614 (Spain, 695 g), k-3745 (Italy, 675 g), k-3412 (Syria, 660 g), k-3791 (Turkey, 640 g), k-3612 (Italy, 640 g), k-3626 (USA, 620 g), k-3689 (Portugal, 640 g), k-3609 (Greece, 640 g), k-3647 (Spain, 640 g) and k-431 (Mexico, 630 g).

The weight of seeds per plot for standard varieties Volgogradsky 10 and Krasnokutsky 36 was 418 g and 463 g, respectively; on average for the collection, it was equal to 409 g (ranging from 30 to 1302 g for some specimens), and 180 and 289 specimens surpassed varieties Krasnokutsky 36 and Volgogradsky 10, respectively. There specimens with significant exeedance of this parameter were identified: 1,302 g for specimen k-3407 from France, 835 g for specimen k-3782 from Syria, and 772 g for specimen k-2144 from Afghanistan. This character had Cv = 28.0 %, i.e. it was more stable than the weight of seeds per plant (Cv = 71.5 %), because less number of the plants sprouting on a plot was compensated by larger weight of the seeds harvested from them.

Bean shattering for standard varieties Volgogradsky 10 and Krasnokutsky 36 was less than 10 % (1 point) and more than 10 % (2 points), respectively. By this character, 330 of the collection chickpea specimens studied were referred to the same group as the best of the standards (variety Volgogradsky 10, less than 10 %, 1 point); for 202 specimens, the analyzed parameter was more than 10 % (2 points). 96 chickpea specimens which turned out to be resistant to bean shattering (0 points) are valuable for breeding

Damage by fusarium disease for various specimens was assessed to be within 0–7 points (0 points—no damage, 7 points—severe damage). Standard variety Krasnokutsky 36 was characterized by the absence of damage (0 points), and variety Volgogradsky 10 was slightly damaged (1-3 points on average). 147 of the studied chickpea specimens were not damaged by fusarium disease; 120 specimens were damaged very slightly (1 point); 132 specimens were found to be slightly damaged (3 points); 227 specimens were characterized by medium damage (5 points), and 2 specimens were severely damaged (7 points).

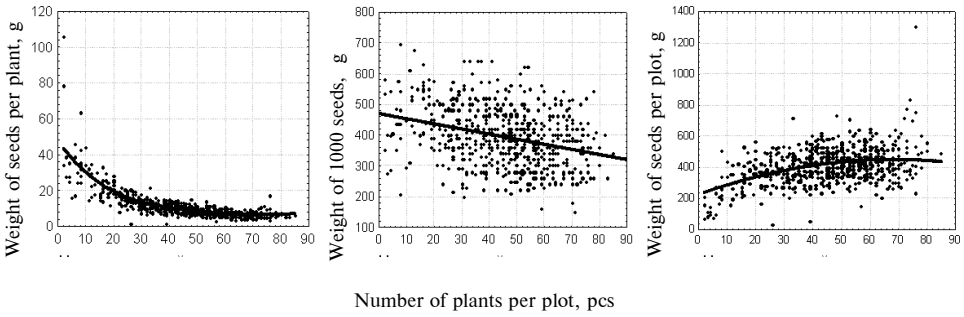

Fig. 1. The weight of seeds of chickpea (*Cicer arietinum* L.) specimens from VIR's collection, depending on the number of plants per plot (the curves were plotted using the weighted least-squares method; Tambov Region, 2011).

The number of germinating plants ranged from 2 to 85 per plot (45 on

average). All studied characters, except for sprouts-blossom period duration, were more or less dependent on the number of plants per plot (especially, in case of 2-30 plants) (Fig. 1). The strongest dependence on sprout density was observed for plant dry weight: for the number of plants less and more than 30, its average value was 35.6 and 12.5 g, respectively. In the same cases of sprout density, the weight of seeds per plant was equal to 21.6 and 8.7 g, respectively. Similar nonlinear variation was observed for the number of beans and seeds per plant, as well as for the number of secondary branches. For density of up to 30 plants, increase of their number by one led to increase of the weight of seeds per plot by 8.6 g on average; for larger density, the corresponding increment was equal to 1.6 g. Damage by fusarium disease on plots with up to 30 plants corresponded to 1 point on average, or 3 points for more than 30 plants; vegetation period duration decreased in a weak-linear fashion with the growth of seeding density (correlation coefficient $r = -0.36$), and decrease was also noted for plant height ($r = -0.30$) and weight of 1000 seeds ($r = -0.30$).

1. The correlation coefficients of yielding capacity elements for chickpea (*Cicer arietinum* L.) specimens from VIR's collection (Tambov Region, 2011)

Character / Character	Sprouts-blossom period	Blossom-ripening period	Vegetation period	Plant height	Height of lower bean attachment	Number of primary branches	Number of secondary branches	Plant dry weight	Number of beans on one plant	Number of seeds on one plant	Weight of 1000 seeds	Damage by fusarium disease
Weight of seeds from one plant	0.11	0.10	0.31	0.34	–0.14	0.26	0.48	0.60	0.50	0.45	0.27	–0.34
Sprouts-blossom period		–0.77	0.27	0.53	0.57	–0.04[a]	0.06[a]	0.09	0.11	0.09	–0.28	–0.41
Blossom-ripening period			0.41	–0.25	–0.53	0.11	0.22	0.24	0.19	0.18	0.30	0.13
Vegetation period				0.38	0.01[a]	0.10	0.42	0.48	0.44	0.40	0.04[a]	–0.39
Plant height					0.62	0.09	0.37	0.42	0.39	0.36	0.03[a]	–0.60
Height of lower bean attachment						–0.24	–0.18	–0.20	–0.19	–0.20	–0.20	–0.30
Number of primary branches							0.27	0.35	0.39	0.38	0.09	–0.08
Number of secondary branches								0.78	0.76	0.75	0.11	–0.33
Plant dry weight									0.94	0.91	0.27	–0.34
Number of beans on one plant										0.98	0.03[a]	–0.32
Number of seeds on one plant											0.02[a]	–0.31
Weight of 1000 seeds												–0.01[a]

N o t e: 629 specimens from 44 originating countries were studied; a—significant values.

The analysis of relationships between yielding capacity elements (Table 1) has shown that plant dry weight positively correlates with the number of beans per plant ($r = 0.98$) and number of seeds ($r = 0.91$), as well as with the number of secondary branches ($r = 0.78$). We observed medium relationship with vegetation period duration ($r = 0.48$), plant height ($r = 0.42$), the number of primary branches ($r = 0.35$) and damage by fusarium disease ($r = -0.34$). Plant height positively correlated with the height of lower bean attachment ($r = 0.62$) and negatively correlated with damage by fusarium disease ($r = -0.60$); it also correlated with sprouts-blossom period ($r = 0.53$) and the number of secondary branches ($r = 0.37$). The weight of 1000 seeds weakly, but significantly correlated with blossom-ripening period ($r = 0.30$). Medium inverse correlation was noted between the degree of damage by fusarium disease and sprouts-blossom period ($r = -0.41$), the strong one being observed between sprouts-blossom and blossom-ripening periods ($r = -0.77$).

The weight of seeds per plant differed for specimens with dissimilar bean shattering rates, being equal to 14.7 (shattering-resistant), and 11.1 and 9.7 g (shattering is less and more than 10%, respectively). The analysis of variance has revealed significant differences in the weight of seeds per plant for shattering-

resistant specimens (no significant differences were found for non-resistant specimens). The weight of 1,000 seeds significantly differed between three groups (440.1, 396.6 and 368.6 g for specimens with shattering rates of 0, 1, and 2 points, respectively).

The factor analysis of the components of productivity and interphase periods has shown that 73 % of the diversity of the studied characters is explained by three factors. The first factor, which provided the greatest differentiation of the collection, determines 38 % of the diversity within the collection and includes the block of the correlated characters associated with the number of seeds per plant (number of primary and secondary branches, number of beans per plant, plant dry weight); the second one (duration of sprouts-blossom and blossom-ripening periods and the corresponding values of the height of lower bean attachment and plant height) determines 25 % of the diversity; the third, independent factor explaining 10 % of the diversity is the weight of 1,000 seeds. The weight of seeds per plant stronger correlated with the first factor ($r = 0.65$), than with the third one ($r = 0.34$), and absolutely did not correlate with the second factor ($r = 0.02$). In other words, the large weight of seeds per plant is generally determined by the large number of productivity capacity elements, and, to a lesser extent, by the large weight of 1,000 seeds.

Based on the results for specimens from various countries (Table 2), we have compared valuable breeding characters for the countries represented by at least five specimens. See Figure 2 for the obtained values (minimum, first quartile, median, third quartile and maximum) providing an insight into the distribution shape and allowing visualization of the results.

2. The comparison of yielding capacity elements for chickpea (*Cicer arietinum* L.) specimens from VIR's collection, depending on the country of origin (Tambov Region, 2011)

Origin	Number of specimens, pcs	Vegetation period, days			Number of seeds per plant, pcs			Weight of 1000 seeds, g			Weight of seeds per plant, g			Weight of seeds per plot, g		
		$\bar{x}$	min	max	$\bar{x}$	min	max	$\bar{x}$	min	max	$\bar{x}$	min	max	$\bar{x}$	min	max
Tested specimens																
Azerbaijan	18	69.2	68	70	17	7	34	353	240	440	8	4	14	375	211	628
Algeria	5	70.8	69	77	23	9	47	483	370	620	9	7	12	371	272	476
Argentine	1	69.0			19			480			20	20	20	489		489
Armenia	6	69.7	69	70	21	11	34	321	240	400	6	5	8	351	235	455
Afghanistan	23	69.6	68	71	21	8	97	324	150	480	9	4	14	451	269	772
Bulgaria	10	70.2	69	73	27	6	100	330	280	410	14	6	36	376	142	553
Hungary	1	70.0			45			350			20			391		
Guatemala	1	68.0			5			280			8			270		
Germany	1	75.0			7			280			7			328		
Greece	4	69.3	69	70	18	7	28	568	510	640	12	4	19	426	300	520
Georgia	2	70.5	70	71	24	22	26	285	280	290	7	5	10	332	304	360
Israel	8	69.3	68	75	25	7	61	444	270	555	14	8	35	443	331	572
India	16	68.9	68	70	27	11	81	393	315	575	10	6	20	462	266	605
Jordan	9	69.2	68	70	13	5	25	404	290	540	9	5	13	408	287	562
Iraq	1	69.0			17			420			11	11	11	456		
Iran	24	69.8	68	77	20	6	33	377	200	560	10	5	19	411	234	753
Spain	14	70.8	68	77	27	5	95	443	270	695	14	4	35	343	68	529
Italy	13	68.9	68	70	20	7	80	459	240	675	11	6	22	441	226	702
Kazakhstan	4	69.8	68	72	16	10	22	280	180	340	7	4	11	408	282	500
Canada	1	68.0			11			500			11	11	11	474		
Cyprus	7	68.4	68	70	20	10	32	390	360	430	9	7	13	420	371	528
Kirghizia	3	68.7	68	69	22	11	31	333	280	370	10	6	15	419	410	434
Colombia	2	70.5	68	73	44	14	73	323	240	405	11	6	16	215	79	351
Morocco	9	70.6	68	77	15	7	31	437	330	520	7	3	14	350	151	552
Mexico	34	68.7	68	73	19	7	55	410	220	630	11	5	33	446	193	646
Moldova	5	71.2	68	78	43	21	113	346	280	440	10	7	17	368	100	617
Pakistan	14	68.7	68	70	19	9	33	359	220	500	9	6	19	377	260	468
Palestine	1	68.0			16			440			10			511		
Poland	1	68.0			46			480			14			362		
Portugal	4	68.8	68	69	13	10	20	490	360	640	10	6	14	416	287	615

Table 2 (continued)

Russia	32	70.1	68	76	24	9	67	318	205	540	11	3	34	397	204	616
Syria	165	69.5	68	77	24	6	203	411	220	660	13	1	106	418	50	835
USA	20	70.7	69	78	33	6	86	520	435	620	21	1	78	396	30	600
Tajikistan	2	68.5	68	69	42	26	58	415	370	460	11	10	12	417	374	459
Tunisia	2	69.5	69	70	16	14	18	445	380	510	15	8	22	378	264	491
Turkmenia	1	69.0			7			400			8			520		
Turkey	65	69.0	67	76	18	6	74	434	280	640	10	5	39	427	167	640
Uzbekistan	34	69.8	68	76	21	5	59	344	160	460	9	4	42	405	224	556
Ukraine	33	70.1	68	74	23	6	55	303	220	470	9	5	25	353	223	565
France	4	68.8	68	70	18	8	25	368	280	550	11	6	17	662	383	1302
Croatia	1	73.0			61			515			4			300		
Czech Republic	3	71.3	69	75	123	9	292	243	215	275	16	5	27	279	82	421
Chile	22	69.0	68	71	14	6	37	456	260	560	10	5	21	396	309	479
Ethiopia	2	68.5	68	69	13	13	14	285	260	310	5	5	6	357	265	449
Standards																
Volgogradsky 10 (ST 1)	65 replicates	69.4	68	74	25	16	40	278	260	295	12	10	18	418	245	600
Krasnokutsky 36 (ST 2)	65 replicates	72.5	69	74	28	22	31	278	275	280	13	5	18	463	222	691
Total	632	69.8	67	78	23	5	292	394	150	695	11	1	106	414	30	1302

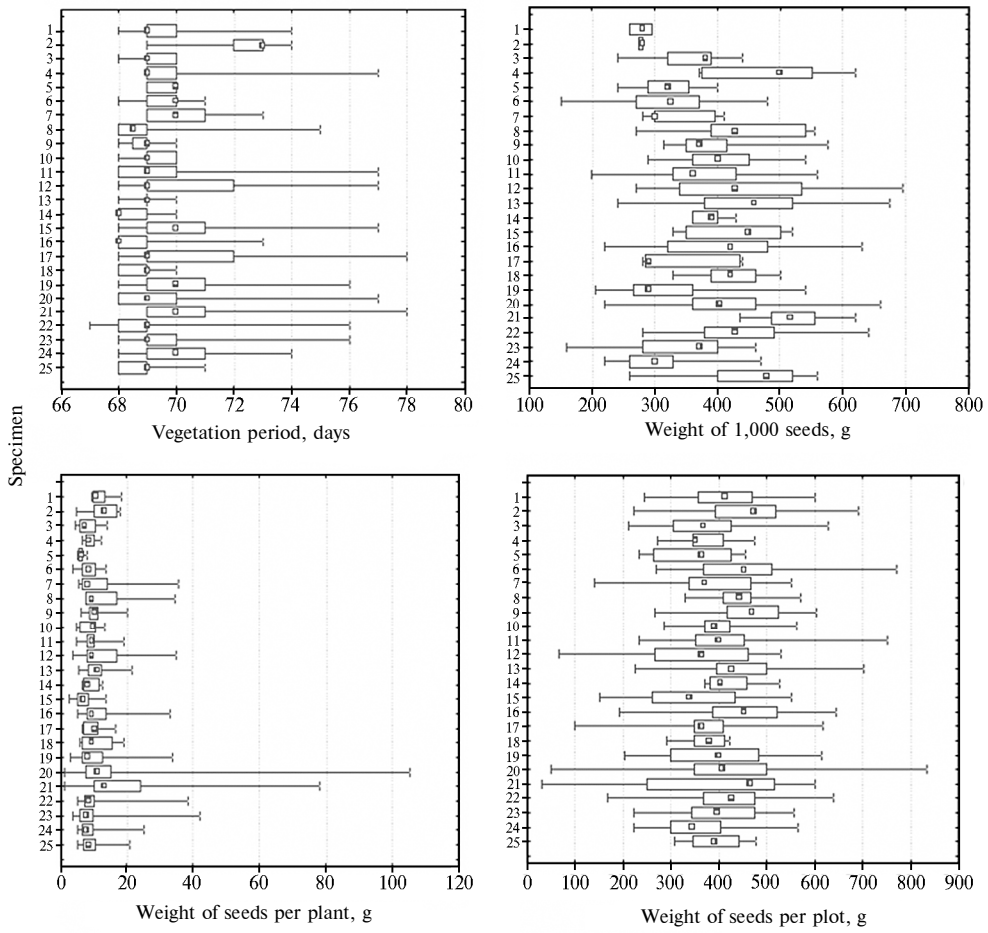

Fig. 2. Comparison of four economic characters for the chickpea (*Cicer arietinum* L.) specimens of various origin: 1 — ST 1 (variety Volgogradsky 10), 2 — ST 2 (variety Krasnokutsky 36) (standards for the Russian Federation), 3 — Azerbaijan, 4 — Algeria, 5 — Armenia, 6 — Afghanistan, 7 — Bulgaria, 8 — Israel, 9 — India, 10 — Jordan, 11 — Iran, 12 — Spain, 13 — Italy, 14 — Cyprus, 15 — Morocco, 16 — Mexico, 17 — Moldova, 18 — Pakistan, 19 — Russia, 20 — Syria, 21 — USA, 22 — Turkey, 23 — Uzbekistan, 24 — Ukraine, 25 — Chile; □ — median, ▭ — first and third quartiles, ├─┤ — minimum and maximum values (for the countries represented in VIR's collection by at least five specimens; Tambov Region, 2011).

The analysis of variance and subsequent pair-wise comparisons by the least significant difference have shown the absence of countries with the vegetation duration significantly shorter than in case of standard variety Volgogradsky 10 (69 days). The largest number of seeds per plant was noted for specimens from Moldova (43 pcs), USA (33 pcs), Spain (27 pcs), Bulgaria (27 pcs), India (26 pcs) and Israel (25 pcs), however they were not significantly different from the standards (varieties Volgogradsky 10 and Krasnokutsky 36; respectively, 25 and 28 seeds per plant). The weight of 1,000 seeds for the standards (278 g) was the least among average values for large originating countries. The average weight of 1,000 seeds for specimens from Iran, Cyprus, India, Jordan, Mexico, Syria, Pakistan, Turkey, Spain, Israel, Chile, Italy, Algeria and the USA was significantly higher than that of the standards. The weight of seeds per plant for a group of specimens from the USA was significantly higher than that of the standards (21 g). The largest weight of seeds per plot was observed for standard variety Krasnokutsky 36 (463 g); specimens from India (462 g), Afghanistan (451 g), Mexico (445 g), Israel (443 g) and Italy (441 g) were comparable and were not significantly different from it.

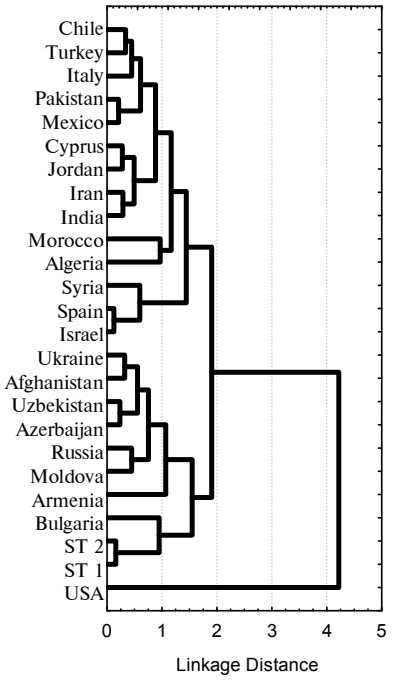

Fig. 3. The dendrogram reflecting degree of closeness of the chickpea (*Cicer arietinum* L.) originating countries: ST 1 and ST 2 — varieties Volgogradsky 10 and Krasnokutsky 36, respectively as standards for the Russian Federation (data on the weight of seeds per plant and weight of 1,000 seeds were analyzed for the countries represented in VIR's collection by at least five specimens; Tambov Region, 2011).

The degree of similarity of the chickpea-originating countries represented in VIR's collection by at least five samples (with regard to the weight of seeds per plant and weight of 1,000 seeds for the studied forms) was investigated by the cluster analysis method (Euclidean distance, UPGMA). As a result, three groups have been distinguished (Fig. 3): first, USA; second, Russia, ex-USSR countries, Bulgaria and Afghanistan; third, the other countries. Specimens from the USA were characterized by the large weight of 1,000 seeds and weight of seeds per plant; in the second group, the average weight of 1,000 seeds and weight of seeds per plant was small, whereas both parameters for the other countries were close to average values.

We identified 180 chickpea collection samples surpassing the more productive standard (variety Krasnokutsky 36) in the weight of grains per plot. With regard to the origin, the analysis of these specimens has shown (Table 3) that they were 53 specimens from Syria (34.6 % of 153 Syrian specimens studied), 17 specimens from Turkey (26.2 % of 65 specimens studied), 16 specimens from Mexico (47.1 % of 34 specimens studied), 11 specimens from Russia (34.4 % of 32), 11 specimens from Afghanistan (47.8 % of 23), 11 specimens from Uzbekistan (32.4 % of 34), 10 specimens from the USA (50.0 % of specimens from this country).

With regard to the weight of 1,000 seeds, the vast majority of specimens surpassed the standards (278 g), except for only 56 specimens.

3. The characteristics of yielding capacity elements for the chickpea (Cicer arietinum L.) specimens of various origin which surpass the standard (variety Krasnokutsky 36) in the weight of seeds per plot (in ascending order of parameter value; Tambov Region, 2011)

Origin	Number of specimens, pcs	Weight of seeds per plot, g	Vegetation period, days	Number of seeds per plant, pcs	Weight of 1,000 seeds, g
Krasnokutsky 36		463.0	72.5	27.7	278.3
Pakistan	1	468.0	68.0	19.7	320.0
Canada	1	474.0	68.0	11.0	500.0
Algeria	1	476.0	69.0	8.7	550.0
Chile	1	479.0	69.0	36.7	480.0
Argentine	1	489.0	69.0	19.3	480.0
Tunisia	1	491.0	69.0	14.0	380.0
Greece	2	497.0	69.0	23.2	560.0
Uzbekistan	11	497.1	69.4	18.7	385.9
Kazakhstan	1	500.0	68.0	22.0	340.0
Spain	3	500.7	69.7	19.1	333.3
Palestine	1	511.0	68.0	16.3	440.0
Ukraine	3	512.3	69.0	17.8	376.7
Bulgaria	3	518.7	69.3	15.7	370.0
Turkmenia	1	520.0	69.0	7.3	400.0
Israel	2	522.5	68.5	8.2	500.0
Jordan	2	527.5	70.0	11.7	480.0
Cyprus	1	528.0	70.0	23.7	360.0
Russia	11	528.6	69.5	22.0	331.5
Afghanistan	11	531.4	69.3	19.3	346.4
USA	10	531.5	69.7	18.9	509.0
Azerbaijan	3	533.3	68.7	12.9	360.0
Mexico	16	533.6	68.3	16.8	416.3
Turkey	17	537.4	68.8	17.3	436.5
India	8	538.3	68.8	27.2	365.6
Iran	6	546.3	69.2	19.8	418.0
Syria	53	551.4	69.5	24.9	411.2
Morocco	1	552.0	72.0	31.3	490.0
Italy	4	564.8	69.3	16.6	510.0
Portugal	1	615.0	69.0	12.3	500.0
Moldova	1	617.0	68.0	21.0	280.0
France	2	939.5	69.0	16.0	425.0
Total	180	539.2	69.2	20.5	410.5

Thus, in 2011, the heat provision and moisture content in the Tambov Region made it possible to differentiate chickpea forms by their breeding value for the region. As a result, 178 of 629 specimens studied had been distinguished as most promising for further investigation which was carried out in 2011-2013 (data are not presented). The large number of the studied specimens and assessed parameters has allowed us to arrive at substantiated conclusions about regional regularities of yield buildup for the crop forms of various geographical origin.

So, in the conditions of the Tambov Region, the significant breeding characters determining chickpea plant productivity should include the number of beans and seeds on one plant, number of secondary branches, plant height and vegetation period duration. The weight of 1,000 seeds positively correlated with blossom-ripening period duration. The weight of seeds per plot substantially correlated with the number of germinating plants and achieved maximum values at 70-80 plants per plot. Based on the most significant economic characters (weight of seeds per plant and weight of 1,000 seeds), we have distinguished three groups of chickpea-originating countries using the cluster analysis method. Specimens from the USA are characterized by the large weight of 1,000 seeds and weight of seeds per plant. Specimens from the former Soviet states, Bulgaria, and Afghanistan have the small weight of 1,000 seeds and

weight of seeds per plant. For the other originating countries represented in the collection of the N.I. Vavilov Research Institute of Plant Industry (VIR), both parameters are close to average values. With regard to breeding for seed productivity in the conditions of the Tambov Region, the following specimens are most promising: k-3720, k-3721, k-3740, k-3771, k-3783, k-3785 from Syria; k-604, k-2340 from Turkey; k-431, k-437, k-2176 from Mexico; k-1188, k-1335, k-1480, k-2197, k-2397 from Russia; k-2144 from Afghanistan; k-1491, k-1724, k-1727 from Uzbekistan and k-2597, k-2949 from the USA. In total, 178 of 629 specimens studied had been distinguished as most promising for further investigation which was carried out in 2011-2013.

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GENETIC RESEARCH OF QUANTITATIVE TRAITS OF INBRED LINES OF WINTER RYE (*Secale cereale* L.) IN DIALLEL CROSSINGS

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Abstract

One of effective methods for estimation of inbred lines of winter rye is the diallel analysis allowing to spread out genotypic variance to general (GCA) and specific (SCA) combinational abilities and to estimate quantitatively the contribution of the basic gene interactions in expression of traits. The purpose of our research was studying genetic features of inbred lines of rye by means of diallel crossings. Tested 5 inbred lines of winter rye and 10 interline F_1 hybrids were obtained according to incomplete diallel scheme (B. Griffing, 1956). Parental lines and F_1 hybrids were compared in 2011 and 2012 in a field experiments under the scheme of a Latin square (6×3×3) on the plots of 8.8 m^2 in 3 replicates at a sowing rate of 500 seeds per 1 m^2. Comparative estimation of gene effects has been carried out for 17 traits. The genetic parameters D, H1, H2, √H1/D, H2/4H1 and h^2 according to B.I. Hayman (1954) were evaluated, and also the contribution of GCA and SCA effects into genotypic variance was assessed. It was shown that according to effects of GCA to SCA ratio the traits can be divided into two groups. The first group contained 5 traits with low heritability (h^2 = 0.10-0.22), exactly the productivity, number of productive stalks per 1 m^2, number of grains in an ear, height of plant and the content of starch. In this group the contribution of GCA effects was rather low and varied from 7.0 up to 36.4 %, and the expression of traits strongly depended on domination and epistasis. In the second group there were 12 traits with rather high heritability (h^2 = 0.28-0.62) for which an additive dispersion exceeded the component of domination and varied from 50.3 up to 82.0 %. These high-hereditable traits were the winter hardiness, weight of 1000 grains, a grain unit, number of falling, amylogramm peak, viscosity of water extract, temperature of pasting starch, fluidity of dough, loaf volume, the content of protein in grain, sensitivity to snow mould and brown rust. For six traits of this group a strong genotype—environment interaction was characteristic. The conclusion is made that cumulative accumulation of valuable genes by means of recurrent selection is advisable for improvement of inbred lines on these traits in the course of breeding.

Keywords: winter rye, inbred lines, quantitative traits, diallel analysis, general and specific combinational ability, genetic parameters.

One of top-priority areas in winter rye breeding is the use of a heterosis effect and obtaining of F_1 hybrids based on Cytoplasmic Male Sterility (CMS) [1]. Now, the rye hybrids created in Germany occupy about 70 % of the total area under this crop and have put a significant pressure on population varieties in most of the Central European countries where the rye is cultivated. H.H. Geiger and T. Miedaner [2] report that, from 1982 to 2005, annual average increase in yield due to breeding of hybrid and population varieties was 51 and 30 kg, respectively. In addition, the superiority of hybrids over population varieties is evident not only in yielding capacity, but also in shorter stem length, better lodging resistance, higher grain quality, etc. [3].

For efficient selection of inbred lines of the winter rye, it is very important to know what kinds of gene interactions are involved in provision of hereditary polymorphism of characters, what the nature of their expression is, in what correlation relationship they are, what the share of contribution from specific genetic effects (in particular, additive, dominant and various epistatic effects) is, and in what extent such effects are modified by environmental factors. In order to solve this problem, diallelic analysis is widely used in breeding. It allows us to

break the genotypic variance of the character down into components — General Combining Ability (GCA) and Specific Combining Ability (SCA), and quantitatively estimate the contribution of some or other gene interactions to character expression.

Virtually all selected characters of the rye are polygenic by nature, and their phenotypic manifestation is a sum of the effects of allelic and nonallelic interaction in respect to many genes (such effects are significantly corrected by environmental conditions). The heterosis effect with regard to selected characters in the system of diallelic crossings of inbred rye lines is poorly known yet. It is reported for the majority of characters that the GCA variance is significantly higher than the SCA variance. H.H. Geiger [4] presents the summary of GCA variance shares for a number of crops; it follows from these data that this value for the rye is equal to 21 %, which is 2.0-2.5 times less than the corresponding value for the corn and sugar beet. Therefore, the assessment of winter rye lines by GCA is considered to be more important than by SCA, and in practice, SCA effects are taken into account only at the final stage of experimental hybrid investigation [2].

It has been noted [5] that phenotypic expression of many characters for inbred lines per se may serve as a ground for prediction of their GCA. Therefore, it is proposed to actively select selfed lines for their own high productivity and repeatedly test them for GCA [1]. However, significant correlation between the productivity of inbred lines and the productivity of their hybrids shows itself for not all of characters. The reason is that they have different degrees of heritability. It is highly heritable characters that lead to noticeable genetic shift in case of indirect selection of lines in the process of breeding [6]. If dominance effects prevail in genetic control of a character, selection shall be based not on line productivity per se, but on the productivity of F_1 interline hybrids [7].

Researchers do not fully understand a genetic determination mechanism for the yielding capacity character and associated structural components. The same can be said about rye grain quality characters. Quite controversial conclusions have been drawn with regard to the structure of the heritable variance of the selected characters and share of contribution from specific genetic effects, in particular, additive, dominance and epistatic ones. H.H. Geiger [5] has determined that, for most of winter rye characters, the additive variance is much greater than the sum of all other components of the genetic variance. It is believed that, even in case of grain yield, nonadditive effects contribute to the genetic variance less than additive ones, although some hybrids obtained by crossing of high-GCA lines may be characterized by significant dominance effect. Lines may vary in adaptiveness to cultivation conditions and have a substantial influence on the variance of genotype-environment interaction. Other researchers also note the predominant contribution of the additive variance [6, 8, 9].

The significant part of nonadditive variance of the rye is accounted for the epistatic interaction of genes [10]. Depending on a genotype and environmental conditions, this component of the genotypic variance may significantly increase or reduce the character value. In the latter case, the breeder has to seek ways to minimize negative consequences of nonallelic interaction so that to use SCA effects to the maximum extent possible [11].

Our task was to investigate the combining ability of inbred lines of the winter rye in the system of diallelic crossings and comparatively assess basic kinds of the gene interactions affecting the value of important breeding characters.

Technique. The parent material was represented by 5 inbred lines of the winter rye (H-649, H-1078, H-1179, H-451, H-842) and 10 F_1 interline hybrids obtained in accordance with an incomplete diallelic pattern (Method II accord-

ing to B. Griffing) [12]. The studied lines were consecutively subjected to repeated inbreeding (S_{14}-S_{16}) and were deeply homozygous. The lines were crossed under the conditions of the Moscow Region in 2010 in 25 m^2 isolating houses where the CMS analogs of above-listed lines were seeded near fertile forms for cross pollination. Parent lines and F_1 interline hybrids were studied in a field trial established in 2011-2012 based on a Latin rectangle design (6×3×3) on 8-row plots with the area of 8.8 m^2, in three replicates, at the seeding rate of 500 grains per 1 m^2.

The following parameters were taken into account for each plot: yielding capacity (t/ha), winter hardiness, number of productive stems per 1 m^2, number of grains per spike, plant height, weight of 1,000 grains, grain unit, falling number, amylogram height, aqueous extract viscosity, starch gelatinization temperature, dough shape stability (H/D), pan bread volume, protein and starch content in grain, and resistance to damage by the snow mold and brown rust. The general and specific combining abilities of lines were determined according to B. Griffing [12], and the genetic analysis of the diallelic complex was performed according to B.I. Hayman [13]: genetic parameters D, H_1, H_2, $\sqrt{H_1/D}$, $H_2/4H_1$ and h^2 were determined, and the share of contribution from GCA and SCA effects to the genotypic variance of the character was calculated.

Statistical data processing was carried out using the Agros software program (2.13).

Results. The field trial was carried out in the years with different weather conditions. Hot and dry weather was predominant in June and July 2011. It accelerated the phases of earing, blossom and grain filling; no grain lodging was observed; harvesting occurred 10 days earlier than the date according to long-term observations. In 2012, plants overwintered well, and the density of the formed stand was high, but abundant precipitation in June (150% of mean annual value) led to early (in the blossom phase) crop lodging, which had a negative impact on yield and grain quality.

Statistical processing of initial data has revealed significant differences between the parent lines and hybrids with regard to all the characters studied. The analysis of variance of the combining ability has shown that significant contribution to the heritable variance of characters was both from GCA and SCA. However, the share of these components greatly varied depending on a character.

Taking a relative contribution of GCA and SCA to the genotypic variance as a basis, we have divided all the characters studied into two groups. The first group included 5 characters with low (h^2 = 0.10-0.22) heritability (yielding capacity, number of productive stems per 1 m^2, number of grains per spike, plant height and starch content), for which the contribution from effects of GCA was lower than from those of SCA (average contribution varied within 7.0-36.4% in the years of the studies). The second group comprised 12 characters with relatively high heritability (h^2 = 0.28-0.62), for which the additive variance component exceeded the dominance component and varied from 50.3 to 82.0%. These characters were winter hardiness, weight of 1,000 grains, grain unit, falling number, amylogram height, aqueous extract viscosity, starch gelatinization temperature, hearth bread shape stability, pan bread volume, protein content in grain, and damage by the snow mold and brown rust (Table 1).

1. The groups of quantitative characters by the value of contribution from general and specific combining abilities to their genotypic variance for the winter rye (*Secale cereale* L.) by observation years (Moscow Region)

Character	2011	2012	Average
Group 1 (GCA < SCA)			
Yielding capacity	4.4/94.5	9.5/87.9	7.0/91.2
Number of productive stems per 1 m^2	43.9/56.1	20.6/68.4	32.3/62.3

Table 1 (continued)

Number of grains per spike	43.0/50.4	29.8/63.1	36.4/56.8
Plant height	20.1/79.3	23.8/74.9	22.0/77.1
Starch content	41.1/56.0	17.3/79.8	29.2/67.9
Group 2 (GCA > SCA)			
Winter hardiness	73.5/25.6	82.1/8.6	77.8/17.1
Weight of 1,000 grains	54.8/43.1	66.7/31.1	60.8/37.1
Aqueous extract viscosity	66.5/33.1	82.2/15.1	74.4/24.1
Falling number	45.5/54.4	69.3/29.6	57.4/42.0
Amylogram height	77.1/22.9	86.9/12.2	82.0/17.6
Starch gelatinization temperature	53.4/38.7	85.1/9.7	69.3/24.2
Grain unit	47.2/52.6	53.4/42.8	50.3/47.7
Dough shape stability	29.4/58.8	83.2/16.6	56.3/37.7
Volume yield of bread	74.4/23.2	60.8/37.4	67.6/30.3
Protein content	55.7/42.4	58.6/37.9	57.2/40.2
Snow mold resistance	82.5/15.2	48.4/47.3	65.5/31.3
Brown rust resistance	67.8/20.6	74.2/18.0	71.0/19.3

N o t e: The shares (%) of General Combining Ability (GCA) variance and Specific Combining Ability (SCA) variance are shown before and after the slash, respectively.

The GCA of parent forms may be indicative of their breeding value. The lines studied in our experiments had both positive and negative estimates of GCA effects (Table 2). With regard to yielding capacity and some other characters, relatively high GCA was noted for lines H-649, H-1179 and H-451. In breeding programs, they may be valuable components for synthesis of high-yielding hybrids with high quality of grain. The differentiation between the lines allows us to conclude that each of them contains a specific complex of genes having a different impact on the combining ability. Strong contrast between inbred lines and F_1 hybrids with regard to yielding capacity additionally confirms that the genetic variance of this character is primarily determined by intralocus dominance and nonallelic interaction of genes.

2. The effects of general combining ability (g_i) for winter rye (*Secale cereale* L.) inbred lines by groups of quantitative characters (Moscow Region, average for 2011-2012)

Character	H-649	H-1078	H-1179	H-451	H-842	*r*
Group 1 (GCA < SCA)						
Yielding capacity:						
centners/ha	22.9	21.0	34.5	28.7	28.9	0.10-0.55
g_i	4.6	–0.8	2.1	–1.9	–4.0	
Number of productive stems per 1 m^2:						
pcs	287	362	443	399	512	0.73-0.79
g_i	–33.0	13.4	38.5	–31.6	12.8	
Number of grains per spike:						
pcs	30.3	35.4	29.9	37.6	34.2	0.74-0.78
g_i	0.3	–0.6	–3.4	2.3	1.4	
Plant height:						
cm	91.7	89.0	117.5	90.7	94.2	0.91-0.98*
g_i	–0.88	–4.3	6.5	–0.65	–0.72	
Starch content:						
%	56.7	57.2	55.5	57.4	56.4	0.89-0.95*
g_i	0.28	0.15	–0.67	0.59	–0.35	
Group 2 (GCA > SCA)						
Winter hardiness:						
%	90.6	77.9	94.8	73.5	94.3	0.95-0.98*
g_i	0.9	–3.4	4.6	–5.4	3.4	
Weight of 1,000 grains:						
g	26.6	16.8	26.2	19.2	16.8	0.96-0.99*
g_i	2.4	–1.8	1.7	–0.3	–2.0	
Aqueous extract viscosity:						
cP	8.0	5.5	7.1	3.4	3.9	0.98-0.99*
g_i	1.19	–0.18	0.82	–1.03	–0.86	
Falling number:						
s	103	174	235	313	161	0.95-0.99*
g_i	–24.4	–12.5	9.1	41.8	–14.1	
Amylogram height:						
amylograph units	169	132	224	594	114	0.91-0.98*
g_i	5.2	–83.3	–9.2	137.1	–49.8	

N o t e: GCA — General Combining Ability, SCA — Specific Combining Ability, g_i — GCA effect value for the *i*-th line.
* Reliable at 5 % significance level.

An important criterion is the value of the coefficient of correlation (r) between the quantitative aspect of the character of homozygous lines per se and GCA effects. In our experiments, it was highly significant for all the characters studied except for yielding capacity, number of productive stems per 1 m^2 and number of grains per spike (see Table 2). This creates difficulties for breeding because early prediction of GCA using these characters is impossible for inbred lines, and intensive testing of F_1 hybrids derived on their basis is required. The reason is that the correlation between own productivity of lines and their advantage in crossings depends on which part of the variance is determined by additive genes [14]. In our experiments, the share of GCA contribution to the yielding capacity character variance turned out to be low (7.0 % on average). Therefore, when this character is used as an indirect criterion for selection, the probability of obtaining highly heterotic hybrids based on lines with the best yielding capacity will be low. In this respect, the rye is similar to the corn, for which the productivity of lines per se is also not a reliable indicator of their GCA [15]. However, the high yielding capacity of inbred lines should not be neglected because it is important for their seed breeding.

The analysis of genetic variability parameters has shown (Table 3) that the potential of rye productivity and grain quality characters depends on the effects exerted by three types of gene interactions: additive interaction (when effects are summarized for multiple loci), dominance (intralocus interaction of genes) and epistasis (nonallelic interaction of genes). However, their share of contribution to the character variance greatly varied by years and was a function of close interaction of the tested genotypes with the limiting environmental factors (precipitation, lodging) that altered the spectrum of the efficient genes determining the average value and genotypic variance of characters.

3. The comparative assessment of genetic variability parameters of quantitative characters with different ratios between general and specific combining abilities of the winter rye (*Secale cereale* L.) by observation years (Moscow Region)

Character	GCA/SCA ratio	Genetic parameters				
		D	H_1	H_1/D	$H_2/4H_1$	h^2
		2011				
Yielding capacity	0.1	9.3	2,268*	15.6	0.247	0.10
Number of productive stems per 1 m^2	0.5	4,492*	67,274*	3.9	0.217	0.18
Number of grains per spike	0.6	22.7	148.9*	2.6	0.212	0.22
Starch content	0.4	1.9*	23.2*	3.5	0.241	0.10
Plant height	0.3	206*	1,092*	2.3	0.240	0.10
Amylogram height	4.7	48,615*	86,184*	1.3	0.198	0.62
Winter hardiness	4.5	281.9*	321.9*	1.1	0.210	0.49
Aqueous extract viscosity	3.1	4.9*	13.2*	1.6	0.240	0.49
Weight of 1,000 grains	1.6	12.2*	46.9*	2.0	0.243	0.54
Falling number	1.4	5,478*	12,957*	1.5	0.207	0.29
		2012				
Yielding capacity	0.1	41.7	1,331*	5.6	0.238	0.10
Number of productive stems per 1 m^2	0.5	13,887*	44,926*	1.8	0.178	0.04
Number of grains per spike	0.6	15.3	246.7*	4.0	0.233	0.05
Starch content	0.4	2.1*	9.5*	2.1	0.245	0.22
Plant height	0.3	104*	899*	2.9	0.240	0.10
Amylogram height	4.7	28,052*	14,855*	0.7	0.178	0.61
Winter hardiness	4.5	17.9*	7.0	0.6	0.190	0.69
Aqueous extract viscosity	3.1	2.7*	1.8	0.8	0.250	0.54
Weight of 1,000 grains	1.6	36.1*	43.4*	1.1	0.206	0.31
Falling number	1.4	7,282*	7,996*	1.1	0.213	0.37

N o t e: D, H_1, H_2, and h^2— according to B.I. Hayman [13].
* Reliable at 5 % significance level.

The above division of the studied characters into groups makes it possible to use a differentiated approach to the assessment of their genetic systems and substantiation of methods for improvement by breeding.

The distinctive property of characters from Group 1 was strong depres-

sion in case of inbreeding, low contribution of additive gene effects to heritability, relatively high (except for starch) hypothetic heterosis in case of interline crossings (34.4-174.6 %), as well as a point where the regression line crosses the W_r axis, which is located below the datum point (Fig. 1).

The expression of characters from this group strongly depends on effects of dominance in loci and on interaction of epistatic genes. The significant effect of epistasis was revealed in both years of the studies with regard to yielding capacity, plant height, number of grains per spike and starch content. It is to be supposed that it had a substantial influence on the dominance component, which led to increase in the potential of the nonadditive genetic dispersion reflecting the high SCA variance.

As for two characters from Group 1 (yielding capacity and plant height), the genetic control system turned out to be relatively stable, and coefficients of correlation (r) between the sum $W_r + V_r$ (covariance + variance) and average value of parent character X_p were consistently negative by years, which is indicative of high expression of these characters under control of dominant genes. However, in respect to the number of productive stems per 1 m^2 and number of grains per spike, coefficients r for 2011 were unreliable, which gives evidence of genetic system instability due to the influence of weather conditions. A similar peculiarity was also noted for starch content in 2012. It manifested itself as strong genotype-environment interaction when dominant genes had different expressivity due to different lodging resistance of lines. Therefore, with regard to the two mentioned characters, it is reasonable to make a selection assessment of inbred lines through testing in various environmental conditions.

Group 2 turned out to be more numerous than Group 1. It is quite consistent with H.H. Geige's conclusion [5] that, for most of winter rye characters, the additive variance is much greater than the sum of all other components of the genetic variance. The specific feature of characters from this group consists in relatively high contribution of additive genetic effects to character heritability, as well as in weak inbreeding depression and relatively low (except for falling number, amylogram height and aqueous extract viscosity) hypothetic heterosis in case of interline crossings. We did not observe stable effects of epistasis for any of these characters.

A predominant role in their determination is played by additive effects of genes, and it should be noted that dominant genes provided positive increase in almost all grain quality characters, except for protein content (Fig. 2).

In case of six characters from this group (winter hardiness, weight of 1,000 grains, grain unit, falling number, aqueous extract viscosity, protein content), weather conditions had little effect on the genetic control system in the years of the studies. This system turned out to be relatively stable because coefficients of correlation (r) between $W_r + V_r$ and X_p were significant and were consistent in sign by years, i.e. the trend of dominance remained unchanged. However, strong genotype-environment interaction was observed in respect to other six characters (amylogram height, starch gelatinization temperature, H/D ratio, pan bread volume, as well as damage by the snow mold and brown rust). The environmental instability of genetic systems for these characters was indicated by coefficients of correlation (r) between X_p and $W_r + V_r$, which were unreliable in different years, as well as by the genetic parameters and location points of lines on the Hayman's plot, which were substantially different by years. It is indicative of the lability of the mentioned genetic systems under the influence of the limiting environmental factors. We may suppose that genetic information about these characters is implemented in such an interaction with

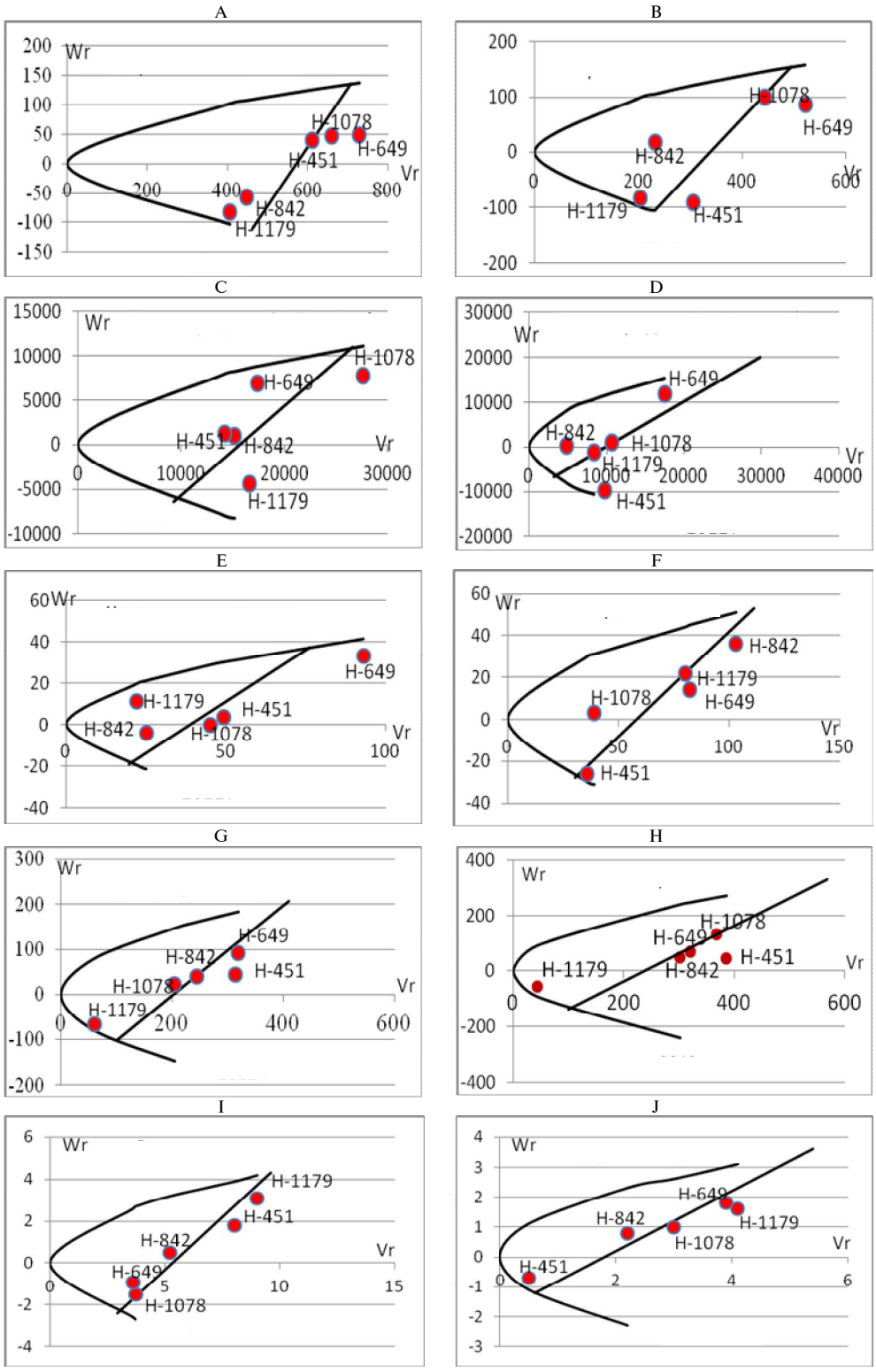

Fig. 1. The plots of regression W_r/V_r (covariance/variance) **for winter rye (*Secale cereale* L.) lines H-649, H-1078, H-1179, H-451 and H-842 by the characters, for which the variance of general combining ability does not exceed the variance of specific combining ability** (Group 1): A, B — yielding capacity; C, D — number of productive stems per 1 m^2; E, F — number of grains per spike; G, H — plant height; I, J — starch content; on the left and right — 2011 and 2012, respectively (Moscow Region).

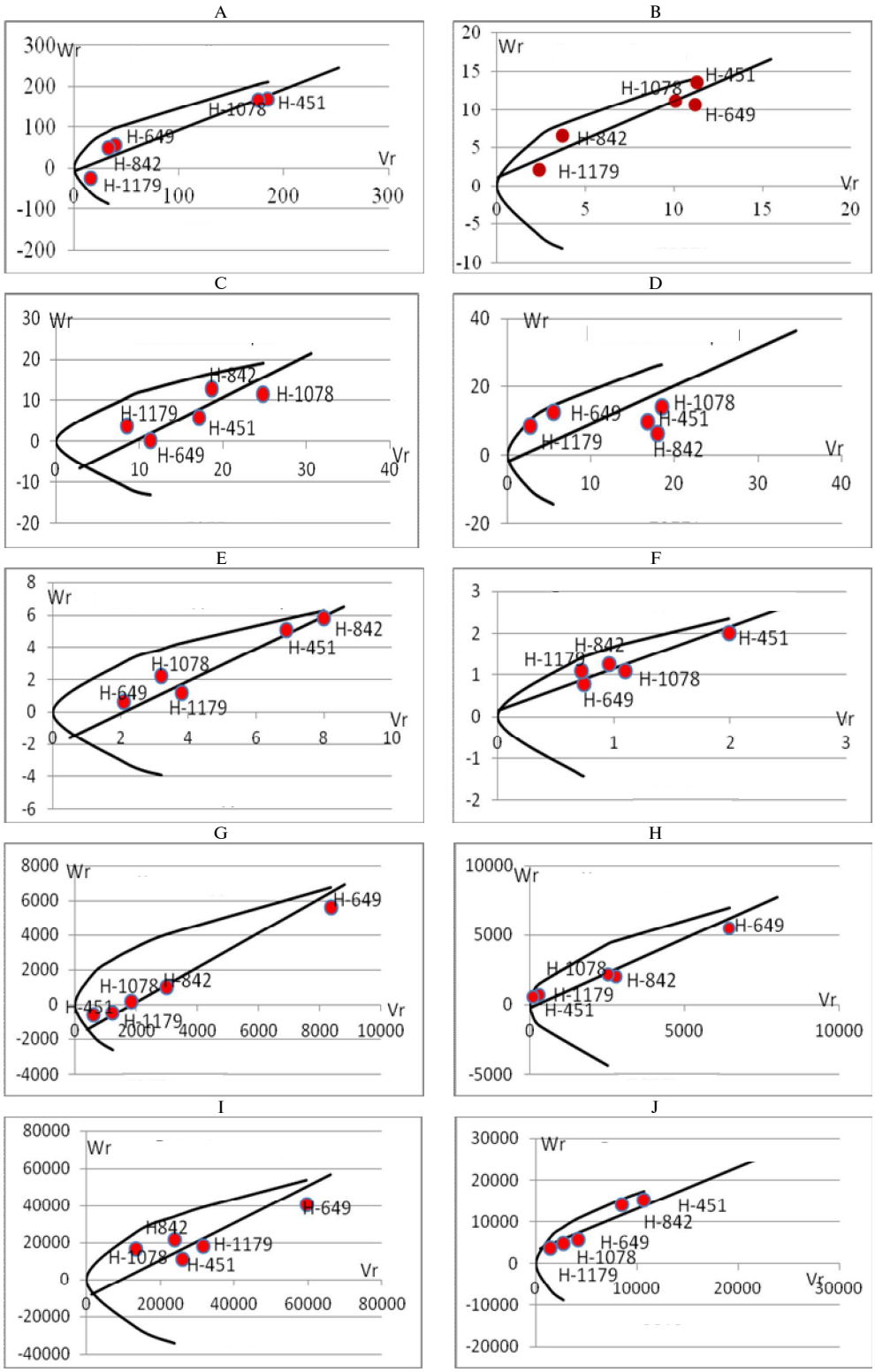

Fig. 2. The plots of regression W_i/V_r (covariance/variance) **for winter rye (*Secale cereale* L.) lines H-649, H-1078, H-1179, H-451 and H-842 by the characters, for which the variance of general combining ability exceeds the variance of specific combining ability** (Group 2): A, B — winter hardiness; C, D — weight of 1,000 grains; E, F—aqueous extract viscosity; G, H — falling number; I, J — amylogram height; on the left and right—2011 and 2012, respectively (Moscow Region).

the environment when both causes determining it become hardly separable from each other. By this reason, expression of the indicated characters in different years may be determined both by dominant and recessive genes, which complicates the work for their improvement in the course of breeding.

Thus, in order to more efficiently improve low-heritable characters (Group 1), it is necessary to use heterosis breeding methods to the maximum extent possible, and preference shall be given to selection of inbred lines with high Specific Combining Ability (SCA) with regard to yielding capacity and low SCA with regard to plant height. It is reasonable to select inbred lines by highly heritable characters (Group 2) based on the principle of cumulative buildup of valuable genes in them, using the recurrent selection method. It is especially important to adhere to this principle when selecting inbred lines for winter hardiness because the hypothetic heterosis by this character is poorly manifested in interline crossings. The strategy for breeding of lines by the environmentally unstable characters prone to redefining of their genetic formula under the influence of environmental factors (amylogram height, starch gelatinization temperature, dough shape stability, bread volume, damage by diseases) shall be based on early prediction of the general combining ability of parent forms in accordance with the degree of phenotypic expression of such characters in them.

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PRODUCTIVITY AND ECOLOGICAL PLASTICITY OF PLUM (*Prunus domestica*) VARIETIES UNDER ENVIRONMENTAL INSTABILITY

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Abstract

In perennial crops, particularly in plum trees, a genotypic (varietal) ecological plasticity as the ability to respond to external changes and anthropogenic factors is little studied. Plum tree is one of the most adaptive and wide spread fruit crop peculiar in its drought resistance, winter hardiness, high and sustainable fruit production. However, plum varieties significantly differ in valuable traits and ecological plasticity. Here we show the data on yield, fruit size and plasticity of differently originated plum varieties under conditions of four soil and climatic zones of Krasnodarskii krai. The data were obtained by statistical processing results of varietal testing. The main regional varieties Kabardinskaya rannyaya, Renklod Altana, Vengerka italianskaya, Anna Shpet, Vengerka azhanskaya and Tuley Gras were assessed. The role of the genotype in adaptation to environment indicated by fruit size and yield as an integrated parameters of the response to adverse condition was studied. In some varieties the yield varied from 18.0 до 50.0 centner per ha but did not exceed an average value for all varieties in each zone. Higher average yield for all zones was observed in Stenley variety at 165-170 centner per ha, in Anna Shpet variety at 115-145 centner per ha, in Kabardinskaya rannyaya variety at 130.4-147.9 centner per ha, and it was a little bit lower in Renklod Altana and Tuley Gras while the lowest in Vengerka azhanskaya and Vengerka italianskaya varieties. The yield was shown to be specific to genotype as a varietal biological peculiarity influencing the yield at 51.6 % rate, and also it depends on weather condition of the year with 28.7 % influence, and on their interaction at 17.2 % influence. Definite effects were shown for additional factors such as scheme of planting, type of crown, mineral nutrition, protection measures against diseases and pests. Fruit size was shown to be a varietal parameter closely related to the weather conditions and stresses during vegetation such as heat and water deficit at the phases of ovary and fruit formation and maturation. According to fruit size the rate of influence for weather, variety and their interaction was 41.6 %, 37.5 % and 20.8 %, respectively. Calculated regression coefficient b_1 for the yield value was > 1, thus the tested plum varieties were characterized as intensive and responding to new technologies such as dense planting schemes, flattened crowns, integrated protection, etc. The Kabardinskaya rannyaya and Stenley plum varieties being the most plastic, adaptive and productive were selected as basic for commercial assortment of plum trees in the conditions of the southern gardening.

Keywords: fruit crops, plum, *Prunus domestica*, variety, genotype, plasticity, efficiency, stability, technology.

The strategy of modern horticulture shall be based on the use of the adaptive potential of fruit crops and varieties [1-6]. The role of plastic crops becomes more important under unfavorable environmental conditions [7-12]. Therefore, resistance of fruit (perennial) plantings can be improved due to proper selection of more plastic varieties and optimal growing zones [12-17].

The ecological plasticity is defined as genotype (variety) response to change of environmental and anthropogenic factors, and it is understudied with regard to perennial crops, such as the plum tree. The plum tree is one of the most adaptive (wide spread) stone fruit crops; it differs from many other fruit crops in its drought resistance, winter hardiness, high productivity and stable fruiting [8, 18]. However, not all of plum tree varieties exhibit valuable charac-

ters and ecological plasticity [1, 7, 8].

Our study was aimed at assessing plum tree varieties of different environmental and geographical origin in respect to productivity and ecological plasticity in the horticultural zones of Krasnodar Territory.

Technique. The leading released varieties Kabardinskaya Rannyaya, Renklod Altana, Vengerka Italianskaya, Anna Shpet, Vengerka Azhanskaya and Tuleu Gras were studied. The data used for analysis included the results of yielding capacity and fruit weight determination for six plum varieties obtained at state crop testing sites in the Kuban, piedmont, steppe and Black Sea horticultural zones of Krasnodar Territory. The genotype-environment interaction was investigated using the analysis-of-variance method [19]. The parameters of ecological plasticity (response b_1, stability S_1^2) were determined according to V.Z. Pakudin [20]. The yielding capacity and fruit quality were assessed as described [21].

Statistical data processing was carried out using the Statistika-99 application software package. The variance was calculated in accordance with the method described by B.A. Dospekhov [19].

Results. For some varieties, the yield varied from 18.0 to 50.0 centners/ha, but did not exceed an average value for all varieties in each zone. For variety Anna Shpet in the Kuban and piedmont zones, it was 70.6 and 60.0 centners/ha, respectively; for variety Kabardinskaya Rannyaya in the steppe zone and variety Tuleu Gras in the Black Sea zone, it was 60.8 and 50.0 centners/ha, respectively. Insignificant yield capacity variation was observed for variety Vengerka Azhanskaya in the Kuban and piedmont zones, variety Vengerka Italianskaya in the steppe zone and variety Kabardinskaya Rannyaya in the Black Sea zone. High average yielding capacity was noted in all fruit-growing zones for varieties Stenley (165-170 centners/ha), Anna Shpet (115-145 centners/ha) and Kabardinskaya Rannyaya (130.4-147.9 centners/ha). Yielding capacity was somewhat less in case of varieties Renklod Altana and Tuleu Gras and low in case of varieties Vengerka Azhanskaya and Vengerka Italianskaya (Table 1). High variability of yielding capacity by varieties was observed both within the zone and between zones. This parameter ranged from 18.2 % in the piedmont zone to 65.6 % in the steppe one.

1. The influence of the «variety» and «year» factors on yielding capacity and the parameters of yielding capacity for different plum tree varieties in the fruit-growing zones of Krasnodar Territory

Variety, factor	The influence of the factor, %	Yielding capacity parameter			
		lim	R	*X*	*V*
Kuban zone					
Variety (A)	53.8				
Year (B)	28.6				
Interaction (A + B)	15.6				
Kabardinskaya Rannyaya		120.5-170.0	49.5	145.3	34.0
Renklod Altana		100.0-130.8	30.8	115.3	26.7
Vengerka Italianskaya		50.0-90.0	40.0	70.0	57.1
Anna Shpet		110.0-180.6	70.6	145.8	48.3
Vengerka Azhanskaya		70.0-90.0	20.0	80.0	25.0
Tuleu Gras		100.0-150.0	50.0	125.0	40.0
Stenley		150.0-190.0	40.0	170.0	23.5
Piedmont zone					
Variety (A)	49,8				
Year (B)	29,7				
Interaction (A + B)	17,8				
Kabardinskaya Rannyaya		125.0-170.8	45.8	147.9	30.9
Renklod Altana		93.0-136.0	43.0	114.5	37.4
Vengerka Italianskaya		60.0-90.0	30.0	75.0	40.0
Anna Shpet		90.0-150.0	60.0	120.0	50.0
Vengerka Azhanskaya		75.0-95.0	20.0	85.0	23.5
Tuleu Gras		98.0-145.0	47.0	121.5	38.5
Stenley		150.0-180.0	30.0	165.0	18.2

Table 1 (continued)

Steppe zone					
Variety (A)	55,4				
Year (B)	27,9				
Interaction (A + B)	16,8				
Kabardinskaya Rannyaya		100.0-160.8	60.8	130.4	46.7
Renklod Altana		60.0-100.0	40.0	80.0	50.0
Vengerka Italianskaya		60.0-74.0	14.0	67.0	20.8
Anna Shpet		95.0-143.0	48.0	119.0	40.3
Vengerka Azhanskaya		45.0-89.0	44.0	67.0	65.6
Tuleu Gras		105.0-121.0	16.0	113.0	14.5
Stenley		140.0-190.0	50.0	165.0	30.3
Black Sea zone					
Variety (A)	49,3				
Year (B)	28,9				
Interaction (A + B)	18,6				
Kabardinskaya Rannyaya		130.0-160.1	30.1	145.1	20.8
Renklod Altana		95.0-128.0	33.0	111.5	29.4
Vengerka Italianskaya		98.0-130.0	32.0	114.0	28.1
Anna Shpet		90.0-140.0	50.0	115.0	26.1
Vengerka Azhanskaya		65.0-90.0	35.0	77.5	44.9
Tuleu Gras		100.0-120.0	50.0	105.0	18.8
Stenley		140.0-180.0	40.0	160.0	25.0

N o t e: lim — yielding capacity variation, centners; R — variation range, centners; X — average yielding capacity, centners/ha; V — yielding capacity variation, %.

Plum tree productivity depended on the variety and cultivation zone. Thus, high yielding capacity in all fruit-growing zones was noted for varieties Stenley, Anna Shpet and Kabardinskaya Rannyaya, medium yielding capacity was detected for varieties Renklod Altana and Tuleu Gras, and low yielding capacity was shown for varieties Vengerka Azhanskaya and Vengerka Italianskaya (see Table 1).

The calculation of the share of influence for the «variety» (A) and «year» (B) factors has shown that that the largest effect on yielding capacity in all fruit-growing zones was exerted by the plant variety or biological potential determining adaptation both to environmental factors and agrotechnological conditions of cultivation. Thus, the influence of factor A on yielding capacity was 49.3 % in the Black Sea zone, 55.4 % in the steppe zone, 53.8 % in the Kuban zone, 49. % in the piedmont zone, and 52.1 % on average for the fruit-growing zones (see Table 1). The influence of factor B combining biotic and abiotic conditions in the plum tree vegetation period on the yielding capacity of the varieties was 28.6-29.7 %. On average, the share of the influence of the year (B) on plum tree yielding capacity was 28.7 %.

The interaction of factors A and B was also substantial. Based on the results of the studies, the share of its influence on plum tree yielding capacity ranged within 15.6-17.8 % (see Table 1).

The fruit size is one of the key elements determining the yielding capacity of a variety. This parameter closely correlated with environmental conditions, especially in the fruit formation period. The range of character variation by fruit-growing zones turned out to be rather high: from 1.3 for variety Vengerka Azhanskaya in the piedmont zone to 9.0 g for variety Vengerka Italianskaya in the piedmont zone (Table 2).

The range of fruit size variation in the piedmont zone turned out to be significant and was from 5.2 to 26.5 %. It was somewhat less in the Kuban (8.6-16.6 %) and Black Sea (5.4-22.9 %) zones, and the least values (3.7-13.9 %) were observed in the steppe fruit-growing zone (see Table 2).

With regard to fruit weight, varieties Kabardinskaya Rannyaya, Stenley and Anna Shpet were distinguished in all zones. Medium fruit sizes were noted for varieties Renklod Altana and Tuleu Gras, whereas small sizes were observed for varieties Vengerka Azhanskaya and Vengerka Italianskaya. Varieties Vengerka

Italianskaya and Kabardinskaya Rannyaya exhibited high variability of the fruit weight, and the least variability was noted for Anna Shpet and Stenley. The significant variability of the plum fruit size depending on the environmental conditions of cultivation was indicative of the large share of the influence of external factors (see Table 2).

2. The share of the influence of the “variety" and “year” factors on fruit weight, as well as the parameters of the "fruit weight" character for different plum tree varieties in the fruit-growing zones of Krasnodar Territory

Variety, factor	The influence of the factor, %	«Fruit weight» parameter			
		lim	R	*X*	*V*
Kuban zone					
Variety (A)	33.8				
Year (B)	43.8				
Interaction (A + B)	18.9				
Kabardinskaya Rannyaya		38.0-5.0	7.0	41.5	16.6
Renklod Altana		32.5-38.1	5.6	35.3	15.8
Vengerka Italianskaya		30.1-35.1	5.0	32.6	15.2
Anna Shpet		33.5-36.5	3.0	35.0	8.6
Vengerka Azhanskaya		23.1-25.2	2.1	24.2	8.8
Tuleu Gras		25.5-29.2	3.7	27.4	13.7
Stenley		34.5-38.2	3.7	36.4	10.2
Piedmont zone					
Variety (A)	33.6				
Year (B)	46.2				
Interaction (A + B)	16.3				
Kabardinskaya Rannyaya		37.5-43.5	6.0	40.5	14.8
Renklod Altana		33.1-37.9	4.8	35.8	13.3
Vengerka Italianskaya		29.3-38.3	9.0	33.8	26.5
Anna Shpet		33.4-36.6	3.2	35.0	9.1
Vengerka Azhanskaya		24.5-25.8	1.3	25.2	5.2
Tuleu Gras		23.9-26.8	1.9	25.8	7.3
Stenley		35.8-38.8	3.0	37.3	8.1
Steppe zone					
Variety (A)	35.6				
Year (B)	41.2				
Interaction (A + B)	19.3				
Kabardinskaya Rannyaya		35.5-39.5	4.0	37.5	10.6
Renklod Altana		29.8-31.1	1.3	30.5	4.1
Vengerka Italianskaya		26.2-30.1	3.9	28.2	13.9
Anna Shpet		32.4-35.6	3.2	35.0	9.1
Vengerka Azhanskaya		22.1-24.2	2.1	23.2	9.1
Tuleu Gras		22.7-29.8	7.1	26.3	3.7
Stenley		32.0-35.9	3.9	34.0	11.4
Black Sea zone					
Variety (A)	34.8				
Year (B)	39.5				
Interaction (A + B)	22.7				
Kabardinskaya Rannyaya		36.5-44.0	7.5	40.3	18.8
Renklod Altana		29.3-33.7	4.4	31.5	13.8
Vengerka Italianskaya		28.1-34.2	7.1	30.7	22.9
Anna Shpet		33.4-36.6	3.2	35.0	9.1
Vengerka Azhanskaya		23.3-25.6	2.3	24.5	9.4
Tuleu Gras		25.5-28.9	3.4	27.2	12.5
Stenley		35.8-37.8	2.0	36.8	5.4

Note: lim — fruit weight variation, R — variation range, *X* — average fruit weight, *V* — fruit weight variation, %

Thus, on average for the zones, the share of the influence of factors A and B on the fruit weight was 33.8-35.6 % and 39.5-46.2 %, respectively; with regard to interaction of the variety and conditions of the year, this parameter ranged within 16.3-22.7 %.

The index of yielding capacity formation conditions for plum tree varieties ranged from –15 to +45 centners/ha, which indicates that the influence of weather conditions was large enough. It should be noted that the weather conditions in the period of the studies were diverse, incuding droughts in 2005-2007, frosts to –37 °C in the winter of 2005-2006, annual recurrent spring frosts from –2.5 to –4.0 °C, more often in the blossom period.

The yield capacity regression coefficient (b_1) for all varieties turned out to be higher than 1, which characterizes them as adaptive and intensive crops (i.e. they are responsive to the improvement of cultivation technology conditions). Thus, the biological potential of variety Vengerka Italianskaya was best perfomed in the Black Sea and piedmont zones, where this crop accumulated about 20 % sugars, consistently fruited and yielded a good harvest. The high regression coefficient (1.48) obtained based on the «yielding capacity» parameter for variety Vengerka Italianskaya confirms that it is especially demanding of cultivation conditions.

The stability of yielding capacity (S_1^2) for the studied varieties significantly varied from 250 for variety Stenley to 850 for variety Vengerka Italianskaya (Table 3).

3. Ecological plasticity of different plum tree varieties in the fruit-growing zones of Krasnodar Territory

Character, variety	Ecological plasticity parameter		
	L_i	b_1	S_1^2
Yielding capacity, centners/ha	−15/0/+45		
Variety:			
Kabardinskaya Rannyaya		1.21	350.0
Renklod Altana		1.36	600.0
Vengerka Italianskaya		1.48	850.0
Anna Shpet		1.25	280.0
Vengerka Azhanskaya		1.12	360.0
Tuleu Gras		1.11	289.2
Stenley		1.28	250.0
Fruit weight, g	Decrease from 31 to 29		
Variety:			
Kabardinskaya Rannyaya		1.31	550.0
Renklod Altana		1.33	340.0
Vengerka Italianskaya		1.51	800.5
Anna Shpet		1.21	380.0
Vengerka Azhanskaya		1.43	625.3
Tuleu Gras		1.32	369.8
Stenley		1.27	220.4

N o t e: L_i — index of year conditions (with regard to yielding capacity, it means decrease, no influence or increase), b_1 — regression coefficient, S_1^2 — stability of yielding capacity.

The low absolute stability index was indicative of the high degree of response and adaptability to changing environmental conditions for varieties Stenley, Anna Shpet and Kabardinskaya Rannyaya. Varieties Renklod Altana and Vengerka Italianskaya were characterized by higher absolute stability parameters and lower adaptiveness.

For the varieties under study, we have not found any decrease of response to changes of conditions in fruit-growing areas.

The indices of fruit weight formation conditions greatly varied depending on the year conditions or variety, but to a lesser extent than in case of yielding capacity formation. The conditions for fruit formation were almost similar in all four zones, which is confirmed by the corresponding indices (i.e. a decrease from 31 to 29 g) (see Table 3).

The coefficient of fruit weight regression (b_1) for varieties was higher than the similar parameter for yielding capacity, which is indicative of the significant influence of year conditions on character variation (see Table 3). Maximum (1.51) response to environmental factors was noted for varieties Kabardinskaya Rannyaya and Anna Shpet, whereas a minimum (1.40) value was observed for variety Vengerka Italianskaya. High stability of fruit weight was noted for all the plum varieties studied.

The assessment of plum varieties with regard to plasticity in the fruit-growing zones of Krasnodar Territory has revealed insignificant differences between varieties Kabardinskaya Rannyaya, Renklod Altana, Vengerka Italian-

skaya, Anna Shpet, Vengerka Azhanskaya, Tuleu Gras and Stenley. Fruit weight stability (S_I^2) was rather high for varieties Stenley, Anna Shpet and Renklod Altana, and lower for varieties Kabardinskaya Rannyaya and Vengerka Italianskaya (see Table 3). The strongest influence on fruit weight in all zones was observed for year conditions with quite high involvement of the genotype.

High yielding capacity, response to improvement of cultivation conditions and insignificant deviation of the «yielding capacity» and «fruit weight» characters from the regression line were noted for varieties Kabardinskaya Rannyaya, Renklod Altana, Vengerka Italianskaya, Anna Shpet, Vengerka Azhanskaya, Tuleu Gras and Stenley cultivated in different environmental conditions (see Table 3).

So, the obtained results have shown that the plum tree is an ecologically-plastic stone fruit crop which can be cultivated in all fruit-growing zones of Krasnodar Territory and can produce a good yield, provided that its varieties are properly selected. We have confirmed a varietal specificity and a need for an ecological variety study in order to distinguish the most adaptive and plastic varieties for a particular zone of cultivation. Based on the obtained results, varieties Kabardinskaya Rannyaya, Stenley and Anna Shpet can be referred to plastic ones, i.e. to the crops which are adaptive and high-yielding under the conditions of Krasnodar Territory.

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VARIABILITY OF MORPHOMETRIC PARAMETERS IN NATURALIZED AND CULTIVATED *Hydrangea macrophylla* Ser. PLANTS UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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Abstract

Plants cultivated in humid subtropics of Russia are mostly exotic species. One of them, *Hydrangea macrophylla* Ser., can grow in city parks and gardens, as well as in the forest of Sochi suburb as a component of adventive flora, thus, the *H. macrophylla* habitat conditions differ significantly. To estimate the genetic resources, implement breeding programs and optimize plant cultivation, an adaptability and intraspecial variability of plants must be evaluated. In our observation in Sochi region during 2006-2008 carried out on the Black Sea coast (in the garden-museum «Tree of Friendship» and in Kuban Subtropical Botanic Garden) and in the mountain area (Krasnaya Polyana and Solokh-Aul settlements), a phenotypic variability of morphological traits was analyzed in naturalized and cultivated *H. macrophylla* plants (Madame Faustin Travouillon and Madame Faustin Travouillon cultivars), and an influence of the external conditions to the range of variations was investigated. It was shown that genotype considerably influenced the plant height (63.5 %), leaf length (82.0 %), inflorescence diameter (19.3 %) and bract length (84.1 %). Climatic factors of the year also affected leaf width in both cultivars (22.5 %). With increase in height above sea level (400-600 m in settlements Krasnaya Polyana and Solokh-Aul) most of the morphometric parameters increased. Maximum changes were observed in inflorescence diameter and bract length (30.1-55.4 % in Madame Faustin Travouillon and 26.5-71.5 % in Madame Maurice Hamard), indicating optimization of a complex of abiotic and edaphic factors at this high-rise level. The lowest variability (5.5-12.3 %) was indicated in shoot length both in cultivated and naturalized plants. In general, naturalized and cultivated plants differed considerably. The bract length and inflorescence diameter were the most variable, at 37.5-80.1 % and 17.9-31.2 %, respectively, when compared in *H. macrophylla* from city biocoenoses and settlements of Krasnaya Polyana and Solokh-Aul being higher in naturalized plants. Leaf length variation in cultivated plants ranged from 15.8 to 20.7 % being at 34.9 % in plants grown in Solokh-Aul. Thus, the range of variability on most of the tested morphological traits was wider in the naturalized plants if compared to the cultivated plants with slightly changed parameters. Therefore, the plants growing in city biocoenosis are more leveled on an individual development.

Keywords: morphometric parameters, *Hydrangea macrophylla*, plant introduction, wild, cultigenic and city coenosis.

The humid subtropics of Russia are referred to the region where natural vegetation has been almost displaced by urban phytocenoses. Most of the plants growing in this territory belong to alien species. A considerable number of them take their origin from the East-Asian floristic region where the climatic conditions are similar to those in the considered area.

At present, in connection with at first spontaneous and then systematic introduction of plants in botanical gardens, as well as with the wide use of exotic species in landscape gardening and amateur horticulture, the facts when alien plants run wild become increasingly frequent [1]. Formation of introduced plant populations is observed all over the world [2], and the humid subtropics of Russia are not an exception in this respect.

One of the alien species, which belong to the family *Hydrangeaceae* and are used for landscape gardening, is the large-leaved hydrangea, *Hydrangea macrophylla* Ser. The intraspecies diversity of this bush is represented by a large

number (about 800) of cultivars and garden forms [3]. Under the conditions of the region, *H. macrophylla* plants are found both in urban cenoses (parks, public gardens, etc.) and as the component of the adventive flora in the suburban forests of Big Sochi. Feral plants of the species *H. macrophylla* usually grow on clear-boled forest plots and on river banks [4].

It is known that morphological characters of species may vary depending on environmental conditions [5-15]. Studying the degree of morphological parameter variation allows us to reveal the most stable characters, which is important in breeding work. However, the scientifically grounded description of characters is impossible without the assessment of their variability [16].

The purpose of this work was to investigate the phenotypic variability of morphological characters of naturalized and cultigenic *Hydrangea macrophylla* plants, as well as the influence of habitat conditions on the variation range of these characters.

Technique. The studies were carried out in 2006-2008 (the size of each sample was about 250 plants) on cultivars Madame Faustin Travouillon and Madame Maurice Hamard which are most common in the region and grow under cultivation on the Black Sea coast in the Friendship Tree Garden Museum (Sochi) and Kuban Subtropical Botanical Garden (Sochi), as well as in mountainous areas in the settlements of Krasnaya Polyana and Solokh-Aul (Sochi). Measurements and calculations were carried out with regard to five morphological characters: sprout length (l, cm), leaf length (l_1, cm), leaf width (b, cm), inflorescence diameter (d, cm), bract length (l_2, cm). Mature leaves were taken from the middle part of sprouts on the southern side. The inflorescence diameter and bract length (sample size of 2,500 pcs) were also measured on the southern side.

The *H. macrophylla* morphological variability in different environmental conditions was assessed based on the value of the variation coefficient in accordance with the following scale: < 7 % as very low, 8-15 % as low, 16-25 % as medium, 26-35 % as increased, 36-50 % as high, > 50 % as very high [16, 17].

The obtained data were processed using statistics methods [18].

Results. The comparative analysis of naturalized and cultigenic *H. macrophylla* plants has shown that the environmental conditions of the habitat have a significant positive influence on the linear characters associated with the generative sphere. The greatest variability was observed for the bract length and the diameter of the inflorescences collected from plants under the conditions of urban cenoses and from naturalized plants near the settlements of Krasnaya Polyana and Solokh-Aul.

High variation coefficient values (37.5-80.1 %) were noted for the bract length character (Table 1). The amplitude of inflorescence diameter variability for *H. macrophylla* plants ranged from 17.9 % (medium) to 31.2 % (increased).

1. Morphological parameters of *Hydrangea macrophylla* plants depending on growth conditions ($X \pm S_x$, Sochi, 2006-2008)

Parameter	Friendship Tree Garden Museum	Kuban Subtropical Botanical Garden	Krasnaya Polyana	Solokh-Aul
	Madame Faustin Travouillon			
l, cm	126.5±2.1	134.7±1.4	189.8±2.1	224.5±2.4
Cv, %	5.4	4.9	10.1	14.4
l_1, cm	17.5±0.9	18.6±1.0	18.3±0.8	19.1±0.9
Cv, %	15.8	20.7	23.8	24.5
b, cm	12.3±0.4	12.7±0.9	13.0±0.7	13.3±1.1
Cv, %	20.3	25.2	27.8	34.9
d, cm	18.0±1.4	18.5±1.1	17.3±1.5	17.5±1.1
Cv, %	17.9	21.1	28.7	31.2

Table 1 (continued)

l_2, cm	0.9±0.2	0.9±0.2	0.8±0.1	0.9±0.1
Cv, %	37.5	41.0	48.7	62.0
		Madame Maurice Hamard		
l, cm	120.3±2.4	123.6±1.9	175.5±1.9	213.8±1.8
Cv, %	6.3	5.4	6.9	11.5
l_1, cm	13.6±0.3	13.9±0.4	13.1±0.7	14.0±0.6
Cv, %	19.3	17.5	31.8	32.2
b, cm	12.3±0.8	12.5±0.9	11.8±0.8	12.0±1.0
Cv, %	23.1	22.3	25.0	28.7
d, cm	18.3±1.0	18.6±1.2	19.0±0.2	18.5±0.4
Cv, %	17.9	23.9	25.9	27.1
l_2, cm	2.1±0.2	2.2±0.2	3.0±0.8	3.9±0.9
Cv, %	53.8	37.6	62.8	80.1

Note: l—sprout length, l_1—leaf length, b—leaf width, d—inflorescence diameter, l_2—bract length, $X \pm S_x$—average with error, *Cv*—variation coefficient.

The characters associated with linear dimensions of leaves also varied. Thus, medium variability was noted for the leaf length of the plants growing under the conditions of urban cenoses (from 15.8 to 20.7 %). In *H. macrophylla* populations in the lower mountain belt, the amplitude of the variation of this character was somewhat higher and ranged from 23.8 to 32.2 %. The variability with regard to the leaf width in plant populations from the settlement of Solokh-Aul was 34.9 %.

The instability of morphometric characters of *H. macrophylla* leaves may be due to variations of soil humidity, air temperature and humidity in the period of spring and summer plant sprout growth. Also, these parameters may depend on the age of sprouts, the ratio between generative and vegetative organs on sprouts, or the age-specific condition of the plants growing in different environmental conditions.

The high variation amplitude for 3 years of observations is explained by substantial climatic differences of these years. Particularly, 2006 was the most favorable year for the majority of the investigated characters in the populations under study (Table 2).

2. Morphological parameters of naturalized *Hydrangea macrophylla* plants in different years (Solokh-Aul, Krasnaya Polyana and Sochi)

Parameter	2006		2007		2008	
	$X \pm S_x$	*Cv*, %	$X \pm S_x$	*Cv*, %	$X \pm S_x$	*Cv*, %
	Madame Faustin Travouillon					
l, cm	161.0±4.5	9.3	198.3±15.8	26.3	224.1±6.8	10.8
l_1, cm	19.6±1.0	17.3	18.3±1.2	21.3	16.9±1.2	24.3
b, cm	14.2±1.2	27.5	13.1±1.1	27.5	12.5±1.3	34.4
d, cm	16.8±1.1	22.6	17.8±1.6	28.7	17.8±1.5	30.1
l_2, cm	0.9±0.1	22.2	0.8±0.1	50.0	0.8±0.1	62.5
	Madame Maurice Hamard					
l, cm	165.5±5.8	11.5	188.3±3.9	6.9	196.5±3.2	5.4
l_1, cm	13.0±0.8	21.5	12.7±1.2	32.3	12.9±1.2	31.0
b, cm	12.9±0.8	21.7	11.0±1.0	29.6	11.8±1.0	28.8
d, cm	19.6±0.1	17.9	19.0±1.6	27.9	18.9±1.5	25.4
l_2, cm	3.9±1.0	35.9	3.0±0.8	86.7	2.8±0.6	75.0

Note: l — sprout length, l_1 — leaf length, b — leaf width, d — inflorescence diameter, l_2 — bract length, $X \pm S_x$ — average with error, *Cv* — variation coefficient.

In that year, the amount of precipitation was by 25.2 % larger than the long-time annual average value, which had a positive impact on the stability of the studied characters of plants in populations.

The sprout length variability was low (5.4-11.5 %). The year of 2007 was an exception because well-marked differences between plants in the population were noted for cv. Madame Faustin Travouillon with regard to this character (26.3 %).

Naturalized *H. macrophylla* plants demonstrated the variation of linear

dimensions of leaves by years from 17.3 % (medium) for cv. Madame Faustin Travouillon in 2006 to 32.3 % (high) for cv. Madame Maurice Hamard in 2007.

Variability was found with regard to the leaf width, in 2008 from increased (28.8 %) for cv. Madame Maurice Hamard to high (34.4 %) for cv. Madame Faustin Travouillon. The inflorescence diameter ranged from 22.6 (2006) to 30.1 % (2008) for cv. Madame Faustin Travouillon and from 17.9 (2006) to 27.9 % (2007) for cv. Madame Maurice Hamard. Very high variability was noted with regard to the bract length in 2007-2008, being 50.0-62.5 % for cv. Madame Faustin Travouillon and 86.7-75.0 % for cv. Madame Maurice Hamard.

Within the period of the studies, no strong development of *H. macrophylla* plants was observed under the conditions of urban cenoses in the absence of competition. The morphological variability for the cultivated plants was less than that in populations of naturalized plants (Table 3).

The sprout length variability for the studied cultivars was shown to be low (3.8-6.6 %), the variation of linear dimensions of leaves being from medium (16.8 %) to increased (25.2 %). Similar results were obtained with regard to the inflorescence diameter. High variability was only noted for the bract length (33.3-58.3 %).

3. The parameters of morphological characters of *Hydrangea macrophylla* plants in urban cenoses in different years (Sochi)

Parameter	2006		2007		2008	
	$X \pm S_x$	*Cv*,%	$X \pm S_x$	*Cv*,%	$X \pm S_x$	*Cv*,%
Madame Faustin Travouillon						
l, cm	133.5±2.0	5.2	130.9±1.8	4.5	129.9±1.5	3.8
l_1, cm	19.0±0.9	16.8	18.5±1.1	20.0	17.6±1.2	23.3
b, cm	13.1±0.9	22.1	12.3±0.8	20.3	12.7±1.0	25.2
d, cm	19.0±0.8	14.7	17.5±1.1	21.1	17.5±0.9	17.1
l_2, cm	0.9±0.1	22.2	0.9±0.1	37.5	0.8±0.1	41.1
Madame Maurice Hamard						
l, cm	125.0±2.5	6.6	126.1±2.1	5.4	120.7±1.8	4.8
l_1, cm	14.4±0.8	19.4	13.1±0.8	19.8	13.5±0.7	16.3
b, cm	12.5±0.9	24.0	12.8±0.8	21.9	12.4±0.9	23.4
d, cm	18.7±1.0	17.1	17.5±1.2	21.7	18.6±1.0	17.2
l_2, cm	2.3±0.2	34.5	2.1±0.2	33.3	2.4±0.4	58.3

Note: l — sprout length, l_1 — leaf length, b — leaf width, d — inflorescence diameter, l_2 — bract length, $X \pm S_x$ — average with error, *Cv* — variation coefficient.

4. The parameters of morphological characters in the populations of naturalized and cultivated *Hydrangea macrophylla* plants (Solokh-Aul, Sochi, 2006-2008)

Parameter	*Cv*, %	
	naturalized	cultivated
Madame Faustin Travouillon		
l, cm	12.3	5.5
l_1, cm	24.2	18.3
b, cm	32.4	22.8
d, cm	30.1	19.5
l_2, cm	55.4	39.3
Madame Maurice Hamard		
l, cm	9.2	5.9
l_1, cm	32.0	18.4
b, cm	26.9	22.7
d, cm	26.5	20.9
l_2, cm	71.5	45.7

Note: l — sprout length, l_1 — leaf length, b — leaf width, d — inflorescence diameter, l_2 — bract length, *Cv* — variation coefficient.

The comparison of average data (2006-2008) on linear dimensions and the morphological variability in the populations of naturalized and cultivated *H. macrophylla* plants has made it possible to reveal the sprout length as the most stable of these characters (Table 4).

As for the other studied characters, we found substantial differences between the *H. macrophylla* plants growing in dissimilar environmental conditions. The leaf length was characterized by medium variability (18.3-18.4 %), and the same can be said of the inflorescence diameter. Increased variability was observed with regard to the leaf width (22.8 %). In case of cultivated plants,

the high variability was only noted for the generative character, the bract length (39.3-45.7 %).

At the same time, morphological characters in the populations of naturalized *H. macrophylla* plants demonstrated the increased or very high variability, for example, 55.4-71.5 % in case of the bract length.

The influence of the genotype and year conditions on morphological characters of garden forms was assessed using the two-factor analysis-of-variance method (Table 5). The influence of the *H. macrophylla* genotype on all studied characters turned out to be statistically significant. The contribution of «genotype» factor variance was from 19.3 % (inflorescence diameter) to 84.1 % (bract length). The contribution of «year conditions» factor variance ranged from 1.8 % (bract length) to 22.5 % (leaf width). Only 3.2 % was accounted for the variance of «leaf length»—«year conditions» factor interaction, whereas the contribution of the variance of «leaf width»—«year conditions» factor interaction was equal to 47.3 %.

5. The structure of morphological variability for *Hydrangea macrophylla* garden plants, based on the results of the analysis of variance (Sochi, 2006-2008)

Factor	df	mS	*F*	Variance	Factor contribution,%
		Plant height, cm			
Genotype	3	37.991	97.6*	1121	63.5
Year conditions	2	3.645	9.5*	99	5.6
Interaction	6	1.814	4.7*	162	9.2
Unaccounted	120	383	–	383	21.7
		Leaf length, cm			
Genotype	3	285.0	244.1*	8.7	82.0
Year conditions	2	19.1	16.4*	0.4	3.8
Interaction	6	4.1	3.6*	0.3	3.2
Unaccounted	120	1.2	–	1.2	11.0
		Leaf width, cm			
Genotype	3	4.5	3.9*	0.102	2.4
Year conditions	2	42.7	36.6*	0.943	22.5
Interaction	6	18.6	16.0*	1.987	47.3
Unaccounted	120	1.2	–	1.200	27.8
		Inflorescence diameter, cm			
Genotype	3	17.9	11.0*	0.490	19.3
Year conditions	2	4.4	2.7*	0.060	2.3
Interaction	6	4.7	2.9*	0.350	13.8
Unaccounted	120	1.6	–	1.630	64.6
		Bract length, cm			
Genotype	3	45.79	323.5*	1.320	84.1
Year conditions	2	1.46	10.3*	0.030	1.8
Interaction	6	0.90	6.4*	0.090	5.5
Unaccounted	120	0.14	–	0.140	2.6

Note: df — number of degrees of freedom for factor, mS — mean square, *F* — Fisher's variance ratio. Dash means no data.
* $P < 0.05$.

Therefore, the genotype had the most significant influence on the variation of such morphological characters as plant height, leaf length and bract length. The largest influence on the variation of the leaf width of the studied cultivars was noted in case of the year conditions, which was significantly confirmed by the high percentage characterizing the interaction of these factors.

The values of most of the morphometric characters increased with the increase of altitude above sea level (the settlements of Krasnaya Polyana and Solokh-Aul). At the altitude of 400-600 m, generative characters (inflorescence diameter and bract length) had the largest variation coefficient for cv. Madame Faustin Travouillon (30.1-55.4 %) and cv. Madame Maurice Hamard (26.5-71.5 %), which indicates the optimization of conditions with regard to a set of abiotic and edaphic factors. The lowest variability (5.5-12.3 %) was noted for the

sprout length in the populations of both cultivated and naturalized plants.

The obtained results are consistent with data from other researchers who have demonstrated that the variability of the characters attributable to the number of any particular plant organs or components is not similar to the level of variability in the linear parameters [19-21].

Thus, adventive *Hydrangea macrophylla* plants in the natural environment substantially differ from cultigenic plants in absolute morphometric parameters. The variation range for most of the studied characters is larger for the first group of plants than for the latter one. The morphometric characters for the cultivated plants vary insignificantly, i.e. the *H. macrophylla* plants growing under the conditions of urban cenoses were more uniform with regard to the individual development.

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BACTERIAL STRAINS ANTAGONISTIC TO *Pyrenophora tritici-repentis* in vitro DEMONSTRATE DIFFERENT EFFICACY ON WHEAT SEEDLING IN GREEN HOUSE

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Abstract

Yellow leaf spot is a wide spread diseases of soft and hard wheat. Numerous publications and the authors' own research show that its epiphytotics occur in different countries (Australia, Canada, USA, India, England, Belgium, Romania, Czech Republic, Kazakhstan) with crop losses reaching 65 %. In Russia the disease is most common in the North Caucasus. Development and application of new high-performance environmentally friendly biological products is regarded as one of the most effective biologized approach to wheat protection against the disease. In searching potential bacterial agents for use as protective means, their in vitro antagonistic activity should be accompanied by the ability to provide effective protection for seeds and seedlings. Herein we studied the repression of yellow spot development during early stages of plant vegetation in a greenhouse as influenced by the bacterial strains which were in vitro antagonistic to *P. tritici-repentis* (Died.) Drechsler. Winter wheat cultivar Bat'ko susceptible to the pathogen was used as test plant. Six isolates of the *Bacillus* family (*Bacillus* sp. BZR 18, *B. subtilis* BZR 336 s, *B. subtilis* BZR 336 g, *B. subtilis* BZR 436, *B. subtilis* BZR 517, B. *licheniformis* BZR 59), as well as *Ochrobactrum* sp. BZR 417 from the collection of All-Russian Research Institute of Biological Plant Protection were used as candidate bio agents. For comparison, liquid Fitosporin-M was used as a biological preparation (LLC Scientific Innovation Enterprise «BashInkom», Russia) and Prozaro emulsion concentrate was used as a chemical fungicide («Bayer CropScience», Germany). Liquid cultures of antagonistic bacterial strains were applied in three modes, namely before inoculation (prophylactic treatment), at early signs of the disease (on the day 3 after inoculation) and by their combination. All treatments were performed with inlaying and without inlaying grain with liquid bacterial culture. All studied bacterial strains except *Ochrobactrum* sp. BZR 417 showed considerable biological efficacy of leaf spot inhibition. The *Bacillus* sp. BZR 18 and *B. subtilis* BZR 517 were the most inhibiting strains which repressed leaf spot development at 68.5 to 83.0 % and 55.6 to 64.0 % rate, respectively, in all variants except treatment at early signs of the infection without inlaying grains when the efficiency was 26.8 and 35.9 %, respectively. *B. licheniformis* BZR 59 provided for 52.6 to 68.9 % leaf spot inhibition. Liquid culture of *B. subtilis* BZR 336 g strain ensured efficiency from 51.5 to 58.3 % in all variants except a preventive treatment without inlaying grain when the efficiency was 40.9 %. *B. subtilis* BZR 336 s, if used at early signs of infection with inlaying grain and in combination of prophylactic treatment with application at early signs of infection and inlaying grain, caused 60.2 and 60.3 % leaf spot repression; in other cases a 14.7 to 44.3 % repression was observed. BZR 436 *B. subtilis* effectiveness was from 28.4 % under preventive treatment with preliminary grain inlaying to 73.4 % under preventive treatment together with application at early signs of infection and inlaying grain. The efficiency of *Ochrobactrum* sp. BZR 417 strain does not exceed 45.4 % in all variants. Depending on antifungal activity of the bacterial agent, a combination of grain pre-treatment, prophylactic treatment and treatment at early signs of infection proved to be the most effective.

Keywords: *Pyrenophora tritici-repentis*, antagonistic bacteria, biological effect, winter wheat, tan spot disease, seedlings.

Yellow spot of wheat leaves is economically important in many regions where this crop is cultivated, such as Australia, Canada, USA, India, England, Belgium, Romania, Czech Republic, Kazakhstan, etc. [1-8]. In Russia, this dis-

ease is most common in the North Caucasus. In the Krasnodar Territory, the pathogen was first found in 1985 [9] and has been noted every year since then [10-12]. In case of epiphytotic development, crop losses may achieve 40-65 % [13, 14]. The causal agent of this disease is homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechsler, the imperfect stage of *Drechslera tritici-repentis* (Died) Shoem. The pathogen has a wide range of gramineous host plants, including both cultivated and various wild-growing forms.

At present, pesticides are the most popular means of plant protection (including against yellow spot of wheat leaves). However, as toxic agents, they have a negative influence on agrobiocenoses, which gives rise to serious concern. The existing limitations on the use of pesticides have encouraged the search for alternative means of plant protection with the emphasis on biological preparations based on living microorganism cultures. The range of the biological preparations known for their activity against the phytopathogens of the genus *Pyrenophora* (*Drechslera*) is quite narrow. According to the List of Pesticides and Agrochemicals Permitted for Use in the Russian Federation [15], Planriz, Alirin B, Baktofit and Gamair are recommended against barley net blotch (helminthosporiosis), however none of them is referred to preparations against yellow spot. At the same time, it is reported that some biocontrolling microorganisms exhibit antifungal activity in respect to both the causal agents of this disease and other representatives of the genus *Pyrenophora* (*Drechslera*).

Thus, T. Taechowisan et al. [16] report about antifungal action of the 3-methylcarbazoles produced by endophytic strain *Streptomyces* sp. LJK109 with regard to a number of phytopathogens, including *Drechslera* sp. Several strains of *Trichoderma* spp. were tested against *P. tritici-repentis* (*D. tritici-repentis*) by Argentinean researchers in field conditions. The use of bioagents for presowing treatment of seeds and vegetation treatment led to reduction in spread of yellow spot disease by 16-56 %, depending on the conditions of the year, methods of application and variety of the wheat [17]. Earlier, studying the microflora of wheat leaves, the same authors extracted 13 isolates of fungi, two isolates of yeasts and one strain of *Bacillus* (Bw/97), which, in laboratory tests, turned out to be active against the pathogens causing leaf diseases, in particular, *Alternaria triticimaculans*, *Bipolaris sorokiniana*, *Drechslera tritici-repentis* and *Septoria tritici*. The largest inhibiting effect in these studies was observed for *Aspergillus niger*, Bw/97 and *Nigrospora sphaerica* [18]. Substantial antifungal activity of strains of *Pseudomonas fluorescens* bacteria against fungi of the genus *Pyrenophora* was noted with regard to the barley. It must be stressed that the efficiency of bioagent application in greenhouse experiments depended on treatment time, in fact, protective action was more pronounced in case of use before pathogen inoculation [19].

Endophytic micromycete *Chaetomium globosum* also exhibits biological activity against the yellow spot pathogen. It has been established that culture filtrate on its basis promoted the production of extracellular protein and, therefore, retardation of yellow spot development on wheat leaves [20, 21].

In general, however, it can be concluded that information about biocontrol of the diseases caused by pathogens of the genus *Pyrenophora* (*Drechslera*) is quite fragmentary and scarce, and the biological preparations recommended for use are, as a rule, not referred to target ones. For example, one of such products is Planriz, which was developed based on *Pseudomonas fluorescens* AP-33 (Institute of Genetics and Cytology of the National Academy of Sciences of Belarus, Minsk). At present, it is produced by some Russian companies (PA Sibbiopharm Ltd, Berdsk; Biotekhagro LLC, Timashevsk) and widely used on various crops in different regions of the country, including for control of leaf spot diseases of grain

crops. In the studies of V.P. Borovaya [22], biological efficiency of Planriz against barley net blotch was 82 %. The same author reports about high efficiency of Psevdobakterin-2 based on *Pseudomonas aureofaciens* (84 %) and Baktofit based on *Bacillus subtilis* (70 %) against the mentioned pathogen. Cedomon (Bio-Agri, Sweden) based on *Pseudomonas chlororaphis* (strain MA 342) can be distinguished among foreign preparations effective against the causal agents of the genus *Pyrenophora* (*Drechslera*). Its antifungal action is mainly related to production of phenazine antibiotics. The preparation is intended for control of barley and oat seed infections and used for presowing treatment. Manufacturer Cedomon states that such treatment on the barley is more efficient than the application of chemical treaters, such as Fungazil A, Panoctine Plus 400, Cevex 300, Robust [23-25].

Along with antagonistic activity in vitro, an important property of potential protective bioagents is their capability to provide efficient protection of seeds and seedlings.

In this connection, in a greenhouse trial, we have studied the influence of the bacterial stains exhibiting antagonistic properties with regard to *Pyrenophora tritici-repentis* in vitro on yellow spot development in case of wheat plants in the seedling phase.

Technique. The objects of study were represented by seven promising bacterial strains from microorganism collections of the All-Russian Research Institute of Biological Plant Protection, which exhibited antagonistic properties with regard to the pathogens causing fusarium disease, *Fusarium graminiarum* Schwabe and *F. culmorum* (Sm.) Sacc., and yellow spot of wheat leaves (*Pyrenophora tritici-repentis*) in vitro [26-28]. Six of the studied strains belonged to the genus *Bacillus* (*Bacillus* sp. BZR 18, *B. subtilis* BZR 336 s, *B. subtilis* BZR 336 g, *B. subtilis* BZR 436, *B. subtilis* BZR 517, *B. licheniformis* BZR 59), and one was from the genus *Ochrobactrum* (*Ochrobactrum* sp. BZR 417).

The plants of winter soft wheat variety Batko susceptible to the pathogen were hydroponically grown in greenhouse conditions (under daylight, at 20-24 °C) using Knop's solution to the 2-leaf stage and were infected by virulent isolates of *P. tritici-repentis* from the collection of the All-Russian Research Institute of Biological Plant Protection using water spore suspension with the density of 5×10^3 conidia/ml.

Treatment with liquid culture based on antagonistic strains was carried out in three ways: 1 — before inoculation (preventive treatment), 2 — upon appearance of the first signs of disease (on the 3rd day after inoculation), 3 — preventive treatment and application of the preparation upon appearance of the first signs of disease development. These treatments were carried out both with and without inlaying of grains with the liquid culture based on bacterial strains. The liquid culture based on the promising strains was applied at the rate of 1-3 l/ha. Chemical and biological standards were fungicide Prozaro (emulsion concentrate, 0.6 l/ha) (Bayer CropScience, Germany) and Phytosporin-M (liquid, 1 l/ha) (NVP Bashinkom LLC, Russia), respectively. A total of 50 plants were accounted for in each trial case. Disease development on the plants infected and treated with liquid bacterial culture was compared with the control (without treatment). The number of spots was counted; a reaction type was assessed according to the 5-point scale of Rees et al. (1987), and disease development percentage (2) was evaluated for one plant on the 7^{th} day after inoculation. Biological efficiency was calculated by Ebbot's formula [29].

Statistical processing was carried out using the standard Microsoft Excel software.

Results. The reduction of point-based reaction type evaluation allows us to assess the degree of inhibition of plant tissue colonization by the fungal

pathogen; decrease in the number of spots characterizes the bacterial strain ability to inhibit *P. tritici-repentis* infection on plants, and the variation of disease development percentage reflects the capability of limiting infection contamination and pathogen development in plants.

In case of infection, the average number of spots per plant in a control (without treatment) was 15.6 pcs with the reaction type under disease development at 4.6 points and the disease development of 26.4 % (Table 1).

1. The characteristic of protective action of the strains exhibiting antagonism in vitro with regard to *Pyrenophora tritici-repentis* on wheat plants of sensible variety Batko in the seedling phase in greenhouse conditions ($X \pm s$)

Antagonistic strain, preparation	Treatment					
	preventive		upon the first signs of disease		preventive + upon the first signs of disease	
	1	2	1	2	1	2
Number of spots per plant, pcs						
Bacillus sp. BZR 18	6.1±1.2	7.8±1.2	5.7±0.9	12.5±1.4	6.2±1.1	7.3±0.9
Bacillus subtilis:						
BZR 436	10.9±1.8	8.8±1.2	9.6±1.2	9.1±1.3	6.8±1.8	10.4±1.5
BZR 517	4.5±1.1	7.5±1.7	10.2±1.1	12.4±1.7	8.8±1.9	9.7±1.2
BZR 336 s	10.1±1.0	10.8±1.5	8.0±1.3	12.3±2.1	8.5±1.3	10.1±1.1
BZR 336 g	9.0±1.1	9.5±1.7	7.2±1.5	8.4±1.8	13.3±1.4	11.8±1.3
B. licheniformis BZR 59	9.4±1.2	10.3±1.1	7.0±1.3	6.7±1.1	8.2±1.5	7.9±0.9
Ochrobactrum sp. BZR 417	10.5±1.9	10.5±1.1	11.9±1.8	12.3±1.7	10.1±1.1	10.3±1.1
Phytosporin-M, liquid	7.7±0.9	10.5±1.2	9.7±1.4	8.3±1.3	7.9±1.1	11.3±1.4
Prozaro, emulsion concentrate	–	4.3±1.0	–	6.9±1.1	–	4.3±1.1
Control (without treatment)	15.6±1.8	15.6±1.8	15.6±1.8	15.6±1.8	15.6±1.8	15.6±1.8
Type of reaction to infection contamination, points						
BZR 18 *Bacillus* sp.	3.2±0.4	3.2±0.5	2.8±0.5	4.0±0.4	2.7±0.5	3.2±0.5
Bacillus subtilis:						
BZR 436	4.0±0.5	3.8±0.5	3.5±0.4	3.8±0.6	3.1±0.5	3.8±0.5
BZR 517	3.4±0.6	3.6±0.8	3.6±0.8	4.1±0.6	3.2±0.5	3.6±0.5
BZR 336 s	4.3±0.5	4.5±0.6	3.9±0.5	4.4±0.6	3.8±0.6	4.3±0.7
BZR 336 g	4.1±0.9	4.1±0.5	3.6±0.7	3.9±0.8	4.1±0.8	4.0±0.8
B. licheniformis BZR 59	4.1±1.0	3.6±0.4	3.6±0.5	4.0±0.8	3.4±0.7	3.6±0.6
Ochrobactrum sp. BZR 417	4.1±0.9	4.2±0.7	3.8±0.6	4.3±0.5	3.6±0.6	4.1±0.6
Phytosporin-M, liquid	3.9±0.5	4.0±0.7	3.6±0.5	3.6±0.6	3.5±0.5	3.6±0.5
Prozaro, emulsion concentrate	–	2.4±0.5	–	3.3±0.6	–	2.3±0.5
Control (without treatment)	4.6±0.5	4.6±0.5	4.6±0.5	4.6±0.5	4.6±0.5	4.6±0.5
Disease development on one plant,%						
BZR 18 *Bacillus* sp.	4.4±2.5	8.2±4.5	4.6±3.1	19.3±4.8	4.6±3.5	8.3±4.3
Bacillus subtilis:						
BZR 436	18.9±4.7	11.5±4.5	10.1±4.1	13.3±4.2	7.0±4.0	13.8±4.5
BZR 517	9.5±3.6	9.5±4.0	11.7±3.9	16.9±4.5	9.4±4.8	11.4±4.0
BZR 336 s	14.7±4.1	22.0±6.1	10.5±4.0	22.5±5.2	10.5±4.5	22.5±5.8
BZR 336 g	11.8±3.9	15.6±5.5	12.8±3.5	11.0±4.7	12.0±4.6	12.5±3.8
B. licheniformis BZR 59	11.4±4.0	12.5±5.1	8.9±5.0	8.2±3.8	8.5±4.8	8.5±4.0
Ochrobactrum sp. BZR 417	15.0±4.1	15.5±3.9	14.4±4.5	18.5±4.0	14.8±5.2	15.0±4.6
Phytosporin-M, liquid	14.0±4.3	15.8±5.0	15.3±5.1	16.2±4.5	14.7±3.5	16.0±4.2
Prozaro, emulsion concentrate	–	4.5±2.8	–	7.7±3.0	–	5.1±3.1
Control (without treatment)	26.4±5.6	26.4±5.6	26.4±5.6	26.4±5.6	26.4±4.6	26.4±4.6

Note: 1 — with additional seed inlaying, 2 — without inlaying. Dashes mean that the variant was not used. In control no preparations were used.

The analysis of biological efficiency of bacterial preparations has revealed differences depending on the properties of the strain used and modes of application. The highest inhibition of spot formation and development was noted for *Bacillus* sp. BZR 18 (68.5-83.0 %) and *B. subtilis* BZR 517 (55.6-64.0 %) in all cases, but treatment upon appearance of the first signs without preliminary inlaying of grains (in this case, the indicator was equal to 26.8 and 35.9 %, respectively), as well as for *B. licheniformis* BZR 59 (52.6-68.9%) in all variants (Table 2).

2. The biological efficiency (relative to the control,%) **of the strains exhibiting antagonism in vitro with regard to *Pyrenophora tritici-repentis* on wheat plants of sensible variety Batko in the seedling phase in greenhouse conditions**

Antagonistic strain, preparation	Treatment					
	preventive		upon the first signs of disease		preventive + upon the first signs of disease	
	1	2	1	2	1	2
By number of spots on leaves						
Bacillus sp. BZR 18	60.8	50.0	63.4	19.8	60.2	53.2
Bacillus subtilis:						
BZR 436	36.0	43.6	38.5	41.6	56.4	33.3
BZR 517	71.1	51.9	34.6	20.5	43.5	37.8
BZR 336 s	35.8	30.7	48.7	21.1	45.5	35.8
BZR 336 g	42.3	39.1	53.8	46.1	46.7	43.5
B. licheniformis BZR 59	39.7	33.9	55.1	57.0	47.4	49.3
Ochrobactrum sp. BZR 417	32.7	32.7	23.7	21.1	35.8	33.9
Phytosporin-M, liquid	50.6	32.7	37.8	46.8	49.3	27.6
Prozaro, emulsion concentrate	–	72.4	–	55.7	–	72.4
By type of reaction to infection contamination						
BZR 18 *Bacillus* sp.	30.4	30.4	39.1	13.0	41.3	30.4
Bacillus subtilis:						
BZR 436	13.0	17.3	23.9	17.3	32.6	17.3
BZR 517	26.0	21.7	21.7	10.8	30.4	21.7
BZR 336 s	6.5	2.1	15.2	4.3	17.3	6.5
BZR 336 g	10.8	10.8	21.7	15.2	10.8	13.0
B. licheniformis BZR 59	10.8	21.7	21.7	13.0	26.0	21.7
Ochrobactrum sp. BZR 417	10.8	8.6	17.3	6.5	21.7	10.8
Phytosporin-M, liquid	15.2	13.0	21.7	21.7	23.9	21.7
Prozaro, emulsion concentrate	–	47.8	–	28.2	–	50.0
By disease development degree						
BZR 18 *Bacillus* sp.	83.0	68.9	82.5	26.8	83.0	68.5
Bacillus subtilis:						
BZR 436	28.4	56.4	61.7	49.6	73.4	47.7
BZR 517	64.0	64.0	55.6	35.9	64.3	56.8
BZR 336 s	44.3	16.6	60.3	14.7	60.2	14.7
BZR 336 g	55.3	40.9	51.5	58.3	54.5	52.6
B. licheniformis BZR 59	56.8	52.6	66.2	68.9	67.8	67.8
Ochrobactrum sp. BZR 417	43.1	41.2	45.4	29.9	43.9	43.1
Phytosporin-M, liquid	46.9	40.1	42.0	38.6	44.3	39.4
Prozaro, emulsion concentrate	–	82.9	–	70.8	–	80.6

Note: 1 — with additional seed inlaying, 2 — without inlaying. Dashes mean that the variant was not used. In control no preparations were used.

Treatment with the liquid culture based on strain *B. subtilis* BZR 336 g provided efficiency within 51.5-58.3 % in all cases, except for preventive treatment without preliminary treatment of grains (40.9 %); for *B. subtilis* BZR 336 s, this indicator was almost equal and quite high (60.2 and 60.3 % in treatments upon appearance of the first signs with preliminary inlaying of grains and in case of preventive application in combination with treatments upon appearance of the first signs and with preliminary inlaying, respectively), whereas, in the other cases, it was significantly lower and strongly varied (from 14.7 to 44.3 %). The efficiency of strain *B. subtilis* BZR 436 ranged from 28.4 % (preventive treatment with preliminary grain inlaying) to 73.4 % (preventive treatment in combination with application upon appearance of the first signs and preliminary inlaying). The efficiency of strain *Ochrobactrum* sp. BZR 417 did not exceed 45.4 % in all cases.

The chemical standard has shown maximum protective effect against the yellow leaf spot pathogen with regard to the number of spots (55.7-72.4 %), type of disease manifestation (28.2-50.0 %) and the disease development level (to 78.0-82.9 %). The biological efficiency of Phytosporin-M (liquid) was less than that of the chemical standard, but matched the efficiency of other strains, particularly from 27.6 to 50.6 % with regard to decrease in the number of spots,

from 13.0 to 21.7 % with regard to the type of reaction to disease development, and from 38.6 to 46.9 % with regard to the disease development.

It has been noted that basic mechanisms for biocontrol of phytopathogens by rhizobacteria, including the studied new agents, comprise the following: competition for ecological niches and nutrient sources; enzyme activity leading to lysis of phytopathogen cells; production of substances of antibiotic nature, and induction of resistance to phytopathogens [30, 31].

The previously obtained data allow us to assume that the substantial protective effect in all cases of winter wheat plant treatment with test samples of biological preparations upon appearance of the first signs of yellow leaf spot is associated with the synthesis of mycolitic enzymes of chitinase, lipase and protease groups, as well as with the production of antibiotic substances [26, 27].

It is important to note that high protective action indicators under preventive treatment with test samples of developed biological preparations may be presumably associated with a capability of biological agents to cause Induced Systematic Resistance (ISR) of plants [32].

Thus, all the studied bacterial strains, except for *Ochrobactrum* sp. BZR 417, on average, exhibited biological efficiency above 50 % with regard to a capability of restraining the development of yellow spot of wheat leaves in various variants of treatments in the seedling phase. The best results were observed for the combination of preliminary seed inlaying with subsequent preventive treatment and application of preparation upon appearance of the first signs of disease (depending on the antifungal activity of the bacterial agent).

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ARTIFICIAL ASSOCIATIVE SYMBIOSES BETWEEN TOMATO PLANTS AND FUNGISTATIC *Rhizobium*

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Abstract

Biomethods in plant protection against pests and diseases considered the most prospective alternative to chemicals which pollute soil and water causing concern about public health. The possibility of creating an artificial association of nodule bacteria with plants to protect them from the adverse effects of pathogenic fungi can be realized using one of the specific mechanism of nodule bacteria attachment to the roots of leguminous plants by plant lectins, able to recognize and specifically bind with different carbohydrates, particularly polysaccharides of rhizobia cell wall. In our study we used the composite plants of tomato (*Lycopersicon esculentum*) Dubok variety and bacterial strains associated with the roots of wild legumes from the collection of *Institute of Biochemistry and Genetics* (IBG USC RAS). «Hairy rooted» tomato plants were obtained by treatment with *Agrobacterium rhizogenes* ATCC 15834, containing vehicle gene construction pCambia 1305.1 with inserted pea lectin gene *psl* under cauliflower mosaic virus 35S promoter. The antagonistic activity of bacteria towards pathogens was tested by dual culture study. The ability of microorganisms to produce siderophores and cyanide was analyzed. Few isolates were identified by sequencing of the 16S rRNA gene fragments. By screening of the collection of isolates from nodules of wild legume from tribe *Viceae* the candidate strains were detected, particularly *Rhizobium leguminosarum, Pseudomonas* sp. and *Stenotrophomonas rhizophila,* with fungistatic activity against *Fusarium solani, F. oxysporum, Fusarium* sp. and *F. oxysporum* f. sp. *lycopersici*. Prodiction of siderophores was detected in two members of *Pseudomonas genus*, *S. rhizophila* and *R. leguminosarum*. Two *Pseudomonas* strains, the 14M and 103, and *S. rhizophila* were shown to produce cyanide. It was also found that treatment of roots transgenic by *psl* gene with *R. leguminosarum* 116 strain reduced the amount of hyphae of the pathogen *F. oxysporum f.* sp. *lycopersici* in the rhizosphere of tomato plant that could potentially contribute to plant defense against pathogenic fungi. Thus, the use of lectins as transgenes in roots allows us to obtain artificial association with rhizobia in non-symbiotic plants such as tomato, which in combination with the use of microorganisms possessing fungistatic activity can more effectively protect the plant root system o against pathogens.

Keywords: rhizobia, phytopathogenic fungi, transgenic plants, lectins, associative symbiosis.

Phytopathogens in the agricultural industry are usually controlled using various pesticides, which leads to soil and water pollution. Moreover, accumulation of such compounds in plants is hazardous to human health. Biological methods of plant protection provide an alternative to chemical techniques. They include the use of the soil microorganisms exhibiting the protective effect, most of which belong to the genera *Bacillus* and *Pseudomonas*. The strains capable of inhibiting the growth of fungi were also found among bacteria of the family *Rhizobiaceae* [1-3], which is associated with various protection mechanisms, such as synthesis of toxic substances [4] and cyanide [5], induced systematic resistance of plants [6[, release of siderophores [7] and some others [8].

Although (with rare exception) *Rhizobiaceae* are able to enter into endosymbiosis only with legumes, there are studies revealing their potential as associative microsymbionts for nonleguminous crops [9], including the tomato [10,

11]. The colonization of nonleguminous plant roots by rhizobia is improved by various methods, including the use of the transgenic plants that synthesize the substances involved in signaling at early stages of legume-rhizobia symbiosis. Such substances include lectins, the secreted proteins that can recognize and selectively bind to various carbohydrates [12], in particular, to polysaccharides on rhizobium cell walls, which provides the fixation of microorganisms on the root surface [13]. Earlier, several groups of scientists carried out works to change symbiosis specificity using lectins of leguminous plants [14-16]. Bacteria were found both on the external surface of roots and in intra- and intercellular space. Thus, the roots of the plants transgenic for lectin genes, potentially, may be specifically colonized only by the rhizobia which perform the functions useful for plants, for example, protection against phytopathogens.

Soil bacteria *Agrobacterium rhizogenes* carrying Ri-plasmids can cause the formation of transgenic «hairy roots» in many dicotyledon species. «Composite» plants with such roots, which carry target genes, are used in order to study plant interactions with microorganisms, fungi and nematodes [17]. In particular, the «hairy roots» of tomatoes, where a pea lectin gene is expressed [18], may become a good model to study the possibility of plant protection against pathogens through improvement of the efficiency of associative symbiosis with rhizobia.

The purpose of this work was to investigate the possibility of creating artificial targeted associations of bacteria with the roots of agricultural nonsymbiotrophic plants in order to protect them against phytopathogenic fungi by the example of the tomato (*Lycopersicon esculentum*) and nodule bacteria *Rhizobium leguminosarum*.

Technique. The object of study was represented by the tomato (*Lycopersicon esculentum*) of variety Dubok. Plants were transformed using strain *Agrobacterium rhizogenes* ATCC 15834 taken from the collection of the All-Russia Research Institute for Agricultural Microbiology (St. Petersburg) with previously introduced vector pCambia 1305.1, where field pea lectin gene *psl* was incorporated under the control of 35S promoter of cauliflower mosaic virus [19]. This work also included the use of the strains of the bacteria associated with the roots of wild-growing legumes in the territory of the Republic of Bashkortostan (collection of the Institute of Biochemistry and Genetics of Ufa Scientific Centre of Russian Academy of Sciences).

Composite tomato plants were obtained using the method described by R. Collier et al. [17]. The seed surface was sterilized for 2 min in 7 0% alcohol and then for 15 min in 15 % sodium hypochlorite solution with addition of several drops of Tween 20. The plant transformation experiment involved the use of 2-day cultures of *A. rhizogenes* (pCambia 1305.1-*psl*) and *A. rhizogenes* (initial strain) grown at 28 °C in TY liquid medium (0.1 % yeastrel, 1.0 % bactotryptone, 0.1 % $CaCl_2$) with addition of kanamycin (100 mg/l) and acetocyringone (200 μM) in the first case and only acetocyringone in the second case.

Prior to relocation of plants with «hairy roots» into substrate (sterilized mixture of soil and sand) and 1 week after the relocation, the roots were histochemically analyzed for GUS activity [20]. Fragments were incubated in X-Gluc reagent containing 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (1 mg/ml), 0.5 % Triton X-100, Na_2EDTA (100 mM), methanol (20 %), $K_3Fe(CN)_6$ (0.5 mM), $K_4Fe(CN)_6$ (0.5 mM) and Na-phosphate buffer (50 mM, pH 7.0) (Sigma-Aldrich, USA). The roots were held at 37 °C over night, and blue staining was detected.

In PCR analysis, DNA was extracted from «hairy roots» using the phenol-chloroform method. Lectin gene *psl* was detected in preparations using the primers (5′-ATAATGGCTTCTCTTCAA-ACCC-3′ and 5′-GCAAAAAAACT-ATGCATCTGCA-3′) flanking the site of this gene, as well as standard kits of reagents for amplification (Helicon, Russia). The PCR was carried out in a Tertsik MS2 amplifier (DNA-Technology, Russia) as per a protocol according to manufacturer's recommendations at optimal annealing temperature for the mentioned pair of primers. The positive control was represented by plasmid pCambia 1305.1-*psl.*

In order to identify symbiotic bacteria with fungistatic activity, isolates were screened using the test cultures of fungi *Fusarium solani*, *F. oxysporum* and *Fusarium* sp. from the collection of the Ufa State Petroleum Technological University, as well as *F. oxysporum* f. sp. *lycopersici* (F-140) from the All-Russian Collection of Microorganisms (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms of Russian Academy of Sciences, Pushchino). The fungistatic activity of microorganisms was assessed using the dual-culture method. Bacteria were introduced in a cross pattern in the center of a Petri dish, dividing it into four sectors. A piece of agarized growth medium with fungus mycelium was put in the middle of each sector. The dishes were placed into a thermostat and incubated at 27 °C. After 3 days, fungal colony radius was measured in directions towards bacteria (R_1) and dish edge (R_2), and a degree of fungal growth inhibition was calculated by the formula [21]:

$$T = (R_2 - R_1)/R_2 \times 100\ \%.$$

The specific identity of the bacteria was determined based on the analysis of 16S rRNA gene sequence [22].

When bacterial strains were checked for siderophore synthesis, minimal medium with CAS reagent (blue agar) was prepared as described [23]. The bacteria grown on YM medium were relocated to blue agar and grown within 5 days. The change of agar color to yellow, orange or pink was indicative of siderophore release. In order to reveal cyanide synthesis, bacteria were grown in Petri dishes within 1 day on YM medium with addition of glycine (4.4 g/l). Then, filter paper impregnated with 0.5 % aqueous solution of picric acid with 2 % Na_2CO_3 was put on the covers of the Petri dishes. The dishes were wrapped with Parafilm and incubated for 4 days at 28 °C. In case of cyanide release, paper color changed from yellow to orange or brown due to picrate formation [24].

For joint inoculation of plants by bacteria and fungi, suspension of spores of *F. oxysporum* f. sp. *lycopersici* was obtained. The fungus was grown in a Petri dish with YM medium for 5 days. Then, it was poured with 20 ml of sterile water and put to a refrigerator over night. The number of washed-off spores was counted in a Goryaev chamber. *R. leguminosarum* bacteria were accumulated at 28 °C within 1 day in YM liquid medium to titer of 10^7 CFU/ml. Plant roots were placed into the obtained bacterial suspension for 1 day. Then, they were washed out in sterile water; the plants were relocated to soil containing 10 ml of fungus spore suspension (10^5 pcs/ml) and grown within 3 days. After that, the plant roots were washed out and stained with toluidine blue for 1 hr (fungus hyphae changed color to violet, and plant cells to blue), and then washed out again in citrate buffer [25] and examined using an Axio Imager M1 microscope (Carl Zeiss AG, Germany).

Results. After plant treatment with *A. rhizogenes,* «hairy roots» began to form after 10-12 days on 90 % of plants (Fig. 1). Adventitious roots emerged during the first week; they were removed using a scalpel.

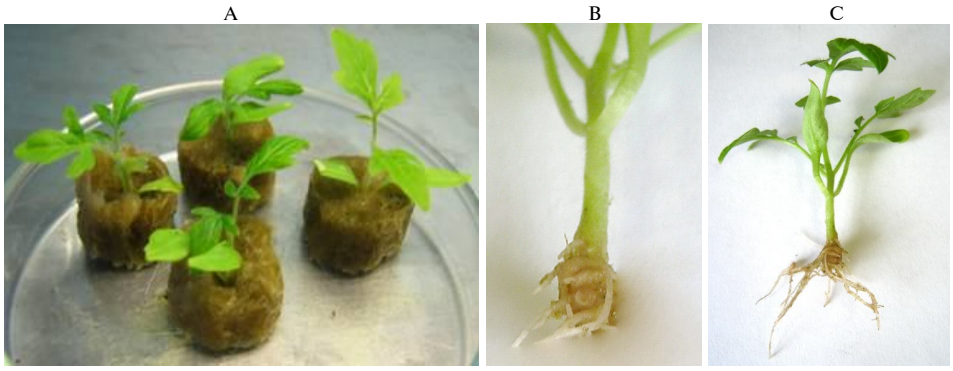

Fig. 1. Composite tomato (*Lycopersicon esculentum*) plants of variety Dubok after transformation with *Agrobacterium rhizogenes* ATCC 15834 carrying pCambia 1305.1 with incorporated field pea lectin gene *psl* under the control of 35S promoter of cauliflower mosaic virus: A — plants in mineral wool, B — «hairy roots» after 10-day growth, C — «hairy roots» after 3-week growth.

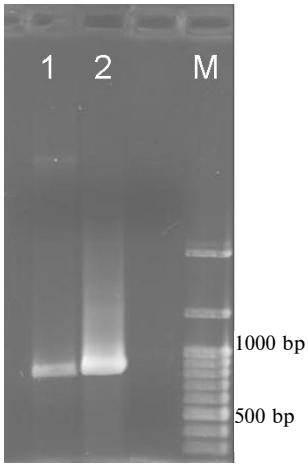

Fig 2. The PCR analysis of the field pea lectin gene *psl* in DNA from the «hairy roots» of composite tomato (*Lycopersicon esculentum*) plants of variety Dubok, obtained using pCambia 1305.1: 1 — «hairy roots», 2 — pCambia 1305.1; M — molecular weight marker 100 bp + 1.5 Kb + 3 Kb (Sibenzyme, Russia).

After 2 weeks, the roots were checked for GUS activity, and the plants were relocated to sterile mixture of soil and sand and grown for 1 more week. GUS-stained roots were found in 53% of the plants. The PCR analysis of DNA from these roots revealed the presence of the lectin gene (Fig. 2).

Earlier, we obtained completely transgenic tobacco plants, as well as the chimeric rape and tomato plants expressing the pea lectin gene [26-28)]. On such plants, we found the number of *R. leguminosarum* 1078 bacteria increased 37-, 14- and 10-fold, respectively, as compared to the non-transgenic plants. This fact gave evidence of interaction of rhizobia with lectin on the surface of transgenic roots. The colonization of roots by bacteria with fungistatic activity with the increase in the number of the latter ones could potentially protect plants against pathogens. In nature, the plants belonging to the tribe *Vicea* are the most frequent symbionts with *R. leguminosarum.* Because rhizobia with fungistatic activity were found earlier in the nodules of some wild-growing plants [29], we have screened the bacterium collection obtained on the wild vegetation belonging to the mentioned tribe [30] in order to reveal strains with such properties. A total of 568 isolates were investigated. Fungistatic activity with regard to the studied fungi was found in seven strains (Table).

The coefficient of colony growth retardation (T, %) **for *Fusarium* fungi in the presence of natural isolates of bacterial symbionts of *Vicea* wild plants**

Microsymbiont	*Fusarium* sp.	*F. oxysporum* f. sp. *lycopersici*	*F. solani*	*F. oxysporum*
Rhizobium leguminosarum 116	17	41	65	70
Pseudomonas sp. 2	0	0	25	0
Pseudomonas sp. 102	40	29	53	53
Pseudomonas sp. 103	0	21	22	33
Pseudomonas sp. 15.2	0	13	17	19
Pseudomonas sp. 14M	0	25	19	25
Stenotrophomonas rhizophila	40	0	33	18

The specific identity of bacterial strains was confirmed by the analysis of

16S rRNA gene sequence (data are not presented).

The fungistatic activity of the bacteria may be caused by secretion of siderophores, theproteins capable of forming a complex with iron ions, making them inaccessible to fungi; also, some rhizobium strains release hydrogen cyanide (HCN), which has a negative impact on fungal growth [2, 5]. The microsymbionts studied by us had the following ability to synthesize these fungistatic metabolites (siderophores/cyanides): *Rhizobium leguminosarum* 116 — «+»/«–», *Pseudomonas* sp. 2 — «+»/«–», *Pseudomonas* sp. 102 — «+»/«–», *Pseudomonas* sp. 103 — «–»/«+», *Pseudomonas* sp. 14M — «–»/«+», *Stenotrophomonas rhizophila* — «+»/«+». I.e., among the studied strains, the ability to synthesize siderophores was found in two representatives of the genus *Pseudomonas*, *S. rhizophila* and *R. leguminosarum*, and cyanide was released by two strains of *Pseudomonas* (14M and 103) and *S. rhizophila*. Thus, strains with the highest fungistatic activity (*R. leguminosarum* 116 and *Pseudomonas* sp. 102) synthesized siderophores, but not cyanide. However, it should be noted that parameters for *S. rhizophila* secreting both substances were not the highest. It is possible that the fungistatic activity of the strains is associated with any other mechanisms (for example, with synthesis of antibiotics).

Strain *R. leguminosarum* 116 with the highest fungistatic activity with regard to *F. oxysporum* f. sp. *lycopersici*, which was found as a result of the studies, was used as a microsymbiont in further experiments to create artificial symbiotic associations.

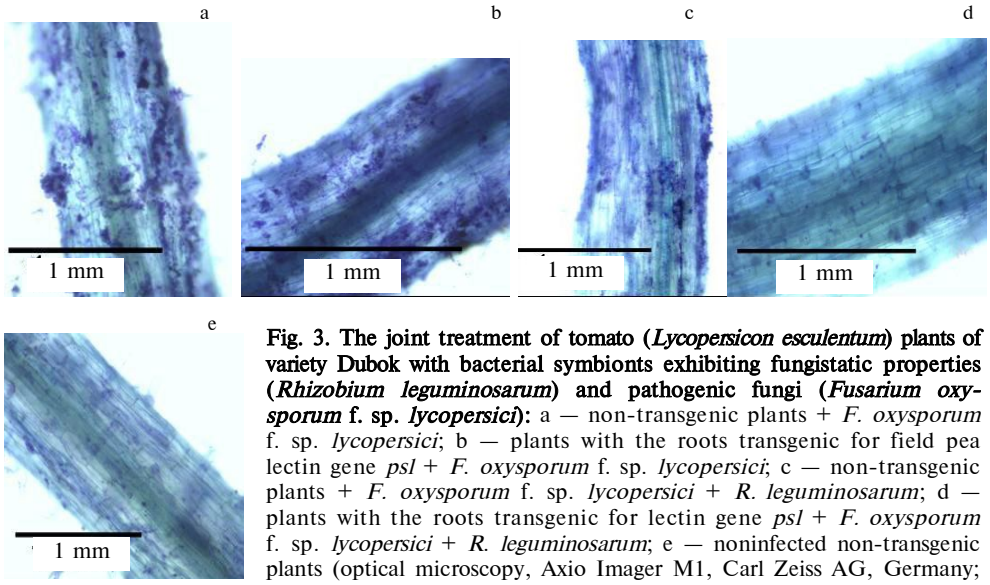

Fig. 3. The joint treatment of tomato (*Lycopersicon esculentum*) plants of variety Dubok with bacterial symbionts exhibiting fungistatic properties (*Rhizobium leguminosarum*) and pathogenic fungi (*Fusarium oxysporum* f. sp. *lycopersici*): a — non-transgenic plants + *F. oxysporum* f. sp. *lycopersici*; b — plants with the roots transgenic for field pea lectin gene *psl* + *F. oxysporum* f. sp. *lycopersici*; c — non-transgenic plants + *F. oxysporum* f. sp. *lycopersici* + *R. leguminosarum*; d — plants with the roots transgenic for lectin gene *psl* + *F. oxysporum* f. sp. *lycopersici* + *R. leguminosarum*; e — noninfected non-transgenic plants (optical microscopy, Axio Imager M1, Carl Zeiss AG, Germany; staining with toluidine blue).

After inoculation of the composite tomato plants having the roots with the lectin transgene, with non-transgenic plants as a control, by the suspension of strain *R. leguminosarum* 116 and their relocation to the soil containing the spores of fungus *F. oxysporum* f. sp. *lycopersici*, optical microscopy examination confirmed (Fig. 3, a-e) that the treatment of the roots transgenic for gene *psl* with strain *R. leguminosarum* 116 reduces the number of the hyphae of pathogen *F. oxysporum* f. sp. *lycopersici* in the rhizosphere (see Fig. 3, d). The same effect, but to a far less extent, was observed for control plants with less effective rhizobium adsorption on their roots (see Fig. 3, c).

Earlier, it was shown in a number of studies that *Bradyrhizobium japonicum*, *Sinorhizobium meliloti* and *R. leguminosarum* are able to retard *F. solani*

growth in experiments with the sunflower and okra [1], and *S. meliloti* and *R. trifolii* can be used for biocontrol of *F. oxysporum* infecting the sunflower and tomato [31].

So, the use of legume lectin genes as transgenes for non-symbiotrophic plants, such as the tomato, makes it possible to create artificial root associations with the rhizobia exhibiting fungistatic activity. The obtained results can find application in creation of artificial associative symbioses for biocontrol of phytopathogens.

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ENHANCEMENT OF ADAPTIVE CAPACITY OF SUGAR BEET CROPS BY MICROBIAL BIOPREPARATIONS UNDER BIOTIC AND ABIOTIC STRESSES

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Abstract

Diseases caused by pathogenic micromycetes and impact of herbicides lead to lower productivity and quality of sugar beet. Biopreparations based on *Bacillus* Cohn are promising environmentally friendly agents for plants protection under biotic and abiotic stresses. Meanwhile it is important to search of bioregulators for application on sugar beet and identify the mechanisms of their actions for effective use. This study summarizes the results of analysis of the effect of *Bacillus* Cohn based biopreparations Fitosporin-M, Albit, Vitaplan on structure of pathogenic mycromicetes complex in sugar beet (*Beta vulgaris* L., cv. KVS) rhizosphere, enzymatic activities in soil and leaves, sucrose content in roots and productivity under pathogenic and herbicides pressing. Evaluations were carried out using classical microbiological, physiological and biochemical methods. It was revealed that Fitosporin-M, Albit, Vitaplan decreased the abundance and frequency of rhizosphere pathogenic species and thus lead to activation of useful microflora. Partilularly, a total of 8 pathogenic micromycetes were found in control of which *Alternaria tenuis*, *Aspergillus niger* and *Penicillium glabrum*, the last two being strong toxigenic isolates, were dominant. After a single treatment with Fitosporin-M only *Penicillium glabrum* was found in the rhizosphere, in case of Albit there was only *P. citrinum*, and at Vitaplan application *Alternaria tenuis*, *Aspergillus niger*, *Fusarium oxysporum*, *F. solani* and *Penicillium solitum* were identified. In plants treated with Fitosporin-M and Albit, no Black Leg pathogens were observed. After doubled treatment the abundance decreased 1.5-3.0-fold, and the frequency decreased 2.0-4.0-fold compared to control. Catalase activity reflecting intensity of soil microbiological processes exceeded control from 1.4 to 3.7 times after the first treatment with Fitosporin-M and Albi, respectively, and from 1.4 to 1.2 times after the second one. The data on increasing oxidoreductases and hydrolases activities in rhizosphere confirm this conclusion. Also, biopreparations decreased activities of hydrolases in leaves and the activity of their inhibitors indicating induction of protective reactions against pathogens. The maximum values were recorded when Fitosporin-M and Albit were used. The protective actions of biopreparations were also indicated by activation of peroxidase in leaves up to 0.175-0.250 OD_{490} per 1 g. Furthermore Fitosporin-M supported a relatively high level after reintroduction indicating a prolonged action on the antioxidant system during vegetation. The positive effect of biopreparations under pathogenic and herbicides pressing was reflected in increasing productivity and sucrose content in sugar beet roots, and the maximum effect was achieved after 2-fold treatment of sowings with Fitosporin-M.

Keywords: *Bacillus* Cohn based biopreparations, oxidoreductases, hydrolases, peroxidase, sucrose, pathogenic microbiota, herbicides pressing, resistance, productivity, *Beta vulgaris* L.

The sugar beet (*Beta vulgaris* L.) is one of the most important industrial crops for sugar production [1]. At the same time, its potential productivity remains unrealized because the plants are adversely affected by various stress factors of both biotic (diseases and pests) and abiotic (exposure to high and low temperatures, moisture deficit, pesticide pressing, etc.) nature, which leads to decrease in root crop weight gain intensity and sugar content [2-4]. Significant crop losses (especially in recent years) are caused by the diseases of the root system (root rots) and leaves (necrotic spots), the pathogens of which may later cause clamp rots [5, 6], as well as by the widespread use of herbicides in the

plant vegetation period [2]. In this connection, it continues to be very important to select various environmentally safe bioregulators having a positive influence on the phytosanitary condition of sugar beet crops and increasing the degree of plant cell homeostatic state and tolerance under stress conditions [2, 3].

As compared to chemical pesticides, microbial biopreparations used for plant enhancement and protection against adverse environmental factors have a number of advantages associated with their environmental safety and systemic immunomodulatory action [7-11]. Along with low toxicity, biopreparations based on living bacterial cultures are characterized by a polyfunctional effect and a broad spectrum of action with regard to various plants and pathogens, and are not expensive [12, 13]. It is reported that associative microorganisms perform a series of functions which are useful for the host plant, particularly, they promote plant growth and development (synthesis of hormones, vitamins), facilitate biocontrol of pathogens (synthesis of antibiotics, induction of systematic resistance), and increase the intensity of root assimilation of nutrients including nitrogen, phosphorus and potassium [14-19]. Interaction with the microbial community of the rhizosphere has a positive influence on the plant, improving its resistance to biotic [15, 16, 20, 21] and abiotic environmental factors [22-26]. In particular, it is known that biopreparations based on endophytic bacteria *Bacillus* Cohn improve the adaptive potential of wheat plants under abiotic stresses [22, 25, 27] and have a favorable influence on plant immunity [12, 20, 28, 29]. Plant treatment with biological preparations based on symbiotic and endophytic microorganism strains with a set of properties improving the microbial diversity of the rhizosphere is a promising way to increase the productivity of agricultural crops [9, 13, 30, 31]. However, in spite of numerous studies, the sequence of plant reactions of the stress resistance induced by bacillar biopreparations is not completely understood and requires further investigation. In addition, there are limited data on their influence on sugar beet plants in conditions of various stress factors, in particular, pathogen and herbicide pressing, which makes it difficult to more effectively use the already existing microbial biopreparations for the purpose of targeted control of plant resistance.

The purpose of this work was to assess the influence of biopreparations based on *Bacillus* Cohn and their metabolites on the structure of the complex of pathogenic micromycetes in the plant rhizosphere, on enzymatic activity in soil and leaves, as well as on the dynamics of saccharose accumulation in the root crops of the sugar beet and their productivity under the influence of pathogenic soil microflora and herbicides.

Technique. Field trials were carried out in 2010-2011 on crops of the sugar beet (*Beta vulgaris* L.) of KWS hybrid (Singenta LLC, Russia) under the conditions of the Pre-Ural steppe zone of the Republic of Bashkortostan (Chishmy Agroinvest LLC). The soil characteristics of the trial field were as follows: leached chernozem, pH 5.4, Hg at 5.64 meq/100 g of soil, humus content of 8.72 %, phosphorus and potassium level at 24.8 and 30 mg/100 g of soil, respectively. The sugar beet cultivation technology was the same in all trial variants and corresponded to the one commonly used in the region. Crop protection against weeds was provided by triple treatment with a complex of herbicides Lontrel-300 (0.3 l/ha; Avgust, Russia), Betanal 22 (1.5 l/ha; Bayer CropScience, Russia) and Fusilade Forte (1 l/ha; Singenta LLC, Russia). The trials were established in compliance with procedural guidelines [32]. The following preparations were used: Phytosporin-M (rate of application at 1 l/ha, endophytic strain of *Bacillus subtilis* 26D as active agent; NVP BashInkom, Russia), Vitaplan (40 g/ha, mixture of *Bacillus subtilis* strains; CJSC Agrobiotekhnologiya, Russia), Albit (40 ml/ha; poly-β-hydroxybutyric acid produced by soil bacteria *Ba-*

cillus megaterium and *Pseudomonas aureofaciens*; NPF Albit LLC, Russia). The preparations were applied in tank mixtures with herbicides two times (1st and 2nd treatments at the stages of 2-3 and 4-6 pairs of true leaves, respectively). In each trial variant the area of production plots was 0.5 ha, 3 replicates; and the area of record plots was 25 m^2, 4 replicates. Assessments were made at the stages of 2-3 and 4-6 pairs of true leaves.

Isolation and quantitation of microorganisms on trial plots were carried out in accordance with a method for soil suspension inoculation on solid agarized media [33]. Species of micromycetes were identified using guides [34, 35]. Specific names of fungi were clarified using the updated lists published in the Index Fungorum database (http://www.indexfungorum.org).

On the days 4 and 10 after the start of the experiment, the part of leaves from the test plants was fixed in liquid nitrogen for further biochemical studies. The samples were triturated in porcelain mortars in 0.05 M Na-phosphate buffer (PB, pH 6.2) (1:5 weight/volume) and, after extraction of proteins for 60 min at 4 °C, centrifuged for 10 min at 15,000 g.

The activity of peroxydase in aliquots was determined using a micromethod. An aliquot of enzyme sample (0.075 ml) prediluted in 0.01 M Na-phosphate buffer (at sample to buffer rate of 1:50) and 0.025 ml of 0.5 mg/ml o-phenylenediamine solution was added in each well of flat-bottom immunology plates. After addition of 0.025 ml of 0.016 % H_2O_2, staining was stopped after 2 min by adding 0.05 ml of 4 N H_2SO_4. The plate was scanned at λ = 490 nm on an IFA-Reader spectrophotometer (Poland) [14]. The activity of hydrolases was determined in the plant extracts after homogenization in buffered solution (0.05 M Tris-HCl, pH 8.0, and 0.05 M NaCl) for 12 hrs in the ratio 1:30 (sample weight, g/extractant volume, ml) at 4 °C. The activity of proteases was taken into account using gelatin plates as recommended [36]. A layer of agarose gel was formed on the gelatin plate surface; 5 mm diameter wells were cut out in it; then, their edges were melted, and 50 μl of protein extract was added into each well. Upon completion of incubation, agarose gel was removed, and the plate was washed with running water in order to remove the hydrolyzed areas which then were easily seen against a dark background in the form of round light spots. The circle diameter was used as a basis for estimating the size of the hydrolyzed area, and the activity was recounted from the curve of the trypsin standard solutions pretitrated on the same plate. The lower limit of activity quantification for the commercial trypsin preparation was 0.5-1.0 μg. The activity of catalase, dehydrogenase, polyphenoloxidase, peroxydase, protease and urease in soil from the zone adjacent to the rhizosphere was measured in extracts by the method proposed by the F.Kh. Khaziyev (37); saccharose content in sugar beet root crops was determined using a polarimeter (P161-M, Russia) by the cold water digestion method [38].

Statistical processing of the obtained results was carried out using the analysis-of-variance method [39]. The calculations of the experimental data was performed using the Microsoft Excel software. The histograms show average values and their standard deviations.

Results. High yields of agricultural crops largely depend on both the agrochemical condition of soil and the presence of appropriate microflora in it [30]. Thus, the microbial community in the rhizosphere of the plant improves its resistance to biotic [15] and abiotic stress factors [8, 24], but the large number of pathogenic and opportunistic microorganism species produces a negative effect due to development of various diseases leading to reduction in productivity and quality of root crops, as well as their storage life.

The significant decrease in sugar beet productivity due to the diseases of

the root system (rots) and leaves (necrotic spots) has been noted in recent years. In future, the corresponding pathogens may cause clamp rots [4, 6]. The works to reduce losses due to diseases, in particular, root rots, with the use of microorganism-based biopreparations are being performed very actively now [2, 3, 6, 40], and a special emphasis is put on the study of the microbiological processes occurring in soil and plant rhizosphere. In this regard, the interest to the rhizosphere as a zone of maximum accumulation and functional activity of soil microorganisms becomes more profound [30, 41, 42]. Our study of the influence of microbial biopreparations Phytosporin-M, Vitaplan and Albit on a complex of micromycetes in the sugar beet rhizosphere has demonstrated that, at the stage of 2-3 pairs of true leaves, it was possible to extract up to 38 microfungus species from the mentioned soil zone, and 28 strains of them were identified as representatives of three genera, *Aspergillus*, *Penicillium* and *Fusarium* (5, 20 and 3 strains, respectively). The genera *Alternaria*, *Rhizopus* and *Trichoderma* were significantly less represented (Table 1).

1. The change of pathogenic mycobiota composition in the rhizosphere of sugar beet (*Beta vulgaris* L., KWS hybrid) root crops after treatment with biopreparations against the use of herbicides (Chishmy Agroinvest LLC, Republic of Bashkortostan, 2010-2011)

Species	Abundance of species, unit/frequency, %			
	control	Phytosporin-M	Albit	Vitaplan
Single treatment at 2-3 true leaves				
Alternaria tenuis Nees[1]	12.1/40	–	–	5.3/20
Aspergillus niger Tiegh.[1]	9.1/40	–	–	5.3/20
Aspergillus parvulus G. Sm.[2]	3.0/20	–	–	-
Fusarium oxysporum var. *Orthoceras* Appel & Wollenw.[2]	6.1/40	–	–	5.3/20
Fusarium solani Appel[2]	–	–	–	2.6/20
Fusarium solani var. *agrillaceum*[2]	6.1/40	–	–	–
Penicillium aurantiogriseum Dierckx[1]	15.2/80	–	–	–
Penicillium glabrum Wehmer[2]	9.1/40	3.1/20	–	–
Penicillium citrinum Thom[2]	–	–	8.1/40	–
Penicillium solitum var. *crustosum* Thom[1]	–	–	–	2.6/20
Rhizopus microsporus Tiegh.[2]	6.1/20	–	–	–
Double treatment at 6-8 true leaves				
Alternaria tenuis (Fr.) Keissl.[1]	7.0/40	5.9/20	–	–
Aspergillus flavus Link[2]	3.5/20	–	–	–
Aspergillus niger Tiegh[1]	10.5/80	3.0/20	2.6/20	2.8/20
Fusarium oxysporum Schltdl.[2]	3.5/40	–	–	–
Fusarium solani var. *agrillaceum* C. Booth[2]	7.0/60	5.9/20	–	–
Penicillium aurantiogriseum Direckx.[1]	14.0/80	–	5.1/40	5.6/20
Penicillium glabrum Wehmer[2]	19.3/100	–	–	–
Penicillium solitum var. *crustosum* Thom[1]	–	3.0/20	–	–

Note: Dashes mean that species was not found; 1 — sugar beet Pythium disease pathogen according the classification proposed by A.V. Kornienko [28], 2 — clamp rot pathogen.

As may be inferred from the presented data (see Table 1), 8 species of pathogenic micromycetes were found in soil from control plots which were not treated with the biopreparations; according the classification proposed by A.V. Kornienko [5], they include 3 sugar beet Pythium disease pathogens (*Penicillium aurantiogriseum*, *Alternaria tenuis*, *Aspergillus niger*) and 5 root system disease pathogens (clamp rot) (*Penicillium glabrum*, *Fusarium solani* var. *agrillaceum*, *F. oxysporum*, *Aspergillus parvulus*, *Rhizopus microsporus*). *Alternaria tenuis*, *Aspergillus niger* and *Penicillium glabrum* were dominant species. It should be noted that the last two of them are strong toxin producers: they release substances which can weaken and subsequently kill plants and cause the reduction of root crop quality [43].

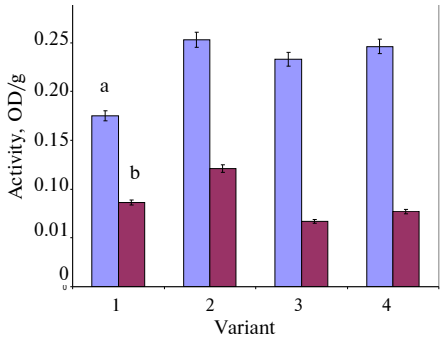
Sugar beet plant treatments with biopreparations Phytosporin-M, Albit and Vitaplan promoted the change of microfungus species composition in the rhizosphere of the sugar beet. Thus, after single treatment of plants, pathogenic

fungi in the root crop rhizosphere were represented by only one species, *Penicillium glabrum,* in case of Phytosporin-M, *P. citrinum* in case of Albit, and 5 species (*Alternaria tenuis*, *Aspergillus niger*, *Fusarium oxysporum*, *F. solani* and *Penicillium solitum*) in case of Vitaplan (see Table 1). It is also important that there were no Pythium disease pathogens on the plots treated with Phytosporin-M and Albit.

The mycobiota species composition in the rhizosphere of sugar beet control plants at the stage of 6-8 leaves remained almost unchanged, except for the appearance of the species *Aspergillus flavus* Link causing clamp rot (see Table 1). At the same time, only two Pythium disease pathogens, *Aspergillus niger* and *Penicillium aurantiogriseum*, were revealed after double treatment of crops with biopreparations Albit and Vitaplan, and their occurrence rate was 4 times less as compared to the control. Three Pythium disease pathogens and one clamp rot pathogen were found in case of treatment with Phytosporin-M. In this trial variant, the abundance of species and their occurrence rate were 1.5-3.0 times less and 2.0-4.0 times less, respectively, than in the control. Thus, plant treatments with the biopreparations reduced the abundance of species and occurrence rate of pathogenic micromycetes in the rhizosphere of the sugar beet.

The activation of beneficial soil microbiota under the influence of biopreparations, undoubtedly, leads to changes in the biochemical condition of the soil, in particular, its enzymatic activity, which may have a significant impact on plant productivity [19]. Earlier, we demonstrated that sugar beet plant treatments with biopreparations Albit and Phytosporin-M had a positive influence on the activity of oxidoreductases and hydrolases in the rhizosphere of the sugar beet [42]. In the experiment, the activity of catalase reflecting the intensity of microbiological processes in soil was 1.4 and 3.7 times higher than that of the control after the first treatment with Phytosporin-M and Albit, respectively; after the second treatment, it was 1.4 and 1.2 times higher, respectively.

Plant treatments with Phytosporin-M and Vitaplan stimulated the development of agronomically beneficial microbiota under sugar beet crops, leading to increase in the total quantity of microorganisms consuming organic nitrogen forms, as well as increase in the number of microorganisms breaking up complex polymeric compounds and the number of nitrogen-fixing bacteria [42]. The obtained results are consistent with the published data on mechanisms for induction of productive qualities of plants under the influence of microorganisms [30, 44].



The dynamics of peroxidase activity in sugar beet (*Beta vulgaris* L., KWS hybrid) leaves under plant treatment with biopreparations against the use of herbicides: a — 1st treatment, b — 2nd treatment (Chishmy Agroinvest LLC, Republic of Bashkortostan, 2010-2011).

It is known that, in response to harmful impacts, an oxidative stress is developed in plants, and peroxidase plays an important role in its neutralization [14, 16]. In all variants, statistically significant activation of peroxidase in leaves was observed after plant treatment with biopreparations (Fig.). Earlier we noted such increase in peroxidase activity for wheat and potato plants under the influence of biopreparations [12, 14]. After reapplication of biopreparations, decrease in peroxidase activity was observed in all samples, except for the variant with Phytosporin-M, where this parameter remained higher than in the control, which is probably indicative of the long-lasting action of the preparation on the anti-

oxidant system of the plant.

The formation of protective mechanisms against pathogens in plants largely depends on hydrolytic enzymes (proteases, amylases, pectinase) and their inhibitors. Earlier, in model experiments on potato tubers, we demonstrated that prolonged activation of protease inhibitor synthesis improved the protection of the tubers treated with Phytosporin-M against penetration and development of pathogenic microorganisms [36].

Sugar beet plant treatment with biopreparations reduced the activity of hydrolases in leaves (Table 2) and, conversely, increased the activity of their inhibitors, which is indicative of induction of protective reactions against pathogens in plants; maximum values were registered in case of treatments with biopreparations Phytosporin-M and Albit.

2. The dynamics of hydrolytic enzyme activity (IU/g fresh weight) **in sugar beet (*Beta vulgaris* L., KWS hybrid) leaves after treatment with biopreparations against the use of herbicides** ($X \pm x$, Chishmy Agroinvest LLC, Republic of Bashkortostan, 2010-2011)

Variant	4th day after treatment			10th day after treatment		
	protease	amylase	pectinase	protease	amylase	pectinase
Single treatment with herbicides and biopreparations						
Control	6.43±0.23	7.12±0.14	4.46±0.12	6.67±0.10	8.22±0.11	5.76±0.24
Phytosporin-M	3.12±0.13	2.57±0.23	2.76±0.12	2.77±0.12	3.68±0.13	4.11±0.15
Albit	2.89±0.13	3.03±0.21	3.33±0.23	3.67±0.24	3.88±0.22	3.58±0.12
Vitaplan	3.67±0.14	3.43±0.12	3.86±0.21	3.21±0.11	3.76±0.10	3.34±0.21
Double treatment with herbicides and biopreparations						
Control	5.78±0.22	6.65±0.23	4.03±0.31	6.77±0.12	8.72±0.11	5.76±0.25
Phytosporin-M	3.23±0.10	3.21±0.12	3.03±0.32	2.77±0.19	3.68±0.22	3.21±0.17
Albit	3.43±0.09	2.67±0.14	2.78±0.22	3.57±0.12	3.58±0.24	3.58±0.21
Vitaplan	2.48±0.13	3.26±0.12	3.24±0.12	3.21±0.12	3.76±0.13	3.34±0.12

The resultant indicators of physiological-biochemical and microbiological processes in plants throughout the vegetation period are the indices of productivity and quality of sugar beet root crops. Thus, the application of the studied biopreparations led to increase in root crop productivity by 1.6-5.0 t/ha as compared to the control and helped in more intensive accumulation of sweeteners in the crops. Whereas the saccharose content in the control achieved 16.2 % by the time of harvesting, it ranged within 17.0-18.8 % between the variants of the trial, and the highest values were obtained in case of double treatment of crops with Phytosporin-M.

So, the obtained data support the conclusion that the biopreparations based on *Bacillus* Cohn and their metabolites improve the yielding capacity and quality of sugar beet root crops by the correction of microflora composition in the rhizosphere, in particular, due to reduction in the number and species diversity of pathogenic micromycetes, which, in turn, reduces the probability of plants being infected by root system and leaf disease pathogens. In addition, an important contribution to the formation of productivity and the growth of plant resistance to adverse environmental factors (in particular, to pathogen and herbicide pressing) is probably made by the increase of enzymatic activity both in the rhizosphere and leaves of the sugar beet due to the influence of biopreparations. In general, it can be concluded based on the results of the study that the application of such biopreparations as Phytosporin-M, Albit and Vitaplan leads to reduction in plant disease incidence rate and increase in productivity and quality of sugar beet root crops under the influence of pathogenic micromycetes and herbicide pressing. In this regard, the most profound effect was noted for Phytosporin-M, which allows us to recommend using it in technologies for intensive cultivation of the sugar beet.

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PHOTOSYNTHESIS AND PRODUCTIVITY OF BASIL PLANTS (*Ocimum basilicum* L.) UNDER DIFFERENT IRRADIATION

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Abstract

Improving the efficiency of growing plants in phytotrons is largely linked to the introduction of advanced technologies, providing the optimization of the light conditions. The use of modern light sources such as light emitting diodes (LEDs) or induction lamps can reduce the energy consumption for growing plants due to the high light output, long work and control of the spectrum of irradiation. Comparative studies of growth processes and activity of the photosynthetic apparatus of plants of Basil (*Ocimum basilicum* L.) variety Ararat, when using LEDs and induction lamps with an energy capacity of 64 and 150 W, respectively were done. The light intensity was 80-85 μmol photons · m^{-2} · s^{-1} under LEDs white light and 240-260 μmol photons · m^{-2} · s^{-1} under induction lamps. CO_2 gas exchange, the content of pigments and growth processes in plants grown in hydroponic conditions were estimated. The rate of photosynthesis under induction lamp was more than 2 times higher than under LEDs (2.6±0.4 and 1.2±0.3 μmol CO_2 · m^{-2} · s^{-1}, respectively), although there was a slight decrease in the content of the chlorophylls (a + b) to 0.71±0.01 mg/g dry weigh compared to 0.83±0.03 for LEDs. More than twofold increase in the rate of photosynthesis did not result in the same increase in the accumulation of plant biomass that may be connected with different light saturation of growth processes and photosynthesis. The efficiency of biomass accumulation per 1 W of energy power for a period of 40 days under LEDs was 1.7 times higher than under the irradiation of induction lamp. Significant difference in photosynthetic efficiency was not detected. At elevated concentrations of CO_2 the rates of photosynthesis were comparable as a result of higher values of the quantum yield of photosynthesis, activity ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) and the efficiency of carboxylation in LEDs plants. The investigation of structural and functional parameters of the photosynthetic apparatus and growth processes under the action of different light intensity showed the complex nature of the changes of some processes during long-term exposure to light of different intensity and spectral composition.

Keywords: basil, photosynthesis, LED.

Numerous basil (*Ocimum basilicum* L.) varieties enjoy well-deserved popularity in Russia. The leaves and stems of some basil species contain flavors and therefore are used both fresh and dried as a spice. At the same time, this plant is valued as a source of antioxidants contributing to human organism protection under various unfavorable conditions [1].

Improving the efficiency of plant cultivation under artificial lighting is largely linked to the introduction of the advanced technologies providing, in particular, the optimization of light conditions. Luminaries with sodium and mercury lamps are widely used now; they have maximum absorption in the area of 550-600 and 450 nm [2, 3]. The use of modern light sources, such as Light Emitting Diode (LED) illuminators, makes it possible to drastically reduce energy requirements for plant cultivation due to high luminous efficiency, long operating life and adjustable radiation spectrum. LEDs can serve as supplementary illuminators or completely replace conventional light sources in plant cultivation [4, 5]. Positive results with the use of LEDs have been obtained for various crops [6-9].

Induction lamps also become more common in plant growing under artificial lighting. The operating principle of these energy-saving light sources is based on electromagnetic induction and gas discharge for generation of visible light. The key difference of these lamps from the existing gas discharge lamps is in the electrodeless design, which significantly extends their operating life. As compared to diode illuminators (operating life of 50,000 hours), the induction lamp is designed for 100,000 hours of operation. A technique has been developed for the manufacture of special high-efficiency induction lamps with high luminous flux in the red and blue spectral regions needed for plants.

The reported data are mainly referred to the study of the effect of LEDs with various spectral distributions on growth processes and photosynthetic characteristics [10-12], as well as the influence of light of various intensity on the production process of plants [13]. Nevertheless, in spite of significant interest in the problem, there is still a lack of information about the effect of modern illumination sources on plant growth and development, photosynthetic apparatus activity and, finally, yielding capacity.

The purpose of this work was to carry out a comparative study of growth processes and photosynthetic apparatus reactions for basil plants in case of the use of a LED illumination source and an induction lamp.

Techniwue. The experiments were carried out on basil (*Ocimum basilicum* L.) plants of variety Ararat. Seeds were preliminary dipped in weak solution of potassium permanganate for 10 min and solution of biopreparation Albit (NPF Albit LLC, Russia) (1 g/ml) for 3 h and then sown directly in hydroponic units developed by us (All-Russia Research Institute of Biotechnology).

The plants were cultivated using nutrient solution of our own formulation including all necessary macro- and microelements. Room air temperature was maintained at 24-26 °C. Relative air humidity ranged from 60 to 75 %. Concentration of CO_2 during cultivation was 380-400 μmol · mol^{-1}. Full plant growth cycle from sprouts to harvesting was 38-40 days on average.

The light sources were induction and LED illuminators with energy capacity of 150 and 64 W, respectively. The light intensity of the white-light LED illuminator (Focus LLC, Russia) and induction lamp (GK BSKA LLC, Russia) was 80-85 μmol photons · m^{-2} · s^{-1} and 240–260 μmol photons · m^{-2} · s^{-1}, respectively. Each variant included 15 plants for analyses.

The rate of CO_2 gas exchange in leaves was measured in situ, at plant growth sites, using an LCPro$^+$ portable photosynthetic system (ADC BioScientific Ltd., Great Britain). In order to plot carbon-dioxide curves, carbon dioxide concentration in air was set within 0-1,600 μmol CO_2 · mol^{-1} using the microprocessor of the gas analyzer. The carbon-dioxide curve of CO_2 gas exchange was analyzed based on the model proposed by G.D. Farquhar et al. [14] in modification [15-17]. The dependence of apparent photosynthesis rate (Φ_B) on light intensity was determined within the range of Photosynthetically Active Radiation (PAR) from 0 to 1,600 μmol photons · m^{-2} · s^{-1} at CO_2 concentration in air equal to 400 μmol · mol^{-1}. In order to determine the light dependence of CO_2 gas exchange in leaves, light intensity was gradually increased from 0 to 1200 μmol photons · m^{-2} · s^{-1}. The light curve was approximated using the model proposed by J.L. Priol and P. Chartier (18).

The pigment content was estimated after homogenization of leaves in a porcelain mortar with addition of $CaCO_3$ and pigment extraction with 80 % acetone. The chlorophyll absorption was registered using a Spekol-11 spectrophotometer (Carl Zeiss, Germany) at wave lengths of 662 nm (chlorophyll a), 644 nm (chlorophyll b) and 470 nm (carotenoids) and was calculated as described [19].

A total of 15 plants in each variant were collected in order to determine growth parameters. The plants were separated into aboveground and underground parts, weighed and dried at 70 °C.

The tables and figures show arithmetic mean values with a standard error. The significance of differences was determined based on Student's *t*-test at P = 0.95.

Results. The basil plants grown under illumination with the induction light source were characterized by greater activity of the photosynthetic apparatus. The photosynthesis rate for the leaves of 30-day plants was more than 2 times higher than that for the plants grown under the LED illuminator (2.6±0.4 and 1.2±0.3 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively).

In the conditions which did not limit the process with regard to CO_2 (at increased CO_2 concentration in air), the photosynthesis rate was the same (Table 1).

1. The parameters of approximation of carbon-dioxide curves for CO_2 gas exchange in the leaves of the basil (*Ocimum basilicum* L.) of variety Ararat with the use of the model proposed by G.D. Farquhar et al. [9] ($X \pm x$)

Parameter	Induction lamp	LED's
Maximum CO_2 absorption rate, μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$	8.78±2.53	8.02±1.61
CO_2 dark release rate, μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$	1.05±0.04	3.27±0.80
Maximum carboxylation rate, μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$	7.20±0.82	9.65±0.40
Carboxylation efficiency, μmol $CO_2 \cdot m^{-2} \cdot s^{-1} \cdot Pa^{-1}$	0.10±0.04	1.74±0.30
Light-saturated electron transport rate, μmol $\cdot m^{-2} \cdot s^{-1}$	21.6±0.5	14.9±2.2
Triosophosphate utilization rate, μmol $\cdot m^{-2} \cdot s^{-1}$	4.32±0.60	1.74±0.40
Carbon dioxide compensation point, μmol $CO_2 \cdot mol^{-1}$	124±10	230±12

Note: The energy capacity of the induction lamp and LED illuminator is 150 W and 64 W, respectively. The light intensity of the white-light LED illuminator (Focus LLC, Russia) and induction lamp (GK BSKA LLC, Russia) was 80-85 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and 240-260 μmol photons $\cdot m^{-2} \cdot s^{-1}$, respectively.

The same photosynthesis rate at increased carbon dioxide concentration (1,200 μmol $CO_2 \cdot mol^{-1}$) could be associated with higher quantum yield for plants under the LED illuminator (i.e. 0.088 μmol $CO_2 \cdot \mu mol^{-1}$ photons against 0.056 μmol $CO_2 \cdot rmol^{-1}$ photons for the plants grown with the induction lamp). Thus, one of explanations with regard to the observed comparable values of plant photosynthesis rates in both variants may be the increase in quantum yield at higher CO_2 concentration in the intercellular space of plants under the LED illuminator. In addition, the light of different intensity and spectral distribution had different effects on the activity of light and dark reactions in the photosynthetic apparatus of plant leaves. Under LED illumination, maximum carboxylation rate and carboxylation efficiency were higher than similar parameters for the plants grown under the induction lamp, although electron transport and triosophosphate utilization rates turned out to be lower (see Table 1).

As a result, in spite of relatively low values of light intensity under LED illumination as compared to the induction lamp, photosynthesis rates of these plants at increased CO_2 concentration were comparable due to higher quantum yield of photosynthesis, as well as high activity of ribulose bisphosphate carboxylase/oxygenase (RBPC/O) and carboxylation efficiency in case of the plants illuminated by LED's. Along with changes in the functional activity of the photosynthetic apparatus under LED illumination conditions, some increase in the sum of chlorophylls a + b took place (0.83±0.03 as compared to 0.71±0.01 mg/g dry weight under the induction lamp). The ratio of chlorophylls a + b was also higher (4.73±0.31 under the LED illuminator and 4.47±0.22 under the induction lamp). At the same time, reduction in carotenoid content was observed: 0.12±0.01 as compared to 0.16±0.01 mg/g dry weight under the induction lamp.

In spite of some decrease in chlorophyll content when plants were cultivated under the induction lamp, biomass accumulation in this case was higher

2. The accumulation of fresh and dry biomass and growth parameters for the variety Ararat basil (*Ocimum basilicum* L.) plants grown under LED and induction illuminators

Variant	Weight, g						Plant height, cm	Number, pcs		Root length, cm
	leaves		stems		roots			leaves	nodes	
	fresh	dry	fresh	dry	fresh	dry				
Induction luminary	1.538±0.134	0.143±0.024	0.531±0.056	0.041±0.005	0.667±0.159	0.057±0.009	17.43±1.19	28.27±3.82	5.53±0.23	13.73±1.80
White LEDs	0.997±0.106	0.102±0.014	0.545±0.112	0.039±0.011	0.615±0.171	0.048±0.008	14.17±0.70	23.27±0.93	5.40±0.16	8.51±0.74

N o t e: The energy capacity of the induction lamp and LED illuminator is 150 W and 64 W, respectively. The light intensity of the white-light LED illuminator (Focus LLC, Russia) and induction lamp (GK BSKA LLC, Russia) was 80-85 μmol photons · m^{-2} · s^{-1} and 240-260 μmol photons · m^{-2} · s^{-1}, respectively

than that for the plants grown under LED illumination due to the higher activity of the photosynthetic apparatus, as well as due to the formation of larger leaf surface (Table 2). Alongside with that, more than double increase in photosynthesis rate did not lead to equivalent increase in biomass accumulation in plants, which may be associated with different light saturation of growth processes and photosynthesis as noted as early as in the study by N.N. Protasova and V.I. Kefeli [20]. In addition, relatively higher plant productivity at low light intensities may be explained by the X.G. Tooming's concept [21] regarding maximum productivity at adaptation radiation intensity when maximum efficiency of incident radiation utilization is observed.

In assessing the effect of various sources of illumination, it is important to determine their energy efficiency. In our situation, higher efficiency of light energy utilization by plants was observed in case of cultivation under the LED source of illumination.

So, the following indices were obtained with the use of the induction lamp (240 μmol photons $\cdot m^{-2} \cdot s^{-1}$) and LED illuminator (80 μmol photons $\cdot m^{-2} \cdot s^{-1}$): plant biomass was 1,538±134 and 997±106 mg, respectively, efficiency of leaf biomass accumulation per 1 W for 40 days was 7.70±0.7 and 13.3±1.4 mg, respectively, photosynthesis was 2,600±400 and 1,200±300 nmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively, and photosynthetic efficiency of energy capacity utilization (per 1 W) was 13±2 and 16±4 nmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively. I.e. the efficiency of leaf biomass accumulation per 1 W of energy capacity for a period of 40 days under the LEDs was 1.7 times higher than under the induction lamp. We have not found significant differences in photosynthetic efficiency of energy capacity utilization.

Thus, we have revealed the regularities of productivity formation in basil plants under different illumination conditions using the LEDs and induction lamp. Increase in light intensity due to the use of the induction lamp led to greater biomass accumulation as compared to that for the LED source of illumination, however, the energy efficiency in the latter case was higher. The study of the structural and functional indicators of the photosynthetic apparatus and growth processes depending on lighting conditions has shown a complex behavior of the part of them during long-term exposure to the light of different intensity and spectral distribution.

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