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PLANT DEFENSINS: BIOLOGICAL FUNCTION, MECHANISMS OF ACTION AND METHODS OF ANALYSIS (review)

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Abstract

One of the actual problems of modern agriculture is crop loss due to various biotic and abiotic factors. In plants there is a multicomponent protection system, including the formation of protective barriers, activation the reaction of hypersensitivity and synthesis of antimicrobial peptides, which are low molecular weight compounds showing broad spectrum activity against fungi, bacteria and viruses. This group consists of several groups of peptides, including defensins, which are one of the most common classes of antimicrobial peptides and are detectable in all living organisms. Defensins are small (45-54 amino acids), cysteine-rich peptides involved in a different protective responses (B.P. Thomma et al., 2002). Genes which are coding plant defensins are expressed in different organs of plants, where their products are necessary for biotic and abiotic stresses. Thus, these peptides are extremely important in terms of getting crop lines that are resistant to pathogens and abiotic stresses. Defensins are characterized by a strong sequence variability that seems to correlate with a variety of mechanisms of action of these peptides that can induce pathogen's cell death by penetrating into a cell or by being on its surface (T.M. Shafee et al., 2016). Most of the plant defensins are characterized by the antifungal activity. Some defensins have antibacterial activity, which may be combined with activity against fungi. For a small number of plant defensins their participation in the processes of resistance to heavy metals, cold stress, drought, salinity, and in the development process is indicated. Modern approaches of molecular and computational biology allow an effective search for new forms of defensins activity by studying the wild, non-model plant objects. The development of next-generation sequencing methods («Next Generation Sequencing») make possible the intensive study of the transcriptomes of such objects. However, the correct annotation of the sequences of peptides, characterized by small size and high variability, can be done by usage the special programs, such as SPADA (Small Peptide Alignment Discovery Application) (P. Zhou et al., 2013). SPADA makes multiple sequence alignment of all known paralogous genes within a gene family and builds a predictive model for the search of new members of the same family. Prediction of newly identified active defensins and identification of conserved amino acids can also be performed by computational biology methods. An approach based on a multiple sequence alignment and subsequent cluster analysis allows dividing defensins into groups with similar functional activity (N.L. van der Weerden et al., 2013). Thus, the combination of modern methods of molecular and computational biology allows carrying out the study of this group of protective peptides with high efficiency.

Keywords: defensins, cysteine-rich peptides, defense reactions, biotic and abiotic stress, next-generation sequencing

Various damages caused by insects, fungi, viruses or physical factors lead to crop losses. To fight against negative environmental exposure, plants use a multicomponent system that includes a hypersensitivity response, consolidation of protective barriers using cell wall components, activation of the production of immune proteins and antimicrobial peptides [1]. Antimicrobial peptides are the low-molecular compounds (12-95 amino acids), which are synthesized in the cells of all living organisms. The structure and action mechanisms of antimicrobial peptides are much diversified. Plant antimicrobial peptides are divided into several families — cyclotides, defensins, thionines, lipid-carrying proteins, hevea-like proteins, etc. [1-3].

Plant defensins are small cysteine-rich peptides consisting of 45-54 amino acids. They are located in majority of the Plant Kingdom. Structurally and functionally, defensins of the plants, insects, mammals and fungi are similar [4-6]. Genes of plant defensins are expressed in different organs, e.g. seeds [7, 8], leaves [9], roots [10], flowers [11, 12] and symbiotic tubercles [13]. There is both constitutive and inducible expression of genes, encoding plant defensins [11, 14]. The main function of defensins is to inhibit fungal disease [8, 11, 15], but for some peptides there are antibacterial activity [16, 17], inhibition of trypsin [18], and participation in the formation of resistance to heavy metals [19], cold stress [20, 21], drought [22], salinity [23, 24] and in developmental processes [25].

Structure of plant defensins. Modern studies show that, based on the structural organization, defensins of animals, plants and fungi can be divided into two superfamilies (cis- and trans-defensins) with independent evolutionary descent [26]. In cis-defensins, two parallel disulfide bridges bind the α-spiral to β-bands, while in trans-defensins similar disulfide bridges are oppositely oriented and are connected with different structural elements. Plant defensins are the main members of cis-defensins family. Using nuclear-magnetic resonance spectroscopy, it was shown that in plant defensins the basis of the molecule is formed by a three-layer element consisting of one α-spiral connected with cysteine bridges to β-bands (Fig. 1) [7, 8, 27, 28].

Fig. 1. Amino acid sequence of mature defensin MtDef4. The lines connecting the cysteine residues are disulfide bridges. The spiral and arrows indicate the location of the α-spirals and β-bands, respectively. Conserved cysteine residues are in bold.

All plant defensins are formed during the processing of precursor proteins, which may not be the same in different species. Defensins with 8 or 10 conserved cysteine residues have been identified in plant organisms. It is supposed that such residues are necessary to maintain the conformation of the molecule, whereas the revealed high variability in functional regions of defensins ensures their specificity related to the targets and determines the diversity of functions of this protein family [26, 29].

Functional characteristics of some defensins. For most known defensins, antifungal and (or) bactericidal activity is specific. Defensin NaD1 was segregated from the outer cell layers of the decorative tobacco flower *Nicotiana alata*, which correlates with its protective function in the reproductive organs. In vitro studies have shown that NaD1, presented as a dimer, suppresses the growth of *Botrytis cinerea* and *Fusarium oxysporus* [11]. ω-Chordothionine and γ-chordothionine, found in the endosperm of barley grains, inhibit translational activity in both eukaryotic cell-free mammalian systems (lysates of mouse liver cells and rabbit reticulocytes) and prokaryotic systems (*Escherichia coli*), but

do not exert the same effect in relation to translation processes in plant systems [30]. AlfAFP from the seeds of *Medicago sativa* is antifungal peptide and provides resistance to fungal pathogen *Verticillium dahlia* in transgenic potato plants [11, 31]. Defensins from *Raphanus sativus* (RsAFP) are accumulated as the seeds ripen and are released after the integrity of seed membranes is destroyed to create a microenvironment that suppresses the growth of fungi, with a decrement in the elongation of hyphae and their enhanced branching [8, 32]. MsDef1 from *M. sativa* seeds significantly inhibits the growth of *F. graminearum* in vitro [33]. These defensins are examples of peptides with 8 conserved cysteine residues. Another subclass (with 10 conserved cysteine residues) includes defensins PhD1 and PhD2 (*Petunia hybrida* defensin 1 and 2, respectively) isolated from petunia flowers. In their three-dimensional structure, there is an additional disulfide bond [11, 34], which does not change the conformation as compared to other defensins, but probably gives additional thermodynamic stability [34]. In vitro experiments, PhD1 and PhD2 blocked the growth of *F. oxysporum* and, in a lesser extent, *B. cinerea* [11].

In addition to antifungal activity, some plant defensins have antibacterial properties. Thus, Cp-thionine II, identified in seeds of *Vigna unguiculata*, acts against Gram-positive and Gram-negative bacteria, *Staphylococcus aureus*, *E. coli* and *Pseudomonas syringae* [16]. Fabatins isolated from *Vicia faba* beans also inhibit the growth of various bacteria, but are inactive against yeast *Saccharomyces cerevisiae* and *Candida albicans* [17]. PsD1 (from *Pisum sativum*) is an antibacterial peptide that is localized in nuclei of the treated *Neurospora crassa* cells and interacts with proteins involved in control and termination of cell cycle, for example, cyclin F [35].

It is shown that 8 genes of *M. truncatula MtDefNS* are specifically expressed in nodules formed in symbiosis with *Sinorhizobium meliloti*, and (with the exception of one gene) are in the chromosome 8, which suggests some specialization of these sequences. Also, most of them are clustered phylogenetically and form a separate clade in relation to other classical defensins [13]. However, the specific function of defensins in symbiotic relationships is unknown by now.

For a small number of defensins, participation in plant adaptation to abiotic factors has been described. Thus, increased concentrations of NaCl influenced the change in expression of genes encoding defensins in *Arachis hypogaea* [23] and *Arabidopsis thaliana* [24]. The effect of water deficiency on changes in the expression of genes encoding defensins is shown in *Glycine max* [22, 36]. Cold also affects the expression of defensin genes [20, 21]. Winter wheat has *Tad1* gene, the expression of which is induced specifically under cold stress [21]. The hyper-accumulator of zinc and cadmium, *Arabidopsis halleri*, has gene, encoding defensin AhPDF1.1, which is actively studied [19, 37-40]. Expression of this gene in *A. thaliana* and *S. cerevisiae* leads to an increase in resistance to zinc, but not to cadmium, cobalt, iron or sodium. *A. halleri* shows a zinc dependence of the pool of defensins both at transcript and peptide levels. Compared to *A. thaliana*, *A. halleri* shows an increase in the defensin amount in seedlings [19].

Some defensins are involved in a response to several stressors. In *Brassica rapa*, the expression of the genes encoding defensins BrDLFP and BrBetvAFP was significantly altered at cold stress, drought and salinity [41]. When studying the effect of pre-treatment of arabidopsis plants with nontoxic concentrations of silicon and cadmium on *B. cinerea* infection, the enhancement of expression of the gene encoding defensin PDF1.2 [42] was shown. Expression of the defensin gene NbDef2.2 in *N. benthamiana* increases not only after infection of *Pseudomonas syringae* pv. *tabaci*, but also in wounding and ethylene treatment [43]. Thus, plant defensins can be components of overlapping re-

sponses to abiotic and biotic agents.

Action mechanisms of plant defensins. The strong variability of plant defensin sequences seems to correlate with the variety of their action mechanisms [26]. By now, the action mechanisms of defensins with antifungal activity have been thoroughly studied. Plant defensins can interact with specific binding sites (receptors) — components of the target cell membrane, for example, bacterial lipid II, sphingolipids [44] and phospholipids of fungi [45]. In this case, different plant defensins interact with different classes of sphingolipids [46-49]. Thus, RsAFP2 from the radish binds to the glycosphingolipids of *Pichia pastoris* and *C. albicans* [50]. Defensin NaD1 interacts with various phospholipids, but not with sphingolipids [45]. The presence of specific sphingolipids on the surface of the membrane is necessary to mediate the cell death of fungi, since yeast mutants defective in the genes, involved in the synthesis of sphingolipids, are resistant to the defensins that bind them [47, 48, 51]. After interaction with the receptors, the defensins either penetrate the interior of the fungal cell and interact with intracellular targets, or remain on the surface (for example, as MsDef1) and induce cell death by inducing the signal cascade (Fig. 2) [52]. An example of defensin penetrating cell is MtDef4 (see Fig. 2). In its sequence, the RGFRRR motif is identified, which apparently serves as a translocation signal necessary for penetration into the fungal cell. When replacing this motif with AAAARR or RGFRAA, MtDef4 loses its ability to penetrate the interior of the cell [53]. Nevertheless, there is no evidence that this sequence is in other plant defensins penetrating fungal cells [52].

Recently, more and more information has appeared on peptides that cause cell death, stimulating the production of reactive oxygen species (ROS) [54-56]. The formation of ROS and induction of oxidative stress were observed in target cells at treatment with defensins RsAFP2, DmAMP1 [54], HsAFP1 [57] and NaD1 [55].

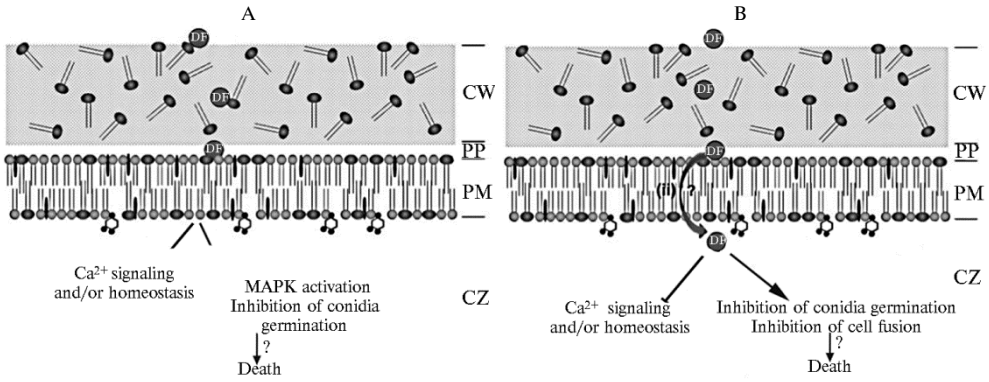

Fig. 2. Suggested mechanisms of anti-fungal action on the example of defensins MsDef1 (A, does not penetrate the fungal cell) **and MtDef4** (B, penetrates into the fungal cell)**:** DF — defensin, CW — fungus cell wall, PP — periplasmic space, PM — plasma membrane, CZ — cytosol, MAPK — mitogen-activated protein kinase (cit. ex 52 with changes).

So far, molecular mechanisms of defensin action in abiotic stress remain unknown. This is due to a small number of studies (when compared to the role of defensins in biotic stress), the complexity of such experiments and the fact that defensins vary greatly in both structure and action mechanisms. The works are mostly devoted to the study of activation of certain genes encoding defensins and discussion on their putative role in the resistance to certain factors [39].

Use of sequencing and bioinformatics methods for the study of defensins. The protective properties manifested by defensins make them helpful in crop selection for resistance to various pathogens. Seeking for new

forms of activity of defensins in wild plants that do not belong to model ones is also prospective. With the development of NGS (next generation sequencing), it became possible to quickly identify genomes and/or transcripts sequences of many plant species, including non-model ones [58, 59]. At the same time, the problem of the annotation and functional characteristics of sequences obtained becomes urgent [60]. Due to their high divergence in defensins of one group and a limited number of experimentally detected peptides, the commonly used tools for searching similar sequences and gene detection do not mostly provide reliable identification of such short peptides [61]. For reliable detection of genes encoding short peptides, special algorithms are developed. These include SPADA (Small Peptide Alignment Discovery Application). The SPADA algorithm allows finding all related paralogic genes within a gene family, multiple alignments of sequences and construction of a predictive model for searching. In SPADA, several approaches to searching for similar sequences are used, i.e. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and HMMER (http://hmmer.org/), as well as tools for predicting genes, for example Augustus (http://bioinf.uni-greifswald.de/augustus/), which significantly increases the efficiency of annotation of gene families with a large number of members. It is shown that almost all representatives of gene families encoding short cysteine-rich proteins in *A. thaliana* and *M. truncatula* are annotated more effectively in SPADA, than in other programs [61]. Thus, SPADA can be successfully used to identify genes encoding defensins, including those of non-model objects.

With the advent of the ability to quickly obtain information about the genome and/or transcriptome of the studied object, and due to the development of algorithms for proper annotation, the number of identified defensin-related sequences is steadily growing. Within specific groups of peptides, it is necessary to study their biological activity, but a full analysis of the activity of defensins detected by traditional methods has almost never been carried out. Only certain aspects of the action against certain fungi or bacteria have been found out. When analyzing a large number of defensin genes, the methods of computational biology can be used to predict the activity of newly detected peptides and to detect conserved amino acids. Thus, the approach based on multiple sequence alignment and subsequent cluster analysis allowed to separate the defensins of different species, presented in the NCBI database (National Center for Biotechnology Information, USA) into 18 groups [29]. It turned out that defensins from one group possess similar functional activity. It is worth noting that, due to low similarity of the sequences, the comparative analysis of defensins carried out by classical direct sequence comparison may have extremely low efficacy. To align more correctly such complex sets of sequences, special methods based on the barcoding of conserved sections of cysteine sequences [62] should be used. This allows comparative analysis of even very different sequences, including defensins.

The presentation of the results of different research teams in databases with free access increases the effectiveness of research. Thus, using the expression atlases, it becomes possible to identify the expression profile of certain groups of genes. This allows us to estimate the organ or tissue specificity of the expression of a particular gene and its involvement in certain processes [63, 64].

Thus, plant defensins (a group of peptides with antifungal and bactericidal activity, which also participate various responses to abiotic effects) are extremely important in view of obtaining resistant crop varieties. Representatives of this peptide group are characterized by an extremely low similarity of the primary structure (with the exception of conservatively located cysteine residues), however modern methods of computational biology in combination

with sequencing technologies accelerate the identification and analysis of sequences encoding defensins, including those of non-model plants.

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INTERACTIONS OF PLANTS WITH NOBLE METAL NANOPARTICLES (review)

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Abstract

Gold and silver nanoparticles are used in a variety of biomedical practice as carriers of drugs, enhancers and/or converters of optical signal, immunomarkers, etc. The review examines a decade publications (2007-2016) pertaining to the various influence of nanoparticles of noble metals (gold and silver) on growth and productivity of higher plants. In fact, possible phytotoxicity of these nanoparticles is being actively studied for over 10 years. The topicality of this field of research is due to the detection of a number of natural and human-caused factors resulting in interactions of plants with nanoparticles (B.P. Colman et al., 2013; N.G. Khlebtsov et al., 2011). A positive or negative impact of nanoparticles on plants is little known, and the information is very contradictory (P. Manchikanti et al., 2010; M. Carrière et al., 2012; C. Remédios et al., 2012; N. Zuverza-Mena et al., 2016). In the study both model (*Arabidopsis thaliana*) and cultivated plants (soy, canola, beans, rice, radish, tomato, pumpkin, etc.) were involved. The discussed data are indicative of both positive and negative effects of metal nanoparticles on plants, as well as of the chemical nature, size, shape, surface charge, and the dose introduced being the major factors that are responsible for the processes of intracellular nanoparticle penetration. In general terms, it was mentioned that silver nanoparticles were more toxic as compared to gold ones being due to more active silver ion diffusion from the silver nanoparticle surface. Silver ions are known to inhibit effectively biosynthesis of ethylene — a phytohormone controlling processes of plant stress, aging etc., wherein gold ions do not influence ethylene biosynthesis and signaling. Considered all, metal ion toxicity exceeds considerably a toxicity of nanoparticles. The mechanism of the nanoparticle phytotoxic action is often connected with accumulation of active oxygen species in plant tissues. The use of cell suspension cultures may be a promising approach to study plant-nanoparticles interaction (E. Planchet et al., 2015). The period during which these studies are conducted is still small for elucidating all aspects with regard to biosafety. Contradictory (often conflicting) information on the impact of nanoparticles, in our opinion, is a result of diverse experimental conditions used. It is noted that while being clearly incomplete and contradictory, the obtained data suggest that a coordinated research program is needed that would detect correlations between particle parameters, experimental design, and the observed biological effects.

Keywords: gold nanoparticles, silver nanoparticles, toxicity, biological effects, plants

In recent decades, both scientists and the world community have paid much attention to nanotechnology, based on the use of objects no larger than 100 nm in the synthesis, assembly and modification of substances, materials and structures with unusual (often unexpected) properties. The specific characteristics (physical and chemical, structural, optical, etc.) and adequate methods of obtaining, studying and using such materials and compositions are responsible for definitions of phenomena and concepts in this dimension area [1]. Toxicological effects and the effects of metal nanoparticles and their oxides on biological systems [2] are of particular interest, since the physical and chemical characteristics of nanoparticles are signif-

icantly different from those for larger particles and massive materials [3].

Gold and silver nanoparticles are used in a variety of biomedical practices as drug carriers, amplifiers and/or converters of the optical signal, immunomarkers, etc. [4, 5]. Thanks to the development of industrial nanotechnologies, the accidental impact of artificially created nanoparticles on plant and animal cells is becoming increasingly probable, which requires the impact analysis. According to approximate estimations, the annual production of silver nanoparticles (Ag NP) is 3-20 tons in the United States [6)], 5.5 tons in Europe [7], and global use is about 800 tons [8]. Nanoparticles may cause difficult-to-predict (including undesirable) ecological effects in the environment [9]. Gold nanoparticles (Au NP) are most actively used biomedical purposes [5], necessitating investigations of pharmacokinetics, biodistribution in organs and tissues and possible toxicity of these compounds [10, 11]. But if a rather large number of papers have been focused on the ways of nanoparticle penetration into cell, the subsequent transformation and elimination from the cell and the organism as a whole, the toxicity of nanoparticles for microorganisms and animals [10-15], the interaction of nanoparticles with plant cells largely remains terra incognita.

There is very little evidence about the positive or negative effects of nanoparticles on plant cells and plants, and these data are highly controversial [16-20], although there are many nano-scale particles with which plants can come into contact under natural conditions. In addition, technogenic nanoparticles in their physical and chemical properties are often close to nanoparticles of natural origin. For example, recently it has been revealed that nanostructures, i.e. nanospheres and nanoplates, similar in size and shape to those produced at the laboratory are formed under the influence of climatic factors in geological deposits enriched with gold [21]. The approaches have also been reported for use of plants as a tool for biotechnological synthesis of nanoparticles ("green chemistry") [22]. It is suggested that the nanoparticle formation can serve as an anti-toxicant mechanisms in plants at metal-contaminated soils [23].

In this review, we summarized the information evolved recently on the interaction of higher plants with nanoparticles of the most widespread noble metal, such as gold and silver.

The penetration of nanoparticles into plant cells and tissues. Recently, several reviews have been published on the interaction of metallic nanoparticles with higher plants [24-27] and algae [28, 29]. It turned out that algae, especially unicellular ones, in particular *Dunaliella salina* Teod., are a convenient model to investigate the effects of Au NP and Ag NP on living cells [30, 31].

One of the first papers on penetration of nanoparticles into tissues and cells of plants, fungi and algae was presented by E. Navarro et al. [32]. It is known that the cell wall (a structure specific for plants, fungi and algae) limits the flow of large molecules and particles, while allowing the small ones to pass. It serves as the primary step in the interaction of a cell with nanoparticles and a barrier for their penetration. The pores which average 5-20 nm in diameter limit the size of nanoparticles that can pull through the cell wall. However, there is evidence that nanoparticles can themselves modulate the pore size and, removing thereby rigid structural limitations, reach the plasmalemma [32]. It is assumed that at the next stage, nanoparticles are able to enter the cell through endocytosis, but this process has not been adequately studied in plants. There is evidence that Au NPs penetrate tissues of *Oryza sativa* L. and *Solanum lycopersicum* L. due to both clathrin-dependent and clathrin-independent endocytosis [33].

To investigate the endocytosis in the growing pollen tubes of the *Nicotiana tabacum* L., negative and positively charged Au NPs were used [34]. Electron

microscopy has convincingly shown that endocytosis enables the Au NPs to be rapidly captured and found in membrane vesicles. Apical growth of pollen tubes is a rather rapid process, in which the cortical actin cytoskeleton and plasmalemma are constantly changing. Perhaps in such systems (and in protoplasts), nanoparticles can be delivered to the cell through endocytosis [35]. Other types of nanoparticles, such as gold nanostars [36], paramagnetic nanoparticles [37], nanoparticles of silicon oxide [38] and magnesium oxide [39], and carbon nanotubes [40], are presumably appear in the plant tissues the same way.

A number of studies have shown [41-44] that Au NPs were never found in the aboveground parts of radish, pumpkin, barley, poplar, and wheat plants unlike tobacco, tomato, alfalfa, ryegrass, maize, bamboo and rice. In addition to the plant species, the efficiency of Au NPs penetration into the tissues depends on the size and surface charge of the nanoparticles. Positively charged Au NPs are actively absorbed only by plant roots, while negatively charged Au NPs are also able to actively move from roots to stems and leaves [33, 45]. Involvement of both plasmodesma and the vascular system of plants in this process has been under debate [41, 46]. Small nanoparticles penetrate into the aerial parts of plants more readily than large ones and, in addition, they are more toxic. For Ag NPs, this fact is probably due to a greater tendency to dissociation in small particles and the toxic action of metal ions [13, 47].

Intriguing data were obtained using mass spectrometry and X-ray fluorescence to study the intake of 5, 10 and 15 nm Au NPs in *N. tabacum* tissues [48]. Au NPs were found not only in the leaves but also in the tissues of tobacco hornworm (*Manduca sexta*), which ate them. Using artificial aquatic ecosystem as a model, it was shown [49] that gold nanorods more rapidly penetrate into the tissues of mollusks, shrimps and fish than that of aquatic plant *Spartina alterniflora* Loisel. At the same time, the toxicity of Ag NPs in the cells of the algae and crustaceans were detected at much lower concentrations compared to mammalian cells (~ 0.1 mg Ag/l vs ~26 mg Ag/l) [13].

Biological effects in plants exposed to metal nanoparticles. Various toxic effects of nanoparticles on plants have been discussed in several comprehensive reviews [50-56], but data on the mechanisms of phytotoxicity reported in these publications are scarce and controversial.

S. Arora et al. [57] after studying the effect of nanoparticles on the growth and yield of mustard (*Brassica juncea* L.) in a field trials sprayed the plants with the Au NP suspensions of different concentration. The presence of Au NPs in the tissues was detected by atomic absorption spectroscopy. The authors reported the positive effect of Au NPs: the length and diameter of the stem, as well as the number of leaves and shoots increased, and the yield grew. Similar results were obtained for germination of mustard seeds on growth medium [58] and with Ag NPs [59]. Using a synchrotron X-ray microanalysis and high-resolution transmission electron microscopy, it has been shown that Au NPs 3.5 nm in diameter penetrate the *Nicotiana xanthi* plants through the roots and move along the vascular system. The Au NP aggregates of 18 nm in size were detected only in the cytoplasm of the root cells [60]. Necrotic lesions of the leaves were observed 14 days after exposure to small Au NPs, but in the presence of large Au NPs, no difference vs control was observed. A lack of effect or a minor physiological effect at very high concentrations of Au NPs was described for *Glycine max* L. [61] and freshwater aquarium plants [62, 63].

When used at the early ontogenesis of *Brassica napus* L., Ag NPs significantly promoted the growth of roots and stems, while the energy and rate of seed germination were somewhat decreased [64]. At the same time, the treatment of *Boswellia ovalifoliolata* N.P. Balakr & A.N. Henry seeds with Ag NPs

markedly accelerated the germination and seedling growth [65]. A similar effect was observed after exposure to Ag NPs in the *Asparagus officinalis* L. seeds [66]. In addition, the authors noted an increase in the ascorbic acid and chlorophyll content in seedling treated with Ag NPs. A decrease in germination, a slowdown in the root nodules formation (due to a decreased number of the symbiotic bacteria *Rhizobium leguminosarum*) and shoot growth, and smaller root length in *Vicia faba* L. are described when Ag NPs were added to the culture medium [67). In hydroponic culture, the germination rate in *Solanum lycopersicum* [68] and *Raphanus sativus* L. did not decrease in the presence of Ag NPs, but root and shoot length decreased, and the photosynthetic activity was slightly reduced [69]. According to C.L. Doolette et al. [70], the potential risk of negative effects of Ag NPs on the *Lactuca sativa* L. plants is very low: the Ag content in the edible parts of the plant was < 1 % of the total amount added to the soil. Similar findings were obtained on *Lactuca sativa* and *Cucumis sativus* L. for Ag NPs, Au NPs, and iron oxide nanoparticles [71. The authors reported low or no toxicity for all types of nanoparticles. The negative effect of the dispersion medium was sometimes more significant than that of nanoparticles, which may be due to the diffusion of metal ions into the medium. For example, after three months of storage the Ag content in nanoparticles decreased by 7 % [72].

The effect of nanoparticles on the development of plants can be dose-dependent. It was found [73] that when the *Oryza sativa* seeds were germinated on the culture medium containing Ag NPs (30 mg/ml), the root growth was enhanced, but when the concentration of nanoparticles increased to 60 mg/ml, the growth of seedlings slowed down compared to the control. In addition, the number of rhizosphere microorganisms decreased due to the damage to the bacterial cell wall under the influence of Ag NPs. Inhibition of growth depending on the Ag NP dose and exposure was demonstrated for *Lemma minor* L. [74]^ signs of oxidative stress and changes in the structure of chloroplasts were observed at high concentrations. In *Phaseolus radiates* L. and *Sorghum bicolor* L. the growth inhibition due to the addition of Ag NPs was more pronounced on the culture media, but not in the soil [75]. The particle size also affects toxic effects. Thus, small (6 nm) Ag NPs proved to be more toxic than large ones (20-1000 nm) for *Spirodela polyrhiza* L. [76]. It has also been shown that colloidal silver, in comparison to solid metal, inhibited the growth of *Cucurbita pepo* L. to a larger extent [77].

A number of the studies on the mechanisms of the nanoparticle penetration and phytotoxicity have been performed on *Arabidopsis thaliana* (L.) Heynh., a classical model in plant physiology [78-81].

There is evidence of the Au NPs influence on *A. thaliana* seed germination [82]. Addition of Au NPs with a diameter of 24 nm (10-80 mg/l) to the growth media led to a 3-fold increase in the total germination of the Arabidopsis seeds compared to control, as well as to a marked increase in the length and diameter of the stem and roots. Interesting results were presented by A.F. Taylor et al. [83]. They revealed that the root length in *A. thaliana* when grown on an agar medium containing Au NPs (100 mg/l) was reduced by 75 %. This was accompanied by an increase in the expression of genes that encode proteins involved in plant response to stress, i.e. glutathione transferase, glucosyltransferase, peroxidase and cytochrome P450. At the same time, the expression of genes that encode aquaporins and proteins involved in the transport of copper, cadmium, iron, and nickel ions has slightly decreased.

A much more pronounced genotoxic effect was revealed for gold chloride [84]. In general, the toxicity of metal ions significantly exceeds the toxicity of nanoparticles [85], and the phytotoxic effect of Ag NPs is higher than that of

Au NPs [86]. The toxic effect was manifested in inhibition of elongation of the arabidopsis roots, an increase in the vegetation period by 2-3 days, a decrease in seed germination rate in the offspring, and, according to the authors, was due to the diffusion of silver ions from the Ag NP surface.

However, another study [87] showed that sublethal concentrations of Ag NPs (up to 1 mg/l) had a pronounced stimulating effect on arabidopsis plants. The authors observed an increase in root length and wet weight, and the intensification of evapotranspiration in the seedlings. In this case, silver nitrate had a toxic effect even at a concentration of 0.05 mg/l.

The effect of the shape and size of Ag NPs on the expression of phytohormone genes and the development of seedlings in arabidopsis was reported [88] and found to dose-dependent. These nanoparticles induced the expression of the auxin-dependent transcription factor gene and one of the key enzymes for the synthesis of abscisic acid. In addition, Ag NPs reduced the inhibition of root growth in the arabidopsis seedlings caused by ACC (aminocyclopropane-1-carboxylic acid, the precursor of ethylene synthesis), and also reduced the expression of ACC synthesis gene (*ACS7*) and gene of ACC conversion to ethylene (*ACO2*). It was concluded that Ag NPs act at the ethylene reception stage and adversely affect its biosynthesis [88]. It is known that silver ions effectively inhibit the biosynthesis of ethylene, which is a phytohormone, regulating the processes under stressens, aging (maturation), etc., while gold ions do not affect the ethylene biosynthesis and signaling [89, 90]. It was shown that the plasma membrane of *A. thaliana* is susceptible to Ag NPs, and when their concentration in a growth medium is 300 mg/l, root elongation and leaf growth are inhibited, and the photosynthetic efficiency decreases [91]. There is a suggestion [92] that phytotoxicity is due to the accumulation of reactive oxygen species in the tissues (mainly under the influence of silver ions diffusing from Ag NP surface).

The research of the nanoparticle penetration into cells and cytotoxicity is often carried out on suspension cultures of animal and human cells [10-12, 93]. Given that the protective mechanisms against adverse factors have much in common for all living organisms, we suggest that the use of plant cell suspension cultures can be quite effective in studying the effect of metal nanoparticles on plant cells. A distinctive feature of plant cell suspension cultures is their higher susceptibility to a wide range of compounds and abiotic effects compared to the whole plant [94, 95]. These properties are determined by the peculiarities of the physiological state of cells, including the ability to reproduce various responses at metabolic and gene regulation levels. The development of biochemical and physiological responses in the cell culture occurs over a short period and fairly evenly throughout the population (unlike entire plant or its organs). In addition, one can expect more pronounced effects of nanoparticles on cells of the suspension culture due to the absence of specialized protective structures, such as cuticle, epidermis, etc.

It has been shown that the addition of Au NPs and Ag NPs of 20 nm in a diameter to growth media positively influences the biomass gain in *A. thaliana* cell suspension. It has been found out that the nature of changes in pH of the culture media containing Au NPs and Ag NPs is not identical, since Ag NPs caused severe acidification while Au NPs alkalinized the culture medium. In the presence of metal nanoparticles, the specific respiratory activity of the *A. thaliana* cells in the suspension was insignificantly, though steadily decreased, and a decrease in the intracellular pool of free amino acids (alanine, γ-aminobutyric acid, valine) was seen, which is characteristic for responses to abiogenic stresses [96]. Moreover, the addition of nanoparticles changed the patterns of extracellular proteins and the structure of the actin cytoskeleton in the *A. thaliana* cell culture [97].

From our point of view, contradictory (often conflicting) data on the effect of gold and silver nanoparticles on plants (Table) are due to differences in design of experiments (i.e. unequal sizes and charges of nanoparticles, doses, time of observiton, etc.).

The effects of noble metal nanoparticles on plants

Diameter, nm	Conditions			Plant	Effect	Reference
	dose	substrate	time			
			Au nanoparticles			
10	10 mg/l	H	2 weeks	Barley	No effects on the seed germination; decrease in biomass and root length	[43]
5, 10, 15	30, 100 mg/l	H	3-7 days	Tobacco	Increased growth	[48]
3.5	48 mg/l	H	2 weeks	Tobacco	Necrotic lesions of leaves	[60]
18	76 mg/l				No effects	
10-20	0-100 mg/kg	FE	50-70 days	Mustard	Increase in height and diameter of the stem, number of leaves and shoots, yield	[57]
50	0-400 mg/l	CM	10 days	Mustard	Increased root length	[58]
5, 10, 20	0-17 μM	S	2 weeks	Soybean	No effects	[61]
24	10-80 mg/l	CM	15 days	Arabidopsis	Increased total germination of seeds	[82]
7, 18, 49, 108	0-400 mg/l	CM	2 weeks	Arabidopsis	Reduced root length	[83]
20	50 mg/l	CM	72 hrs	Arabidopsis	Increased biomass of the suspension cell culture	[97]
			Ag nanoparticles			
30	0-400 mg/l	CM	1 week	Mustard	Increased root length, enhanced photosynthesis	[59]
10	0.75-18 μM	CM	1 week	Rapeseed	Increased weight of roots and stems; reduced energy and rate of seed germination	[64]
30-40	10-30 mg/l	CM	3 weeks	Boswellia	Accelerated seed germination and growth of seedlings	[65]
20	100 mg/l	CM	25 days	Asparagus	Increased content of ascorbic acid and chlorophyll	[66]
5-50	800 mg/kg	Soil	5 weeks	Beans	Reduced germination, slowing down the formation of root nodules, inhibition of shoot growth and root length	[67]
10-15	0-1,000 mg/l	H	6 days	Tomatoes	Significant root growth inhibition, reduced photosynthesis	[68]
2	0-500 mg/l	CM	5 days	Radish	No effects on germination; decreased length of roots and shoots	[69]
20	30, 60 mg/l	CM	1-3 weeks	Rice	Concentration-dependent increase/decrease in root growth	[73]
10, 100	5 mg/l	CM	2 weeks	Duckweed	Dose- and exposure-dependent inhibition of growth; oxidative stress, changes in the chloroplast structure	[74]
5-25	0-40 mg/l	S, CM	5 days	Sorghum, beans	Growth inhibition	[75]
6, 20, 1000	0.5-10 mg/l	CM	72 hrs	Spirodela	Growth inhibition depending on the particle size	[76]
100	0-500 mg/l	H	1 week	Pumpkin	Decreased biomass	[77]
20-80	67-535 mg/l	H	2 weeks	Arabidopsis	Inhibition of root elongation, vegetation period is 2-3 days longer, a decrease in seed germination rate in the offspring	[86]
5, 10, 25	0.01-100 mg/l	H	6 weeks	Arabidopsis	Increased length of roots, raw biomass, and evapotranspiration	[87]
8, 45	0-100 μM	CM	3 days	Arabidopsis	Increased root length; activation of the expression of genes involved in cell proliferation, metabolism, and hormone signaling	[88]
40	300-5,000 mg/l	CM	4-10 days	Arabidopsis	Inhibition of root elongation and leaf growth, decreased photosynthetic efficiency	[91]
100	50-100 μM	CM	10 days	Arabidopsis	Oxidative stress	[92]
20	30 mg/l	CM	72 hrs	Arabidopsis	Increased biomass gain in suspension cell culture	[97]

Note. H — hydroponics, CM — culture medium, S — soil, FE — field experiment.

To summarize, active studies on the toxicity of nanoparticles in plants are being carried out for no more than 10 years. This period seems to be still small to clarify all the aspects of the problem as required by the biosafety principles but sufficient for some preliminary principal conclusions. The data obtained indicate both positive and negative effects of metal nanoparticles on plants [98]. Factors that undoubtedly determine the processes of intracellular penetration of

nanoparticles are their chemical nature, size, shape, surface charge, and dose. The available methods for the determination of metals in organs, the localization and identification of nanoparticles at the cellular and subcellular levels, as well as for in vitro estimation of cytotoxicity are relevant, and has been robustly tested. The cell suspension cultures may serve a promising tool for studying the effect of metal nanoparticles on plants.

Therefore, the problem of metal nanoparticles-plants interaction, despite the urgency, is far from a convincing solution. We need a coordinated research program that would reveal the correlations between the parameters of the particles (size, shape, surface functionalisation), the design of experiments (model, dose, method and timing, duration of observations, studied organs, cells, subcellular structures, etc.) and the observed biological effects, including these in trophic chains. In addition, concerted efforts are required to introduce standards for particles and methods used to test the toxicity of nanomaterials

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TARGET GENES FOR DEVELOPMENT OF POTATO (*Solanum tuberosum* L.) CULTIVARS WITH DESIRED STARCH PROPERTIES (review)

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Abstract

Starch is an important organic feedstock easily available for human in industrial scale. Optimal physical and chemical properties of amylose and amylopectin molecules comprising starch significantly vary in dependence on the technical scope. Molecular and supramolecular composition as well as structure of the molecules are genetically regulated and may be considered as traits for selection. Combining genes in certain composition one may program potato plant to produce starch of predetermined structure and properties. The main goal of the review is analysis of chain sequence industrial application→starch properties→enzymes→coding genes and discussion of genes and gene compositions programming synthesis of certain starch modifications in potato tubers. Potato genotype may be changed in a controlled manner by classical combination breeding or marker-assisted selection as well as genetic engineering approaches, including the new breakthrough genome editing technologies. Starch biosynthetic pathway in tuber cells requires participation of at least seven main enzymes in cytosol and plastids and of about ten more enzymes in starch granule surface or inner space. Thus, granule-bound starch synthase gene (*GBSS*) knockout drastically increases amylopectin content up to > 98 %. That is the namely reason why cultivars with *GBSS* knockout turned out the first genetically modified forms of potato with corrected starch, field-tested as a technical crop. High amylopectin starch gives gels with high optical clearance, stability during centrifugation, and demonstrates valuable increase of maximum and final gelatinization temperature as well as different rheological behavior. If both *GBSS* and starch synthases genes *SSII* and *SSIII* are inhibited, the starch gives the gel, which is much more stable in prolonged freezing, or multiple freeze—thaw cycles compared to ordinary starch gel. The *SBEI* gene encoding the main starch branching enzyme being inhibited does not increase amylose content in modified potato. But simultaneous inhibition of both *SBEI* and *SBEII* genes results in high (60-89 %) amylose starch with minor amylopectin content. Elevation of *SBEII* expression allows obtaining starch characterized by increased amylopectin branching with shorter end chains. On contrary, amylopectin from potato plants with inhibited SBE synthesis has longer polysaccharide chains with lower branching. *GWD* gene knockout results in amylopectin with reduced phosphate content and, accordingly, reduced viscosity gels from the modified starch. Low phosphate starch demonstrates also a reduced rate of biocatalytic hydrolysis. Overexpression of *SSIV* results in increased tuber starch content in both greenhouse and field grown plants. Starch granule morphology and crystallinity may be corrected on genetic level as well. Typically, morphological traits including physical and chemical properties of starch are regulated by not one or two genes, but a certain gene network. So, discovery of qualitative trait loci and identification of diagnostic markers for them allows application of marker-assisted selection for developing potato cultivars with predetermined starch properties as an optimal feedstock for certain industries.

Keywords: potato, starch, biosynthesis genes, starch synthase, amylose, amylopectin, branching enzyme, physical and chemical properties

Starch is one of the few complex organic substances readily available for human economic activity on an industrial scale. From the point of view of the composition homogeneity, with rich possibilities for modifying its constituent

molecules of amylose and amylopectin [1, 2], starch outperforms other sources of organic raw material, such as oil, which contains mostly simpler hydrocarbon molecules in difficult-to-separate mixtures, or lignocellulose, which requires chemical processes for the separation of its constituent heterogeneous poly- and oligomers. The variety of products that can be obtained from starch as a result of chemical or biotechnological transformations determines the range of fields of its application [3]. It is an accessible, eco-friendly and economical biopolymer that is widely used in a native or modified form (Fig. 1). In Europe, 70-80 % of starch go to non-food needs [4, 5]. In Russia, native starches are widely used in the food industry (confectionery, bakery, in sausage and canned food production), as well as in the production of corrugated board [6].

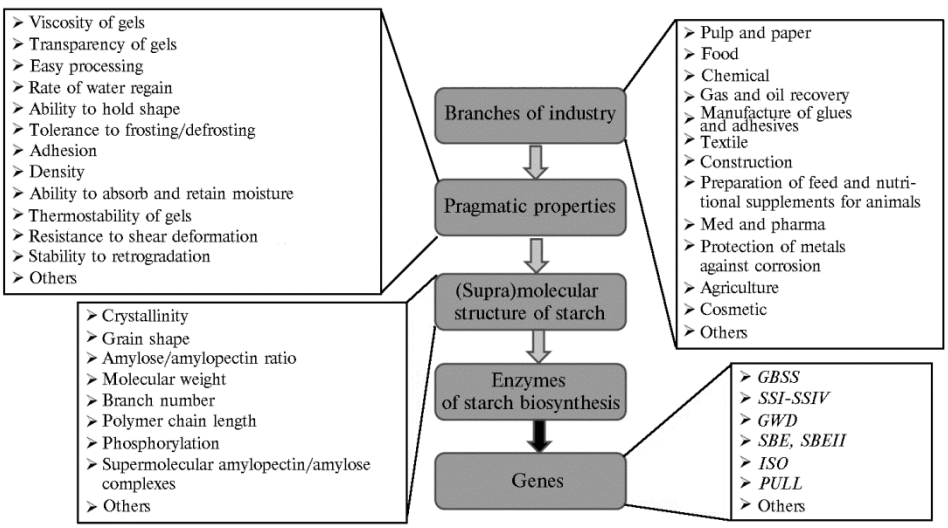

Fig. 1. Molecular and supramolecular characteristics, which are determined by genes controlling the enzymatic biosynthesis of starch in a cell, and responsible for its properties and practical applications. The target genes of starch synthases (*GBSS* and *SSI-SSIV*), H_2O-dikinase (*GWD*), starch branched enzymes (*SBEI* and *SBEII*), isoamylase (*ISO*), pullulanase (*PULL*) are indicated.

Starch is a complex carbohydrate of plant origin, consisting of two types of polymer molecules (amylose and amylopectin), each of which is a homopolymer formed from identical monomer units (glucose residues), with general formula $(C_6H_{10}O_5)_n$. The molecular composition and structure, as well as the supramolecular assembly of these molecules are regulated by the genes of starch biosynthesis through the corresponding proteins and, therefore, can be considered as phenotypic signs for selection. A combination of certain variants of such genes allows the potato plant to be programmed to produce starch with a given structure and properties.

Practical interest is seen in the amylose-amylopectin ratio, their branching and molecular weight, crystallinity, granule size and porosity, the amount of phosphate residues in polymer chains, the rheological and optical properties of starch gels (see Fig. 1). Molecular and supramolecular characteristics of native starch are responsible for qualities, due to which one or another variety of this natural polymer can find its application in a certain branch of industry. An optimal combination of physical and chemical properties of the molecules that make up starch varies considerably depending on the field of its application.

The purpose of this review is to discuss variants of genes or their combinations that program starch biosynthesis in different modifications, depending on the planned practical result with a sequential task definition at the stages as follows: commercial application of starch→starch properties→proteins

(enzymes)→encoding genes.

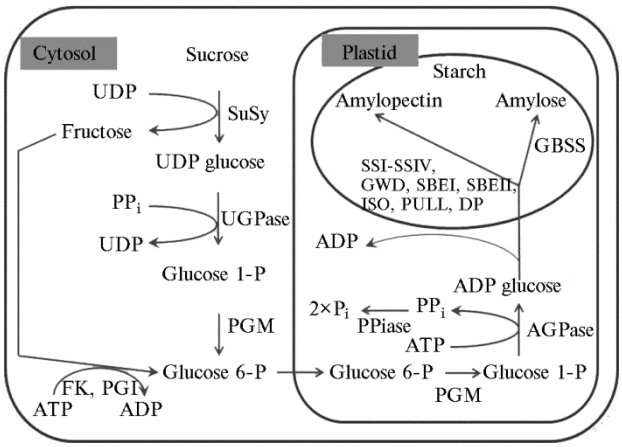

Fig. 2. A diagram of starch biosynthesis in a cell of the potato tuber pulp (7, 8): SuSy — sucrose synthase; UGPase — UDP-glucose pyrophosphorylase; FK — fructokinase; PGM — phosphoglucomutase; PGI — phosphoglucose isomerase; PPiase — pyrophosphatase; AGPase — ADP glucose pyrophosphorylase; GBSS — granule-bound starch synthase; SSI-SSIV — starch synthases; SBEI-SBEII — starch branching enzymes; GWD — H_2O-dikinase; ISO — isoamylase; PULL — pullulanase; DP — disproportionating enzyme; PP_i — pyrophosphate; P_i — phosphate.

The biosynthesis of starch in the tuber pulp cells occurs with the participation of 7 major enzymes in the cytosol and plastids and about 10 more enzymes on the surface or inside the starch grains located in plastids (Fig. 2).

Understanding mechanisms of biosynthesis allows to influence the process in order to adjust the physical and chemical properties of starch so that they better match the needs of certain industries. This effect can be implemented either using the combining selection in its classical concept and diagnostic DNA markers (MAS, marker-assisted selection), or with the involvement of genetic engineering, including for the production of non-transgenic plants with predetermined properties based on safe technologies for genomic editing [9, 10]. Information on target genes is needed for marker-assisted selection and genomic editing. One of the challenges with biological production of starch with predetermined properties is that the measured physical and chemical properties (signs) of starch are usually formed by the gene network as a result of different loci performance. Sometimes it is feasible to identify several of the most important controlling genes, the inhibition or activation of which leads to a significant change in a certain property of starch, and only in exceptional cases a simple “one gene-one chemical trait” correlation can be observed.

Despite the fact that the legislative regulation of the Russian Federation currently does not support the commercial production of genetically modified plants, their production in a laboratory setting is advisable for a number of reasons [11] One of them is the confirmation of the relation between the expression of one or more genes and the properties manifested in the organism obtained. Let us consider the known examples of the influence of the genetic modification of *Solanum tuberosum* L. potato on some of the physical and chemical properties of starch.

High content of amylopectin. Potato starch contains about 80 % of branched amylopectin polysaccharide and 20 % of linear amylose polysaccharide. The main gene responsible for the synthesis of amylose is *GBSS* which encodes granule-bound starch synthase. A modification of this gene dramatically changes the ratio of amylose and amylopectin (up to the amylopectin content > 98 %). It was the genotypes with knockout of the indicated *GBSS* gene that were obtained earlier than other genetically modified forms of potato with altered starch and tested in the field as a technical crop for industrial use, e.g. Amflora (EH92-527-1) produced by BASF Plant Science GmbH (Germany) [12, 13] or the variety by Avebe U.A. (Netherlands) [14]. More recently, a

knockout of the *GBSS* gene was successfully performed using CRISPR/Cas9, the state-of-the-art system for genomic editing. The approach used by the authors allowed the site-directed mutagenesis to be carried out without inserting a foreign DNA into the potato genome. This means that modified, but not transgenic potato plants were obtained. At the same time, in 2 % of the regenerants, genetic editing took place in all four alleles of the *GBSS* gene. The presence of at least one functional allele of the *GBSS* gene is sufficient for a significant amount of amylose to be synthesized in the tuber, and only inhibition of all four alleles results in the formation of starch containing mainly amylopectin [15], which significantly affects the technical characteristics of starch. Starch with a high content of amylopectin produces gels with enhanced optical transparency and stability during centrifugation, and also shows an increase (by 5-6 °C) in the maximum and final gelatinization temperature [16] and a change in rheological properties [17]. If, in addition to *GBSS*, the genes of the *SSII* and *SSIII* starch synthases are inhibited, the tuber starch would be low in the amylose content, with the shortened terminal chains of amylopectin. Gels obtained from such starch are much more stable than those obtained from conventional starch, both during prolonged freezing and in several freeze-thaw cycles [18]. Low amylose starch is better suited for obtaining thickening agents for the textile and pulp and paper industries [19].

High content of amylose. To switch off the synthesis of amylopectin, which is a branched polymer, it is necessary to inhibit the *SBE* gene which encodes a starch branching enzyme (SBE). However, repression of *SBEI*, the major branching enzyme gene, in practice did not result in an increase in the amylose content in the modified potato [20]. A knockout of the *SBEII* gene responsible for the synthesis of the minor form of the SBEII branching enzyme, resulted in a moderate increase in the relative content of amylose (by 38 %) [21]. And only concurrent inhibition of both *SBEI* and *SBEII* genes made it possible to obtain starch with a high portion of amylose (60-89 %) and an addition of a small amount of branched amylopectin. Thus, the SBEII enzyme is required for the synthesis of normal branched amylopectin, and possibly enhances the SBEI activity, though the role of SBEI has not yet been elucidated in detail, given some differences in the mechanisms of action of these enzymes [5, 22]. Field trials conducted for a number of years have confirmed that in potato varieties obtained by inhibition of both branching enzymes the high amylose content of starch still persists.

Inhibition of the *SBEI* and *SBEII* genes also results in a 5-6-fold increase in the number of phosphate groups in the molecules. In addition, with an increase in the amylose content in starch from 20 to 60-80 %, its accumulation in tubers becomes noticeably decreased (from 22-23 to 10-15 %), and the granule size also reduces (from 50-63 to 32-39 μm) [23].

The branching of amylopectin. In potato, only two isoforms of SBE are responsible for the branching of the amylopectin molecule: the major SBEI and the minor SBEII branching enzymes. The construction of a cis-gene, consisting of complementary and genomic DNA fragments (cDNA and gDNA) of the *SBEII* gene under the control of a strong promoter of another potato gene, *GBSS*, made it possible to enhance the expression of the SBEII enzyme. In the modified forms of potato, the branching of the amylopectin molecules was higher (a degree of polymerization 6-12, mostly closer to 6), and the chains are shorter than in the selection varieties [24]. And on the contrary, with suppressed synthesis of SBE, the degree of amylopectin branching decreases [25], and the number of elongated phosphorylated chains with a degree of polymeri-

zation above 14 increases.

When the expression of the *SBE* gene is inhibited, starch acquires the ability to gelatinize in lowered temperatures (by 3 °C) and lower urea concentration (gelation agent), its viscosity is reduced and the volume in water regain is increased [24].

The content of phosphate groups. Some polysaccharide chains in starch granules contain covalently attached phosphate groups. In industry, starch is purposely phosphorylated by treating with orthophosphoric acid, which results in obtaining a food additive E1410, i.e. starch with an increased content of phosphate groups. Phosphorylated starch with negatively charged phosphate groups, which are repelled from each other in solution, much more rapidly increases its viscosity, gives more transparent and viscous gels less prone to retrogradation. In addition, phosphate residues can effectively bind to metal ions, making phosphorylated starch an effective ligand.

In potato starch, 0.2-0.5 % glucose monomers are phosphorylated which is several times higher than in starches obtained from other sources [26]. The most important enzyme for phosphorylation of starch is α-glucan-H_2O-dikinase [27, 28], encoded by the *GWD* gene. It has been reported that knocking out the *GWD* gene results in the synthesis of amylopectin with reduced amounts of phosphate groups and, correspondingly, decreased viscosity of gels obtained from such a starch [27, 29]. It has also been established that the rate of biocatalytic hydrolysis of starch, which is characterized by the reduced content of phosphate groups, decreases [30]. For arabidopsis, another phosphorylating enzyme, phosphoglucan-H_2O-dikinase (PWD), has been shown, which converts the C6-phosphorylated fragment of the molecule to C3-phosphorylated [31]. However, so far there has been no data on whether an enzyme with a similar effect is available in potatoes.

Importantly, with a decrease in the number of the glucan polymer chain branches, i.e. when the activity of the SBE branching enzymes is inhibited, the degree of chain phosphorylation increases (20), sometimes up to 5-6-fold [5].

The method of association mapping revealed genetic markers associated with the degree of phosphorylation of starch at C3 and C6 positions in the residues of D-glucose. A significant association of the trait with certain SNP (single nucleotide polymorphism) in the genes encoding α-glucan-H_2O-dikinase (GWD), starch branching enzyme (SBEI) and starch synthase (SSIII) has been shown. In addition, a link between a polymorphism of a simple sequence repeat (SSR) within the gene encoding the SBEII branching enzyme has also been found. At the same time, the SNP in the *GWD* gene is associated exclusively with C6 phosphorylation, in the *SSIII* gene with C3 phosphorylation, and the polymorphic sites in *SBEI* and *SBEII* are associated with both C3 and C6 phosphorylation [32].

Consequently, potato modifications aimed at enhancing the expression of the *GWD* gene and/or inhibiting the *SBE* and *SSIII* genes activity would allow to produce much-needed in industry starch with an increased content of phosphate groups, bypassing the stages of chemical processing of natural raw materials, i.e. to obtain an environmentally friendly product with a reduced production cost.

Starch content. The starch content of tuber depends on the enzymatic reactions aimed at both the synthesis and the cleavage of starch. Three main stages of starch formation are controlled by three enzymes: ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS) and starch branching enzyme (SBE). And it is AGPase (encoded by the *glgc-16* gene) that catalyzes the reaction which limits the rate of starch biosynthesis. Indeed, the expression of the *glgc-16* transgene in potato plants increased the starch content by 30 % [33, 34].

Enhanced expression of SSIV increased the amount of starch in the tubers of transgenic forms both in the greenhouse and in the field from 94 g to 98-137 g per plant, providing an increase in the product yield from 4.25 to 4.40-6.10 t/ha (depending on the cultivar) [35].

The cleavage of the polymer chains of starch is regulated by amylases. Due to their activity, the starch content of potato tuber decreases and storage time increases. Recently, it has been shown that the *SbAl* gene inhibits amylase, slowing the hydrolysis of starch and controlling its content in tuber. At the same time, the amount of reducing sugars decreases. As a result, chips made from such tubers have a less intense brown colour [34]. Phosphate groups in amylopectin molecules promote the hydrolysis of starch, therefore their reduced amount leads to the accumulation of this polysaccharide. In potatoes, the overexpression of a gene that regulates the phosphorus content in starch in arabidopsis (*AtPAP2*, encodes purple acid phosphatase) results in the increased yield (2-3 times) of tubers and their size due to solids and starch content [36].

Breeding for regulation of the activity of genes responsible for the phosphorylation and synthesis of polymer chains of starch, as well as the inhibition of amylases, can be applied to obtain technical varieties of potatoes with a high content of starch.

Crystallinity of granules. Tuber starches, including potato starches, are characterized by the presence of B-polymorphs in crystalline domains [37], usually well-structured, compact and responsible for the formation of starch granules with a visually smooth surface. It is believed that it is the supramolecular organization of the amylopectin branches in starch that is responsible for its crystallinity [38]. Due to the well-ordered structure, the starch granules are very resistant to amylolytic degradation [39]. In fact, there were no differences in the dimensions of amylopectin crystallites, the thickness of the crystalline lamellae of the granules, and the polymorphic structure of starch from potato tubers deficient for the expression of the *GBSS* (encodes starch synthase controlling the amylose synthesis) or *GWD* genes and unmodified plants. The reduced content of the *GBSS* or *GWD* encoded enzymes affects only the pattern of the defects in amylopectin crystallites [40]. The impact of starch synthase (SSII) in rice [41] associated with starch granules (GBSS) in the representatives of *Chlamydomonas* [42] and in the SGP (starch granule protein) in wheat [43] on the crystallinity of starch granules has been described in the literature, although experimental findings on potatoes are limited. Therefore, it is still rather difficult to propose a strategy for increasing the bioavailability and reactivity of starch grain through selection methods or gene modification. However, it should be borne in mind that facilitating these processes on an industrial scale could lead to a reduction in energy costs, saving of water, reagents and operational time, and also provide the deeper processing of starch.

Morphology of granules. Of interest, the expression of a foreign starch branching enzyme (SBE from *Escherichia coli*) does not enhance the branching of amylopectin in potatoes, but significantly changes the morphology of the starch granules: hummocky granules with deep pores are formed in an amylose-containing mutant, and tuberous agglutinated granules in the amyloseless line [43, 44].

Not every species of bacteria or yeast can destroy the supramolecular organization of polymer molecules of starch to involve them into biochemical transformations. Due to the limited availability of this polysaccharide, the degree of its modification during chemical processing also remains low, and to increase it, a temperature higher than the gelation temperature is required. In starch granules with the altered morphology, increased surface area and a larger pore

size, the reaction centers are more accessible for chemical reagents and enzymes, and therefore may exhibit increased reactivity, including at low temperatures.

Genetic markers of quantitative traits. Despite the fact that the properties of starch in potato tubers can be modified by affecting a certain number of genes, the general nature of displaying the expression activity of DNA regions during formation of quantitative traits is much more complicated. Using the method of interval mapping of genomes in a population of diploid potatoes, it was shown that starch phosphorylation is regulated and/or controlled by five quantitative trait loci (QTL) on chromosomes 2, 5 and 9, and the content of amylose by six loci on chromosomes 2, 3, 5, 7 and 10. Similarly, loci controlling the starch grain size, the starch content of potato tuber and the temperature of starch gelatinization were discovered [45, 46]. Many of the identified QTLs coincided by their localization with the known genes that encode the enzymes of starch biosynthesis, but, in addition, loci have also been discovered in which no one of the starch biosynthetic genes was previously mapped. The nucleotide sequences corresponding to these QTLs have not yet been deciphered and the molecular mechanisms through which they influence the alterations in the properties of starch are still not clear. However, the stable association of such loci with traits and the presence of DNA markers closely linked to these loci makes it possible to use the results obtained for marker-assisted selection. Indeed, for tetraploid potato, three markers had been identified, associated with the traits of productivity, i.e. the yield of tubers per hectare and the starch content of tuber [47], which were later used for marker-assisted selection [48]. Another study showed that single nucleotide substitutions in the genes encoding the Pain1 and InvCD141 invertase enzymes, the SSIV starch synthase, the StCDF1 transcription factor and the LapN aminopeptidase are associated with the yield of tubers and starch and starch content of tuber [49]. Identified SNPs can also be used for controlled selection of potato forms possessing the specified technological properties. In addition, markers associated with the desired quantitative traits in potato would be possibly found in its plastid and mitochondrial genomes. For example, it has been shown that the W/y-type cytoplasm in European potato varieties correlates with a high content of starch [50]. Findings on the effects of the starch biosynthesis genes on its properties are summarized in Table 1.

1. The genes of starch biosynthesis, the corresponding enzymes, their effects on the properties of starch in potato (*Solanum tuberosum* L.) (7, 50-52)

Gene	NCBI No.	Linkage group	NCBI Reference	Product	EC	Starch properties
SuSy	102577594	VII, XII	Baroja-Fernández E. et al. Plant Cell Physiol, 2003, 44(5): 500-509	Sucrose synthase	2.4.1.13	Content in tubers
UGPase	102577726	XI	Katsube T. et al. Biochemistry, 1991, 30(35): 8546-8551	UDP-glucose pyrophosphorylase	2.7.7.9	Content in tubers
PGM	102585015	N/A	None	Phosphoglucomutase	5.4.2.2	Content in tubers
FK	102577816	VI	Smith S.B. et al. Plant Physiol., 1993, 102(3): 1043	Fructokinase	2.7.1.4	Content in tubers
PGI	102577825	N/A	None	Phosphoglucose isomerase	5.3.1.9	Content in tubers
PPiase	102584131	VIII, IX, XII	du Jardin P. et al. Plant Physiol., 1995, 109(3): 853-860	Pyrophosphatase	3.6.1.1	Content in tubers
AGPase	102577790 (small subunit)	I, IV, VII, VIII, XII	du Jardin P. et al. Plant Mol. Biol., 1991, 16(2): 349-351	ADP-glucose-pyrophosphorylase	2.7.7.27	Content in tubers
GBSS	102577459	VIII, II	van der Leij F.R. et al. Mol. Gen. Genet., 1991, 228(1-2): 240-248	Granule-bound starch synthase	2.4.1.21	Amylose Content
SSI	102600045	III	Kossmann J. et al. Planta, 1999, 208(4): 503-511	Starch synthase	2.4.1.21	Content in tubers
SSII	102583115	N/A	Kossmann J. et al. Planta, 1999, 208(4): 503-511	Starch synthase	2.4.1.21	Content in tubers
SSIII	102577674	II	Marshall J. et al. Plant Cell, 1996, 8(7): 1121-1135	Starch synthase	2.4.1.21	Content in tubers

Table 1 (continued)

GWD	102577510	V	Lorberth R. et al. Nat. Biotechnol.. 1998, 16(5): 473-477	H_2O-dikinase	2.7.9.4 2.7.9.5	Phosphorylation
SBEI	102596498	IV	Kossmann J. et al. Mol. Gen. Genet., 1991, 230(1-2): 39-44	Starch branching enzyme	2.4.1.18	Branching of amylopectin chains
SBEIIA *SBEIIB*	102590711	IX	Larsson C.T. et al. Plant Mol. Biol., 1998, 37(3): 505-511	Starch branching enzyme	2.4.1.18	Branching of amylopectin chains
ISO	102577466	XI	Sun C. et al. Plant Mol. Biol., 1999, 40(3): 431-443	Isoamylase (hydrolysis at branching points)	3.2.1.68	Isomerization of starch
PULL	102581262	N/A	None	Pullulanase	3.2.1.41	Isomerization of starch
DP	Different options	IV	None	4-α-Glucotransferase	2.4.1.25	Isomerization of starch

N o t e. NCBI — National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

As a reminder, despite the multiple-factor nature of the impact of many genes involved in the biosynthesis of starch, in some cases, through their inhibition or the other way around overexpression, it was possible to change certain properties of starch (Table 2).

2. The effect of inhibition and expression of the genes encoding the biosynthesis of potato starch on its physicochemical properties

Gene or gene combination	Inhibition or expression outcome	Change in physicochemical properties
GBSS	Inhibition: Decrease in amylose content	Increase in the gel optical transparency and stability during centrifugation, increase (by 5-6 °C) in the maximum and final gelatinization temperature
GBSS, *SSII*, *SSIII*	Inhibition: Decrease in amylose content, shortening of the terminal chains of amylopectin	Gel stability during freezing or freeze-thaw cycles, water regain and ability to absorb water
SBEI	Inhibition: Moderate increase in amylose content, increase in phosphate content, increase in amylopectin chain length	Not established
SBEI and *SBEII*	Inhibition: Significant increase in amylose content, increase in phosphate content	Decreases in gelatinization temperature, gel viscosity, increased volume in water regain, increase in reducing sugars content and amount of phosphate groups
SBEII	Expression: Increase in amylopectin branching, decrease in degree of polymerization of external chains of amylopectin	Decrease in gelatinization temperature, viscosity, increase in water regain volume
GWD	Inhibition: Decrease in amount of phosphate groups	Decrease in gel viscosity, reduced rates of biocatalytic hydrolysis
glgc-16	Expression	Increase in starch content of tuber
SSIV	Expression	Increase in starch content of tuber
SbAI	Expression. Inhibition of amylase activity, slowing down the starch hydrolysis, decreased amount of reducing sugars	Increased starch content of tuber, decreased effect of brown colouration during heat treatment

Therefore, the impact of most genes involved in starch biosynthesis in potato is multi-faceted: on the one hand, each gene is involved in the regulation of the manifestation of several starch physicochemical properties, and on the other, almost every trait is affected by several genes. Genetic engineering methods allow verifying assumptions about the role of a particular gene, as well as directly evaluating the outcome using instrumental procedures. If the outcome coincides with the expectations, the approach can be further used in traditional or marker-assisted selection for obtaining technical varieties in which starch will serve as the optimal raw material for the relevant industries.

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ANTHOCYANINS SYNTHESIS IN POTATO (*Solanum tuberosum* L.): GENETIC MARKERS FOR SMART BREEDING (review)

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Abstract

Potato may have anthocyanin-colored tuber skin, tuber flesh, flowers, leaves, stems and eyes. Anthocyanins protect photosynthetic apparatus of plant cell, scavenge free radicals under stress conditions, increase efficiency of phosphorus and nitrogen uptake, possess osmoregulatory function, antimicrobial activity and have a number of other useful properties. Anthocyanins are also known for their health benefit: diabetes type II and cardiovascular diseases protection, anti-inflammatory effect, etc. Thus, anthocyanins are important for adaptation of plants to unfavorable environment conditions as well as for nutritional value when they are taken with food. Since potato *Solanum tuberosum* L. is one of the main crop species, possibility to increase anthocyanin content in tuber flesh is important. Anthocyanin concentration in pigmented tuber flesh is similar to that in blueberries, blackberries, cranberries and red grapes. It is important that cooking as well as long storage of potato tubers doesn't affect anthocyanin content. Coloration traits (red or purple tuber flesh) are included in ongoing breeding programs. Therefore, development of tools (convenient diagnostic PCR-markers for anthocyanin biosynthesis genes) for accelerated and efficient selection is of importance. The goal of the current review is to summarize information on the genes regulating anthocyanin biosynthesis in potato and assess possibility of development of diagnostic marker for prediction of tuber flesh color before tuber formation. Anthocyanin biosynthesis takes place in cytosol with the help of enzymes CHS, CHI, DFR, F3H, F3′H, F3′5′H and ANS, after that anthocyanins are transported to vacuoles. Activation of biosynthesis is controlled by MBW complex consisting of transcription factors MYB, bHLH and WD40. This complex activates transcription of structural genes encoding the enzymes mentioned above. A number of MYB-encoding genes are identified in potato, among them *StAN1* related with anthocyanin biosynthesis. This gene corresponds to the *D* locus previously revealed with genetic dissection approach and mapped to chromosome 10. The genes encoding bHLH (*StJAF13* and *StbHLH1*) and WD40 (*StWD40*) have been revealed only by their homology with similar genes of other plant species, but not by genetic dissection, probably because they have no allelic diversity. Thus, the main gene determining high variability of potato by the coloration traits is *StAN1*. Its allelic variants are described and shown to be related with anthocyanin synthesis efficiency. The *StAN1* alleles can be easily distinguished by PCR fragments lengths, what allows constructing convenient diagnostic markers for selection. In some cases, the lack of anthocyanins is due to mutation of a structural gene. This was described in the literature for the *R* locus encoding DFR enzyme. Mutation of other structural gene, *StF3′5′H* (locus *P*), just partially disrupts anthocyanin synthesis, not effecting red pigments, but blue and purple only. This makes the *StF3′5′H* an attractive target for marker-assisted identification of genotypes with different tuber flesh color — purple or red. Thus, there are two main targets for breeding anthocyanin-colored potato — *StAN1* and *StF3′5′H*.

Keywords: *Solanum tuberosum*, potato, marker-assisted selection, anthocyanins, stress tolerance, nutrition value, genes, diagnostic markers

Anthocyanins are plant pigments of a large family of phenolic compounds, the flavonoids. In potato (*Solanum tuberosum* L.) the anthocyanins can cause the coloration of the tuber peel (pink, red, red, blue and dark purple), the pulp of the tuber (red and violet, solid or patchy, of different saturation), eyes

and flowers (red and blue, unequal intensity), leaves and stems (from weak to very intense) [1].

Color of the stem and leaves is worthy of attention, as these are adaptive signs. Thus, anthocyanins participate in the protection of the photosynthetic apparatus and cell membrane, neutralize free radicals, protect against their adverse effects, increase the efficiency of phosphorus and nitrogen assimilation, enhance the osmoregulatory function, reduce the freezing temperature of cell contents, and provide chelation and sequestration of heavy metal ions. These properties in different combination allow plants to adapt to unfavorable conditions, e.g. excessive UV radiation, drought, extreme temperatures, soil salinity, phosphorus and nitrogen deficiency, toxic effects of herbicides and heavy metals [2-5]. Also anthocyanins have antimicrobial activity [6, 7]. For example, their significant contribution to the resistance of potatoes to wet rot is shown: on sections of tubers with purple pulp, the lesion area was on average 28.6 % less than in potatoes with yellow pulp [8]. In plant tissues in the infection zone, there is a rapid oxidation of phenolic compounds, including anthocyanins, followed by lignification, suberinization and programmed cell death [9].

The anthocyanin content in tubers and, especially, in their pulp is of the greatest importance in potato as a food crop. It is known that anthocyanins coming from plant foods can prevent cardiovascular diseases, type II diabetes, arthritis, obesity, vision pathologies, various cancers and neurodegenerative diseases, have anti-inflammatory effects [10-15]. The molecular mechanisms underlying these properties are not completely clear. For some polyphenol compounds, possible participation in the regulation of gene activity, signaling, exposure to cell receptors and blood proteins involved in the inflammatory response (acute phase proteins) [16-18] is indicated. Antioxidant properties of anthocyanins are also discussed. In particular, it is not clear whether they are able to neutralize free radicals in vivo in the same way as in vitro (the interaction efficiency in vitro of anthocyanins against various reactive oxygen species is almost 4 times higher than that of ascorbic acid and α-tocopherol) [3]. The therapeutic (hypoglycemic) effect of anthocyanins is described, which is associated with the suppression of genes involved in the synthesis of aliphatic acids and triacylglycerol, as well as inhibition of pancreatic amylase and intestinal α-glucosidase [19-21].

Tubers with a purple pulp are a rich source of anthocyanins, in particular of their acylated derivatives (22, 23). Anthocyanidin derivatives, the petunidin, pelargonidin, peonidine and malvidin, are detected in potato cultivars [24] (Fig. 1). The content of anthocyanins in colored tubers is comparable to that in blueberries, blackberries, cranberries and red grapes in which these compounds are synthesized in the largest amount [25, 26]. In addition, flavonoids mainly accumulate in potato peel rich in substances that are of pharmacological interest [27, 28]. It is important that, after cooking, the amount of anthocyanins either decreases insignificantly, or does not change [29, 30]. The same happens when storing potatoes [31].

The properties of anthocyanins stimulate involvement of the potato in breeding programs for a high content of these pigments in the red and purple pulp of the tuber [32, 33]. Increasing the effectiveness of such programs is now associated with the development of DNA markers for target anthocyanin biosynthesis genes based on the use of PCR analysis. The use of DNA markers during the early breeding steps (instead of evaluating biochemical parameters after the manifestation of the trait) allows the selection only of the forms with the desired genes and reduces time and costs for creation of cultivars [34].

The purpose of this review is to analyze data on the genes that regulate the biosynthesis of anthocyanins in potato plants and to evaluate the possibility

of developing DNA markers that predict the nature of the pulp color in early ontogeny prior to the onset of tuber formation.

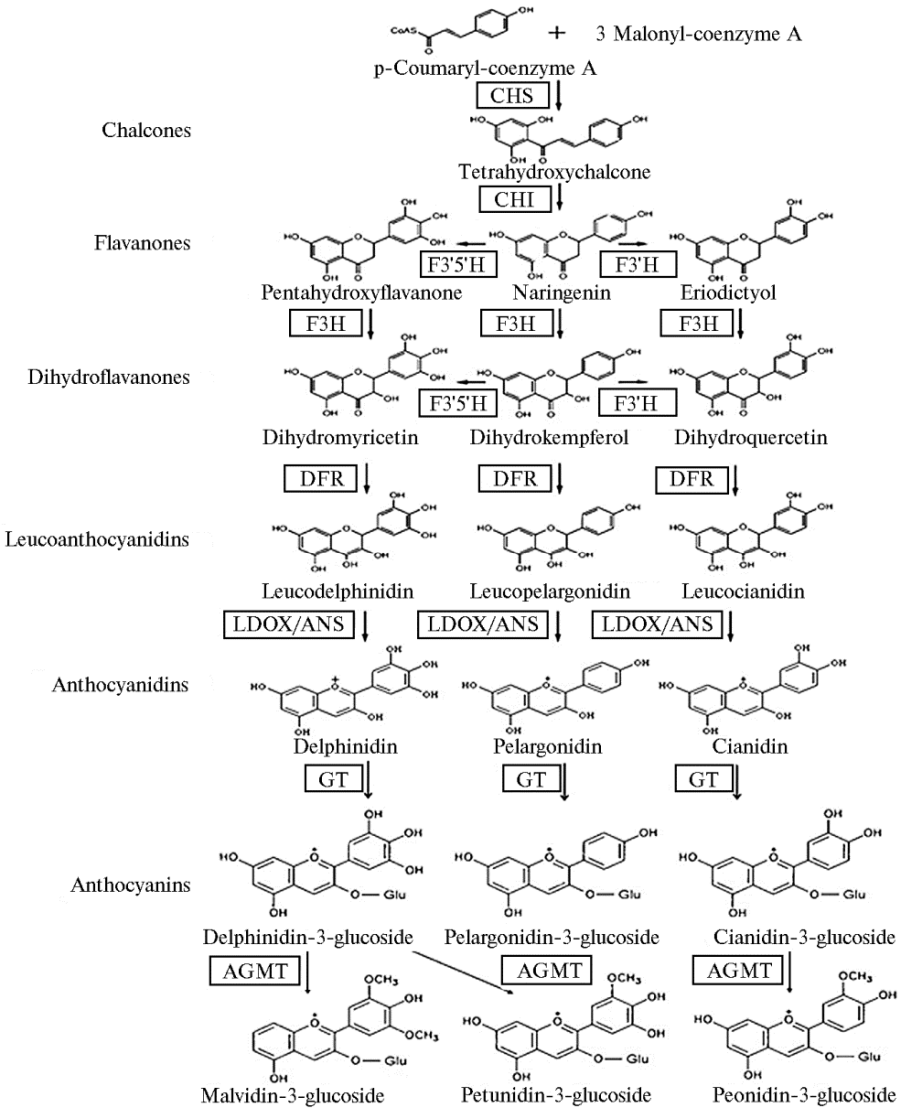

Fig. 1. The biosynthesis scheme for the most common anthocyanin pigments (cited in 51): CHS — chalcone synthase, CHI — chalcone-flavanon isomerase, F3H — flavanon-3-hydroxylase, F3'H — flavonoid-3′-hydroxylase, F3′5′H — flavonoid-3′,5′-hydroxylase, DFR — dihydroflavonol-4-reductase, LDOX/ANS — leucoanthocyanidin dioxygenase/anthocyanidin synthase, GT — glycosyltransferase, AGMT — anthocyanidin glucoside 3′-O-methyltransferase.

Genetic regulation of anthocyanins biosynthesis. The enzymes involved in biosynthesis of anthocyanins and their precursors in higher plants are CHS (chalcone synthase), CHI (chalcone flavanone isomerase), DFR (dihydroflavonol-4-reductase), F3H (flavanon-3-hydroxylase), F3′H (flavonoid-3′-hydroxylase), F3′5′H (flavonoid-3′,5′-hydroxylase), LDOX/ANS (leucoanthocyanidin dioxygenase/anthocyanidin synthase) (see Fig. 1). After synthesis in the cytosol, the phenolic compounds are transported to cell vacuoles [35]. In all plant species analyzed to date, tissue-specific accumulation of anthocyanins is associated with the structural gene expression of abovementioned enzymes regulated by three types of transcription factors (TF), i.e. domain MYB, the basic domain “helix-loop-helix” (bHLH) and WD-repeats (repeats of two amino acid

residues, tryptophan and aspartic acid, WD) [36-39].

The synthesis of anthocyanins in the periderm of tubers of *S. tuberosum* tetraploid potato was shown to be controlled by loci *P* (*purple*) and *R* (*red*) mapped in chromosomes 11 and 2, respectively (40-44). In this case, the locus *P* is epistatic to *R* [40]. Later it was found that *P* and *R* are genes encoding the enzymes of flavonoid biosynthesis (F3′5′H and DFR) [45-48] (see Fig. 1). Now the molecular mechanisms underlying the nature of *P* and *R* inheritance are clear. Red pigments, the derivatives of pelargonidin, can be synthesized in plant cells with functional CHS, CHI, F3H, DFR and ANS enzymes (see Fig. 1), but not in gene *R* mutant with defective DFR. Synthesis of violet pigments, the derivatives of petunidine and its precursor delphinidin, necessitates the enzyme F3′5′H encoded by *P* locus (see Fig. 1). When it is functional, the precursors of the red pigment are used in the synthesis of delphinidin and petunidin thus switching biosynthesis pathway in a manner which leads to epistasis revealed by genetic analysis. Using DNA analysis, the location of genes encoding other enzymes was determined. *CHS* and *CHI* were found on chromosome 5, *ANS* — on chromosome 8 [47, 49, 50].

In addition to structural genes, *S. tuberosum* has loci corresponding to regulatory genes. Locus *D* (*developer*, in diploid potatoe *S. rybinii* Juz. & Bukasov designated as *I* — *inhibitor*) located on chromosome 10 encodes the transcription factor R2R3 MYB, which has a high similarity with the product of the previously isolated petunia (*Petunia hybrida*) regulatory gene — *PhAN2* [27, 40, 52, 53]. Additionally to MYB-like TF, orthologs of the petunia genes encoding bHLH, *PhJAF13* and *PhAN1*, are found in *S. tuberosum* on chromosomes 8 and 9, respectively [47, 49, 50].

Regulatory MYB factors. MYB family is one of the most common groups of TF described in plants. Proteins from this family have two specific domains — conservative N-terminal DNA-binding domain of about 50 amino acids and a non-conservative C-terminal region responsible for regulation of the target gene expression [54]. The common characteristic for all MYB factors is the presence of one to four incomplete MYB repeats that can function together or separately in binding DNA and protein-protein interactions, respectively. By the number of repetitions in the MYB domain, this family can be divided into four classes: 1R, R2R3, 3R and 4R [55]. R2R3 MYB family is the largest group of genes that encode TF in plants. They play an important role in regulation of catalytic enzymes, including the biosynthesis of anthocyanins [54, 56].

Because of tetraploidy and heterozygosity of cultivated potato *S. tuberosum*, the combination of MYB proteins is more complex than in diploid potatoes. In recent years, much attention has been paid to molecular mechanisms and genes controlling the biosynthesis of anthocyanins or their accumulation in potato tubers [57-62], and all researches emphasized the role of gene *StAN1-ANTHOCYANIN1* (GenBank accession number JQ418343) (Fig. 2, A) in controlling the expression of structural genes involved in the biosynthesis of anthocyanins and other phenylpropanoids, especially in the periderm and pulp of pigmented tubers [57, 58, 63]. Besides, *StAN1* is one of the key genes responsible for differences in the biosynthesis of anthocyanins not only in tubers, but in potato leaves [59]. The *StAN1* sequence resembles the petunia gene *PhAN2*, so this locus was originally named similarly, but was later renamed [57]. StAN1 is not only an important regulator of anthocyanins biosynthesis, but also a key factor in the synthesis of other phenylpropanoids in tubers. It is noted that the expression of the *StAN1* gene correlates with accumulation of flavonoids in potatoes under response to drought [63].

Sucrose (see below) is involved in regulation of *StAN1* gene activity [58].

The role of this compound in modulating the transcriptional and post-translational regulation of many genes associated with pigmentation is well known [64]. Sucrose may enhance the synthesis of anthocyanins, but is not capable of causing changes in anthocyanin deficient mutants [65, 66].

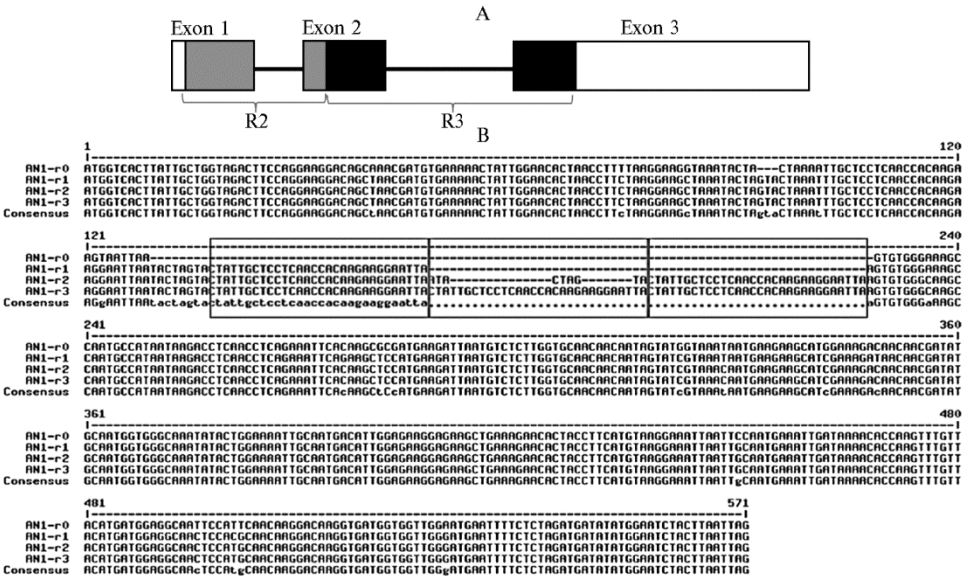

Fig. 2. Regulatory factor StAN1 gene organization: A — exon-intron structure of *StAN1* [57], the DNA-binding domains of R2 and R3 MYB are noted; B — nucleotide sequences of the exon 3 in different alleles (*StAN1-r0, -r1*, *-r2*, *-r3*) of the *StAN1* gene (rectangles indicate motifs r, the number of which affects the efficiency of anthocyanin synthesis).

High intraspecific variability of the *StAN1* gene is noted [59, 61], with mutations detected both in the coding and non-coding regions (i.e. in the intron 2 and the exon 3 of the gene). Some mutations lead to loss of important amino acids, necessary for interaction with DNA. Most mutations of *StAN1* are either silencing, or are in introns. First, $StAN1^{816}$ (AY841128) and $StAN1^{777}$ (AY841130) were found in *StAN1* [57], and it was noted that they are found only in potato cultivars with pigmentation of tuber periderm. Allel $StAN1^{816}$ is 39 bp longer than the $StAN1^{777}$ in exon 3, and 82 bp shorter in the intron 2, so in the PCR analysis, it is easy to distinguish $StAN1^{816}$ from $StAN1^{777}$ by 43 bp shorter amplification fragments. Later, other alleles were detected, *StAN1-r0* (KM822778), *StAN1-r1* (KM822779), *StAN1-r3* (KM822780) [61], and it turned out that the r-motif (see Fig. 2) is a duplication of 30 bp long region (CTATTGCTCCTCAACCACAAGAAGGAATTA, 10 amino acid residues — TIAPQPQEGI) in the exon 3. Thus, the alleles are named depending on whether they have these repeats and how numerous the repeats are. The allele *StAN1-r1* is defined by us as $StAN1^{777}$, and $StAN1^{816}$ is proposed to be designated as *StAN1-r2*, because in its sequence there are two perfect (non-degenerate) r-repetitions (see Fig. 2, B). It is assumed that the presence of one r-motif in the exon 3 is optimal for the activation of anthocyanin synthesis, and the absence of r-motif or its excess (three replicates) decreases the functionality of the protein [61]. The manifestation of the allele with two replicates of the r-motif has not yet been evaluated.

StAN1 is expressed in both normal and pigmented tubers, and the expression level of the gene is not proportional to the amount of flavonoids in the plant. Interestingly, the most *StAN1* transcripts detected in potato cultivars with non-pigmented peel and pulp had a truncated 3′-end. The product of such truncated mRNA is probably unable to activate the biosynthesis of anthocyanins, which may indicate the importance of r-motif as a functional element of StAN1 [61)]. There-

fore, PCR analysis can be developed to accurately identify and map allelic variants of the gene along the DNA amplification fragments. These markers can be used in breeding to select the most effective variant of the regulatory gene *StAN1*.

Allelic differences of *StAN1* affect not only exons and introns. The number of elements associated with sucrose (SURE, sucrose-responsive element) and methyl jasmonate (MeJa, methyl jasmonate-responsive elements) varies in promoters of genes responsible for pigmentation. According to R.S. Payyavula et al. [58], alleles of the *StAN1* gene that cause accumulation of anthocyanins in tubers contain up to six SURE sequences and up to five MeJa motifs in the promoters. In another study, the presence of the SINE retrotransposone in the promoter was shown to be associated with anthocyanin pigmentation of leaves. In genotypes with green leaves (without anthocyanin pigmentation), this element does not appear in the *StAN1* promoter [59]. The presence of mobile elements modifies the expression of genes encoding MYB [67-71], hence further study of the modification effect of SINE on the *StAN1* promoter functions is necessary.

Since some parts (or tissues) of the potato plant (phelloderm, tuber periderm, eyes, leaves, stems, etc.) can be pigmented independently, it has been suggested that locus *D* contains two (or more) tandem duplication of *MYB* gene, at that the phenotype modifications are associated with changes in the sequences of different *MYB* genes [57, 59]. Indeed, by the homology with *StAN1*, another gene was found, first designated as *StAN3*, then renamed to *StAN2*, and also having the synonym *StMYBA1* [57-61]. The predicted amino acid sequence for *StMYBA1* with high similarity corresponds to a protein of the R2R3 MYB class (66 % identity to StAN1) [58]. *StMYBA1* was assumed to be a copy of *StAN1*, lost functionality and turned into a pseudogene, as full transcripts of *StMYBA1* could not be detected [57]. Later it was found out that expression of this gene can be connected with synthesis of anthocyanins in potato tubers, i.e. two allelic variants, *StMYBA1-1* which can be expressed in all tissues irrespective of their pigmentation, and *StMYBA1-2*, active only in violet tubers [61], have been identified. Expression in *StMYBA1* is significantly lower than that of *StAN1*, and its strict correlation with accumulation of polyphenols in tubers is not evident. Probably, *StMYBA1* regulates the transcription of not only anthocyanin biosynthesis genes [57, 63].

In Potato Genome Sequencing Consortium (PGSC) database (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml), other nucleotide sequences for MYB factors conservative in the R2 and R3 domains [58] were identified. One of them, *MYB12B*, is weakly expressed in the pulp of tubers with a clear inverse correlation between transcriptional activity and the amount of phenylpropanoids. Expression of *MYB12B* in the remaining organs and tissues of potatoes was not studied. Perhaps this is a truncated transcript of one of the *MYB* genes found in potatoes.

By homology to *AtMYB113* which positively regulates metabolism of phenylpropanoid in *Arabidopsis thaliana* [72], *StMYB113* [61] was detected and three functionally different gene variants, *StMYBA113-1*, *StMYBA113-2* and *StMYBA113-3*, were identified. *StMYBA113-1* is expressed in both pigmented and nonpigmented potato tissues, while *StMYBA113-3* was active only in the violet peel, but has a shortened protein product due to a 130 bp deletion detected in the cDNA and causing the stop codon at the amino acid 9 position. Compared to other alleles, *StMYBA113-2*, which is expressed only in the red peel, has several deletions leading to amino acid substitutions.

Thus, among MYB-like transcription factors, TF, encoded by the *StAN1* gene for which a clear correlation between the functionality of the alleles and the variability in C-end of the protein is revealed, plays a key role in the regulation of

anthocyanin synthesis. Additionally, *StMYBA1* and *StMYB113* associated with the production of anthocyanins may also have the potential for selection.

Regulatory bHLH factors. As it was noted, various R2R3 MYBs regulate the biosynthesis of phenylpropanoids, at that some of the proteins interact to the bHLH factors [36]. bHLH family form the second largest class of TF in plants. The bHLH domain of about 60 amino acids in length is highly conservative. It consists of two functionally distinct regions. The N-end contains 13-17 amino acids and binds to the E-box (enhancer box, present in some promoter regions in the eukaryotic DNA sequence of CANNTG, where N can be any nucleotide). The HLH consists of two amphipathic α-helices which mainly contain hydrophobic amino acids and are connected by a loop variable in length. Proteins containing the HLH motif often form homo- or heterodimers with other bHLH proteins, which is necessary for DNA recognition. The interaction of bHLH to R2R3 MYB involved in formation of transcriptional complexes with the promoters of anthocyanin biosynthesis genes seems to be rather important. Thus, the MYB transcription factor (*Pp-1* gene) in the absence of bHLH encoded by the *Pp3* gene (*TaMyc1*) is unable to activate the biosynthesis of anthocyanins in the pericarp of wheat weevil [73].

To date, *StbHLH1* (JX848660) and *StJAF13*/*StbHLH2* (KP317176) for two bHLH factors which are involved in regulation of the anthocyanin synthesis in tuber and leaves have been identified in *S. tuberosum* [58, 59]. These genes are extremely conservative in domains that determine the structure of the bHLH domain, but in the others are very different, which makes the amino acid sequences only 43 % similar. The combination of *StJAF13* and *StAN1* functional variants predetermines high activity of anthocyanin biosynthesis structural genes and the presence of a pigment in the leaves and tubers peel [59]. The obtained data indicate a complementary interaction of the regulatory factors StAN1 and StJAF13, as a result of which anthocyanins are synthesized [59]. The level of anthocyanin expression is determined by *StAN1* gene, whereas transcription of *StJAF13* and also *StbHLH1*, another gene encoding bHLH, does not correlate to accumulation of phenylpropanoids, including anthocyanins, in the pigmented tissues [58, 61].

Mutant variants of the *StJAF13* gene, on the basis of which diagnostic markers could be created, are not yet known in potato. For the *StbHLH1* gene, five allelic variants are described and their functional role in certain tissues is assumed [61], but this information is subject to additional experimental verification.

Regulatory WD40 factor. The biosynthesis of anthocyanins is usually regulated by the MBW complex formed by the transcription factors MYB, bHLH and WD40. The first 200 amino acids of bHLH are required for interaction with MYB, and the next 200 residues are involved in interaction with WD40 [74]. WD proteins contain four to eight degenerate tandem repeats and interact with other proteins via the repeats [75].

So far, *StWD40* is known to be the only found tetraploid potato gene, the expression of which correlates to the content of phenolic compounds and anthocyanins [58]. Its expression is 3-5 times higher in red and violet tubers. However, this factor alone can not induce the synthesis of phenolic compounds. That is, WD40 is necessary, but not sufficient to activate the anthocyanin pathway. *StWD40* gene mutations in potato have not yet been described.

Structural gene *F3′5′H*. Pigmentation of tissues and organs in plants primarily depends on enzymes that directly synthesize polyphenolic molecules. For *S. tuberosum*, the key gene that switches the synthesis from red pigments to blue and violet is *StF3′5′H* (see Fig. 1). The cDNA sequence of the *StF3′5′H* gene is known (HQ860267). Introduction of the cDNA of this gene as a

transgene changes the color of the peel of potato tubers from red to violet [45]. Therefore, when selecting the mutants in gene *StF3′5′H*, it is possible to change the color of tuber peel or pulp from violet to red which makes such loci potential targets in programs of potato breeding for anthocyanin composition. Based on the sequencing of the functional and mutant alleles of the *StF3′5′H*, diagnostic markers can be constructed for controlled selection based on the length of fragments in the PCR. *StF3′5′H* seems to be a promising target not only for marker-oriented selection, but also for obtaining modified forms using modern safe editing methods. So, using the CRISPR/Cas9 [76] system it will be easy to knock out the *StF3′5′H* gene to change the color of the tuber peel or pulp from violet to red.

The role of sucrose in the regulation of anthocyanins biosynthesis. The biosynthesis of anthocyanins is usually light-dependent and occurs in the underground parts of the plant as an exception requiring additional mechanisms for activation of the MBW regulatory complex. Such a mechanism in plants is associated with sucrose. Sucrose modulates the regulation of the expression of many genes involved in the synthesis of pigments at the transcriptional and post-translational levels [64]. Sucrose is not only a source of carbon for phenylpropanoid metabolism (through the products of its hydrolysis), but also an anthocyanin biosynthesis regulator [65]. In red and purple tubers, the content of sucrose and glucose is higher than in white or yellow tubers. Sucrose significantly stimulates the expression of *StAN1*, *StbHLH1* and *StWD40*, enhancing the phenylpropanoid potato metabolism [58].

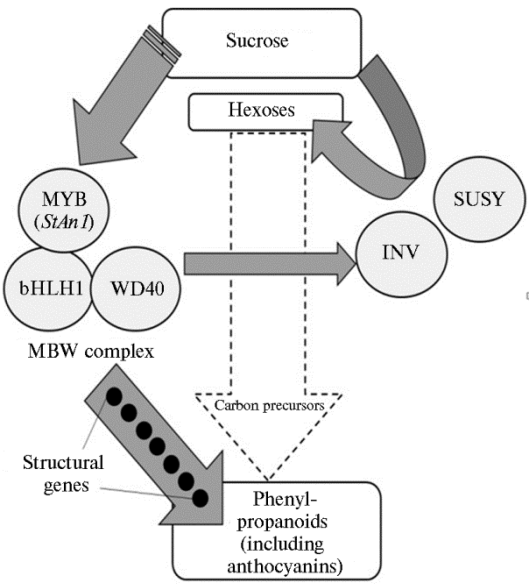

Fig. 3. The proposed model for the interaction of MBW transcription factors complex, sucrose and its metabolism factors in the regulation of the synthesis of phenylpropanoids in potato tubers [58]. Sucrose stimulates the expression of the *StAN1* gene encoding the MYB transcription factor; MYB together with bHLH1 and WD40 (transcription factors of the MBW complex) triggers the synthesis of phenylpropanoids (via activation of the transcription of the structural genes of biosynthetic enzymes) and regulates the expression of the sucrose enzyme (SuSy — sucrose synthase, Inv — invertase) genes. The effect of SUSY and INV factors leads to a decrease in the content of sucrose and accumulation of hexoses, the derivatives of which serve as a substrate for the synthesis of phenylpropanoids.

The presence of SURE elements in the *StAN1* promoter is consistent with the assumption of regulating its expression with sucrose: in potato with purple and red tubers, there are six SURE elements, whereas white and yellow ones are characterized by the presence of a single SURE element [58]. Sucrose treatment with potato seedlings significantly increases the expression of *StAN1* and *StbHLH1*, and to a lesser extent *StWD40*. There is a hypothesis about a possible regulatory loop: sucrose activates the expression of the factors that make up the MBW complex, and that reduces the content of sucrose by inducing the enzymes of its hydrolysis with the release of hexoses, the decay products of which serve as precursors in the synthesis of phenylpropanoids (Fig. 3) [58].

So, the synthesis of anthocyanins in potato tubers is activated by the

MBW complex which is formed by transcription factors MYB, bHLH and WD40. The genes encoding these factors are *StAN1*, *StJAF13* (and *StbHLH1*), and also *StWD40*. Unlike the conservative genes of transcription factors bHLH and WD40, the *StAN1* gene encoding MYB is characterized by a considerable variability. Its alleles are described and their relationship with the efficiency of anthocyanin biosynthesis is shown. *StAN1* alleles can be differentiated by the length of specific PCR products. The formation of red or violet pigments depends on flavonoid-3′,5′-hydroxylase (F3′5′H). Plants with a normal function of MBW regulatory complex and all structural genes of anthocyanins biosynthesis (including *StF3′5′H*) will produce a purple pigment, whereas the presence of the mutant *StF3′5′H* gene leads to red pigment production. Thus, *StAN1* and *StF3′5′H* are currently considered as the main target genes in the selection of anthocyanin synthesizing potato plants.

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DEVELOPMENT OF INITIAL MATERIAL FOR MARKER ASSISTED POTATO (*Solanum tuberosum* L.) PARENTAL LINE BREEDING AT THE DIPLOID LEVEL
(review)

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Abstract

It is important for improving the efficacy of potato breeding to have parental lines that are multiplex for many genes of agronomic characters, first of all genes of resistance to pests and diseases (that have two or more dominant alleles in the locus) (J.E. Bradshaw et al., 1994). Parental lines that are multiplex for several resistance genes occur not often (A.P. Yermishin et al., 2016) and, owing to biological peculiarities of potato crop, they can be produced only by means of breeding at the diploid level. In the presented review the genetic principles are described and an experience of their use by authors in research devoted to the development of the initial material applicable for effective marker assisted potato parental line breeding at the diploid level. The material includes the collection of initial dihaploids originated from potato varieties which were selected for viability, tuber performance, cultivar characters and DNA-markers of pest and diseases resistance genes; the diploid breeding material having wide range of late blight and virus resistance genes, introgressed from wild potato species by means of original methods of overcoming interspecific reproductive barriers; the diploid lines — donors of self-incompatibility gene and high male fertility genes that are effective as in heterozygous as well as in homozygous condition; the diploid lines — donors of genes of male fertile FDR (first division restitution) 2*n*-gamete formation. The best diploid lines having a complex of pest and diseases resistance genes, selected as the result of diploid breeding (on the basis of initial dihaploids, interspecific hybrids and donors of fertility) were used for production of multiplex tetraploid parental lines by means of mitotic chromosome doubling. Diploid parental lines suitable for hybridization with potato varieties (for meiotic polyploidization) were bred using lines-donors of genes of male fertile FDR 2*n*-gamete formation. The diploid initial material developed as the result of the research is of the interest for prospective alternative directions of potato breeding using selection at the diploid level: breeding diploid potato varieties as well as parental lines for production of hybrids and hybrid populations for true potato seed technology.

Keywords: potato, breeding at the diploid level, marker-assisted selection (MAS), multiplex parental lines, male fertility, unreduced gametes

The approach based on development of hybrid populations of high selective value, i.e. hybrid populations where genotypes meeting the requirements to potato varieties are observed more frequently, as compared to random crossings, is considered as the most promising for more effective potato breeding. Such hybrid populations are developed using the known breeding lines with high combining ability with regard to productivity indices, as well as disease and pest resistance gene complex, as one or both parental lines. It is imperative that these breeding lines are multiplex (i.e. having two or more dominant alleles) for several agriculturally valuable genes (first of all, disease and pest resistance genes) [1].

Parental lines that are multiplex for several resistance genes are uncommon among the existing varieties [2]. Owing to biological peculiarities of potato crop, a vegetatively reproduced autotetraploid, effective selection of parental lines that are multiplex for several resistance genes is only possible

by means of breeding at the diploid level.

More simple inheritance of valuable traits at the diploid level makes selection of hybrids with a set of required characteristics much easier, as compared to the tetraploid level. Application of DNA markers allows increasing the effectiveness of diploid selection significantly. Genotypes selected for a complex of resistance gene markers, as well as productivity indices and cultural traits of plants and tubers, may be transferred to the tetraploid level by means of mitotic duplication. As a result, tetraploid parental lines with a complex of genes observed in initial dihaploids are obtained. At that, they are duplex for the respective genes in case of heterozygous dihaploids and quadruplex in case of duplication of chromosomes in homozygotes.

Development of diploid parental lines able to form fertile unreduced pollen by combined selection at the diploid level is considered promising. Their use in crosses with tetraploid varieties allows obtaining of uniform productive environmentally sustainable tetraploid hybrid populations [3], which are mainly not subject to segregation for a complex of traits formed at the diploid level.

Initial breeding material for effective breeding at the diploid level is required for development of the said parental lines. It shall include the collection of dihaploids characterized by a broad genetic basis, sufficient fertility and mutations related to formation of unreduced gametes, which is important for development of diploid parental lines. This material is not available to the majority of potato breeding companies and gene banks. This paper describes the underlying genetic principles of initial material development for diploid potato breeding, as well as the experience of their practical use in research by the authors.

Development of potato dihaploids. Haploids obtained from tetraploids ($2n = 4\times = 48$) are referred to as dihaploids ($2n = 2\times = 24$), while haploids obtained from diploids ($2n = 2\times = 24$) are referred to as monoploids or monohaploids ($2n = \times = 12$). Therefore, for potato the term “dihaploid” does not correspond to the term “doubled haploid” used in genetics of diploid plant species.

Pollination of tetraploid varieties or breeding clones with pollen of haploproducers *Solanum phureja* Juz. & Bukasov is the main method of development of potato dihaploids. Pseudogamy and parthenogenesis are cytological mechanisms of dihaploid embryo development [4]. During pollination with *S. phureja* pollen both nuclei ($n = \times = 12$) of the pollen grain merge with the central nucleus of *S. tuberosum* embryo sac ($2n = 4\times = 48$), which leads to formation of hexaploid endosperm. The egg nucleus ($n = 2\times = 24$) remains unfertilized, but is induced to differentiation. There is evidence [5] of another mechanism responsible for formation of dihaploids which may take place by means of selective *S. phureja* chromosome elimination during formation of hybrid embryos.

Dutch forms of *S. phureja*, IvP 35, IvP 48 and IvP 101, combining high ability to produce haploids with homozygous state of the embryo-spot marker gene responsible for seed lobe anthocyanin coloring, are the most widely used in potato breeding [6]. The embryo-spot is easily detected by a binocular microscope, so introduction of the said haploproducers in the breeding practice made it possible to identify potato dihaploids (by absence of a marker trait) with minimum labor effort. Dihaploids selected from the progeny obtained by pollination of tetraploid varieties or breeding clones with pollen of haploproducers are referred to as primary dihaploids, and the breed obtained by their crossings — as secondary dihaploids.

Studies on selection at the diploid level in potato breeding have been conducted in the potato genetics laboratory of the Genetics and Cytology Institute of the National Academy of Sciences of Belarus since early 1990-s, based

on the material provided by S.A. Lyorek (Ukrainian Research Institute of Potato Cultivation, Kyiv region). During this period a comprehensive collection of diploid breeding material has been developed, which included laboratory primary potato dihaploids, secondary dihaploids on their basis, as well as secondary dihaploids obtained from other research teams. In view of development of DNA markers to detect disease and pest resistance genes in potato, we have been tasked with improvement of quality of the collection by creation of new primary dihaploids with maximum diversity and high frequency of resistance gene markers.

Characteristics of potato varieties of various origins from catalogues have been studied, and varieties, the description of which suggested the presence of several disease and pest resistance genes in the genome, have been selected. The presence of certain genes was confirmed using the respective DNA markers. About 100 of primary dihaploids for several varieties and valuable breeding clones have been obtained using *S. phureja* IvP 35 haploproducer. Some DNA markers observed in initial varieties were lost during transfer to the diploid level due to segregation. However, the frequency of the majority of analyzed markers in the primary dihaploid population obtained turned out to be sufficiently high [7]. New dihaploids of potato varieties, preliminarily evaluated with regard to presence of DNA markers of disease and pest resistance genes, are added to the collection every year. The best dihaploids (apart from DNA markers, viability, tuber performance, nest size, tuber shape, eye depth, and flowering capacity are considered) are included in the program of crossings with diploid lines, which are fertility and 2*n*-pollen formation gene donors, as well as donors of new blight and virus resistance genes introgressed by us into diploid breeding material from wild species.

Diploid breeding material with blight and virus resistance genes introgressed from wild species. More effective involvement of wild and primitive cultivated potato species in valuable gene pool breeding is one of important benefits of selection at diploid level, as compared to tetraploid level. About 70 % of known wild and cultivated potato species are diploids, many of which can be crossed with *S. tuberosum* dihaploids rather successfully. More simple inheritance at the diploid level in backcross generations of interspecies hybrids allows more quick and less expensive selection for certain genes, concentration of desirable and elimination of undesirable wild specie genes [8].

Our study on remote potato hybridization focused on interspecies reproductive barrier mechanisms and effective methods for their overcoming to introgression into breeding material of valuable genes from wild species, which normally do not cross with cultivated potato. At that, achievements in development and implementation of prezygotic incompatibility overcoming approaches, based on the use of segregation for S-genes of parental species, shall be noted. As a result, we involved the tetraploid somatic hybrids of *S. tuberosum* dihaploids with Mexican diploid wild species *S. bulbocastanum* and *S. pinnatisectum* in breeding program [9, 10]. Additionally, we have shown that wild diploid potato species *S. verrucosum* and its laboratory *SvSv*-lines deficient on pistillate S-RNAses, could be successfully used for overcoming pre- and postzygotic incompatibility at interspecies crossing. The use of *S. verrucosum* and *SvSv*-lines allowed involvement of *S. bulbocastanum*, *S. pinnatisectum*, *S. polyadenium*, *S. circaeifolium*, and *S. commersonii* in breeding, as well as overcoming of unilateral incompatibility common to allotetraploid wild species [11-14]. We also observed a new phenomenon, i.e. development of diploid hybrids in crossings of allotetraploid wild potato species *S. acaule*, *S. stoloniferum*, *S. fendlery*, and *S. polytrichon* with *S. tuberosum* dihaploids. Development of such hybrids may greatly simplify the use of allotetraploid species in breeding valuable gene pool [15, 16]. Application of the

above mentioned methods resulted in unique diploid breeding material with a complex of genes of high long-term resistance to blight and viruses [17, 18].

Development of fertility gene donors. It is believed that transfer from the tetraploid level to the diploid level during development of potato dihaploids by self-pollination takes about three generations [19]. As a result, viability of dihaploids mostly decreases and development delays compared to initial varieties. Sterility or decreased fertility is one of the key manifestations of inbreeding depression in primary dihaploids.

About one half of *S. tuberosum* dihaploids developed have rather active flowering required for their inclusion in hybridization. However, flowering genotypes can only be used as female parents, as the majority of male plants is sterile [20]. Though female fertility (ability to set berries and seeds after pollination with fertile pollen of compatible pollinators) is decreased in *S. tuberosum* dihaploids, as compared to tetraploids, it is not considered as the main limiting factor in crossings with dihaploids [21].

Primary potato dihaploids with viable pollen, which could be visualized by acetocarmine staining, cannot mainly serve as pollinators in crossings as their pollen is functionally inactive. Fertility evaluated by the results of hybridization is referred to as "functional male fertility" (FMF) [22]. FMF is determined both by the total amount of pollen and its proportion capable of delivery of male gametes to the embryo sac, i.e. functionally fertile pollen. Several methods are helpful for indirect evaluation of functional pollen fertility (FPF) [23]. According to our experience, the determination of germinated pollen grains abundance on growth medium during a certain period is the most acceptable [24].

The fertility in *S. tuberosum* dihaploids is determined by the initial tetraploid form index and inbreeding [8]. Selection against recessive alleles unfavorable for male fertility is normally not performed in tetraploid potato varieties, as sterile forms may be used as female parents in breeding. Upon development of dihaploids they pass to homozygous state, which is manifested in a decrease in fertility and appearance of sterile forms. Estimates of male fertility in doubled monoploid are in favor of hypothesizing inbreeding as the main reason of male sterility in potato dihaploids [25]. Hybridization of dihaploids with primitive cultivated and wild diploid potato species, which eliminates the inbreeding depression effect, contributes to recovery of their fertility [8]. However, several cycles of crossing at the diploid level are required for development of parental lines suitable for use in potato variety breeding. The genotypes with decreased functional pollen fertility predominate during hybridization of secondary potato dihaploids, especially in combinations with sterile and low-fertility forms [26].

Sterile male progenies are frequent at *S. tuberosum* dihaploids hybridization with diploid potato species. This is due to genetic and cytoplasmatic male sterility, resulting from interaction of dominant nuclear genes observed in the majority of South American diploid species and *S. tuberosum* cytoplasmatic genes [27]. Origin of primary dihaploids from potato varieties carrying certain types of wild specie cytoplasm may also serve as a factor responsible for male sterility. For example, the presence of D cytoplasm from *S. demissum* in varieties correlates to functional pollen sterility, and the presence of W/γ cytoplasm, e.g. from *S. stoloniferum*, correlates to formation of completely abortive pollen [28].

Hybridization of potato dihaploids may fail due to self-incompatibility, which may become a substantial obstacle for implementation of breeding programs related to the use of self-pollination, inbreeding or backcrossing. *Solanum* diploid species are characterized by gametophytic self-incompatibility which is manifested in inhibition and stasis of pollen tubes in the upper third of pistil column [11].

Examination of allelic diversity of potato S-genes has demonstrated its significant limitation [29]. The data obtained in our laboratory suggest that the results of hybridization of certain *S. tuberosum* dihaploids may be explained by the fact that they contain no more than four S-alleles. At that, up to three common S-alleles may be observed in dihaploids originating from two unrelated tetraploid potato varieties [30]. The use of diploid breeding lines with self-compatibility mutations in crossings may contribute to avoiding this problem. Self-compatibility mutations have been identified in *S. tuberosum* dihaploids [31] and in some diploid potato species (*S. goniocalix*, *S. kurzianum*, *S. neohawkesii*, *S. phureja*, *S. pinnatisectum*, *S. raphanifolium*, *S. sanctae-rosae*, *S. stenotomum*) [32]. The mutations *tS1* in *S. tuberosum* dihaploids [31] and *Sli* in *S. chacoense* [33] are best known genetically. *Sli* homozygous donors were successfully used for breeding of diploid inbred lines intended for future development of heterotic hybrids on their basis [34].

The program for development of fertility donors proposed by our laboratory allows solving the problem of decreased fertility in diploid potato plants. This program includes the combination of inbreeding used to eliminate alleles unfavorable for fertility, and hybridization between high fertile genotypes aimed at replacement of unfavorable alleles with alleles that affect pollen development process in potato positively [35].

As a result of examination of a large secondary potato dihaploids collection, IGC 203/5.7 clone based on Polesskii rozovii variety dihaploid and *S. phureja* × *S. vernei* hybrid has been isolated. This clone is male fertile and self-compatible (genetic analysis has demonstrated simple single gene inheritance of this self-compatibility mutation). IGC 203/5.7 clone was involved in hybridization with high fertile forms IGC 92/1.1 and IGC 92/1.2 selected from breed of open pollination of Jubel variety primary dihaploids, which were used as fertility gene donors. The most fertile forms were selected from the obtained hybrids and further IGC 92/1.2 backcross generations (primarily for FPF indices), i.e. mild increase in homozygosity was used, as self-pollination and sibbing produced strongly inbred sterile progeny with low viability. As a result, several high fertile secondary dihaploids have been received to be used for increase in male fertility of initial diploids [35].

Hybridization of male sterile potato dihaploids with fertility donors obtained ensures fertile progeny which can be used effectively in MAS at diploid level. For example, for IGC 01/59.11 line the frequency of high fertile hybrids in the Alpinist variety primary dihaploid progeny amounted to 100 %, and in the Nortena variety dihaploid progeny to 53 % [36]. Currently we use high fertile clones of IGC 10/1.n hybrid population (it was developed in 2010 by self-pollination of IGC 01/61.40 fertility donor line) as fertility gene donors in crossings with primary dihaploids. These clones ensure development of fertile hybrids both in crossings with primary dihaplo-

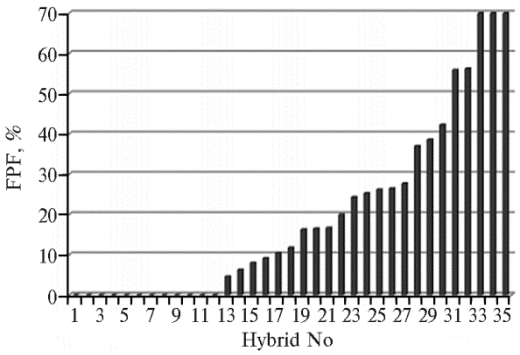

Fig. 1. Functional pollen fertility (FPF**) in hybrids among high fertility diploid potato clones IGC 10/1.1 and IGC 10/1.23**. Despite of similar origin of parental forms (obtained as a result of self-pollination of a relatively homozygous diploid line, the fertility donor IGC 01/61.40), more than half of hybrids is characterized by high (> 10%) FPF.

ids, when fertility genes are heterozygous, and in crossings with each other, when fertility genes are homozygous (Fig. 1).

Thus, hybridization between fertility donors and primary dihaploids selected for viability, productivity, cultivation parameters and a complex of disease and pest resistance gene markers provides initial forms, suitable for breeding for these traits at diploid level. Sufficient male fertility can be easily maintained during such breeding by inclusion of genes with high FPF in hybridization. Effective multiplex tetraploid parental lines can be developed on the basis of the best diploid lines by mitotic chromosome duplication.

Development of diploid parental lines producing unreduced pollen requires slightly different initial forms. Genotypes selected for a complex of agronomically valuable traits shall be suitable for meiotic duplication of chromosomes. According to our experience, FPF and the proportion of 2*n*-pollen shall be at least 10 %. Such genotypes shall appear during final stages of diploid breeding with rather high frequency. It is believed that frequency of genotypes forming unreduced gametes among dihaploids and diploid potato species is sufficient for successful meiotic polyploidization [8]. However, our long-term studies of the large collection of secondary potato dihaploids of various origins suggest that the proportion of genotypes forming fertile unreduced pollen amounted to as few as 0.8 % of the total number of analyzed forms [35], which is clearly insufficient for successful breeding for a complex of traits.

Development of unreduced gamete formation gene donors. Unreduced 2*n* gametes with the number of chromosomes of sporophyte, but not a gametophyte, may be due to meiotic disturbances. Several types of such disturbances resulting in formation of 2*n* gametes are currently known. First division restitution (FDR) mechanism is of the greatest importance for breeding of diploid parental lines of potato.

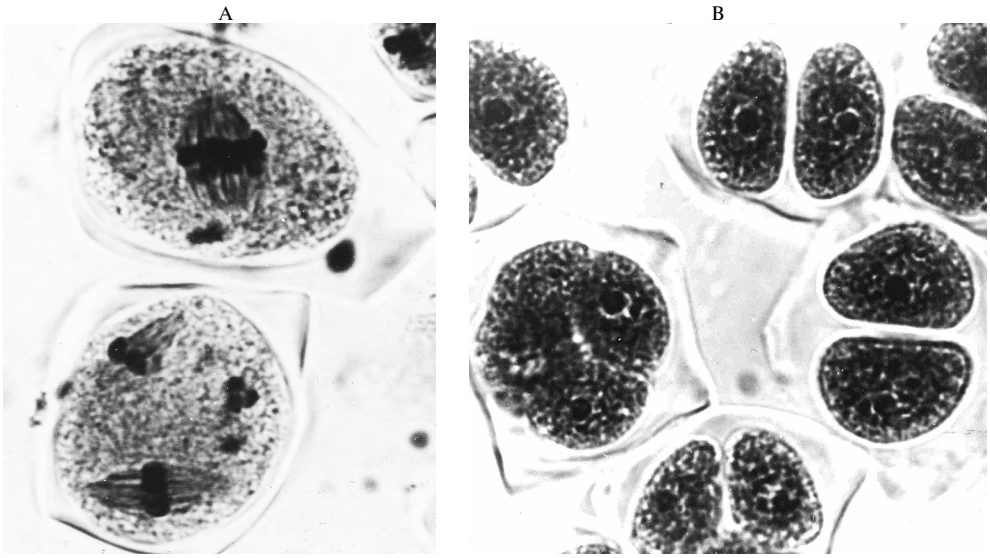

Fig. 2. Meiotic disturbances in potato dihaploids (*Solanum tuberosum* L.) related to formation of unreduced FDR (first division restitution) gametes: A — merged spindles in the second meiotic division due to manifestation of *fs* mutation (top) and two spindles at normal meiosis (bottom); B — formation of 2*n*-microspore dyads as a result of meiotic disturbances related to *fs* mutations (bottom left — normal tetrad of *n*-microspores) (photo by courtesy of V.E. Podlisskykh).

FDR is associated with the occurrence of merged (*fs*) [37] or parallel (*ps*) [39] spindles in metaphase II. Only two poles with two chromosome groups and only one equational division cell wall are formed in mutant cells as a result

of the second meiotic division, which leads to formation of 2*n* microspore dyad (Fig. 2). A special study of the role of spindle coordination in FDR has been conducted in our laboratory. Examination of correlations between *ps* frequency and the frequency of dyads in the forms which produce and do not produce 2*n* pollen, with the use of mathematical modeling, has provided evidence that parallel spindles do not result from meiotic disturbances, and dyad formation is related to *fs* mutation [39]. In case of FDR, chromatids of each chromosome enter different 2*n* spores ensuring preservation of parental heterozygosity in the formed gametes (average degree of parental heterozygosity in FDR gametes is up to 80 %) [40]. It should be noted that one chromatid of each of 24 chromosomes involved in recombination during the first meiotic division, enters 2*n* spores. Thus, all dominant alleles of disease and pest resistance genes observed in unreduced pollen producer are presented in each of them.

The presence or absence of spindle merging during meiosis and formation of 2*n* pollen is a qualitative trait manifested quantitatively. The frequency of 2*n* pollen formation in plants in natural populations of potato species and in breeding samples varies widely, from a fraction to 100 % [38]. Expressivity of *fs* genes manifested as a frequency of 2*n* pollen is determined by the genotype, environmental factors and their interaction [41]. A model of *fs* genetic control has been proposed, where the main genes (gene) interact with polygenes which make a genetic context modifying expression of the main genes [42]. Quantitative trait analysis has demonstrated that the frequency of 2*n* pollen (*fs* expressivity variation) is determined by 2-4 loci with similar effects [43]. The 2*n* pollen rate increases during recurrent selection [44]. Using this approach, we have developed diploid potato lines with 80-100 % of 2*n* pollen [35].

Summarizing our studies on genetic control of unreduced pollen formation during hybridization of potato dihaploids [45], it should be noted that, firstly, the possibility of segregation of hybrids capable of unreduced pollen formation during crossing of dihaploids which do not produce 2*n* pollen is extremely low. At that, the frequency of dyads in hybrids forming unreduced pollen is usually low and insufficient for meiotic duplication of chromosomes. Secondly, crossings where both parents form unreduced pollen ensure development of hybrids, a certain proportion of which forms 2*n* pollen with the frequency sufficient for meiotic duplication. The higher is the unreduced pollen frequency in each parent, the higher is this proportion. Development of hybrids forming 2*n* pollen with higher frequency, as compared to the parent with the best characteristics, is possible. Thirdly, hybridization between dihaploids, one of which produces 2*n* pollen with the frequency of 80-100 % (donor of genes of the trait), while the other does not produce it, does not ensure prodgeny capable of unreduced pollen production with the frequency sufficient for meiotic duplication of chromosomes. A small amount of such hybrids can only be developed in case of presence of genotypes producing 2*n* pollen in the parentage of a parent with normal meiosis.

Thus, in order to identify genotypes capable of functionally fertile unreduced pollen formation with the frequency sufficient for meiotic duplication of chromosomes (along with a complex of agronomically valuable traits) during the final diploid breeding, both parents shall have genes of this trait. So, genes responsible for 2*n* pollen formation shall be introduced in the genetic pool of breeding forms at early diploid breeding. To do so, lines which act as donors of fertility genes and formation of unreduced gametes of a certain type (FDR based on *fs* mutation) at the same time are required. The fertility donor lines developed by us normally do not form 2*n* pollen. In order to develop donor lines for

fertility genes and 2*n* gamete formation the fertility donors were involved in hybridization with the above mentioned lines, capable to produce FDR 2*n* pollen at high frequency (80-100 %) due to *fs* mutation. As a result, several hybrids with high FPF and 2*n* pollen frequency of 20-40 % were selected. They can successfully pollinate both primary and secondary dihaploids, as well as tetraploid varieties and breeding clones [2].

According to the program of breeding diploid parental lines, forming fertile unreduced pollen, donors of fertility and 2*n* gametes were crossed with primary dihaploids possessing a complex of disease and pest resistance genes. As per results of tests in 2015-2016 several promising seedlings have been selected, combining high productivity (at the level of reference varieties), agronomical traits, and presence of markers to several disease and pest resistance genes, which are male fertile and capable of unreduced pollen formation. For example, in 2015 43.2 % of seedlings selected for productivity formed functionally fertile pollen, and some of them produced 2*n* gametes (most of them had 2*n* pollen producers in the parentage). This allowed us to identify a sufficient number of pollinators for MAS program for breeding parental lines at the diploid level (Table).

Characteristic of some diploid potato hybrids (*Solanum tuberosum* L.) selected for use as pollinators in marker-mediated breeding of parental lines at the diploid level

Hybrid, variety	Tubers per plant		Average tuber weight, g	FPF, %	*2n* pollen frequency, %	DNA markers to resistance genes			
	weight, g	number, pcs				*H1* TG689 [46]	*Gro1-4* Gro1-4 [47]	*Ryadg* RYSC3 [48]	*Sen1* Nl25 [47]
IGC 12/42.1	902.3±107.8	18.5±2.6	57.9±15.3	25.8	11.0	0	1	1	0
IGC 12/43.3	838.2±156.4	14.6±1.9	62.1±13.3	49.5	0	1	0	1	0
IGC 12/48.2	802.0±122.7	22.0±3.2	36.5±1.5	47.2	0	1	0	1	1
IGC 12/42.5	672.0±137.9	16.7±3.4	44.5±6.2	44.6	16.0	0	1	1	0
IGC 12/42.2	573.7±110.9	14.3±2.4	40.0±3.7	11.1	9.0	0	0	1	0
IGC 12/42.3	529.5±92.3	10.5±1.2	50.9±6.6	56.5	0	0	1	1	0
IGC 12/45.11	453.0±95.3	16.5±3.3	28.0±2.3	14.0	5.0	0	1	1	0
IGC 12/45.12	379.5±54.9	20.2±1.9	19.0±2.37	21.3	10.0	0	1	1	1
Lilea	651.6±93.1	6.9±0.7	88.8±8.3			1	1	0	1
Skarb	894.3±152.8	14.2±1.3	61.3±7.7			1	0	0	0
Ragneda	1137.7±216.9	16.8±2.3	59.7±7.4			1	0	0	1

N o t e. Average values for 2015-2016 are presented for hybrid productivity, in triplicate, reference variety tubers (early Lilea variety, mid-ripening Skarb variety and middle-late Ragneda variety) in 2015 were planted as a single block, in 10 replications, in 2016 — as three randomized blocks of three plants each (X±SEM; experimental field of the Genetics and Cytology Institute of the National Academy of Sciences of Belarus, Minsk). Functional pollen fertility (FPF) was evaluated as described [24]. Pollen with the diameter above 26 μm was considered as unreduced (2*n*) (as per cytological preparations stained with 2 % acetocarmine). Formation of 2*n* pollen was not examined in standards (blank spaces in the table). "1" and "0" — the marker is present or absent, respectively.

Thus, the breeding material developed has all properties required for MAS at diploid level with development of valuable genotypes, suitable for meiotic duplication at the final stage of diploid breeding.

The prospects of use of marker-mediated selection at the diploid level in potato breeding. The diploid breeding forms developed by us is primarily intended for creation of diploid and tetraploid parental lines, the use of which will significantly increase the effectiveness of conventional breeding. However, it is also of interest for prospective alternative breeding using selection at diploid level. The first trend is breeding of diploid varieties. The best diploid hybrids are equal to potato varieties in productivity and a complex of traits, and breeding at the diploid level is much more effective as compared to the tetraploid level. The second trend means a development of hybrids or hybrid populations reproduced by true seeds. These may include diploid hybrids from crossing of homozygous parental lines, hybrid diploid populations from crossing of relatively homozygous diploid parental lines, and hybrid tetraploid populations from crossing of diploid parental lines, which form unreduced pollen, with potato varieties.

Reproduction of potato by true potato seeds (TPS) suggests the use of seeds obtained from self-pollination (open pollination) of a variety (parental line) or from hybridization of specially selected varieties (parental lines) instead of tubers. A biennial cultivation scheme is also possible, i.e. tubers are obtained from seed sowing on the first year and then used next year as planting material for commercial yield. Such tubers a smaller in size compared to ordinary seed tubers, and are much more healthy, as there is not enough time for accumulation of pathogens. These trends are considered promising for tropical countries where climate is unfavorable for potato seed breeding, and farmers are not able to purchase imported seed tubers annually and store them, reproducing independently [49].

The task of breeding for TPS technology is to obtain seeds of populations with uniform productivity and the presence of pathogen resistance genes, first of all, virus and blight resistance ones. Conventional approaches, i.e. self-pollination of varieties or intervarietal hybridization, did not match the expectations [49]. However, according to the data provided herein, the problems can be successfully solved by MAS at diploid level with respective diploid parental forms capable or incapable of production of unreduced gametes. It should be noted that TPS technology based on diploid hybrids from crossing homozygous parental lines has been recently considered the most promising for developed countries and capable of drastically change of the existing potato farming system [34, 50]. KWS SAAT SE (Germany), one of the world's largest breeding companies, has made a decision to focus its activities in potato breeding on development of this particular technology [51].

Thus, the use of diploid level selection in potato breeding for development of effective parental lines requires special parental forms. Apart from a broad range of primary dihaploids of various origins, carrying DNA markers to disease and pest resistance genes, and interspecies hybrids with valuable traits, diploid lines which can serve as fertility, self-compatibility and FDR 2*n* pollen formation donors must be involved in crossing. They play a key role in diploid potato breeding, as they may help to obtain hybrid populations with fertility sufficient for effective selection at the diploid level.

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DEVELOPMENT OF METHODS FOR AUTOMATIC EXTRACTION OF KNOWLEDGE FROM TEXTS OF SCIENTIFIC PUBLICATIONS FOR THE CREATION OF A KNOWLEDGE BASE SOLANUM TUBEROSUM

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Abstract

Currently there are hundreds of scientific journals that publish research results in various fields of plant biology and agrobiology. Hundreds of thousands of international patents contain a variety of information on agricultural biotechnology. The number of articles and patents is increasing over time in an exponential progression. For example, there are more than 1.5 million publications devoted to the study of *Solanum tuberosum* that is one of the most important crops in the world. Analysis of such huge number of experimental facts presented in text sources (scientific publications and patents), requires the use of automated methods for knowledge extraction (text-mining). Intelligent automatic text analysis techniques are already widely used in biology and medicine to extract information about the properties and functions of molecular genetic objects. Unlike search engines such as Google, Yandex and others, that search documents by keywords, such text-mining methods are aimed at the automatic extraction of knowledge presented in the documents, knowledge integration and formalization according to the defined ontology. Among the known systems for intelligent knowledge extraction from scientific publications STRING, LMMA, ConReg, GeneMania and others can be listed. For the first time in Russia, we have previously developed a system, named ANDSystem, for automatic intelligent knowledge extraction in biomedicine. ANDSystem contains more than 10 million facts about molecular-genetic interactions extracted from more than 25 million scientific publications. For knowledge extraction in ANDSystem, specially developed semantic and linguistic rules are used for recognition of interactions between biological objects such as, proteins, genes, metabolites, drugs, microRNA, biological processes, diseases and others in natural language texts. However, the problem of development of methods for automatic knowledge extraction from the texts in plant biology, agrobiology and agrobiotechnology remains still unsolved and has a high relevance. The aim of this work was to adapt the methods of automatic knowledge extraction, presented in ANDSystem, to the field of crop production and to create on this basis a SOLANUM TUBEROSUM knowledge base, containing information on genetics, markers, breeding and selection of potatoes, its pathogens and pests, storage and processing technologies and others. The knowledge base ontology contains dictionaries, corresponding to more than 20 types of objects, including molecular genetic objects (proteins, genes, metabolites, microRNA, biological processes, biomarkers, etc.), potato varieties and their phenotypic traits, diseases and pests of potato, biotic and abiotic environmental factors, biotechnologies of cultivation, processing and storage of potato, and others. Also, the ontology contains more than 25 types of interactions that describe various relationships between the above listed objects, including molecular interactions, regulatory events and associative links. More than 5 thousand semantic templates were created to extract information about the interactions. The accuracy and recall of knowledge extraction by the developed method were assessed with the expert manual analysis of the text corpus and reached more than 65 % and 70 %, respectively. The full-scale version of the knowledge base SOLANUM TUBEROSUM will be created on the basis of the developed approaches.

Keywords: *Solanum tuberosum,* ANDSystem, text-mining, database, automatic knowledge extraction from texts

Currently, investigation of molecular genetic systems becomes top priority in genomic, proteomic, metabonomic and transcriptomic studies in different fields of biology, including plant growing [1-4]. New approaches take on special significance to the investigation of the genotype-phenotype relationship. Reconstruction and analysis of gene networks replace traditional approaches based on the search for individual genes responsible for the formation of phenotypic plant characteristics, including complex economically valuable features, such as resistance to diseases and pests, tolerance to abiotic factors, and yield [5-8]. The most important source of information on molecular genetic interactions occurring at the intracellular, intercellular and organismic levels of plant organization are databases that summarize the exploratory results, scientific publications and patents. The number of publications increases exponentially each year. Even a simple search query by the keyword 'potato' in the Web of Science and Google Patents, yields information on more than 60,000 papers and 900,000 patents. Many of them contain data on molecular genetic interactions. The prompt accumulation of new knowledge presented in scientific publications and databases is significantly associated with the development of experimental high-performance 'Omic' technology (genomic, transcriptomic, proteomic and metabolic). The use of high-performance sequencing technology allowed reading the genome of the potato.

The NCBI Genomes database (https://www.ncbi.nlm.nih.gov/genome) contains a version of the potato genome GCA_000226075.1 SolTub_3.0 [9, 10], which includes an annotation of 37,966 proteins. Another database, NCBI Gene (https://www.ncbi.nlm.nih.gov/gene), provides information on the sequences and functions of 33,037 potato genes.

To establish interactions between biological objects, experimental methods of direct analysis of protein-protein interactions (yeast two-hybrid systems), transcriptomic analysis (differential gene expression and co-expression) and others are often used. The GEO database (https://www.ncbi.nlm.nih.gov/gds) presents data from more than 1,300 experiments on the expression of potato genes obtained using transcriptomic technology. For example, Y. Ou et al. [11] in their paper presented in GEO (GSE43237) performed a full-genomic analysis of microRNA targets in tubers stored in the cold. The analysis identified 53 known and 59 new miRNAs, as well as 70 target genes potentially involved in the response to low-temperature storage. Another paper [12], also presented in the GEO database (GSE56333), investigated the effects of the potato Y-virus infection on potato resistance to the Colorado potato beetle larvae using high-performance sequencing.

Databases containing information on molecular genetic interactions obtained based on the analysis of factual databases and scientific publications are being rapidly developed worldwide. In particular, the PlantCyc database [13-16] contains information on molecular genetic networks for more than 22 plant species, including potatoes. The PotatoCyc database (the potato section in PlantCyc) contains information on 558 biological pathways, 5,790 enzymes, 3,122 reactions and 2,413 metabolites. However, this database was created based on the non-automated analysis of scientific publications, which guarantees the yield of high quality data, but inevitably leads to a delayed presentation of facts published in scientific articles.

The current number of publications and patents is the so-called 'big data' (extremely large amounts of data), the effective processing of which requires the use of automated text analysis (text mining). The technology of automatic extraction of knowledge from scientific publications is most rapidly developed in biomedicine [17-21]. Among the widely used systems for text analysis on the specified

subjects, STRING [22-24], LMMA [25] and ConReg [26] can be distinguished. The STRING system includes descriptions of protein-protein interactions that are confirmed experimentally or predicted by various methods (including predictions of the distance of genes in the genome, phylogenetic profiles, or co-expression). The system STRING uses information extracted from databases, as well as obtained from publications using methods of automated text analysis. The LMMA system is designed for the reconstruction of biological networks based on the integration of literature data on molecular interactions and evidence on gene expression obtained in microchip experiments. It is based on estimates of the statistical significance of the concurrent occurrence of biological terms in texts from PubMed. ConReg is a plug-in for the Cytoscape system [27] that focuses on the study of genetic regulation in eukaryotic model organisms. Here the evidence on genetic regulation are taken from different databases and supplemented with information about the predicted binding sites for transcription factors, as well as information extracted using the automated analysis of PubMed texts.

We have previously developed the ANDSystem for the automated extraction of medical and biological knowledge from the PubMed texts using semantic template methods [28-30]. ANDSystem includes a linguistic analysis module that automatically extracts from an arbitrary text flow the factual information related to a specific subject (problem) domain according to a given ontology. The module of linguistic analysis consists of three main parts, such as a morphological analyzer, a problem-oriented ontology and a semantic analyzer. The morphological analyzer implements the following functions: descriptive text markup (recognition in the text the concepts included in the ontology, including terminological word combinations); lemmatization; and POS marking. A problem-oriented ontology forms a conceptual model of the problem domain. The semantic analyzer implements the functions of conceptual search in the text of the document and the user interface. The system operation is provided by two main dictionaries: the grammar dictionary supports lemmatization, POS markup and recognition of word combinations based on the linear context; the ontology supports semantic analysis, including elements of limited logic output. In addition, a defining dictionary is used (a word or a word combination as a concept), integrated into an ontology.

In the present work, the methods of the ANDSystem were adapted and adjusted for automatic extraction of the knowledge on genetics, markers, 'omic' resources, breeding, seed production, diagnostics of disease pathogens, protective means and potato storage technologies in order to create a knowledge base called SOLANUM TUBEROSUM. Setting up ANDSystem involved the creation of a subject domain ontology and semantic linguistic rules (templates) for analyzing natural language texts and extracting knowledge formalized according to a given ontology. Important components of the subject domain ontology are dictionaries of the objects, the information about the interactions between which is extracted from texts using templates. The developed ontology of the SOLANUM TUBEROSUM knowledge base contains dictionaries for more than 20 types of objects. Proteins, genes, metabolites, microRNAs, biological processes, biomarkers, etc. are considered as the molecular genetic objects. Separate dictionaries are potato varieties and their phenotypic signs, including potato diseases. A large section of ontology is devoted to potato pests, as well as to environmental biotic and abiotic factors. The ontology also contains dictionaries of cultivating agrobiotechnology, and biotechnology of potato processing and storage. The analysis of quality of the knowledge extraction using created templates demonstrated appropriate accuracy (65 %) and completeness (70 %).

Ontology-based model. We used the term ontology to mean the

$O = <C, R, F>$ set, where $C = C_t + C_o + C_{sp} + C_{ti} + C_i + C_r$ is the set of concepts of the subject domain (Fig. 1), represented by the following components: C_t is the set of object types, C_o is the set of molecular genetic objects, diseases, processes, cellular components, etc., C_{sp} is the set of organisms, C_{ti} is the set of types of interrelations between objects, C_i is the set of interrelations between objects, C_r is the set of object roles in the interrelations. $R = \{is\_a, role, present, exists\_in\} + R_1$ describes the set of relations between the concepts of a given domain and, in turn, consists of subsets describing the different types of relationships between objects, the admitted roles of objects in relationships of a particular type, the relation linking molecular genetic objects with the organisms in which they occur, etc. The third component is represented by the set $F = \{f: R_1 -> C_i\}$, describing the interpretation function, which consists of the one-to-one mapping of the set of the R_1 relations onto the set of types of objects C_i.

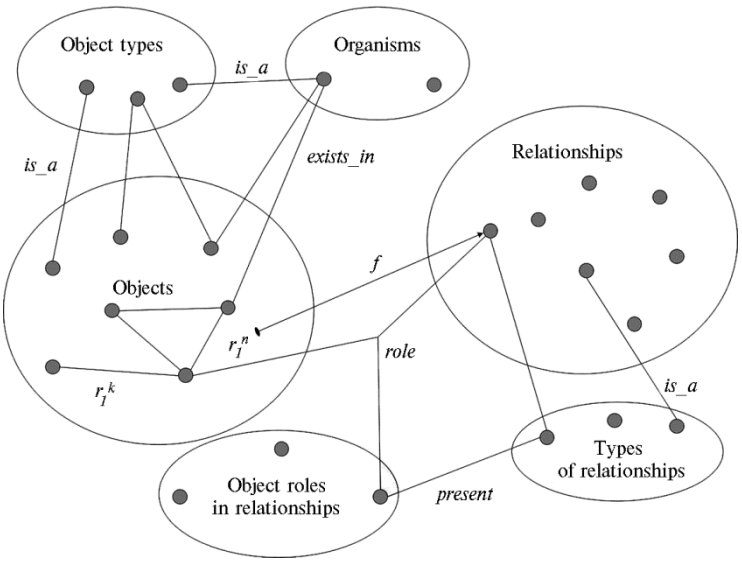

Fig. 1. A graphical presentation of the ontology of associative semantic networks, used to design the SOLANUM TUBEROSUM knowledge base.

The knowledge base structure. Using the developed ontological model of data representation, the SOLANUM TUBEROSUM knowledge base was designed. It includes a database containing molecular genetic information, information about technologies, diseases, environmental factors obtained as a result of the analysis of texts of scientific publications, patents and databases. In addition, the knowledge base contains methods that were used to extract knowledge from texts, and methods designed to analyze the molecular genetic networks available in the database. The MySQL 5.6 relational DBMS was used when developing the knowledge base. The database contains 18 tables describing the following sections: Plant, Potato Pathogens and Pests, Environment, Technology, Bioinformatics, Associative networks (see Fig. 2).

The Plant Section. The Plant section is intended to describe molecular genetic data. In the current version of the knowledge base, this section provides information on potatoes supplemented with information on seven model plants (*Solanum lycopersicum*, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Oryza sativa* Indica Group, *Oryza sativa* Japonica Group, *Zea mays*, *Triticum aestivum*). Molecular genetic data include dictionaries of names and their synonyms for genes (> 140,000 terms), proteins (> 19,000 terms), metabolites (> 42,594 terms), microRNAs (> 10,000 terms), genetic biomarkers (> 20 terms) and biological processes (> 100,000 terms). Separate dictionaries represent potato varieties (206

varieties), selection qualities, economically valuable characteristics and consumer attributes (> 1,300 terms). Specialized dictionaries have been developed that describe more than 100 physiological (phenotypic) signs of potatoes and diseases.

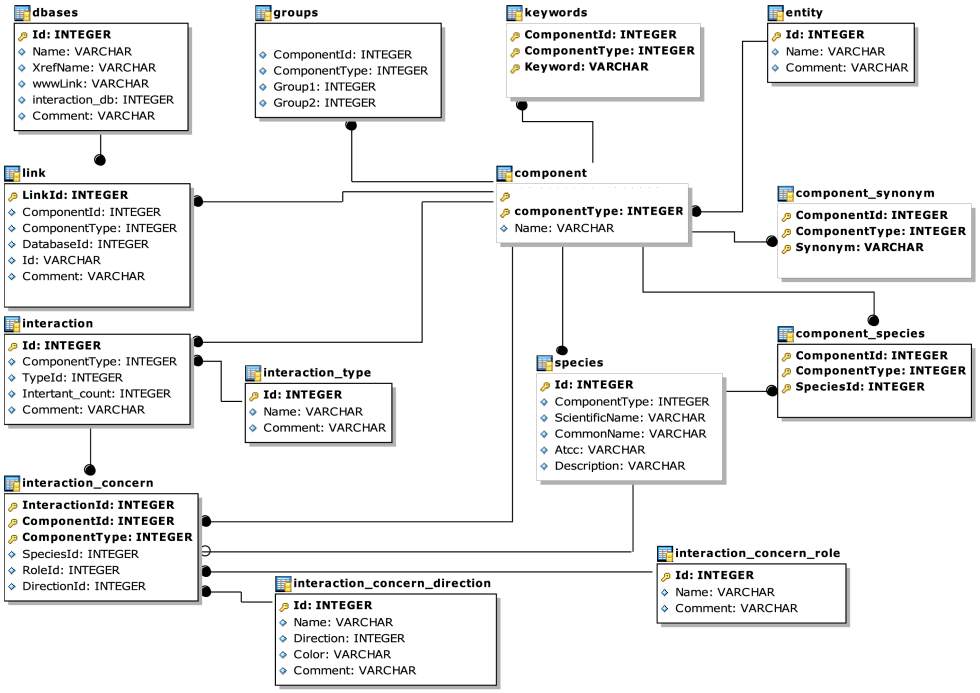

Fig. 2. The structure of the major tables of the relational database in the developed SOLANUM TUBEROSUM knowledge base.

The Potato Pathogens and Pests Section. This contains dictionaries of molecular genetic objects for 24 pathogens and pests of potatoes. Molecular genetic data, similar to the Plant section, are represented by genes (3,451 genes), proteins (476 proteins), metabolites and biological processes. Separate dictionaries describe the markers of resistance to plant protection products, as well as molecular targets for chemical plant protection products.

The Environment Section. This involves the dictionaries for two types of objects, such as biotic and abiotic environmental factors (> 100 and > 50 terms, respectively).

The Technology Section. It should be noted that, along with molecular-genetic objects and environmental factors, various technologies for selection, cultivation, protection and diagnostics of potato diseases, potato processing and storage are presented as independent objects in the developed knowledge base. More than 100 technologies are described in the current version of the knowledge base.

The Associative networks Section. An associative semantic network was used as an informational model of the subject domain, which was in the form of an oriented bipartite graph, the state points of which corresponded to the objects of the domain, and arcs defined relations between them. The relations of the following types are used to describe interactions between molecular genetic objects: 1st type, physical interactions, i.e. the formation of short-lived or permanent molecular complexes; 2nd type, chemical interactions (catalytic reactions and processes) of the substrate-enzyme-product type, in which proteins (enzymes) and low-molecular compounds (metabolites) are involved, including proteolytic cleavage reactions of one protein (substrate) by another protein (proteo-

lytic enzyme), post-translational modifications of proteins (phosphorylation, glycosylation, etc.); 3rd type, regulatory interactions, including regulation of gene expression by transcription factors, regulation of protein activity or function by other proteins, regulation (or implementation) of transport of some proteins by other proteins, regulation of stability or degradation of some proteins by other proteins or metabolites (regulatory events will also be subdivided according to the effect one object exerts onto another one, i.e. the enhancement or weakening of the process); 4th type, co-expression (simultaneous expression of several genes), which was caused by shared regulatory mechanisms that activate expression under varying conditions in the cell; 5th type, associative connections (this category includes unclassified relations between molecular genetic objects, as well as links between molecular genetic objects and objects that match the concepts of breeding, phenomics and seed production, phytopathology, diagnostics, means of protection, agrobiotechnology of cultivation and biotechnology of potato processing and storage). The relations between the concepts of breeding, phenomics and seed production, diseases, diagnostic techniques and means of protection, technologies are based on various types of regulatory links (upregulation and downregulation), as well as links describing the involvement, application, associative links, etc.

The Bioinformatics Section. A specially developed section is closely associated with the knowledge base, which presents bioinformatic methods for analyzing experimental data on molecular mechanisms of functioning of the analyzed biological systems, gene prioritization, prediction of markers, planning experiments, etc. The analysis is performed based on the experimental findings entered by the user, as well as data on the network of molecular genetic interactions automatically extracted from the Associative Networks section.

Currently, bioinformatic analysis of experimental data is actively developing, related to the task of prioritizing the genes when identifying among them those the most important for the studied biological processes (including the response to biotic and abiotic environmental factors), phenotypic (physiological) features, diseases, etc. [31, 32].

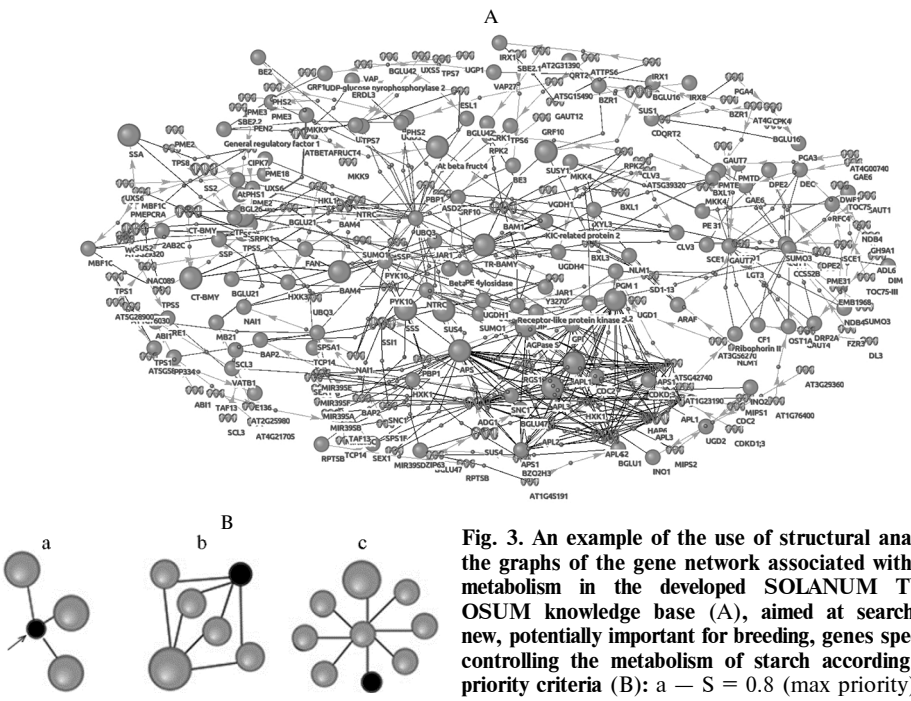

Fig. 3. An example of the use of structural analysis of the graphs of the gene network associated with starch metabolism in the developed SOLANUM TUBEROSUM knowledge base (A)**, aimed at searching for new, potentially important for breeding, genes specifically controlling the metabolism of starch according to the priority criteria** (B)**:** a — S = 0.8 (max priority), the

candidate gene (marked by a black circle) interacts directly with three known key participants in starch metabolism (reference genes, represented by larger circles); b — S = 0.0346, the candidate gene is linked to the reference gene through four proxy genes; c — S = 0.0115 (min priority), the candidate gene is linked to the reference gene through a hub. The identified candidate gene with the highest priority is potato starch branching enzyme 22.1 (marked with an arrow).

To this end, the methods of the well-known GUILD program package (http://sbi.imim.es/web/index.php/research/software/guildsoftware) are integrated into the SOLANUM TUBEROSUM system, based on the analysis of the structure of the gene networks graph [33]. Figure 3 shows an example of the prioritization of genes that specifically control the metabolism of starch, which may be of interest as candidates for breeding. In the present case, the criterion for prioritization was the number of links between candidate genes with reference genes.

Another class of bioinformatic methods, implemented in SOLANUM TUBEROSUM, is based on estimates of the enrichment of biological processes by genes identified experimentally (for example, in transcriptomic analysis). Such methods are widely used in known computer systems intended for interpreting experimental data, e.g. DAVID [34], PANTHER [35, 36], GORILLA [37, 38], etc.

Extraction of knowledge using semantic-linguistic templates. In the ANDSystem, texts are recognized by a linguistic analysis module, which is fed into the input by a textual stream of factual information related to a specific subject (problem) domain. A problem-oriented ontology implemented in SOLANUM TUBEROSUM forms a conceptual model of knowledge. The module of linguistic analysis uses morphological and semantic analyzers. The morphological analyzer performs descriptor markup of the text (recognizing in the text the concepts included in the ontology, including terminological phrases), lemmatization (bringing the word to a normal form), and the POS markup. The semantic analyzer carries out a conceptual search for knowledge in the text, processed by the morphological analyzer, using semantic linguistic templates. A grammar dictionary is used for lemmatization and POS markup. The functional diagram of the system for extracting knowledge about the interactions between ontology objects in the SOLANUM TUBEROSUM knowledge base is given in Figure 4.

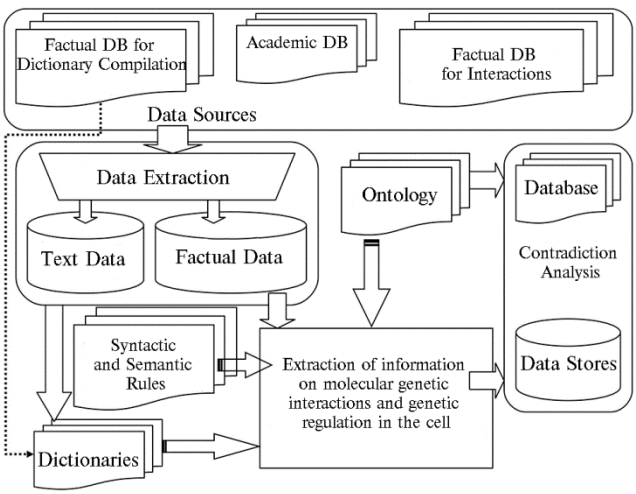

Fig. 4. The functional diagram of the system for extracting knowledge about the interactions between ontology objects in the SOLANUM TUBEROSUM knowledge base.

The initial data are external sources of information, including three groups of factual databases used for compiling dictionaries, extracting knowledge on molecular genetic objects and extracting knowledge about molecular interactions in

the cell and gene networks, as well as a database of bibliographic data (for extracting knowledge using semantic and linguistic templates about interactions between ontology objects of the SOLANUM TUBEROSUM knowledge base).

Semantic and linguistic templates are structured records with information about types of objects, dictionaries, rules for text analysis or regular expressions, and a meta description of interaction semantics. The template structure includes the following main groups of fields: Regular expression, Dictionaries, Interactions, Object Attributes, Interaction Attributes. A regular expression defines the arrangement of object names and special linking words that indicate the specified type of interactions between specific objects in the analyzed sentence. The structure of a regular expression is a sequence of identifiers for object dictionaries and dictionaries of linking words. The symbol "–" is used as a separator character between the identifiers of dictionaries. A regular expression can also specify the admissible number of words that are not object names, which can be placed between object names in the sentence. In addition, a regular expression can contain a negation. We developed a total of about 5,000 such semantic and linguistic templates to be used in ANDSystem for extracting knowledge from the texts of scientific publications.

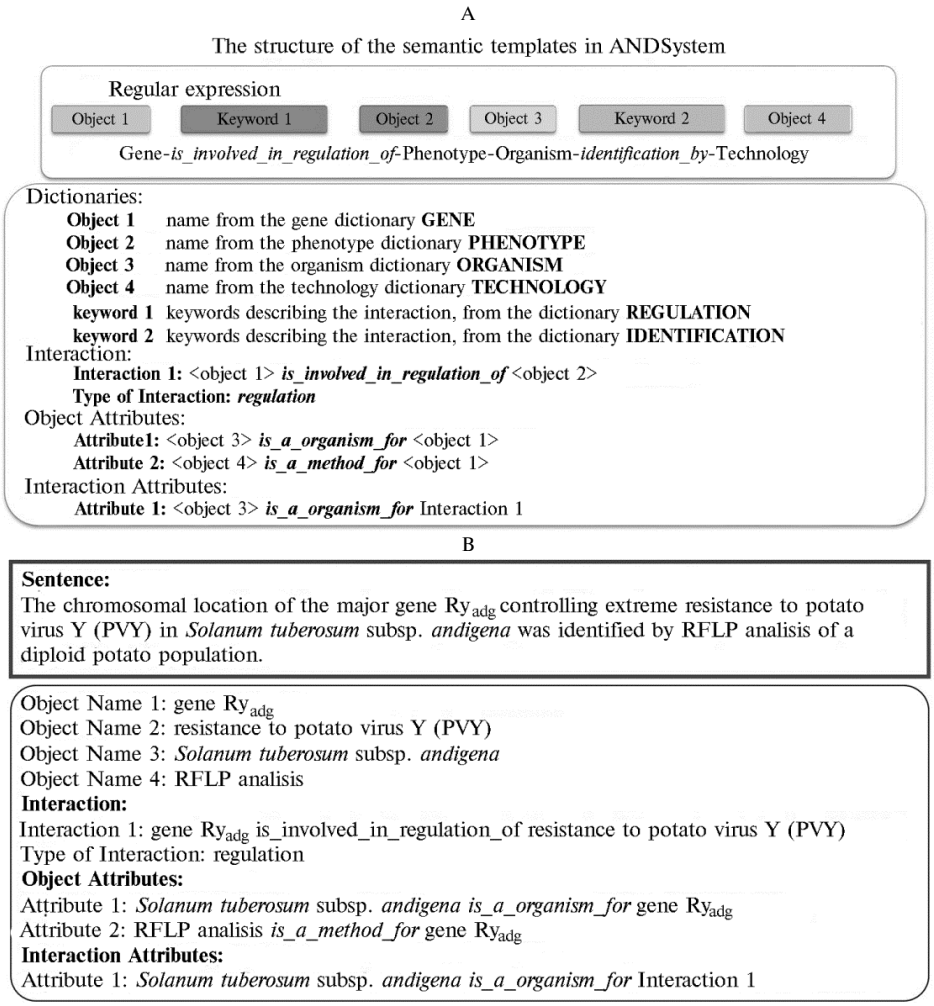

Fig. 5. Examples of the structure of the semantic and linguistic template used in ANDSystem (A), **and computer output of the results of its tryout** (B) **when extracting information about the interactions of objects in the sentence from the paper by J.H. Hämäläinen et al. [39] in the SOLANUM TUBEROSUM knowledge base.**

Let us consider as an example a template for extracting interactions between the genes and phenotypes of the organism (Fig. 5, A), adapted for the SOLANUM TUBEROSUM knowledge base. In this template, the GENE, PHENOTYPE, ORGANISM and TECHNOLOGY dictionaries are used as objects, while the "regulation" and "identification" dictionaries are used as linking words. It follows from the regular expression that object 1 (a gene from the GENE dictionary) is involved in the regulation of object 2 (a phenotype from the FENOTYPE dictionary). As can be seen, the objects and the interactions between these objects have their own attributes. In this example, objects 3 and 4 are, respectively, the organism and technology for object 1. At the same time, object 3 is an interaction attribute for object 1, indicating the organism in which it is performed.

In fact, the template contains all information about the types of objects and the types of their interactions without specifying the names of specific objects. Tryout of a template results in identifying from the texts the specific object names that match a specified regular expression. When applying the considered template to the sentence "The chromosomal location of the major gene Ry_{adg} controlling extreme resistance to potato virus Y (PVY) in *Solanum tuberosum* subsp. *andigena* was identified by RFLP analysis of a diploid potato population" [39] (see Fig. 5, B), the response output is as follows. In the case under consideration, the Ry_{adg} gene corresponds to object 1, the phenotype of resistance to potato virus Y (PVY) to object 2, the organism *Solanum tuberosum* subsp. *andigena* to object 4, and the technology of RFLP analysis to object 5.

Thus, an initial version of the knowledge base has been created for storing information on genetics, breeding, seed production, diagnostics of disease pathogens, protective means and technologies of potato storage, and to do this, an appropriate ontology has been developed (which includes dictionaries of concepts on genetics, breeding, phenomics and seed production, agrobiotechnology of cultivation and biotechnologies for potato processing and storage, diseases, pests, diagnostic methods and means of protection, environmental factors, etc.). The ANDSystem methods were adjusted for extraction of knowledge from the texts of scientific publications, patent and factual databases in the subject domain defined by the created ontology, and previously developed user interfaces were adapted. Using this system, it is planned to conduct a large-scale automatic analysis of the texts of scientific publications and patent databases. It is also expected to significantly expand the volumes of the knowledge base dictionaries by extracting new object names during analysis.

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VALUABLE TRAITS OF POTATO (*Solanum* L.) VARIETIES AS INFLUENCED BY CLIMATE CHANGE IN EUROPEAN RUSSIA

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Abstract

Adaptation of regional assortment of crops to climatic changes necessitates numerical assessment of the observed trends in crop main characteristics and identification of the factors causing this dynamics. In previous research we revealed that valuable traits of cultivated varieties have significantly changed over the last decades. Objective of this research was to summarize our findings on the trends in variability of potato (*Solanum tuberosum* L.) valuable traits in the European Russia territory with special regard to climatic factors as causative ones. It was found out that most valuable traits of potatoes depend on temperature under lack or excess of heat. By correlation-regression analysis, the main agroclimatic factors defining development of potato plants in the European Russia are revealed. Increase in the sum of temperatures above 15 °C and earlier date for temperature to exceed 15 °C were those accelerating the most meteodependent phases, the germination and flowering. The long-time observations on phenology, weigh of marketable tuber per plant and starch content in standard varieties were evaluated in the conditions of regional experimental stations of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR). These were Polar Experimental Station (Murmansk region, variety Hibinskii rannii, 1968-2013), VIR Pushkin laboratories (St. Petersburg, varieties Nevskii, 1984-2004; Elizaveta, Petersburgskii 1999-2010), Maikop Experimental Station (the Republic of Adygea, variety Nevskii, 1990-2012). Correlation and regression analysis was used to determine significant climatic factors. It was shown that the most considerable variability of valuable traits occurred under lack (Polar Experimental Station) and excess (Maikop Experimental Station) of heat. In Murmansk region (Polar Experimental Station), planting to flowering period reduced at a decennial rate of 2.4 days, and the temperature sum for this period also decreased decennially by 15.9 °C; in Adygea (Maikop Experimental Station) the decennial rates were 6.6 days and 73.8 °C, respectively. The time from flowering to harvesting increased at Polar Experimental Station and reduced at Maikop Experimental Station. In Maikop region, the temperature sums during planting to harvesting decreased by 253.4 °C per decade. Under contrast climatic conditions, the temperature sums for interphase periods were less stable indexes than their durations. The tuber weight per plant and starchiness grew at Polar Experimental Station, whereas in the Maikop region the potato productivity increased while starchiness decreased. The obtained models allows to forecast further growth of potato productivity in European Russia caused by earlier flowering and extended period from flowering to harvesting. Earlier planting and involvement of more late-ripening varieties can be offered as a measure to update agro technologies for potato cultivation under climate changes in European Russia.

Keywords: potato, *Solanum tuberosum*, climate changes, phenology, productivity, starch content, stability, sums of temperatures above 15 °C

The period with temperatures comfortable for cultivated plants in Europe has been getting longer since mid-20th century, which has become a significant factor affecting modern agricultural industry [1, 2]. In France potato planting has been performed in average 5 days earlier per 10 years since 1980, in Germany planting and harvesting of cultivated plants accelerate by 2 days per 10 years [3-5]. However, in general, agricultural technologies have not yet adapted to climatic changes [3, 4]. In the European Russia territory the changes observed affect the regional bioclimatic potential, heat supply increases, and the amount of precipitation grows in the north [6, 7].

Heat and water supply conditions significantly affect potato plants both during active growth [8-10] and tuber formation period [8, 11, 12]. Requirements of this culture to climate are as follows: minimum temperature of vegetation start and end is 8-10 °C, cold resistance is up to –2...–3 °C [6, 13], in northern varieties plant growth occurs at 2-3 °C [14]. Potato active vegetation zone is 10-20 °C, growth optimum is 15 °C [15-17], and tuber formation optimum is 16-18 °C [8, 18]. Vegetation continues above 60 days, the sum of average daily temperatures during vegetation must be no less than 1000-1400 °C [7, 8, 19]. Optimum soil moisture is within 75-80 % of full field water capacity in light soils and 40-55 % in heavy soils [8].

It should be noted that agrometeorological models of potato yield in Russia are region-specific. In the west and north-west, where moisture conditions are favorable and average temperatures do not exceed the optimum value during tuber formation, the yield positively correlates with the temperature [20, 21]. In southern regions the yield positively correlates with moisture indices, while correlation with average air temperature is low negative [21, 22]. In central regions the yield is determined by deviation of temperatures and precipitations from optimum for each interphase period [21]. Starch content in potato tubers increases with an increase in temperature and decreases as the day becomes longer and the amount of precipitation increases (optimum amount per tuber formation period is 90-130 mm) [22].

In previous studies we have demonstrated that changes in phenology of previously recognized varieties, their yield and starch content in tubers have been observed in recent decades. These effects were caused by increase in sums of temperatures and precipitation for the period with temperatures above 15 °C and more early temperature increase above this value [23-26].

The long-term data obtained have been summarized for the first time in this paper, revealing the most significant correlation between variability of agriculturally valuable potato traits and the temperature in extreme conditions with regard to this index, i.e. lack or excess of heat. Increase in the sum of temperatures above 15 °C and more early temperature increase above 15 °C, which accelerated the most weather-dependent phases (emergence and flowering), were considered the most important agroclimatic factors of potato development in the European Russia territory. The models developed suggest further increase in potato yields in the European Russia territory due to earlier flowering and longer tuber formation period.

The research was aimed at statistical analysis of observed trends in manifestation of the main agriculturally valuable traits of potato varieties in the European Russia territory with regard to regional climatic changes.

Techniques. Long-term observations of potato plant phenological phase, tuber weight per plant and starch content in tubers of varieties used as reference standards during examination of samples from potato collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) were performed at VIR experimental stations (ES) with contrast climatic conditions, i.e. at VIR Polar ES (Murmansk region, early variety Hibinskii rannii, 1968-2013) and VIR Maikop ES (the Republic of Adygea, middle-early variety Nevskii, 1990-2012), as well as in central part of the country, at VIR Pushkin laboratories (St. Petersburg, variety Nevskii, 1984-2004; middle-early variety Elizaveta and mid-ripening variety Petersburgskii, 1999-2010). Weather conditions during the observation periods were registered based on daily data of the nearest meteorological stations.

Decennial rate of change of analyzed parameters was determined (Δ/10 years). Standard deviations ($\pm$S) and variation coefficients (*Cv*, %) of compared parameters were calculated. Correlation regression analysis was used for identifi-

cation of significant climatic factors. Statistical data processing was performed using Statistica 6.0 software («StatSoft Inc.», USA). Regression models were developed using successive inclusion of variables, determination coefficients R^2 were determined. Monthly temperatures, monthly precipitation totals, dates of stable temperature increase above 10 and 15 °C, duration of periods with temperatures above these limits, sums of active (daily average, above the specified limit) and effective (daily average minus the specified temperature limit) air temperatures, total precipitation, as well as average active and effective temperatures during these periods were examined as possible independent variables. In equations the dates are presented as the number of days from the reference point (April 1). Significance level 5 % ($p = 0.05$) is accepted in the study.

Results. More early change of temperatures has been observed since 1980 in all studied points: for values above 10 °C — with average rate of 0.5; 3.8 and 1.5 days/10 years (VIR Polar ES, VIR Pushkin laboratories and VIR Maikop ES, respectively); for values above 15 °C — for stations by 0.4; 0.6 and 2.9 days/10 years, respectively. The changes of the sum of temperatures for stations amounted to 62.7; 205.1 and 52.2 °C/10 years, respectively, for values above 10 °C; 36.4; 218.4 and 101.1 °C/10 years, respectively, for values above 15 °C. A small increase in total precipitation during the periods with temperatures above 10, 15 °C was observed in all observation points; however, it was statistically insignificant.

1. Trends of agroclimatic factor changes during interphase vegetation periods of reference potato varieties (*Solanum tuberosum* L.) in observation points with optimum and extreme meteorological conditions

Variety	Indicator	Planting—flowering				Flowering—harvesting				Planting—harvesting			
		X	±S	*Cv*	b	*X*	±S	*Cv*	b	*X*	±S	*Cv*	b
VIR Polar Experimental Station													
Hibinskii rannii (1968-2013)	L	51	8	16.6	–4.3*	38	9	23.7	2.3	88	5	5.7	–1.7
	ΣT	607	77	12.6	–15.9*	466	139	29.8	56.7*	1080	122	11.3	8.2
	$T_{cp.}$	12.1	1.1	8.7	0.2	12.4	1.1	9.2	0.6	12.3	1.0	8.5	0.2
VIR Pushkin laboratories													
Elizaveta (1999-2010)	L	59	7	12.3	6.3	27	1	1.8	–1.2	86	5	5.4	–10.1
	ΣT	942	112	11.9	105.5	563	213	37.9	–465.3	1492	178	11.9	–372.6
	$T_{cp.}$	16.0	1.1	6.8	0.7	18.2	1.2	6.9	0.2	16.9	0.8	5.0	–0.2
Nevskii (1984-2004)	L	48	3	6.2	0.8	50	4	8.0	1.4	99	5	5.1	2.5
	ΣT	766	92	12.0	45.1	848	87	10.3	57.1	1617	132	8.2	94.4
	$T_{cp.}$	15.8	1.8	11.5	0.8	16.9	1.1	6.5	0.6	16.4	1.2	7.0	0.5
Petersburgskii (1999-2010)	L	52	9	18.0	–4.8	50	15	29.9	–6.3	101	8	7.8	–11.1
	ΣT	835	90	10.8	232.7	850	245	28.9	–562.7	1694	179	10.6	–321.5
	$T_{cp.}$	15.1	1.0	6.7	0.2	18.2	1.1	6.2	0.0	16.9	0.8	4.8	0.5
VIR Maikop Experimental Station													
Nevskii (1990-2012)	L	61	5	8.1	–6.5*	47	15	31.9	–8.5	108	18	16.4	–6.9
	ΣT	873	111	12.8	–73.8*	941	338	35.9	–179.6*	1840	381	20.7	–253.4*
	$T_{cp.}$	14.2	1.8	12.3	–0.4	20.2	2.6	12.6	–0.5	16.7	1.8	11.0	–0.2

Note. L — duration of period, days; T — sum of average daily temperatures, °C; $T_{av.}$ — average temperature during a period, °C. Average values (*X*), standard deviation (S), variation coefficient (*Cv*, %) and trend (b, units/10 years) are presented. VIR — N.I. Vavilov All-Russian Institute of Plant Genetic Resources.
* The trends are significant at 5 % significance level.

Phenology. Planting dates of the studied varieties did not change significantly, flowering of the majority of varieties (except for Elizaveta variety) was shifted to earlier periods (at VIR Polar ES by 2.6 days/10 years, $p = 0.05$), harvesting was also performed earlier. The duration of planting—flowering period at Polar and Maikop experimental stations decreased significantly (Table 1) at the rate of –4.3 and –6.6 days/10 years. The duration of flowering—harvesting period changed slightly, both toward decrease (at VIR Maikop ES and in Elizaveta and Petersburgskii varieties at VIR Pushkin laboratories) and increase (at VIR Polar ES and in Nevskii variety at VIR Pushkin laboratories). As a result, vegetation

time decreased in four of five studied varieties. Before 2000 vegetation period in Nevskii variety at VIR Pushkin laboratories decreased as well, after which the planting was shifted to earlier periods; as a result, planting—flowering and planting—harvesting periods became longer.

Temperature conditions of potato growing at VIR Polar ES approached the lower limit of optimum zone (see Table 1), average temperature of planting—emergence period amounted to 9.6±2.3 °C, and at planting was 8.2±4.0 °C. At VIR Maikop ES the temperature approached the upper limit of optimum zone and amounted to 20.2±2.6 °C during flowering—harvesting, and 22.3±2.5 °C at harvesting. Temperature conditions at VIR Pushkin laboratories corresponded to optimum conditions. At VIR Polar ES the sum of average daily temperatures decreased significantly during planting—flowering period with reduction of its duration (by 15.9 °C per each 10 years), increased during flowering—harvesting (by 56.7 °C per 10 years), and did not change significantly during the whole vegetation. At VIR Maikop ES the sum of temperatures decreased significantly during planting—emergence, flowering—harvesting and planting—harvesting periods, together with the decrease in duration of these periods (by 73.8; 179.6 and 253.4 °C/10 years). In Nevskii variety, examined in two geographical points, the duration of planting—flowering and vegetation was significantly longer at VIR Maikop ES, as compared to VIR Pushkin laboratories (61 days vs 48 days and 110 days vs 99 days, respectively); sums of temperatures during these periods also differed significantly (1617 and 1840 °C). Thus, in contrast weather and climatic conditions neither interphase periods of potato varieties, nor the sums of the respective temperatures remain constant.

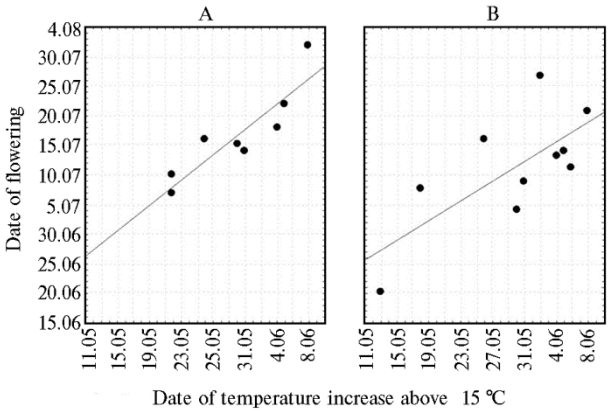
2. Coefficients of correlation (r) between the duration of interphase periods in reference potato varieties (*Solanum tuberosum* L.) and the average temperature during the period in observation points with optimum and extreme meteorological conditions

Variety	Planting—flowering	Flowering—harvesting	Planting—harvesting
VIR Polar Experimental Station			
Hibinskii rannii (1968-2013)	–0.70*	0.25	–0.09
VIR Pushkin laboratories			
Elizaveta (1999-2010)	–0.09	–0.21	–0.06
Nevskii (1984-2004)	–0.17	–0.14	–0.19
Petersburgskii (1999-2010)	–0.38	0.57	0.34
VIR Maikop Experimental Station			
Nevskii (1990-2012)	–0.38	0.01	0.12

Note. VIR — N.I. Vavilov All-Russian Institute of Plant Genetic Resources.
* Significant at 5 % significance level.

Comparison of variation coefficients for the duration of interphase periods and the sums of temperatures during these periods (see Table 1) has demonstrated that the sum of temperatures during the interphase period and the whole vegetation period are generally more variable parameters. The exception was planting—flowering period in several potato varieties at VIR Polar ES and VIR Pushkin laboratories.

Planting—flowering period in potato was regulated by temperature to a greater extent compared to other stages development. The duration of this period in all varieties negatively correlated with the average temperature during the period (Table 2); at that, the strongest correlation was observed at VIR Polar ES (r = –0.70, p = 0.05) at the temperatures approaching the lower limit of optimum zone. Both negative and positive weak and moderate correlations with average temperature were observed for flowering—harvesting and planting—harvesting periods. The strongest positive correlation was observed during flowering—harvesting in mid-ripening variety Petersburgskii (r = 0.57).



Correlation between flowering initiation periods in potato varieties (*Solanum tuberosum* L.) Elizaveta (middle-early; A) **and Petersburgskii** (mid-ripening; B) **and temperature increase above 15 °C** (Pushkin laboratories of N.I. Vavilov All-Russian Institute of Plant Genetic Resources, 1990-2010).

According to our observations, at all stations the temperature above 15 °C was a climatic factor, determining the duration of planting—flowering period to the greatest extent. Direct correlation between the date of temperature increase above 15 °C and the terms of emergence was observed at VIR Polar ES (r = 0.65) and at VIR Pushkin laboratories (r = 0.44 in Elizaveta variety, r = 0.41 in Petersburgskii variety). Apart from that, the periods of budding initiation correlated with temperature increase above 15 °C at VIR Polar ES (r = 0.46) and in Nevskii variety at VIR Pushkin laboratories (r = 0.25); the same was observed for the date of flowering initiation date at the same stations in Elizaveta (r = 0.89) and Petersburgskii (r = 0.71) varieties (Fig.). The duration of planting—flowering (L) at VIR Polar and Maikop experimental stations depended on the sum of active temperatures above 15 °C ($\Sigma T_{act.\ 15}$), at Maikop ES also on the planting date (D_p), at VIR Pushkin laboratories on the sum of effective temperatures above 15 °C ($\Sigma T_{ef.\ 15}$) for Petersburgskii variety, and on the date of stable temperature increase above 15 °S (D_{15}) for Elizaveta variety. In Nevskii variety at VIR Pushkin laboratories the planting—flowering period became longer in case of earlier planting (D_p) (R^2, regression equation determination coefficient):

VIR Polar ES, Hibinskii rannii variety	$L = 64.85 - 0.04\Sigma T_{act.\ 15}$	$R^2 = 0.63$
VIR Pushkin laboratories:		
Nevskii variety	$L = 79.45 - 0.60D_p$	$R^2 = 0.51$
Elizaveta variety	$L = -1.33 + 0.99D_{15}$	$R^2 = 0.69$
Petersburgskii variety	$L = 82.17 - 0.10\Sigma T_{eff.\ 15}$	$R^2 = 0.78$
VIR Maikop ES, Nevskii variety	$L = 90.94 - 1.04D_p - 0.01\Sigma T_{act.\ 15}$	$R^2 = 0.49$

Further elongation in planting—emergence in potato plants may be expected with temperature increase.

With increase of the sum of active temperatures above 15 °C flowering—harvesting period became longer at VIR Polar ES (r = 0.72) and shorter at VIR Maikop ES (r = –0.54). The period from flowering to harvesting in middle-early variety Nevskii at VIR Pushkin laboratories increased in case of earlier planting (r = 0.92), in middle-early variety Elizaveta in case of earlier temperature increase above 15 °C (r = 0.74), in mid-ripening variety Petersburgskii with increase of effective temperatures above 15 °C (r = 0.91). In Petersburgskii variety the duration of vegetation also increased with the increase of average active temperature above 15 °C.

Tuber weight per plant and starch content in tubers. According to the literature, tuber weight, the number and yield of tubers per plant are more variable, as compared to the duration of vegetation period [8]. Tuber weight per plant was the most variable parameter in our study (Table 3). In all varieties, except for Nevskii variety at VIR Pushkin laboratories, tuber weight

per plant increased during the years of study (for VIR Polar ES at 92 g/10 years, p = 0.05). Starch content, examined in three varieties (see Table 3), increased weakly at VIR Polar ES and decreased in other geographic points.

3. Variability of tuber weight per plant and starch content in tubers of reference potato varieties (*Solanum tuberosum* L.) in observation points with optimum and extreme meteorological conditions

Variety	Tuber weight per plant, g				Starch content in tubers, %			
	X	±S	*Cv*	b	*X*	±S	*Cv*	b
VIR Polar Experimental Station								
Hibinskii rannii (1968-2013)	850	223	26.2	92*	11.3	1.2	10.6	0.1
VIR Pushkin laboratories								
Elizaveta (1999-2010)	576	178	30.9	216	14.5	1.2	8.3	–
Nevskii (1984-2004)	915	150	16.4	–82	14.3	1.1	7.7	–0.3
Petersburgskii (1999-2010)	743	208	28.0	226	14.1	1.0	7.1	–
VIR Maikop Experimental Station								
Nevskii (1990-2012)	302	191	63.2	33	13.3	1.8	13.5	–0.3

Note. Average values (*X*), standard deviation (S), variation coefficient (*Cv*, %) and trend (b, units/10 years) are presented. VIR — N.I. Vavilov All-Russian Institute of Plant Genetic Resources. Gaps mean the absence of data.

* Significant at 5 % significance level.

Tuber weight per plant and starch content in tubers correlated with weather conditions to a lesser extent. For example, in Hibinskii rannii variety at VIR Polar ES (1968-2013) tuber weight after harvesting (Y), tuber weight during trial unearthing and average weight of a marketable tuber correlated with the flowering date (D_F) (r = –0.54, r = –0.44 and r = –0.52, respectively) to the greatest extent, i.e. the values of these parameters increased in case of earlier flowering (regression equation for tuber weight per plant at VIR Polar ES is $Y = 2338.134 - 13.675D_F$, $R^2 = 0.30$). In 1991-2004 tuber weight per plant increased due to more late harvesting (r = 0.62). Further increase of yield can be forecasted in conditions of heat supply increase at VIR Polar ES, resulting from earlier flowering and elongation of flowering—harvesting period

At VIR Pushkin laboratories tuber weight per plant in Nevskii variety decreased with increase in average active temperature above 15 °C (T_{15}) (r = –0.71) (regression equation $Y = 1418.355 - 146.292T_{15}$, $R^2 = 0.51$). At VIR Maikop ES in 1990-2012 the weight of tubers per plant of the same Nevskii variety increased in case of earlier budding (D_B): $Y = 1228{,}704 - 16{,}289D_B$, $R^2 = 0{,}48$.

The models developed suggest increase in potato variety yields under earlier budding and flowering and at longer flowering—harvesting period.

Increase in starch content at VIR Polar ES resulted from increase in the sums of active temperatures above 15 °C (r = 0.42) and more early budding (r = –0.40), and slowed down in the years with greater amount of precipitation in August (r = –0.30) and, as per the regression model, total precipitation during the period with temperatures above 15 °C [21]. On the contrary, at VIR Maikop ES starch content decreased in more hot years with early budding (r = 0.66). At VIR Pushkin laboratories no correlation between the starch content and the studied factors was observed.

The main data obtained, important for forecasting and practical recommendations, may be summarized as follows. Variability of agriculturally valuable potato traits and their correlation with temperature was manifested to the greatest extent in conditions of lack (VIR Polar ES) and excess (VIR Maikop ES) heat. Planting—flowering period of reference varieties decreased at the rate of 2.4 days/10 years at VIR Polar ES, 6.6 days/10 years at VIR Maikop ES, and did not change significantly at VIR Pushkin laboratories. At that, the sums of average daily temperatures during the said period decreased significantly at –15.9 °C/10 years and –73.8 °C/10 at VIR Polar and Maikop

experimental stations, respectively, and demonstrated no significant trends at VIR Pushkin laboratories. Decrease in planting—harvesting period was observed in four of five studied potato varieties, also the sum of average daily temperatures during the vegetation period decreased at VIR Maikop ES (at –253.4 °C/10 years). In contrast climatic conditions the sums of temperatures during interphase periods of potato varieties were less stable as compared to the duration of the respective periods.

Thus, temperature increase above 15 °C and more early temperature increase above 15 °C, which accelerate the most weather-dependent phases (emergence and flowering), shall be considered the most important factors of potato plant development in the European Russia territory. The models developed suggest further increase in potato yields in the European Russia territory due to earlier flowering and longer tuber formation period. The variation of starch content in potato tubers has demonstrated no significant trends due to complex regulation of this trait (the increase in temperature increases starch content, while the increase in amount of precipitation decreases it). More early planting of potato may be proposed as a measure of agricultural technology adaptation to climatic changes in the European Russia territory. It is reasonable to continue studying on involvement of more late-ripening varieties for cultivation.

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ANTICIPATORY BREEDING: MOLECULAR MARKERS AS A TOOL IN DEVELOPING DONORS OF POTATO (*Solanum tuberosum* L.) LATE BLIGHT RESISTANCE FROM COMPLEX INTERSPECIFIC HYBRIDS

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Abstract

Potato late blight caused by oomycete *Phytophthora infestans* Mont de Bary remains the most significant agronomic and economic problem of potato husbandry. The unique rate of evolution of this pathogen as well as its migration are a major obstacle to producing varieties with durable late blight resistance. The best way to counter this threat is the anticipatory breeding based on donors that carry genes of resistance to a wide range of pathogen races. Combining multiple genes for late blight resistance in the same plant (pyramiding of genes) makes such resistance durable. The most promising way of obtaining such donors is introgression breeding of interspecific potato hybrids with resistance genes transferred from potato wild relatives — the tuber-bearing species of *Solanum* L. Molecular markers allowing to reliably distinguish between resistance genes of diverse specificity and successfully control their transfer in the process of crossing and selecting, dramatically increase the efficiency of introgression breeding for late blight resistance. We examined about 40 complex hybrids bred in the A.G. Lorkh All-Russian Research Institute of Potato Husbandry, the N.I. Vavilov All-Russian Institute of Plant Genetic Resources and the All-Russian Research Institute of Plant Protection using the germplasm of 16 species of tuber-bearing *Solanum*, and further maintained as clones. Each of these clones carries the genetic material introgressed from two to eight wild species established as the sources of resistance to late blight. Most of these clones have manifested, from year to year, high late blight resistance in field trials under natural infection and in vitro studies when detached leaves were infected with a highly virulent and aggressive isolate of *P. infestans.* SCAR (sequence-characterized amplified region) markers, the fragments of *R* genes of race-specific (vertical) resistance to late blight discerned in various *Solanum* species, were employed to screen the clones of interspecific hybrids. The information on the presence of SCAR markers for six *R* genes, i.e. *R1* (chromosome 5), *R2/Rpi-blb3* (chromosome 4), *R3a* and *R3b* (chromosome 11), *RB/Rpi-blb1* = *Rpi-sto1* (chromosome 8), and *Rpi-vnt1.3* (chromosome 9), was juxtaposed against the indices of late blight resistance. Comparison of the resistance of the clones of interspecific hybrids and potato varieties devoid of *R* gene marker presumes a significant contribution of these genes to general late blight resistance of potato plants. The higher number of *R* gene markers per plant corresponded to superior late blight resistance. Presumably, future use of these clones for pyramiding resistance genes under the control of molecular markers will enable target introgression breeding. Such approach will

streamline developing new potato varieties with durable late blight resistance to maintain high productivity even after significant changes in the populations of *P. infestans*.

Keywords: potato late blight, interspecific potato hybrids, wild *Solanum* species, introgression breeding, *R* genes for late blight resistance, *Avr* genes of *P. infestans*

Late blight caused by oomycete *Phytophthora infestans* Mont. de Bary remains one of the most economically significant potato diseases which results in losses of at least 15 % of yield annually, and global economic damage, including expenses for chemicals for pathogen control, amounts to 10 bln US dollars [1]. Late blight epidemics of new *P. infestans* races due to the pathogen evolution and migration periodically destroy up to 70-100 % of yield [2-4]. Thus, varieties with durable resistance to a wide range of *P. infestans*, ensuring significant preservation of plant productivity in case of changes of the pathogen race composition in agrocenosis, are required for successful potato farming. Development of such varieties may be referred to as preventive breeding.

The sources of resistance to *P. infestans*, the *Solanum* L. species (section *Petota* Dumort.), play the defining role in this approach. Resistance genes have been identified in many representatives of *Solanum* genus from North and South America [5], but only a few of them are available in commercial potato varieties (https://www.europotato.org). Development of interspecies hybrids on the basis of *S. demissum* was one of the first examples of introgression of such race-specific vertical resistance genes (*R* genes) obtained from wild species [6, 7]; however, new races of *P. infestans* quickly overcame the stability obtained using this method [8]. Horizontal late blight resistance [8-10] common to a wide range of tuberiferous *Solanum* species is controlled by numerous race-nonspecific genes. They are mainly mapped as quantitative trait loci (QTL) only and explored to a lesser extent, as compared to race specific genes [5, 11, 12]. Expression of race-nonspecific genes depends heavily on external factors, which significantly complicated the work on breeding for horizontal late blight resistance.

Involvement of genetic sources in development of breeding-valuable trait donors takes place during pre-breeding selection. Collections of cultivated and wild tuberiferous *Solanum* species has been developed in N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg) is one of the world largest ones. Here genetic centers of late blight resistant species formation have been identified [13], and genetic bases for obtaining of interspecies donor hybrids [14] and collection of samples suitable for interspecies hybridization have been developed.

Complex interspecies hybrids with genetic material of several wild *Solanum* species may become the best donors for development of resistant potato varieties [10, 15, 16], and stacking (combining) of different late blight resistance genes in a single plant ensures durability of this resistance. As a rule, wild congeners of potato and hybrids involving them contain several *R* genes with different race specificity [17]; *P. infestans* race sets, with virulence factors determined using Mastenbroek and Black differential varieties, are normally used for their recognition [7, 18]. These differential races only identify 11 *R* genes of *S. demissum* (not all of which are cloned) and do not recognize other genes which may be present in *Solanum* species. Some of these differential plants have more than one *R* gene [19]. Avirulence gene *ipiO*, identifying *RB/Rpi-blb1* gene in potato, which is common to *S. bulbocastanum* and absent in *S. demissum* [20], is represented by two classes in *P. infestans* differential races.

Many problems of this kind have been solved by development of monogenic differential plants [21]. However, in this case the use of *P. infestans* races with known virulence genes will probably fail to provide the same *R* gene identification

accuracy, as compared to their direct identification using molecular markers. Thus, effective search of resistance genes specific for a wide range of *P. infestans* races and their stacking are primarily based on molecular marking.

Markers representing resistance gene fragments are most suitable for review of large genetic collections and control of the results during breeding. Such SCAR (sequence characterized amplified region) markers, unlike flanking markers, completely preserve informative value even in case of significant recombination of genetic material in hybrid genomes. The main problem arising during the use of these markers is the necessity to distinguish functionally active genes from their inactive structural homologs. It should be noted that effectoromics methods [22] ensure more specific recognition of *R* resistance genes in plants: individual pathogen avirulence genes (*Avr* genes) are introduced in a plant as part of a vector, and evident hypersensitivity reaction indicates the presence of the respective *R* gene. This methodological approach has limitations as well, and the best results are achieved by combining of two complementary techniques.

This work presents the first data summary of long-term studies of collections of complex interspecies hybrids and potato hybrids, included in parentage of such hybrids or used as reference standards during evaluation of late blight resistance (some results were previously presented at EuroBlight Workshops, 2013, 2015; http://euroblight.net/). The performed summary allowed us to propose a new approach to development of varieties with durable resistance to late blight. This approach is based on development of a method of controlled introgressive breeding of complex interspecies potato hybrids with high late blight resistance, achieved by stacking of *R* resistance genes, controlled by molecular markers of these genes. A large pool of such hybrids used as breeding donors allows quick response to changes in pathogen population composition.

Application of the approach aimed at preventive breeding and problems related to it will be illustrated below, through the example of two groups of interspecies hybrids.

Techniques. The research included 10 varieties (Alpha, Desiree, Bintje, Early Rose, Eesterling, Escort, Gloria, Elizaveta, Svitanok kievsky and Sarpo Mira), Atzimba variety dihaploid and 39 clones of complex interspecies potato hybrids obtained in A.G. Lorkh All-Russian Research Institute of Potato Husbandry (Moscow region), N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg) and All-Russian Research Institute of Plant Protection (St. Petersburg) with participation of 16 tuberiferous *Solanum* species from various centers of origin.

Late blight resistance of leaves was evaluated in long-term field trials in two regions of the Russian Federation, i.e. Northwest (VIR) and Central (All-Russian Research Institute of Phytopathology, VNIIF) in conditions of natural infection. Varieties Elizaveta, Udacha, Nayada and Petersburgskii (VIR) or Alpha, Bintje, Eesterling, Escort, Gloria, Robijn and Sarpo Mira (VNIIF) served as reference standards. During laboratory tests detached leaves of plants grown in a greenhouse were infected with a highly virulent and aggressive isolate of *P. infestans* (races 1-11) from VNIIF collection, with Santé variety used as a reference standard [23].

Genomic DNA was extracted from leaves using AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, USA). DNA concentration was measured at λ = 260 nm using UV/Vis NanoPhotometer P300 nanophotometer (IMPLEN, Germany). Genomic DNA amplification, cloning and amplicon sequencing methods have been described by us earlier [24]. PCR was performed in a DNA amplifier DNA Engine PTC-200 (Bio-Rad, USA) with 1.0 unit of Taq DNA polymerase (Fermentas, USA), and for R2-2500 marker with

2.5 units of Pfu DNA polymerase (Fermentas, USA). QIAquick Gel Extraction Kit (Qiagen N.V., Germany) was used for elution of target DNA fragments, pGEM-T Easy Vector System I (Promega, USA) was used for cloning; for R2-2500 marker Thermo Scientific™ CloneJET™ PCR Cloning Kit and pJet vector (Fermentas, USA) were used. Sequencing was performed using ABI PRISM 3130xl (Applied Biosystems, USA) or Nanofor 05 (Analytical Instrument Engineering Institute of RAS, Russia). The primers were synthesized by ZAO Syntol (Russia). Sequenced fragments were assembled using SeqMan package, Lasergene 7.0 (available at http://www.dnastar.com).

Correlation analysis was performed using Statistica 6.0 software (StatSoft Inc., USA; available at http://www.statsoft.com/) with evaluation of Pearson's parametric correlation and Spearman's rank correlation, as well as using Wilcoxon's nonparametric test. All correlation coefficients were significant at $p < 0.05$.

Results. Work with clone collections of potato varieties and hybrids [15] maintained in VIR and VNIIF allows comparison of the results of independent test in several laboratories and significantly increases reproducibility of trials. Each interspecies clone studies by us carries genetic material of various late blight resistance sources and has 2-8 wild potato species in parentage (a significant part of them is provided below).

In field conditions at natural *P. infestans* infection the majority of the studied interspecies hybrid clones demonstrated high resistance to late blight every year, which was comparable to that of the reference variety Sarpo Mira (8 points). High resistance of clones (up to 7 points) was registered in laboratory tests as well.

The differences between the field and laboratory test results (1-3 points) for the majority of potato hybrid and variety clones can be explained by more favorable conditions for pathogen development in the laboratory test [3] and the contribution of other plant organs to general resistance [25]. In cases when these differences are especially big, significant activity of race-nonspecific resistance genes can also be expected. Field and laboratory resistance indices are closely related (Pearson's correlation coefficient 0.963 is significant at $p < 0.01$).

1. Sequence characterized amplified region (SCAR) characteristic of potato (*Solanum* L.) *R* late blight resistance gene markers used in the work

Gene	Marker with indication of length (-bps)	Annealing temperature, °C	Forward (F) and reverse (R) primers (5′→3′)	Reference
Rpi-blb1	Rpi-blb1-820	62	F: AACCTGTATGGCAGTGGCATG R: GTCAGAAAAGGGCACTCGTG	[27]
Rpi-sto1 = Rpi-blb1	Rpi-sto1-890	65	F: ACCAAGGCCACAAGATTCTC R: CCTGCGGTTCGGTTAATACA	[28]
R1	R1-1205	65	F: CACTCGTGACATATCCTCACTA, R: GTAGTACCTATCTTATTTCTGCAAGAAT	[24]
R2	R2-2500	62	F: ATGGCTGATGCCTTTCTATCATTTGC R: TCACAACATATAATTCCGCTTC	[19]
R3a	R3-1380	64	F: TCCGACATGTATTGATCTCCCTG R: AGCCACTTCAGCTTCTTACAGTAGG	[24]
R3b	R3b-378	64	F: GTCGATGAATGCTATGTTTCTCGAGA R: ACCAGTTTCTTGCAATTCCAGATTG	[29]
Rpi-vnt1.3	Rpi-vnt1.3-612	65	F: CCTTCCTCATCCTCACATTTAG R: GCATGCCAACTATTGAAACAAC	[30]

Well-verified SCAR markers of 6 race-specific late blight resistance *R* genes localized in six of 12 potato chromosomes were selected for screening: *R1* (chromosome 5), *R2/Rpi-blb3* (chromosome 4), *R3a* and *R3b* (chromosome 11), *RB/Rpi-blb1 = Rpi-sto1* (chromosome 8) and *Rpi-vnt1.3* (chromosome 9) (Table 1). These markers have been developed for late blight resistance *R* genes, initially characterized in four *Solanum* species, *S. bulbocastanum*, *S. demissum*, *S. stolon-*

iferum and *S. venturii*.

Two contrast groups of potato genotypes were selected to illustrate the screening results (Table 2). These are hybrids with 3-4 *R* resistance gene markers corresponding to high late blight (these forms constitute the majority of interspecies hybrids studied by us) and hybrids with high resistance and small number of markers.

2. Distribution of sequence characterized amplified region (SCAR) *R* gene markers in two groups of interspecies potato (*Solanum* L.) hybrids of different origin with high late blight resistance

Genotype	Genotype							Number of markers	Resistance, points		Wild species in the parentage
	1	2	3	4	5	6	7		field	laboratory	
Hybrids with known resistance *R* genes											
2372-60	1	0	1	1	0	0	0	3	8	6.0	adg, chc, dms, sto
12/1-09	0	1	0	0	1	1	0	3	7	6.0	adg, dms, mcd, plt = sto, pnt, sto, vlm
15/13-09	0	1	0	1	1	0	0	3	6	6.0	adg, dms, mcd, plt = sto, sto, vlm
16/27-09	1	0	0	0	1	1	0	3	7	7.0	adg, ber, dms, mcd, phu, plt = sto, sto, vlm
111 (38 KBA)	0	0	1	1	1	1	0	4	7	6.5	adg, dms, mcd, plt = sto, sto
39-1-2005	0	0	0	1	1	0	1	3	7	6.0	aln, dms[a, b]
134-6-2006	0	0	1	1	0	0	1	3	6	5.0	aln = brc[b]
139	1	0	0	0	1	1	0	3	9	6.0	adg, aln, ber, dms, mcd, plt = sto, pnt, sto, vlm
135-1-2006	0	1	1	1	0	0	1	4	7	5.0	aln = brc[b]
Hybrids, possibly carrying unknown resistance *R* genes											
2585-80	1	0	0	1	0	0	0	2	7	6.0	dms
2585-67	0	0	1	1	0	0	0	2	7	6.0	dms
106 (171-3)	0	0	0	1	0	0	0	1	7	6.0	adg, dms, ryb
113 (50/1 KBA)	0	0	0	0	0	0	0	0	7	6.0	adg, dms, phu, sto, vrn and pollen mix from hybrids
118 (118-5)	0	0	1	1	0	0	0	2	8	6.0	adg, dms, ryb
24-1	0	0	0	1	0	0	1	2	8	6.5	aln = brc[6]
134-2-2006	1	0	0	0	0	0	1	2	7	6.0	aln = brc[6]
Atzimba diploid	0	0	0	0	0	0	0	0	7	No data	Unknown

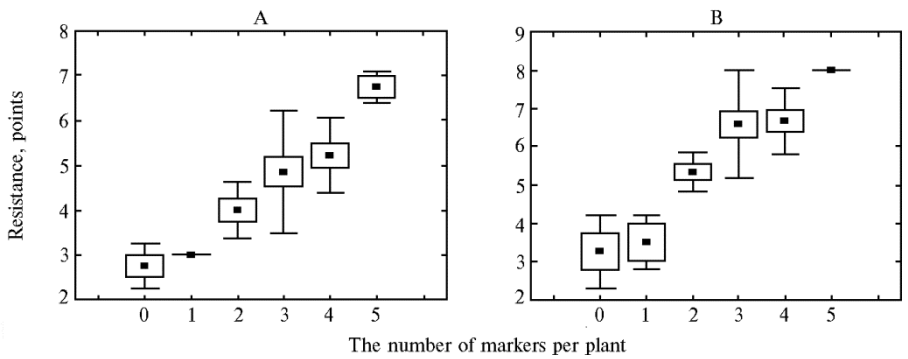
Note. 1 — R1-1205, 2 — R2-2500, 3 — R3a-1380, 4 — R3b-378, 5 — Rpi-blb1-820, 6 — Rpi-sto1, 7 — Rpi-vnt1.3; [a] — Rpi-blb1-820 marker source is unclear, [b] — Rpi-vnt1.3 marker source is unclear; adg — *S. andigenum*, aln — *S. alandiae*, ber — *S. berthaultii*, brc — *S. brevicaule*, chc — *S. chacoense*, dms — *S. demissum*, mcd — *S. microdontum*, phu — *S. phureja*, plt — *S. polytrichon* = *S. stoloniferum*, pnt — *S. pinnatisectum*, ryb — *S. rybinii* = *S. phureja*, sto — *S. stoloniferum*, vlm — *S. vallis-mexici*, vrn — *S. vernei*.

The markers used by us have been developed for late blight resistance *R* genes initially characterized in four *Solanum* species, *S. bulbocastanum*, *S. demissum*, *S. stoloniferum* and *S. venturii*. Comparison of interspecies potato hybrid clones and wild *Solanum* species used during development of these hybrids [24, 26] has confirmed that the composition of *R* genes in hybrids mostly corresponded to their parentages. *R1*, *R2*, *R3a* and *R3b* gene markers were most probably transferred from *S. demissum*, however, according to screening of the large collection of wild *Petota* section species [24], they could originate from other wild species indicated in parentages. Genetic material of *S. bulbocastanum* was nor used for development of the studied hybrids, and in cases where we identify *Rpi-blb1* gene marker, it was transferred from *S. stoloniferum*, a tetraploid species; presumably, one of its genomes originates from *S. bulbocastanum* [27]. The screening results sufficiently correlate with Rpi-blb1-820 and Rpi-sto1-890 markers which correspond to two gene regions *Rpi-blb1* = *Rpi-sto1*, located at a significant distance from each other. The presence of *R1* gene in *S. stoloniferum* has been confirmed by detailed studies of this specie [31], including cloning of a full-size *R1* ortholog (GenBank accession number KU302613, National Center for Biotechnology Information — NCBI, USA); apart from that, the presence of *R1* functional gene in *S. stoloniferum* has been confirmed using effectoromics method [32].

In some cases interpretation of the obtained data is impossible without further in-depth studies. This relates to Rpi-blb1-820 and Rpi-sto1 markers present in hybrids without *S. stoloniferum* specie in parentage. We have also discovered *Rpi-vnt1.3* gene marker in the majority of markers, origin of which is not related to *S. venturii*. This is consistent with the recent report [30] on the presence of structural homologs of the said genes in *S. microdontum* ssp. *gigantophyllum* and *S. phureja* (NCBI GenBank accession numbers GU338312, GU338337). Cloning of SCAR markers of *R* genes from interspecies hybrids performed by us has demonstrated that nucleotide sequences of these markers are 98-100 % identical to prototype genes. In cases where variations are observed, they can be attributed to species (allelic) diversity of *R* genes within *Petota* polymorphic section [33]. It appears that the studied *R* genes are common beyond the species in which they were described for the first time, i.e. more common than it was suggested earlier. The same was confirmed by the results of *Solanum* wild species screening performed by us earlier [17, 24, 26, 33]. Similar results were obtained by H. Rietman using effectoromics method [32].

Correlation of the presence of a marker with availability of a functional *R* gene is the most important question during marker analysis of wild and cultivated potato species. Even complete similarity of marker sequence and prototype gene does not serve as evidence of functional activity: structural differences may be beyond the marker borders, and a single nucleotide substitution is sufficient for change of *R* gene product (kinase) function [34]. Possibly, the use of specific *Avr* genes distinguishing functional *R* genes from their inactive homologs will provide more clear answer [22].

Correlation between the presence of these markers and late blight resistance of plants is a circumstantial evidence of functional activity of *R* genes identified using SCAR markers. The Figure shows the correlation between field and laboratory resistance of 40 potato hybrids and varieties and the number of *R* resistance gene markers per plant as a characteristic of late blight resistance genes stacking. Forty genotypes were selected for calculations, i.e. 10 varieties with low and high resistance and all hybrids, except for forms with presumably unknown resistance genes (see bottom of Table 2). Indeed, in this case resistance of plants as per results of field and laboratory tests is closely related to the presence of *R* gene markers: the respective Pearson's parametric correlation coefficients (0.74 and 0.67) and Spearman's rank correlation coefficients (0.66 and 0.64) were significant at $p < 0.01$, and correlation as per Wilcoxon's test at $p < 0.05$. These data are indicative of evident contribution of race-specific genes to late blight resistance of potato plants [12].



Correlation of laboratory (A) **and field** (B) **late blight resistance of potato (*Solanum tuberosum* L.) plants with the number of *R* gene markers:** ▪ — mean, □ — mean±SEM, ⌶— mean±SD.

Resistance increased with increase in the number of *R* gene markers

transferred by hybridization method (see Fig.). It suggests that further crossings for gene accumulation and selection of the best resistance gene combinations, controlled by *R* gene markers will allow combining more resistance genes in a single plant, as compared to modern genetic engineering techniques [1].

A small group of interspecies potato hybrids, which was not included in the above mentioned statistical analysis, is of particular interest. They are characterized by high late blight resistance, but do not contain the expected large number of *R* gene markers (see Table 2). It was found that these forms include Atzimba variety dihaploid as well. These forms probably carry race-specific and race-nonspecific resistance genes, which cannot be detected with the markers used. Currently we are performing work on development of markers to these genes and their validation for selection screening.

Thus, the best method of prevention of new *P. infestans* races emergence is preventive breeding, i.e. development of breeding donors for late blight on the basis of interspecies hybrids with resistance genes transferred from wild potato congeners. Durable resistance of these hybrids is determined by stacking several *R* genes (jointly they ensure recognition of pathogen races with a wide specificity range and quick response even to rapid changes in *P. infestans* populations). This problem can be solved by search of new initial material among cultivated and wild forms of *Solanum* L., *Petota* section (wild species previously not involved in crossing are of particular interest) and development of interspecies donor hybrids pool with several resistance genes by means of introgressive MAS.

Currently this pool maintained as clone collections of VIR, All-Russian Research Institute of Potato Husbandry and All-Russian Research Institute of Phytopathology contains more than 60 interspecies potato hybrids characterized using morphophysiological, phytopathological and molecular methods, which are unique in agriculturally valuable gene composition and represent ready-to-use breeding material for variety development within short timeframes. They are obtained by traditional hybridization methods, which are more labour- and time-consuming, as compared to genetic transformation using two or three resistance genes with wide specificity. However, the number of agriculturally valuable genes per plant in these hybrids and ability to resist a larger number of *P. infestans* races of the described clones is higher, as compared to forms developed using genetic engineering techniques. Another benefit of breeding donors on the basis of interspecies hybrids is preservation of genetic environment inherited from parental forms (including horizontal resistance genes) for introgressed race-specific resistance genes. This ensures stability of developed varieties and slows down selections of more adapted pathogen forms in crops.

Thus, the use of resistance gene molecular markers in introgressive breeding is a transition to the development of conceptually new breeding donors. We have demonstrated the substantial contribution of *R* genes to late blight resistance of potato plants, which increases significantly in case of stacking genes with different specificity, through the example of potato hybrids carrying genetic material of several wild *Solanum* species. Our studies of interspecies hybrid clones using markers of 6 *R* genes allowed identification of two genotype groups: unique donors for development of new varieties, and the hybrids carrying rare alleles of resistance genes which makes them promising, first of all, as initial material for search and isolation of previously unknown *R* genes. Further stacking of resistance genes by crossings, using thoroughly characterized donors and molecular markers, will allow development of potato varieties able to preserve productivity even in case of significant changes in *Phytophthora infestans* populations in the nearest future. This, in its turn, requires studies of resistant gene specificity and development of reliable markers for

mass selection screening.

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ERADICATION OF VIRUSES IN MICROPLANTS OF THREE CULTIVATED POTATO SPECIES (*Solanum tuberosum* L., *S. phureja* Juz. & Buk., *S. stenotomum* Juz. & Buk.) USING COMBINED THERMO-CHEMOTHERAPY METHOD

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Abstract

A modified method of combined chemo-thermotherapy for eradication the most damaging viruses in potato plants is proposed. The materials for study included 91 clones of 85 accessions of three South-American cultivated species *Solanum phureja* (18 accessions), *S. stenotomum* (26 accessions), *S. tuberosum* (*S. tuberosum* ssp. *andigenum* and *S. tuberosum* ssp. *tuberosum*) (41 accessions) from the VIR field collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources). The results of ELISA for 91 clones from the field collection and RT-PCR analysis of the corresponding microplants, when introducing clones into in vitro culture, have shown that all material was infected by viruses (potato viruses Y, X, S, M and potato leafroll virus — PVY, PVX, PVS, PVM and PLRV) to varying degrees. Single infections were detected in 25.3 % of the 91 clones, and the remaining clones were multi-infected and contained several viruses in various combinations. Based on the termo- and chemotherapy protocols as well as complex therapy used in leading potato genebanks (Leibniz Institute of Plant Genetics and Crop Plant Research — IPK, Germany; International Potato Centre — CIP, Peru), we have developed a modified method of combined therapy comprising three successive cycles of in vitro plant cultivation on Murashige and Skoog basal medium (MS) supplemented with ribavirin (30 mg/l) at 37 °C during 4 weeks. Between the cycles, the microplants were incubated for 2 days at 26 °C on the same medium with ribavirin. Each clone was tested for the presence of viruses by RT (revers transcription) PCR before and after the combined thermo-chemotherapy. In RT-PCR, 42 clones (46 %) were completely free from tested viruses, 44 % were free from some viruses, and 10 % of the clones were unable to improve. On the whole of 91 clones, PLRV was eliminated from 72.7 %, PVY from 71.4 %, PVM from 63.9 % and PVS from 57.4 % of microplants. Differences in elimination frequency between the viruses were not significant ($p > 0.05$). Also, there were no significant differences in elimination of different viruses between the accessions of different cultivated species. The difference of this modification from IPK protocols (Germany) lays in simultaneous effect of high temperature (37 °C) and ribavirin (instead of sequential application), and, unlike the CIP (Peru) scheme, it comprises less steps.

Keywords: potatoes, *Solanum tuberosum*, *Solanum phureja*, *Solanum stenotomum*, microplants, PVY, PVX, PVS, PVM, PLRV, virus eradication, RT-PCR, thermotherapy, chemotherapy

Potato VIR Collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) of about 8,500 samples of breeding varieties, wild and cultivated species, as well as interspecific hybrids [1] is one of the largest and oldest in the world. *S*amples of potatoes of field collections, long reproduced by tubers, can accumulate viral infections. There are about 40 viruses infecting potatoes, the most destructive are potato virus Y (PVY), potato leafroll virus (PLRV), po-

tato virus M (PVM), potato virus S (PVS), potato virus X (PVX), potato virus A (PVA) [2-4)]. On the territory of Russia and the CIS countries, PVY, PLRV, PVM, PVS, PVX are the most frequent, and potato virus F and PVA are less detectable [5]. Under field conditions, viruses are transmitted mainly by insect vectors (mostly aphids), as well as via contacts [3].

For virus eradication from plants one can use apical meristem cultures, chemo-, thermo-, cryo- and electrotherapy, and their various combinations (complex/combined therapy). Apical meristems, which was one of the first developed [6, 7], is relatively low-cost and is still widely used. However, for large collections, numbering thousands of samples, it becomes less popular due to laboriousness and because of development of more effective combined approaches. During the cryotherapy in liquid nitrogen (–196 °C) only meristem cells potentially free from viruses remain viable, and hydrated cells outside the meristem zone die [8]. The method of electrotherapy is based on plant tissues exposure to electrical current, resulting in the degradation of the viral nucleoprotein and the loss of virulence [9, 10].

Thermotherapy in vivo and in vitro is based on a decrease in the titer of viruses in infected plant tissues due to disruption in the synthesis of viral RNA at elevated temperatures (36-40 °C) [12]. The method is less effective against spherical viruses [4], so when mixed infections it is recommended to combine said technique with other ones. Chemotherapy is based on the treatment of infected plants with substances with antiviral activity. These include inhibitors of the synthesis of viral nucleic acids such as tylophorin and its derivatives, the analogues of purine and pyrimidine bases including ribavirin, etc. [11-13], as well as RNase [14], interferon [15], growth stimulants [16] and phenolic compounds, in particular salicylic acid [17]. Ribavirin, a synthetic analogue of guanosine (1-beta-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), is most commonly used for in vitro chemotherapy in potato plants, but it should be remembered that low doses of the drug are ineffective [11], while at high dose it inhibits plant development [8]. The concentrations of 30-50 mg/l [11-13] are considered optimal.

In large centers of plant genetic resources, such as the International Potato Center (CIP, Peru) and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Germany), for the sanitation of collection potato samples, thermo- and chemotherapy schemes different in the number and duration of the stages are used, as well as therapy which provides different combinations of these methods [18-21]. The frequency of virus elimination can be additionally increased by using the culture of the apical meristem together with various combined therapy methods, including thermotherapy [22, 23], chemotherapy [25, 26], and thermo-chemotherapy [12, 26, 27].

It should be noted that antiviral therapy methods are not the same in efficiency, and in some cases the same approaches in different laboratories give contradictory results. Besides, most studies are performed on single varieties or a limited number of them, so it is not known how the proposed methods are applicable to large collections. Thus, the development of methods to restore potato plants from viral infections remains extremely urgent.

Taking as a basis the protocols of thermo- and chemotherapy, as well as the complex therapy of the leading potato genebanks (CIP and IPK), we modified the recovery schemes for large samples of genetically dissimilar potato samples with mixed viral infections in various combinations. Our modification differed from the IPK protocols due to simultaneous rather than a sequential application of elevated temperature (37 °C) and ribavirin, and from the CIP protocols due to a smaller number of stages [21].

The goal of the work is to compare known protocols, and to develop and verify a complex antiviral chemo-thermotherapy scheme for potato plant health sanitation in vitro.

Techniques. We used 91 plants of 85 samples of three South American cultivated potato species (indigenous varieties) — *Solanum phureja* (18 samples), *S. stenotomum* (26 samples), *S. tuberosum* ssp. *andigenum* (= *S. andigenum*) (23 samples), *S. tuberosum* ssp. *tuberosum* (= *S. tuberosum*) (18 samples) from VIR collection. The paper provides the names of the species according to the taxonomy system J. Hawkes (1990) [28] which is currently used most widely; the notation accepted in the VIR collection structured according to S.M. Bukasov (1978) [29] is given in brackets,. The studied samples which represent the expeditionary collections of different years were subsequently reproduced for a long time in the field collection.

The field grown plants were analyzed for the presence of PVM, PVY, PVS, PLRV and PVX by ELISA test using the kits and protocol of Agdia, Inc (USA) (https://orders.agdia.com) (data not provided) to selected clones among the plants of the field collection. To preserve the viral infection, in vitro tissue cultures were derived using apexes large in size (2-4 mm) [30]. The resulting micro-plants were maintained as a clone collection.

Total RNA preparations (including RNA viruses) were extrated from the lower leaves of test tube plants as described [31] using a commercial set for isolation on magnetic particles coated with SiO_2 (Silex, Russia). The preparations were stored at –70 °C. The reverse transcription was carried out using a Synthesis of the first cDNA strand (random) kit (Silex, Russia) in accordance with the attached protocol [32]. The kit includes a mixture of random hexanucleotide primers allowing simultaneous preparation of cDNA for all viral RNAs and for RNA of the tubulin gene as a control of successful matrix synthesis. Specific primers were synthesized in the Evrogen company (Russia), and amplification protocols for PVY, PVM, PVX, PVS and PLRV detection in PCR were as described [33-36). The efficiency of the template in PCR was monitored with primers specific for the tubulin protein gene [36]. The samples without RNA were the negative control. PCR was performed on a Mastercycler nexus gradient thermocycler (Eppendorf, Germany). To increase the specificity of PCR, the used programs included a TOUCHDOWN function: initially, the annealing temperature of the primers was set to 5 °C above the required temperature and then decreased by 1 °C in each of the five amplification cycles. PCR products were separated by electrophoresis in 2.5 % agarose gels, stained with ethidium bromide and visualized in UV light.

Prior to eradication, each clone was RT-PCR tested for viruses. The micro-plant sanitation was carried out by a combined thermo-chemotherapy that we modified. The Murashige-Skoog (MS) growth medium without hormones [30] was used, containing the antiviral drug ribavirin (30 mg/l). Combination therapy included three stages lasting 4 weeks each, in which the plants were affected by ribavirin at elevated temperatures (37 °C), and then kept on ribavirin-containing medium at 26 °C for 1 month.

Statistical processing was carried out using Student's *t*-test at a significance level of $p \leq 0.05$.

Results. Based on analysis of the known methods of virus eradication (Table 1), we proposed a modified protocol of antiviral therapy in vitro (Fig. 1) with the use of RT-PCR to detect infection. The effectiveness of this scheme for the most common viral potato pathogens was monitored in several species representatives of *Solanum*, using the primers specific for different genomic regions of the

corresponding viruses (Table 2).

1. The effectiveness of various sanitation methods for potato cultural plants from viral infections

PVX	PVY	PLRV	PVS	PVM
The apical meristems in vitro culture				
+[b] [22]	+[a] [37]	++ [8]	++[a] [23]	+[a] [23]
++[a] [23]	++ [8]			
Simple therapy				
Treatment with ribavirin				
+[a] [14, 38]	–[a] [11]	–[a] [11]	+[a] [11, 14]	+[a] [14]
++[a] [11]	+[a] [14, 38]		+++[a] [23, 38]	+[c] [12]
	+[c] [12]			++[a] [23]
	++[a] [8, 16]			++++[a] [11]
Treatment with RNase				
+++[a] [39, 40]	–[a] [11]	+[a] [39, 40]	+[a] [39, 40]	+[a] [39, 40]
	+[a] [40]			
Thermotherapy				
++[a] [22, 23]	+[a] [16]	++[a] [8]	–[a] [8]	
+++[a] [41]	++[a] [8, 37]		+[a] [23]	
Cryotherapy				
++[b] [22]	++++[a] [8]	++++[a] [8]		
Complex therapy				
Chemo- and thermotherapy				
++++[a] [11, 23, 28, 41]	–[a] [11, 23]	–[a] [11, 23]	++[a] [23]	++[a] [23]
	++[a] [11, 17]	++[a] [17]	++++[a] [11, 38]	+++[a] [11]
Thermotherapy and meristems culture				
++[c] [22]	++++[a] [8, 42]	++++[a] [8, 42]	++[a] [24]	
++++[a] [23]			++++[a] [23, 42]	
Chemotherapy and meristems culture				
++++[a] [25]	++[a, c] [26]	++[a, c] [26]	+++[a] [25]	
++++[a, c] [26]			++++[a, c] [26]	
Thermo-, chemotherapy and meristems culture				
+++[a] [27]	+++[a] [27]	+++[a] [27]	+++[a] [27]	
	++++[a] [37]			
Thermo-, chemo-, electrotherapy and meristems culture				
+++[a] [9]	+++[a] [10]	++++[a, c] [26]		
		++++[c] [12]		

Note. PVX, PVY, PVS, PVM, PLRV — potato viruses X, Y, S, M, and potato leafroll virus; "–" — lack of effect; "+"— up to 40 % of virus-free plants, "++" — 41 to 60 % of virus-free plants, "+++"— 61 to 89% of virus-free plants, "++++"— 90 to 98 % of virus-free plants. The cited references are provided in brackets. Testing by enzyme multiplied immunoassay ([a]), in PCR with reverse transcription ([b]), using plant indicators or electron microscopy ([c]). Gaps mean the absence of data.

1. RT-PCR test of micro-plants for the determination of viruses
2. Micropropagation of infected plants in vitro
3. Complex chemo- and thermotherapy:
   - 3.1. grafting of micro-plants: for each genotype, 10-20 leafless cuttings (1-21 interstices) are planted on Murashige-Skoog (MS) medium without hormones, kept at 26 °C for 1 day and then cultured at 37 °C for 4 weeks
   - 3.2. obtaining leafless cuttings from plants that passed stage 3.1: micro-plants are cultured on MS medium with the addition of ribavirin (30 mg/l) at 20 °C for 2-3 days, then at 37 °C for 4 weeks
   - 3.3. obtaining leafless cuttings from plants that passed the stage 3.2: micro-plants are cultured on MS medium with the addition of ribavirin (30 mg/l) at 20 °C for 2-3 days, then at 37 °C for 4 weeks
   - 3.4. obtaining leafless cuttings from plants that passed step 3.3: the cuttings are kept at 26 °C for 1 day, then the micro-plants are cultured on MS medium with the addition of ribavirin (30 mg/l) at 20 °C for 4 weeks
   - 3.5. transfer of micro-plants to MS medium without hormones, culture at 20 °C for 2 months
4. Isolation of RNA and RT-PCR test of micro-plants for the determination of viruses
5. Micropropagation of healthy plants

Fig. 1. Modified regime for the recovery of micro-plants of cultivated potato species from viral infections based on complex thermo-chemotherapy. RT-PCR — PCR with reversed transcription.

2. Primers used in the test for viruses determination

Amplified RNA	Pair of primers	Sequence	T, °C	Reference
Potato virus X	PVX1-f	AGGCCACAGGGTCGACTAC	57	[33]
	PVX1-r	TTGTTGTTCCAGTGATACGACC		
Potato virus Y	PVY1-f	ACGTCCAAAATGAGAATGCC	53	[33]
	PVY1-r	TGGTGTTCGTGATGTGACCT		
Potato leafroll virus	PLRV1-f	CGCGCTAACAGAGTTCAGCC	58	[33]
	PLRV1-r	GCAATGGGGGTCCAACTCAT		

Table 2 (continued)

Potato virus S	PVS-f	TGGCGAACACCGAGCAAATG	57	[35]
	PVS-r	ATGATCGAGTCCAAGGGCACT		
Potato virus M	PVM4	ACATCTGAGGACATGATGCGC	59	[34]
	PVM3	TGAGCTCGGGACCATTCAAC		
Control of reverse transcription (RT)	tubul-f	ATGTTCAGGCGCAAGGCTT	54	[36]
	tubul-r	TCTGCAACCGGGTCATTCAT		

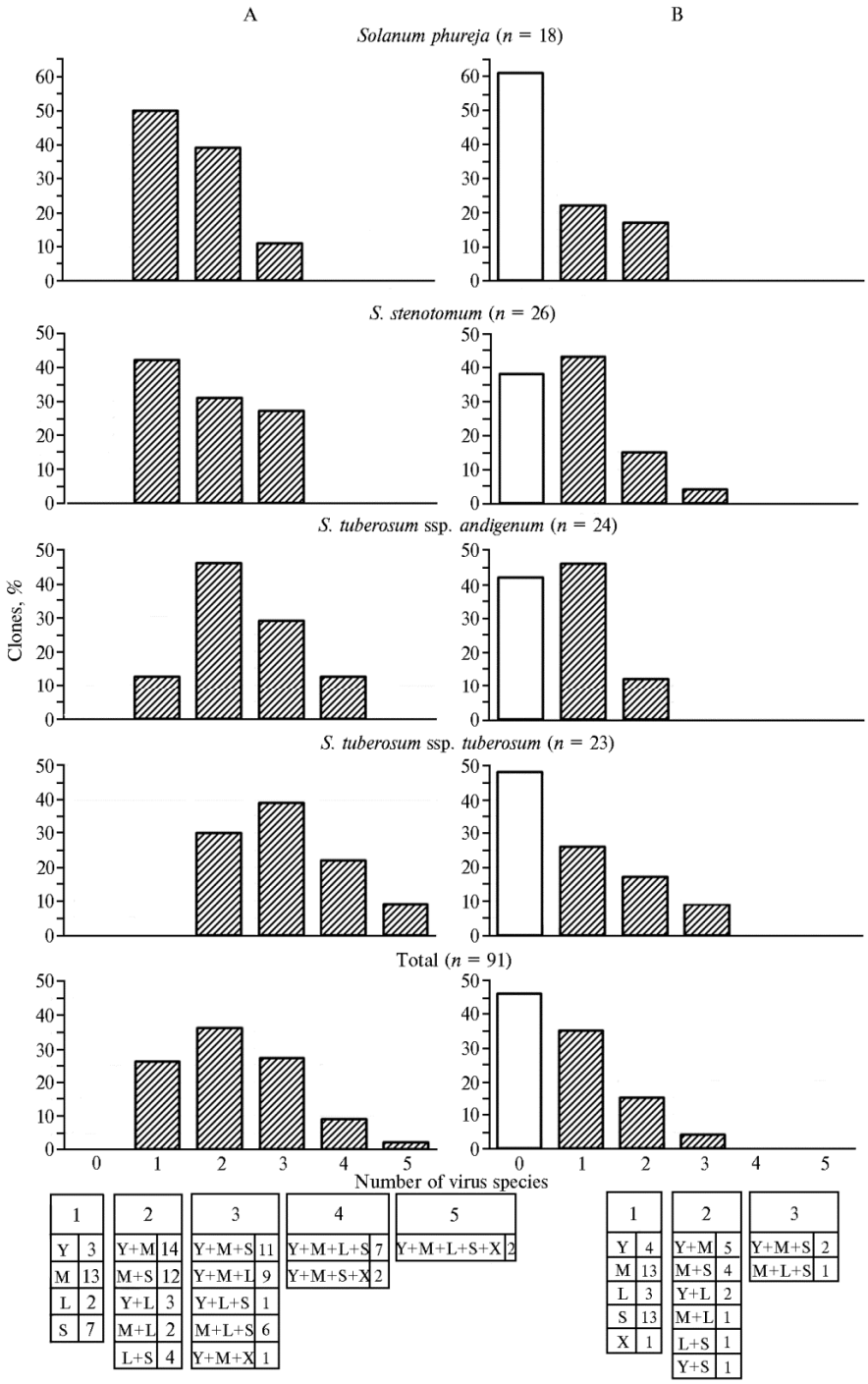

Fig. 2. The percentage of clones (%) among samples of cultivated potato species with various viral infections before (A) and after (B) application of the proposed complex thermo-chemotherapy in vitro protocol: Y, X, S, M are potato virus Y, X, S, and M, respectively, and L is potato leafroll virus.

As it was shown by ELISA for the original plants from the field collection (data not provided) and RT-PCR analysis of derived micro-plants of the same clones, all the samples were affected by viruses to different extents (Fig. 2, A). Simple infections were detected in 25.3 % of the plants, however, the majority of selected clones had mixed infections of several viruses in different combinations, with the maximum number (five virus species) found in *S. tuberosum* ssp. *tuberosum* (see Fig. 2, A).

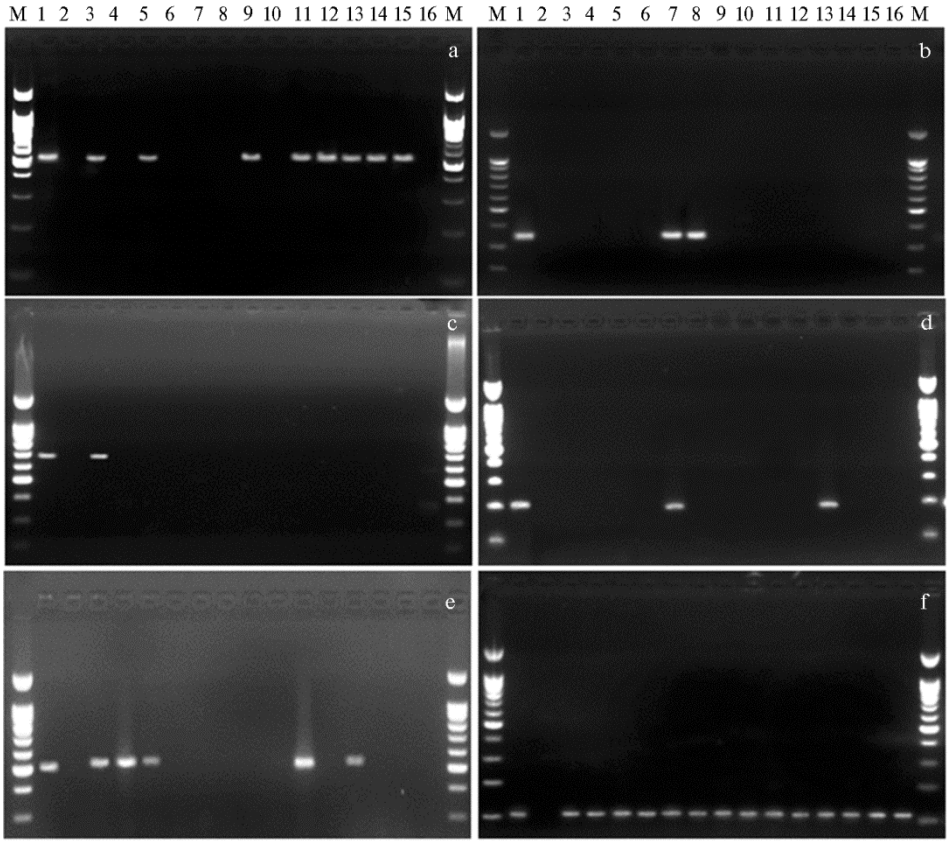

Fig. 3. Example of RT-PCR test of micro-plants of cultivated potato species for potato virus Y (a)**, potato leaf roll viros** (b)**, potato virus X** (c)**, potato virus S** (d)**, potato virus M** (e) **when applying the proposed protocol of complex thermo-chemotherapy with the efficiency control of synthesis of the cDNA** (f) **matrix:** 1 — field plant of *Solanum tuberosum* ssp. *tuberosum* clone (number in VIR collection k-3414), which according to the results of ELISA is affected by all five viruses (positive control), 2 — water (negative control); 3 and 4 — *S. tuberosum* ssp. *andigenum* clone (k-3172), 5 and 6 — *S. tuberosum* ssp. *andigenum* clone (k-3240), 7 and 8 — *S. tuberosum* ssp. *andigenum* clone (k-17961), 9 and 10 — *S. phureja* clone (k-12789), 11 and 12 — *S. phureja* clone (k-5642), 13 and 14 — *S. stenotomum* clone (k-8929), 15 and 16 — *S. phureja* clone (k-9835) (pairwise comparisons of the RT-PCR results before and after treatment); M is a Molecular weight marker 100 bp + 1500 (SibEnzim, Russia). RT-PCR — PCR with reverse transcription. VIR — N.I. Vavilov All-Russian Institute of Plant Genetic Resources.

Combined therapy significantly reduced viral damage of micro-plants (see Fig. 2, B, Fig. 3). Based on the results of RT-PCR analysis, 42 clones (46 %) of these underwent the recovery procedure were completely free from the tested viruses, in 44 % of the clones some virus species were eliminated, and 10 % of the clones failed to recover. Sixteen clones (69.6 %) with a simple infection and 26 clones (38.2 %) with mixed infections were fully revitalized. In other cases after the use of combined therapy, the number of clones with a multiple viral infection decreased. Thus, clones with four to five viral species were not detected,

and the number of micro-plants with two viral species decreased significantly (see Fig. 2, B). These results are consistent with other data [16, 27] obtained in the comparative study of the combined therapy effects at mixed (PVY, PVS, PLRV, PVX) and simple viral infections in potato. However, I.A. Nasir at el. [37] report on the recovery of potato plants with mixed infection from potato viruses S, M, X and note the complete absence of recovery effect from PVY and PLRV.

We did not find any significant differences in the recovery of plants infected by different viruses (see Fig. 2, Table 3). In total, among 91 clones PLRV was eliminated in 72.7 %, PVY in 71.4 %, PVM in 63.9 %, and PVS in 57.4 % of the micro-plants. At that, the differences between the studied potato species for elimination of each virus species were also not significant ($p > 0.05$).

3. Frequency of potato micro-plants (%) free from viruses after application of proposed complex thermo-chemotherapy ($n = 91$, $X \pm x$)

Virus	*Solanum phureja*	*S. stenotomum*	*S. tuberosum* ssp. *andigenum*	*S. tuberosum* ssp. *tuberosum*	Total
PVX	0[в]	0[в]	50.0±50.0[a, b, c]	100.0±0.0[a]	80.0±20.0[a, b]
PVY	33.3±21.1[b, c]	50.0±18.9[b]	93.3±6.7[a]	75.0±9.9[a, b]	71.4±6.5[a, b]
PVS	77.8±14.7[a, b]	23.1±12.2[c]	57.1±13.7[b]	81.8±12.2[a, b]	57.4±7.3[b]
PVM	63.6±15.2[a, b]	66.7±9.8[a, b]	61.1±11.8[b]	63.2±11.4[a, b]	63.9±5.7[a, b]
PLRV	66.7±33.3[a, b]	100.0±0.0[a]	77.8±14.7[a, b]	66.7±11.4[b]	72.7±7.9[a, b]

Примечание. PVX, PVY, PVS, PVM — potato viruses X, Y, S and M, respectively, PLRV — potato leafroll virus. Values marked with the same letters are not significantly different ($p < 0.05$).

Our results on elimination of PVY, PVM and PLRV using the combined thermo-chemotherapy are in line the literature data [9-11, 27, 38, 42] (see Table 1). The proportion of PVS-free plants in our study was 57.4 %, while in other studies [11, 38] almost complete elimination (more than 90 %) was reported. Note that in these studies, the authors used ELISA [11, 38], which is less sensitive than the RT-PCR analysis used by us. As per literature data, in most studies single potato samples [16, 17] or a very small number of samples [38, 42], mostly with simple viral infections [17], were subjected to complex therapy.

So, we proposed a modified protocol for a complex thermo-chemotherapy of potato micro-plants under RT-PCR control of virus infection. This protocol resulted in a comparable eliminating effect for five the most harmful viruses in a genetically diverse 85 samples (91 clones) of three cultivated potato species, which indicates the prospect of further use of the modified thermo-chemotherapy method in the centers of plant genetic resources. The proposed modification differs from the known protocols by simultaneous exposure to elevated temperature and ribavirin, and also by shorter processing cycle.

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A BACTERIAL ISOLATE FROM THE RHIZOSPHERE OF POTATO (*Solanum tuberosum* L.) IDENTIFIED AS *Ochrobactrum lupini* IPA7.2

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Abstract

We present the results of molecular, genetic, physiological and biochemical investigations of a bacterial isolate from the rhizosphere of potato (*Solanum tuberosum* L.) as an object to study plant—microbial associativity, used in particular to improve the existing technologies for the production of high-quality planting material by the method of plant culture micropropagation *in vitro*. To correctly identify the isolate at the species level, we took into account the results of analysis of the current status of prokaryote identification and systematics, reflected in a number of recent reviews. Phylogenetic constructs with strains of the genera *Ochrobactrum*, *Brucella*, *Ensifer*, *Mesorhizobium*, *Rhizobium*, and more (closely related to the isolate under study), which were generated by using DNA sequences of 16S rRNA genes, revealed the isolate in the immediate surroundings of members of the genus *Ochrobactrum*. The isolate turned out to be part of the taxonomic group *Ochrobactrum anthropi* — one of the 1912 taxonomic groups recorded to date, which comprise 6193 prokaryotic species and each include species with coinciding (or almost coinciding) sequences of 16S rRNA genes (http://www.ezbiocloud.net/identify). In accordance with the conceptual propositions formulated in the above-mentioned publications, the species differences within these groups are determined at the level of other molecular genetic (and biochemical and physiological) properties and, with a high probability, by horizontal gene transfer. With account taken of the resulting set of elements of the polyphasic approach, the strain isolated by us was found to be closest to the known type strain *O. lupini* LUP21, which is capable of reinfecting leguminous plants of the genus *Lupinus* and which carries nodulation and nitrogen fixation genes (*nodD* and *nifH*), transferred horizontally into it from rhizobial species. This gave us grounds to identify the isolate being examined as *Ochrobactrum lupini* IPA7.2.

Keywords: plant-microbe associativeness, *Solanum tuberosum* L., bacterial isolate, taxonomic identification, 16S rRNA, polyphasic approach, horizontal gene transfer, *Ochrobactrum lupini*

Symbiosis of microorganisms with plants play a fundamental role in the evolution of life and are of great fundamental and practical interest for the creation of innovative systems of environmentally safe agriculture based on substitution of mineral amendments and pesticides by microbiological preparations [1]. Among the forms of symbiotic interactions, plant-microbial associations occupy an important place with the participation of free-living soil (rhizo)bacteria (plant growth-promoting rhizobacteria — PGPR or plant growth-promoting bacteria — PGPB) that stimulate the plant growth. Such associations have a high potential

as a natural component for modern agrobiotechnologies [2-4]. A good perspective is the creation of plant-microbial associations in vitro based on cellular technologies using mixed cell cultures or plant tissues with bacteria of the PGPB category to obtain high-quality, healthy planting stock of agricultural and ornamental plants with increased resistance ex vitro to adverse environmental conditions [5-7]. The number of bacterial associates is quite large [5-7], but their isolation in situ directly from their habitat and plants, phylogenetic identification and estimation of prospectivity in agrobiotechnology is still of special interest.

Widespread molecular genetic methods, including those based on the use of phylogenetic markers introduced into the standard, the 16S rRNA [8] gene sequenses, are considered sufficiently reliable for solving similar problems. However, in a number of publications in recent years [9-11], more detailed discussion of which may be the subject of a special review, the emerging conceptual and technical problems in the systematics of prokaryotes are analyzed, which should, in our opinion, be taken into account. In particular, it is possible that the studied isolate enters one of the taxonomic groups with coinciding (or almost coinciding) DNA sequences of 16S rRNA genes (http://www.ezbiocloud.net/identify).

This work illustrates some of these problems and concepts on the example of identification of the bacterial isolate from the potato plants rhizosphere of the Nevskii cultivar when screening strains for possible use in reproduction technologies of planting material based on micro-clonal reproduction in vitro [7].

Our goal was to use the sequencing of 16S rRNA gene and polyphase approach [10] to identify the studied strain and predict its properties as a promising bacterial associate.

Techniques. IPA7.2 was isolated in 2012 from the homogenate of washed roots of potato (*Solanum tuberosum* L.) Nevskii cultivar, which grew 3 km to the south-east of Novopushkinskoye settlement (Engels Region, Saratov Ptovince; N 51°21′28.04″ E 46°9′23.82″), at the beginning of tuber formation. Bacteria were isolated on a solid nitrogen-free malate medium NFb (N-free bromothymol blue medium) of the following composition (g/l): hydroxysuccinic acid — 3.8; $K_2HPO_4 \cdot 3H_2O$ — 0.4; KH_2PO_4 — 0.4; $MgSO_4 \cdot 6H_2O$ — 0,2; NaCl — 0.1; $Na_2MoO_4 \cdot 12H_2O$ — 0.002; $FeSO_4$ (in the chelate complex with EDTA) — 0.02; agar-agar — 15 (pH was adjusted with NaOH to 7.0), where the culture was subsequently maintained by periodic replanting.

Optimum growth temperature and pH for the isolate were evaluated in liquid NFb medium supplemented with 1 g/l NH_4Cl at pH 7.0 (4 °C, 20 °C, 28 °C, 35 °C, 42 °C, 50 °C, 60 °C), and pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 (35 °C) by the optical density of the suspension at $\lambda = 660$ nm (a spectrophotometer Specord S 300, Analytik Jena AG, Germany). Urease activity (EC 3.5.1.5) was assessed after 48 hours growth in the medium with 2 % urea as a carbon source (pH 6.8) in the presence of phenol red, nitrate reduction was estimated in a test with Griss reagent after 7 day growth in NFb supplemented with KNO_3 (2 g/l). The ability to utilize various organic compounds as a source of carbon and energy was detected by the growth in 0.1 % peptone water with the addition of citric acid, sodium glutamate, mannitol, D-glucose, maltose, sucrose, gluconate, D-mannose and glycerin (10 g/l each) at pH 7.0.

For specific detection of IPA7.2 on plant roots, the rabbit polyclonal antibodies to 2 % glutaraldehyde-fixed IPA7.2 cells [12] and fluorescent-labeled anti-rabbit antibodies (Alexa Fluor® 532, Invitrogen, USA) were used. Three-day seedlings of white lupine (*Lupinus alba* L.) and wheat (*Triticum aestivum* L.) of Saratovskaya 29 cultivar were incubated in a bacterial suspension of IPA7.2 (10^6-10^8 cells/ml) for 1 day, and then grew for 3 days in sterile water. Immunofluorescence microscopy was performed using a microscope of Leica LMD7000

laser dissector set (Leica Mikrosysteme Vertrieb GmbH, Germany) with light filter № 21 (545±12 nm).

Genomic DNA of IPA7.2 was extracted from cells grown on a solid nutrient medium and purified according to the description [13]. The DNA fragments of the 16S rRNA gene were subjected to PCR amplification using universal primers (529R, 350F and 1492R) under corresponding protocols. The resulting DNA sequence of the 16S rRNA gene of IPA7.2 strain (1403 bp) was deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accession number KU217325).

Initial set of homologous DNA sequences of the 16S rRNA gene of strains closely related to the IPA7.2 was made using BLASTN technology (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=TargLociBlast). Besides, in selecting reference strains for phylogenetic analysis their compliance with the SILVA database (https://www.arb-silva.de/) was taken into account. The bioinformatic resources presented on this website were used to compare phylogenetic constructs based on traditional methods and specially developed methods for aligning the 16S rRNA sequences [14]. To compare DNA and RNA sequences, algorithms of pairwise and multiple alignment were used (http://www.ezbiocloud.net/taxonomy, http://www.ebi.ac.uk/Tools/msa/, http://embnet.vitalit.ch/software/LALIGN_form.html).

Phylogenetic trees from the sequences of 16S rRNA (https://www.arb-silva.de/aligner) or their genes were constructed using the MrBayes method (http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes) and Neighbor Joining from the MEGA v.6 integrated phylogenetic analysis software package (http://www.megasoftware.net/mega.php). For this constructing we applied multiple alignments obtained by the use of the algorithm Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) and the SINA Alignment Service source (https://www.arb-silva.de/aligner) from SILVA portal (https://www.arb-silva.de).

Results. A distinctive feature of the special methods we used in this work for sequence alignment was their comprising the secondary structure of molecules by means of basic alignment on a large data set for 16S rRNA prokaryotes (about 70 thousand sequences; https://www.arb-silva.de/documentation/release-128).

Blasting of the 16S rRNA gene sequence of IPA7.2 isolate using specialized database for bacteria and archaea ribosomal RNA sequences and screening in the SILVA database showed a typical strain *Ochrobactrum lupini* LUP21 [15] to be the closest to the identified isolate by the total count, the maximum similarity assessment in SILVA and the percentage identity (Table 1). The analysis of fragments after pairwise global and local alignment carried out by two independent methods (LALIGN and EzTaxon) [19] for 16S rRNA sequences of these two strains in the regions with detected differences, showed that along the total length of the IPA7.2 16S rRNA (1403 bp), both sequences practically coincided (99.9 % identity) within addition of two (see Fig. 1, A) and one (see Fig. 1, B) gaps.

1. Members of the *Ochrobactrum anthropi* taxonomic group (pairwise identity with sequences of 16S rDNA isolate > 98.65 %)

Typical strain	Reference	Source	GenBank identifier	Identity, %	Score
O. lupini LUP21	[15]	Lupin *Lupinus honoratus* C.P. Sm.	NR_042911	99.93	2586
O. cytisi ESC1	[16]	English broom *Cytisus scoparius* (L.) Link	NR_043184	99.86	2580
O. anthropi ATCC 49188	[17]	Clinical samples from a human	NR_074243	99.86	2580
O. tritici SCII24	[18]	Wheat *Triticum aestivum* L. soil and rhizoplane	NR_028902	99.57	2543

N o t e. The identity and score are as determined in BLASTN.

However, as per http://www.ezbiocloud.net/identify data, both strains

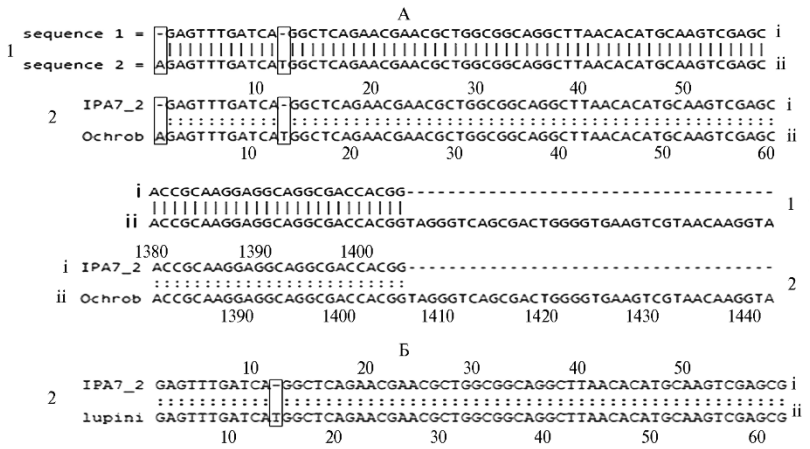

Fig. 1. Fragments of global (A) **and local** (B) **alignment of the 16S rRNA gene sequences of IPA7.2 isolate** (i) **and the *Ochrobactrum lupini* LUP21** (ii)**:** 1 — carried out by the method of O.S. Kim et al. [19], 2 — performed in LALIGN program.

should be included in one of 1912 so-called taxonomic groups (i.e. *Ochrobactrum anthropi*) of 6193 prokaryotes species. Each of these groups comprises the species with coinciding or almost coinciding 16S rRNA gene sequences. Their conservativism turned out to be so high that in each group the bacterial species are virtually indistinguishable from each other on this basis and can not be identified by 16S rRNA analysis without additional genetic or physiological and biochemical studies. The paper of G.E. Fox et al. [20] was probably one of the first reports of the mentioned phenomenon. The high conservativism in the sequences of 16S rRNA genes in this case leads to a kind of "degeneracy" of an attribute with respect to speciation processes controlled primarily by horizontal gene transfer (HGT) for prokaryotes with open pangenome [9, 10]. Such results, therefore, can serve for detection of xenologes in the genomes of the studied microorganisms, which are responsible for species-specific phenotypic characters [21]. In this context, our results of the multiple alignment (MSA, according to the MUSCLE method, http://www.ebi.ac.uk/Tools/msa/muscle/) and pairwise alignment of the 16S rRNA gene sequences for the members of *O. anthropi* group are quite evident. From ten species of this group the members of species *O. lupini*, *O. anthropi*, *O. tritici* and *O. cytisi* were the closest to the studied isolate in the pairwise identity of the 16S rRNA gene sequences estimated in the BLASTN program. For them, this identity turned out to be higher than the conventional border of species separation (98.65 %), which was accepted recently [22] (see Table 1).

Our data on the MSA of 16S rRNA genes of *O. anthropi* and the studied isolate showed practical coincidence (including that for IPA7.2) within the accuracy of four bases in three *O. tritici* strains and terminal gaps that do not play a significant role in taxonomic assessments. Taking into account the results of numerous studies of 16S rRNA gene structure in prokaryotes, generalized, for example, by O. Paliy et al. [23], it can be assumed that the observed differences in the region of high conservativeness of the *O. tritici* 16S rRNA gene (at positions 160-163) can be recognized as insignificant sequencing inaccuracy (about 0.3 % of the total length) of 16S rDNA in *O. tritic* strains. Note that similar results of MSA in this case were obtained using MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft) and TCOFFEE (http://www.ebi.ac.uk/Tools/msa/tcoffee).

Three of four strains from the *O. anthropi* group (see Table 1) belong to soil bacteria actively interacting with leguminous plants (*O. lupini* LUP21,

O. cytisi ESC1) or living on wheat roots (*O. tritici* SCII24), and one (*O. anthropi* ATCC 49188) is clinically isolated from human (blood samples mostly). Since the interspecific index of identity of 16S rRNA genes, determined by M. Kim et al. [22] statistically after processing data for 6787 genomes of prokaryotes of 22 taxonomic groups, falls within the range of < 98.65 %, this parameter could not be the only sign for assignment of the studied isolate to one of these four species. To solve the problem, we used the results of physiological and biochemical study.

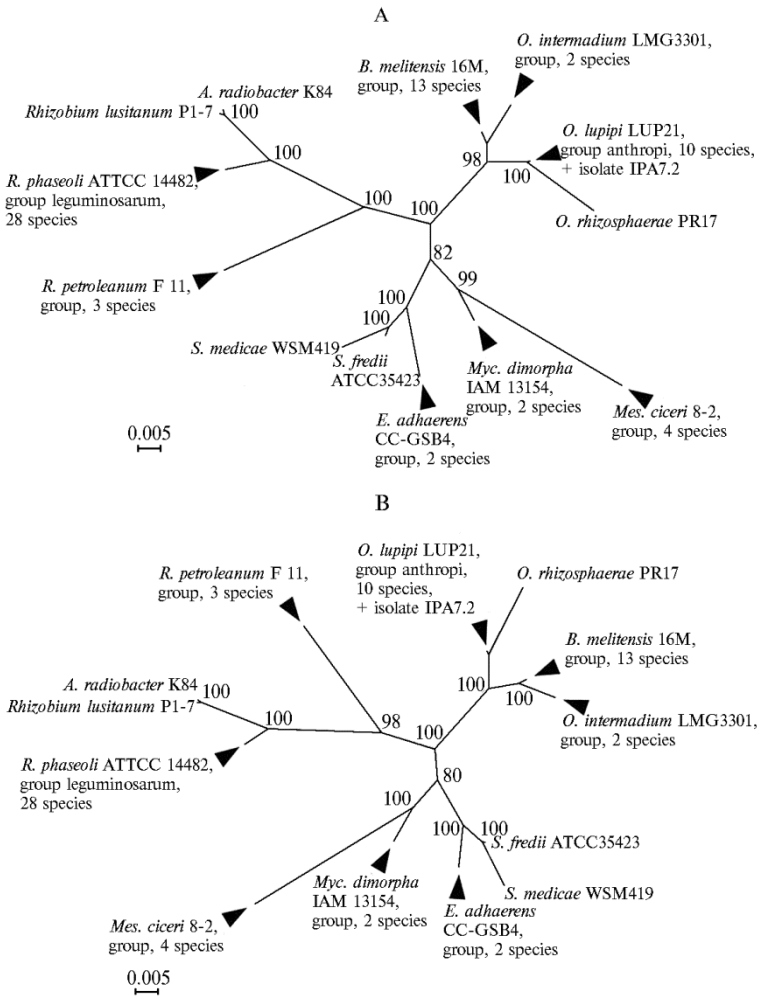

Fig. 2. Phylogram of strains closely related to IPA7.2 isolate, constructed using the method of MrBayes with multiple sequence alignment with Clustal Omega (A**) method and SILVA** (B)**.** Abbreviated names *Agrobacterium*, *Brucella*, *Ensifer*, *Mesorhizobium*, *Mycoplana*, *Ochrobactrum*, *Rhizobium*, *Sinorhizobium*. The numbers show the statistical support of the nodes (Bayesian posteriori probability, %). Black triangles indicate the entry of organisms with the identified 16S rRNA sequences into the corresponding taxonomic groups (http://www.ezbiocloud.net/identify), the number of species in which varies from 2 to 28.

The taxonomic environment of the studied isolate as a representative of the *Rhizobiales* at evolutionary distances, reaching intergeneric level, is characterized by the trees (Fig. 2) constructed from MSA which we obtained by two different methods — Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) (see Fig. 2, A) and offered at the web-resource http://www.arb-silva.de/aligner (see Fig. 2, B). It is reported [24] that the separation at the intergeneric level corresponds to the pairwise identity of the 16S rRNA sequences and their genes

in the range of ≥ 94.5 %. From the initial set of more than 100 sequences (BLASTN, SILVA) satisfying this requirement, we selected 13 reference samples satisfying the following requirements: they were located in typical strains, belonged to the category of complete sequences, or their length was not less than 1380 bp. The comparison (see Fig. 2, A and B) shows that the overall topology of the obtained phylogenetic structures practically coincided (up to equivalent mutual positions of the same branches) under two independent methods of alignment with a high statistical support of the nodes (Bayesian posteriori probability). The observed topology was also reproduced using both MSA and the Neighbor Joining method [25] with support of 70-100 % nodes (at 1000 bootstrapping cycles).

Among the investigated objects, we identified 8 groups with practically identical, according to the conventional border [22, 26], 16S rRNA sequences (> 97 %, http://www.ezbiocloud.net/identify) and the number of species from 2 to 28, combining 64 bacterial species. This more than 5-fold extends the actual list of species which are closely related to IPA7.2 and non-apparently involved in the phylogenetic trees (see Fig. 2). This type constructions reflect, in fact, the contribution of the network component to the generalized phylogenetic constructions [9], which is due to significant contribution of horizontal gene transfer to speciation within these taxonomic groups. In the set of sequences used, one detects the separation of three clusters (monophyletic groups) which group the members of *Brucellaceae*, *Phyllobacteriaceae* and *Rhizobiaceae* families. The resulted clusterization of species is generally consistent with the data of extended taxonomic studies of the *Rhizobiales* sequence based on the results of the genome wide DNA sequencing of strains (https://www.patricbrc.org/view/Taxonomy/356#view_tab=phylogeny).

Information on strains of the close taxonomic environment of IPA7.2, reflected in the annotations and references in GenBank's records, reveal a wide range of habitats for these bacteria (i.e. from the potato rhizosphere, soil samples and rice plants to industrial zones, farm animals and human clinical samples). This testifies to the deep evolutionary connections of the studied isolate with the soil microflora, which is present and functioning in a very diverse ecological niche: from participation in endo- and ectosymbiosis with plants to survival in aggressive ecologically unfriendly environments. There are also a number of evolutionary metamorphoses leading to a very active promotion of individual representatives of the *Ochrobactrum* genus towards establishing a close relationship with the animal world [17].

In order to properly attribute the IPA7.2 strain to one or another species within the taxonomic group *Ochrobactrum anthropi* (see Table 1), additional physiological and biochemical studies were required. Consideration of a set of phenotypic, chemotaxonomic and extended genotypic characteristics of strains, which increases the reliability of the description of new taxa (as well as the identification of isolates among existing ones) is called the polyphase approach [10]. Given the majority of the representatives of *Ochrobactrum anthropi* group belong to soil bacteria actively interacting with plants, we evaluated the ability of the IPA7.2 strain to colonize the roots of lupine and wheat, the macro-partners in symbiosis (association) with plants for *O. lupini* and *O. tritici* representatives. The results of immunofluorescence detection of IPA7.2 cells on the roots of these plants are illustrated in Figure 3. In a series of experiments, it was found that IPA7.2 cells colonize both roots and root hairs of lupine and wheat as multicellular formations — microcolonies or biofilms. At the same time, active colonization of the root surface of both plants with an IPA7.2 strain was noted, indicating that the bacteria had no host plant specificity.

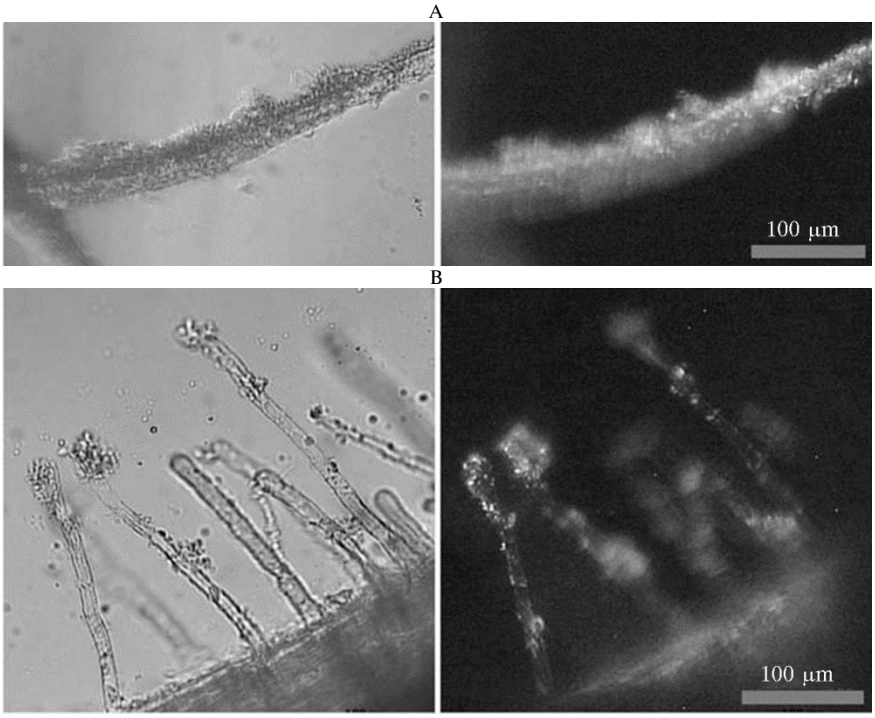

Fig. 3. Immunofluorescence detection of colonization of the lupine white (*Lupinus alba* L.) roots (A) **and wheat (*Triticum aestivum* L.) Saratovskaya 29 cultivar roots** (B) **with an identifiable isolate IPA7.2** (Leica LMD7000, Leica Mikrosysteme Vertrieb GmbH, Germany).

The IPA7.2 isolate showed a high biochemical activity, manifested in the ability to grow on medium with malate, citrate, glutamate, mannitol, D-glucose, maltose, sucrose, gluconate and D-mannose, but not glycerin (the best growth characteristics were noted on media with organic acids), on a nitrogen-free NFb medium with multiple replanting, and also over a wide range of temperatures (from 4 to 42 °C with an optimum at 35 °C) and pH (from 5.0 to 10.0 with an optimum at pH 7.0). The revealed optimum pH is close to the values (pH 7-9) most favorable for *O. lupini* LUP21 cultures.

2. Biochemical characteristics of IPA7.2 isolate and closely related strains from the taxonomic group *Ochrobactrum anthropi* (pairwise identity with sequences of 16S rDNA isolate > 98.65 %)

Trait	IPA7.2	*O. lupini* LUP21	*O. anthropi* ATCC 49188	*O. tritici* SCII24	*O. cytisi* ESC1
Nitrate reduction	–	–	+	+	+
Urease production after 48 hours	+	+	+	+	–
Assimilation of:					
gluconate	+	+	+	+	–
citrate	+	+	–	–	+
D-mannose	+	+	+	–	+

N o t e. "+" и "–" — activity or no activity, respectively.

When comparing the isolate IPA7.2 and the members of the *Ochrobactrum anthropi* group (Table 2), it turned out that by the biochemical properties, as well as by the total score in the blasting, the maximum similarity assessed by SILVA method and the established percent identity (see Fig. 1), IPA7.2 most closely corresponds to strain *O. lupini* LUP21. Note that *O. lupini* LUP21 is a typical strain, it is capable of reinfecting leguminous plants of the *Lupinus* genus and has nodulation and nitrogen fixation genes (*nodD* and *nifH*) obtained from rhizobial species via horizontal transfer [15].

As per polyphase approach [10], IPA7.2 strain is the closest to the *O. lupini* species and can be identified at the current stage of research as *O. lupini* IPA7.2. On this basis, it may be considered advisable to continue its study as a potential associate (inoculum) in improving plant microclonal propagation in vitro [7].

Thus, the performed phylogenetic studies showed that the members of *Ochrobactrum* genus are immediate taxonomic environment of IPA7.2 isolate. As per bioinformatic analysis, IPA7.2 belongs to *Ochrobactrum anthropi* group which comprises 10 species of the genus. This is one of the taxonomic prokaryotic groups, of which each concentrates species with coinciding or almost coinciding 16S rRNA gene sequences. Physiological and biochemical tests, along with bioinformatic analysis allow us to identify the isolate as *Ochrobactrum lupini* IPA7.2. The presence of nodulation and nitrogen fixation genes (*nodD* and *nifH*) in a typical species of the group, *O. lupini* LUP21, makes it possible to predict a bacterial associate properties for IPA7.2, which, however, requires special study.

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PSYCHROPHILIC ENDOPHYTIC *Pseudomonas* AS POTENTIAL AGENTS IN BIOCONTROL OF PHYTOPATHOGENIC AND PUTREFACTIVE MICROORGANISMS DURING POTATO STORAGE

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Abstract

Abundant pathogens which attack seed and food potato tubers are a serious problem of the modern potato growing that causes significant losses during storage. Regular use of chemical fungicides and agrochemicals has led to emergence of resistance and an increased aggressiveness of plant pathogenic microorganisms. New fungal and bacterial strains and races are also appearing which spread rapidly and cause great damage to agricultural production. In this regard, the biologicals based on biocontrol microorganisms, instead of chemicals, are considered particularly relevant to protect seed and food potatoes from infectious diseases. A genus *Pseudomonas* belonging to the group of plant-growth promoting rhizobacteria (PGPR) includes species most effectively colonizing higher plants and used as active agents of biological products. Scientific novelty of our work lies in the fact that this study is the first to report the effect of psychrophilic strain *Pseudomonas* spp. RF13H on the storage of potato tubers. The cold resistant commercial varieties recommended for cultivation in the Leningrad region has been involved. For the first time we estimated the efficiency of tubers protection from a number of pathogenic microorganisms under refrigerated storage, assessed the population dynamics of introduced strain, and visualized the pattern of bacteria distribution and localization on the surface, using fluorescent in situ hybridization and confocal laser scanning microscopy. Using molecular genetic analysis, we have clarified the taxonomic position of several *Pseudomonas* strains, including psychrophilic strain *Pseudomonas* spp. RF13H. This strain possessed fungicidal and bactericidal activity against saprogenic and pathogenic microorganisms and was agronomically and physiologically tested. Its growth at low temperatures and production of auxin-like phytohormones were studied. *Pseudomonas* spp. RF13H influenced positively preservation and biochemical processes in potato tubers under refrigerated storage. Its microcolonies were often localized in different cracks, grooves and recesses on the stored tuber surface that indicated beneficial plant-microbial relations and explained a strategy for tuber colonization at room and low temperature during storage. The occurrence of surface phytopathogenic microorganisms decreased in the presence of *Pseudomonas* spp. RF13H, and the average number of all type-infected tubers was about 50 % lower among those treated with *Pseudomonas* spp. RF13H comparing to untreated ones. This trend continued for 3 month storage, i.e. the amount of infected untreated tubers reached 30 % and reduced up to 10-13 %, when treatment with biocontrol strain. At a temperature of 4 °C, the bacterial counts was quite stable for 5 months, and then significantly decreased (up to trace quantities) to the end of storage. The bacteria concentrated at the boundaries between the individual cells of tuber periderm and in the places of exudation of biochemical substances that serves as nutrients for bacteria. Introduc-

tion of *Pseudomonas* spp. RF13H was shown to contribute to redox processes (activation of antioxidant protection system enzymes — catalase, peroxidase, superoxide dismutase) thus increasing plant resistance to pathogens. Additionally, this strain has a positive effect on the microbiome composition in potato epidermis. Induction of own immunity and creation of a barrier preventing pathogen penetration helped to reduce infection in the potatoes tuber.

Keywords: *Pseudomonas*, potato, biocontrol of plant pathogens, fluorescence in situ hybridization

Rapid increase in plant pathogen resistance and emergence of new species, including those from imported seeds, has been observed in recent years [1, 2]. Both losses during storage and a threat of re-infection of vegetating plants are related to tuber infection in potato farming. According to FAO, per capita consumption of potato in Russia is one of the world's largest, i.e. 130 kg annually [3]. Potato is a crop with high potential yield, but it is extremely sensitive to fungal, bacterial and viral pathogens due to mostly vegetative reproduction.

Increase in resistance of pathogens and disease outbreaks lead to use of increased doses of chemicals, which, in its turn, induces a new increase in resistance of pathogens. Thus, biologicals based on biocontrol microorganisms, i.e. natural ecosystem inhabitants [4] which provide selective action and allow to avoid adverse effects on biocenoses and yield [5], are relevant for environmental stability and protection in plant growing. The majority of such microorganisms synthesize various metabolites initiating a cascade of defense reactions in a plant [6]. Apart from that, during interaction with a plant bacteria can increase its viability, thus regulating the number of plant pathogens. As a result, several protection levels are formed, including direct competition with a plant pathogen, production of biofilms and antibiotic substances, and induction of plant immunity.

Endophytic bacteria [7, 8] inhabiting plant tissues, which makes them less dependent on external factors, are of particular interest. Upon introduction into plant tissues endophytes with a complex of agriculturally valuable traits may contribute to formation of durable macroorganism protection from stresses [9].

The study of microbiome in potato (*Solanum tuberosum* L.) included both rhizosphere and endosphere [10-12]. It has been shown that biodiversity of microorganisms often depends on a variety and plant development phase. Such studies were mostly performed in vegetating parts [13-15], rather than tubers upon harvesting. In one of the studies the authors evaluated bacterial population dynamics in tubers during storage for several taxa [16]. The report on analysis of tuber endophytic microbiome during storage with the use of both conventional methods of microorganism isolation and identification and high-throughput next generation sequencing (NGS) shall be noted among recent publications of particular interest [17]. It should be noted that bacteria isolation from tissue upon surface sterilization is not enough to assign them to endophytes. Bacteria visualization inside a plant is required, e.g. using fluorescent markers (probes or proteins) [18, 19].

Currently, global practice of bacterial product application for potato storage has just started developing, that is why many questions arise on mechanisms of interaction in a complex system plant-pathogen-antagonist. Particularly, what happens to the strain upon introduction and during tubers storage, how its count and distribution on tuber surface change, and how storage temperature affects these processes. Finding answers to these questions will allow forecasting effects and adjusting bacterials application. Involvement of local varieties in such experiments is helpful in development of a comprehensive practical model.

Scientific novelty of this work is examination of effect of *Pseudomonas* sp. RF13H psychrophilic bacterial strain on tuber preservation in cold-resistant potato varieties recommended for growing in Leningrad region (Nevskii and Su-

darynya), performed for the first time. Effectiveness of tuber protection from several plant pathogens during refrigerated storage has been determined, introduced strain count dynamics on tuber surface during the whole storage period has been evaluated. Colonization of tuber surface with *Pseudomonas* sp. RF13H, the nature of which suggests mutually beneficial plant-microbial relations, has been studied for the first time using modern visualization methods. The results obtained using commercial varieties, make it possible to develop practical recommendations on use of *Pseudomonas* sp. RF13H psychrophilic strain in potato production, storage and reproduction.

This work is focused on clarification of *Pseudomonas* sp. RF13H psychrophilic strain taxonomic position and examination of its biocontrol effectiveness (preservation of potato tubers and biochemical processes therein), as well as localization of bacteria on tuber surface under refrigerated storage.

Techniques. Potato (*Solanum tuberosum* L.) varieties Nevskii (middle-late) and Sudarynya (middle-early) were the objects of strain biocontrol effect study. Commercial potato batches were obtained from Belogorka Leningrad Agricultural Research Institute.

Pseudomonad strains, including *Pseudomonas* sp. RF13H, were isolated from *Sphagnum fallax* sphagnum moss tissues as a members of extremophilic endophytic community [20]. The culture was grown for 48 hours at 28 °C in TSB liquid medium (Tryptic Soy Broth, Scharlab, S.L., Spain), and used to prepared working suspension of 10^8 CFU/ml. Potato tubers were sprayed with a bacterial suspension (water in control), dried in air and placed for storage at room temperature and at 4 °C. Test cultures used were the main *Solanaceae* family pathogens, inducing causative agents of vegetative organ diseases in plants during refrigerated storage (*Fusarium solani*, *Alternaria alternata*, *Pseudomonas solanacearum*, *Clavibacter michiganensis* subsp. *sepedonicus*). Micromycetes were grown on potato dextrose agar (PDA) (BD, USA), phytopathogenic bacteria on starvation potato agar medium (All-Russia Research Institute of Agricultural Microbiology — ARRIAM). Strains of phytopathogenic fungi and bacteria were obtained from the collection of Laboratory of microbial preparation technology (ARRIAM).

Standard method of DNA extraction from pure 24-hour cultures of studied bacterial strains (cell lysis using lysozyme, proteinase K and SDS and extraction with phenol:chloroform) was used for analysis of 16S rRNA gene regions V3-V6 variability. PCR was performed in a Bio-Rad C1000 thermal cycler (Bio-Rad, USA). DNA of 16S rRNA gene was amplified using BD1/FD1 primers in the respective mode [21]. Upon gel purification, the fragments were sequenced with internal primers fD1/rD1 to variable regions V3-V6 of 16S rRNA gene according to the protocol of Beckman Coulter, Inc. (USA) for an 8-channel sequencer SEQ8000 using commercial kit SEQ Dye Terminator Cycle Sequencing (DTCS) with Quick Start Kit (Beckman Coulter, Inc., USA).

Identification of isolate species was performed using BLAST (GenBank, http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (http://rdp.cme.msu.edu/) software. Alignment of identified nucleotide sequences with reference sequences from GenBank and phylogenetic tree construction was performed using UGENE (UniPro, Russia).

During examination of physiological properties of strains their fungicidal and bactericidal activity, growth rate at low temperatures (4-10 °C) and production of auxin-like phytohormones were compared. Well method [22] was applied for fungicidal activity assessment, agar blocks [23] for bactericidal activity assessment. The number of introduced and autochthon microorganisms on tuber surface was estimated using microbiological culture [24, 25]. Auxin content

in culture broth extracts was analyzed by a high performance liquid chromatography (HPLC) system Waters ACQUITY UPLC H-class with fluorescence detector (Waters, USA).

Antioxidant protection system activity in tubers was assayed by malondialdehyde (MDA) and peroxydase (PO) content in seedlings. Lipid peroxidation (LPO) was evaluated by the concentration of colored complex formed by MDA in acidic medium at a temperature of about 100 °C [26], catalase activity (CAT) was estimated by hydrogen peroxide degradation rate, PO activity — as optical density of guaiacol oxidation products [27]. For superoxide dismutase (SOD) activity unit, 50 % inhibition of formazan formation in a reaction with nitroblue tetrazolium was taken [28].

Oligonucleotide target probe for fluorescence in situ hybridization (FISH) was designed using IDT Internet resource [28]. The developed probe was marked with 6FAM fluorochrome. Probe hybridization was performed at 60 °C for 90 min. Preparation of plant tissues and hybridization were performed as described [29, 30]. The samples were analyzed using Leica TCS SPE confocal microscope (Leica Mikrosysteme Vertrieb GmbH, Germany). Laser (λ = 488 nm) was used for oligonucleotide probe fluorescence detection, fluorescence was registered within the range of 508-566 nm. Images were visualized using Leica Confocal Software (Leica Mikrosysteme Vertrieb GmbH, Germany).

Each index (fungicidal and bactericidal activity, colony growth rate at low temperatures, enzyme activity and microorganism counts on tuber surface) was evaluated in three independent experiments. Auxin content analysis was performed in triplicate. Statistical processing of numerical values was performed using DIANA software (ARRIAM) at significance level $p = 0.05$.

Results. Taxonomy of studied isolates. *Pseudomonas* genus is extremely diverse, and its taxonomy is still being clarified [31, 32]. Base on the obtained morphological, physiological and biochemical characteristics, 10 studied strains from the laboratory collection were assigned to the group *P. fluorescens–P. asplenii–P. poae*.

Analysis of variable regions V3-V6 of 16S-rRNA gene was performed to obtain more detailed information on taxonomic position of studied strains. The greatest variability was observed for the regions at positions 557-586 and 624-685, corresponding to nucleotide positions in 16S rRNA gene of *Escherichia coli* (Fig. 1, A). Obtained sequences were aligned with available reference sequences from GenBank. Consensus alignments for each proposed specie (*P. fluorescens*, *P. poae*, *P. asplenii*, *P. koreensis*, *P. moraviensis*) were used as references. Phylogenetic tree (see Fig. 1, B) constructed based on the said regions reflected taxonomic position of studied isolates as follows: AF12F, RM13D, AF24C strains were more closely related to *P. koreensis* and *P. moraviensis* subgroup, while RM11C, RM14B were more closely related to *P. asplenii*. RF14J, RF12C, RF14H, RM13C, and RF13H strains shall be possibly considered as representatives of species which have not been described yet.

Ability of psychrophilic growth and development at 0-10 °C, inhibition of phytopathogenic and putrefactive microorganisms, production of auxin-like phytohormones (indolyl-3-acetic, indolyl-3-carboxylic and indolyl-3-lactic acids) was examined in all strains. As it has been shown, pseudomonade strains differed by growth rate at low temperatures which decreased significantly in the majority of the strains at 6-7 °C and was completely inhibited at 5 °C. However, at 7-10 °C all strains formed colonies 3-4 mm in diameter within 7 day growing on R_2A agar. RF13H and RM14B strain growth was observed at positive temperatures (2-4 °C) close to 0°C: in 7 days of low-temperature growth the bacteria formed colonies 1-

2 mm in diameter.

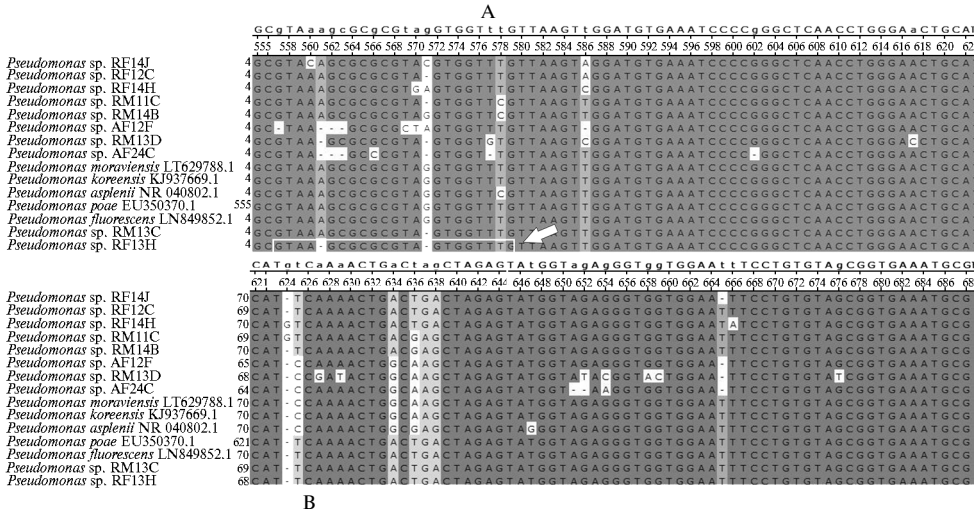

Fig. 1. Regions of variable fragments V3-V6 of 16S rRNA gene of studied pseudomonades with replacements and deletions with regard to reference sequences (A) **and the phylogenetic tree derived from 16S rRNA gene variable regions in 10 studied psychrophilic pseudomonade strains** (B). The arrow indicates the target for Z-SpecPs probe hybridization. The phylogenetic tree is constructed using UGENE software.

A

Strain	Growth at low temperatures, °C												Antagonism				AU
	15	12	10	9	8	7	6	5	4	3	2	1	0	*PS*	*CM*	*FS*	*AA*	
Pseudomonas sp. RM13C														10	12	17	22	-
Pseudomonas sp. RF13H														12	10	22	27	+
Pseudomonas sp. RF14J														7	8	13	15	-
Pseudomonas sp. AF12F														12	10	10	12	-
Pseudomonas sp. AF24C														5	2	7	8	-
Pseudomonas sp. RM13D														10	12	15	17	-
Pseudomonas sp. RM11C														8	12	22	22	-
Pseudomonas sp. RM14B														14	17	17	12	+
Pseudomonas sp. RF12C														1	3	4	4	-
Pseudomonas sp. RF14H														1	2	2	5	-

B

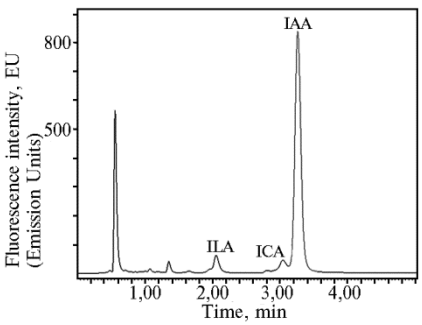

Fig. 2. Physiological and biochemical (potentially adaptive) properties of studied pseudomonade strains: A — psychrophilic growth, diameter (mm) of growth inhibition zones of *Pseudomonas solanacearum* (*PS*), *Clavibacter michiganensis* subsp. *sepedonicus* (*CM*), *Fusarium solani* (*FS*), *Alternaria alternata* (*AA*) and auxin production (AU, presence or absence); B — results of culture broth analysis for RF13H strain grown on L-tryptophan containing medium, by high performance liquid chromatography method (Waters ACQUITY HPLC H-class with fluorescence detector (Waters, USA): ILA — indolyl-3-lactic acid, ICA — indolyl-3-carboxylic acid, IAA — indolyl-3-acetic acid.

Evaluation of antagonistic activity with regard to phytopathogenic and pu-

trefactive potato microorganisms (Fig. 2, A) showed the sign to be clearly manifested in some strains (RF13H, RF14J, RM14B, RF12C) (inhibition of pathogens causing potato losses during refrigerated storage). *Pseudomonas* spp. RF13H strain, selected for a complex of physiological properties, was more prominent in activity against fungi and bacteria, formed optically clear zones without inclusions of test culture mycelium and plant pathogen and produced auxin-like phytohormones (see Fig. 2, B). For this strain considered as prospective for practical use, we have studies physiological activity during colonization of tuber surface.

Pseudomonas sp. RF13H introduced strain count dynamics and impact on storage. Tubers were inoculated with a monoculture of pseudomonade test strain during post-harvesting (treatment) period to create favorable conditions for adaptation of antagonist bacteria and their activity. This period takes 14 days according to the conventional potato storage technique, during which tubers are kept at 18 °C. This mode also ensures active suberization of mechanical damages and reinforcement of tuber surface tissues.

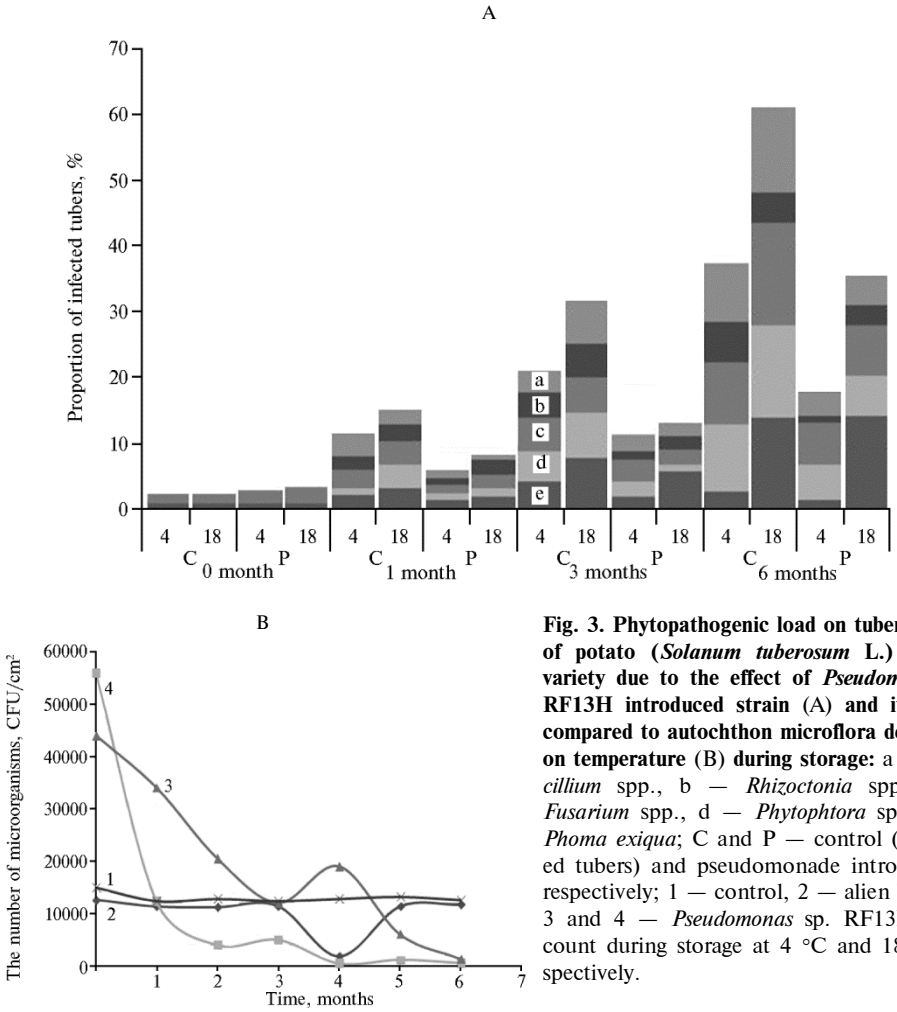

Fig. 3. Phytopathogenic load on tuber surface of potato (*Solanum tuberosum* L.) Nevskii variety due to the effect of *Pseudomonas* sp. RF13H introduced strain (A) **and its count compared to autochthon microflora depending on temperature** (B) **during storage:** a — *Penicillium* spp., b — *Rhizoctonia* spp., c — *Fusarium* spp., d — *Phytophtora* spp., e — *Phoma exiqua*; C and P — control (untreated tubers) and pseudomonade introduction, respectively; 1 — control, 2 — alien bacteria, 3 and 4 — *Pseudomonas* sp. RF13H strain count during storage at 4 °C and 18 °C respectively.

Total count of mesophilic saprotrophic microflora per unit of tuber surface varied within 10^3-10^4 CFU/cm^2. *Pseudomonas* sp. RF13H introduction affected the phytopathogenic compositions (Fig. 3, A), as well as proportion and dynamics of saprotrophic microflora counts (see Fig. 3, B) during long-term storage (for 6 months). The studied microorganism survived good enough on tuber sur-

face and maintained its count during the experiment. It can be noted that refrigerated storage had generally positive effect on RF13H strain due to its physiological properties and characteristics of bacterial population as a whole. It is known that various bacteria species maintain viability on solid growth media at 4 °C during a long period (up to half a year) [33]. Drying of culture media, causing death of a population, serves as a limiting factor. Possibly, the same happens on tuber surface at normal storage temperature: decrease in introduced strain count was most pronounced in this case, and residual amounts of the strain were observed in 6 months. *Pseudomonas* sp. RF13H count at 4 °C was sufficiently stable during 5 months and decreased significantly by the end of the storage, when residual amounts of this strain were observed.

However, effect of the strain on composition and the count of phytopathogenic fungi (see Fig. 3, A) turned out to be significant, according to the phytopathological analysis. It has been found out that development of phytopathogenic micromycetes (*Rizoctonia* spp., *Fusarium* spp., *Penicillium* spp., *Phytophthora* spp., *Phoma exiqua*) was the most pronounced during storage. Their frequency in the population decreased in as little as 30 days (to the greatest extent during refrigerated storage). Average proportion of tubers affected by all types of infections after bacterial culture treatment decreased from 11.5 to 5.9 % at refrigerated storage, and from 15.0 to 8.3 % at room temperature in 30 days. This trend was preserved for 3 months, i.e. the proportion of infected tubers amounted to 30 % in control samples, while introduction of bacterial strain ensured reduction of this index up to 10-13 %. In 6 months significant tuber infection (up to 60.0 %) was observed at room temperature, while at refrigerated storage the parameter was about half as great (37.4 %).

Antioxidant protection (AP) enzymes, which not only control free radical formation, but also perform important signal functions in cells (both in stress and normal conditions), play a special role in cellular activity regulation [34-36]. Oxidation-reduction processes are considered as one of the bases of physiological and biochemical mechanisms determining viability and preservation of potato tubers. Long-term storage or unfavorable conditions leads to lipid peroxidation (LPO) products accumulation, and antioxidant system function decreases [37].

In case of treatment with beneficial bacteria which reinforces this function, resistance to pathogens increases due to increase in plant immunity and formation of a barrier, preventing pathogen infiltration. It is known that microorganisms inhabiting potato surface induce generation of reactive oxygen species (ROS), so beneficial microflora can be used for ensure plant cell defense response to infection. In this context, it was considered important to study the effect of *Pseudomonas* sp. RF13H on ROS formation and antioxidant protection enzyme functioning [38].

Activity of pro- and antioxidant system in tubers of potato (*Solanum tuberosum* L.) Nevskii variety after treatment with *Pseudomonas* sp. RF13H biocontrol strain, depending of storage temperature

Storage period, months	Control (water treatment)				Test			
	LPO	SOD	CAT	PO	LPO	SOD	CAT	PO
Storage at 4 °C								
0	8.4	0.05	0.51	60.8	8.6	0.57	0.57	69.4
1	6.7	0.03	0.34	74.5	6.3	1.61	0.56	104.8
3	6.3	0.07	0.13	50.1	5.8	1.23	0.43	112.6
6	9.8	0.05	0.36	84.3	6.8	1.83	0.78	120.1
Storage at 18 °C								
0	8.7	0.05	0.54	60.2	8.5	0.74	0.56	106.4
1	9.4	0.04	0.38	79.3	6.9	1.83	0.66	120.5
3	10.1	0.06	0.25	67.2	6.2	1.98	0.71	110.3
6	12.3	0.08	0.41	88.9	7.3	2.11	0.84	124.7
LSD_{05}	0.80	0.01	0.50	5.40	2.10	0.30	0.10	7.10

N o t e. LPO — lipid peroxidation, μmol/g; SOD — superoxide dismutase, units; CAT — catalase, mmol/(min · g); PO — peroxydase, μmol/(min · g). LPO, CAT and PO activity is calculated per 1 g of wet weight

Long-term storage led to rapid inhibition of SOD and CAT and accumulation of LPO products (Table), including MDA. Peroxydase activity did not change during storage. High temperature contributed to activation of free radical reactions and LPO, leading to decrease in potato viability and quality. Enzyme activity decreased periodically during 3 months in all samples. At that, enzyme activity growth correlated with LPO increase. Both enzyme and LPO activity increased after 6 months of storage due to end of potato physiological rest stage. In case of biocontrol strain treatment significant activation of all studied enzymes was observed, which is indicative of increase in tuber protective potential during long-term storage.

Strategy of potato tuber surface colonization by *Pseudomonas* sp. RF13H. In order to study survival of introduced RF13H strain at refrigerated potato storage, we have developed oligonucleotide probe Z-SpecPs (5′→3′: CAAACCACTACGCGCGCTTAC), which was binding specifically with the respective unique target during FISH (see Fig. 1, A). In 24 hours after tuber inoculation localization of biocontrol bacteria microorganism visualized using Z-SpecPs probe and confocal scanning laser microscopy was characterized by unordered and uneven distribution on outer layer of tuber surface. Several rows of live (phellogen and phelloderm) and dead (phellem) cells were observed in periderm; the cells were extremely closely packed, had rectangular shape with occasional isodiametric (round) and elongated cells. Suberized covering layer protects tubers from moisture loss, plant pathogen infiltration and other adverse external impacts. The majority of cells of RH13H strain did not occupy certain niches on the surface during the first day, and only a small part was localized in top periderm damage areas.

Unevenness of tuber surface was revealed by confocal microscopy. In 2 months of refrigerated storage of treated tubers the microcolonies of biocontrol strain occurred, which were often located in various surface hollows and niches, skin cracks, scratches, cuts, injury and mechanical damage areas (Fig. 5 A, B). Apparently, the substances accumulated due to mechanical damages (indolylacetic, ascorbic and chlorogenic acids, gibberellin, ATP) [39] which stimulate wound reaction (wound periderm formation) attract the introduced bacteria.

Moreover, high density of inoculum was observed at the border between tuber periderm cells (see Fig. 5 A, a), where the bacteria were usually located along narrow elongated longitudinal cells, differentiated in the secondary surface tissue zone. Most likely, small rectangular slightly thickened and closely packed cells with thin membranes are metabolically active phellogen cells. They have thick viscous cytoplasm and numerous organelles related to synthesis processes, ensuring nuclear and cytoplasm division [40]. It can be assumed that biochemical activity in phellogen contributes to biocontrol strain colony formation. At the same time, it has been found that the strain can actively colonize lenticels, the microscopic surface slots (holes), through which air oxygen is supplied to a tuber and carbon dioxide and water vapor are removed (tuber breathing) (see Fig. 5, D).

Fungal mycelium occupying some local areas of infected periderm was observed during tuber surface microscopy. Introduced pseudomonade bacterial cell clusters were detected in close proximity to mycelium (see Fig. 4, A). Early phase of fungal development was observed in the top cell layer, with formation of intracellular (more rarely intercellular) mycelium. Egg- or lemon-shaped zoosporangia similar to *Phytophthora infestans* zoosporangia had thin smooth membrane and a prominent tubercle on the top, but no rusting color common to late blight was observed on tuber dissection.

It should be noted once again that tuber storage at 18 °C for 14 days might have played a significant role in effectiveness of introduced strain. It has

increased survival and stability of development of biocontrol bacteria, as well as

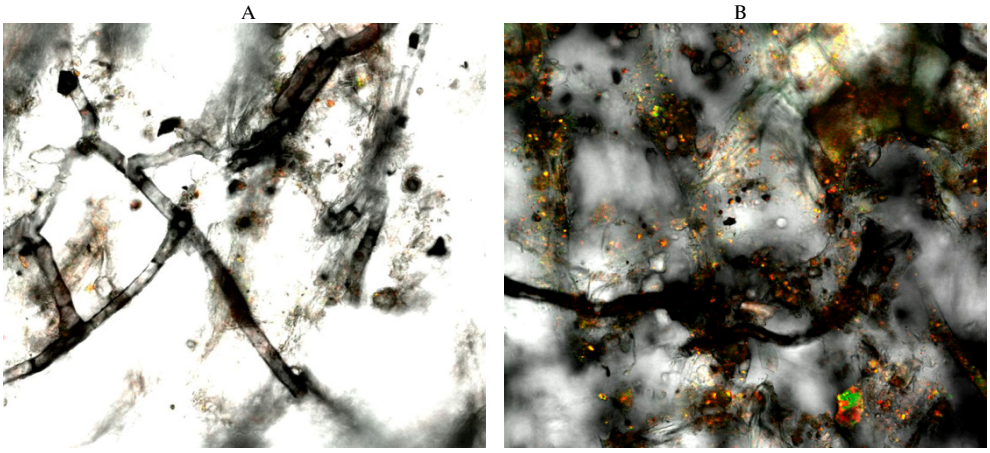

Fig. 4. Localization of introduced *Pseudomonas* sp. RF13H bacteria on tuber surface of potato Sudarynya variety 24 h after inoculation: A — fungal mycelium, fluorescence indicates the presence of RF13H strain cell clusters; B — scattered irregularly localized bacterial microcolonies on tuber surface, high bacterial count per area unit. FISH (6FAM fluorochrome, Cy3) with confocal scanning laser microscopy (Leica TCS SPE, Leica Mikrosysteme Vertrieb GmbH, Germany).

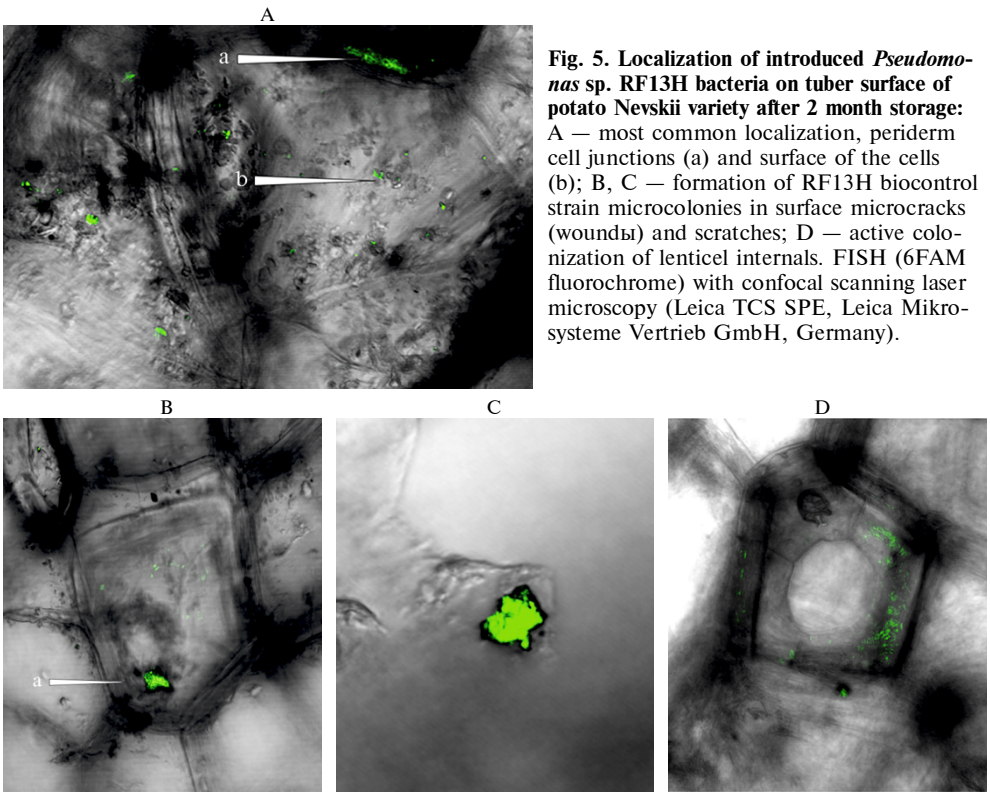

Fig. 5. Localization of introduced *Pseudomonas* sp. RF13H bacteria on tuber surface of potato Nevskii variety after 2 month storage: A — most common localization, periderm cell junctions (a) and surface of the cells (b); B, C — formation of RF13H biocontrol strain microcolonies in surface microcracks (woundы) and scratches; D — active colonization of lenticel internals. FISH (6FAM fluorochrome) with confocal scanning laser microscopy (Leica TCS SPE, Leica Mikrosysteme Vertrieb GmbH, Germany).

their adaptation to the respective ecological niche. It is important for successful competition with already existing native microflora, and synthesis and accumulation of antifungal metabolites. It is known that mechanisms of adaptation and synthesis of bioactive compounds, as well as chemotactic characteristics of population cells correlate to the time of incubation [41]. A host plant, in its turn, also affects the result of biocontrol: quantitative composition of introduced bacteria depends on physiological condition, variety and type of biological object, and secretion of nutrients by tuber cells stimulates chemotaxis of microorganisms.

Hollows, microcracks and niches ensure physical separation of bacteria and serve for accumulation and secretion of metabolites, liquids, etc., creating favorable conditions for physiological activity of biocontrol strain.

Visualization of introduced *Pseudomonas* sp. RF13H strain in vivo on tuber surfaces during storage is indicative of high adaptability of these bacteria at low temperatures, which is important in competition for nutrition sources and localization under conditions of epiphytic microbiome.

Thus, introduced biocontrol bacteria which inhibit pathogen development and reduce losses during storage have a significant potential to ensure environmentally sound products. The ability of *Pseudomonas* sp. RF13H strains to colonize plants and simultaneously protect them from pathogens, observed by us, makes this strain promising for agricultural technologies.

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ECOLOGICAL AND GENETIC EVALUATION OF MORPHOLOGICAL AND BIOCHEMICAL CHARACTERS OF QUALITY IN *Brassica rapa* L. ACCESSIONS FROM VIR COLLECTION

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Abstract

Peculiarity of chemical composition of *Brassica rapa* L. crops (high water content and low content of fats) determine their low-calorie character. They are notable for relatively high content of carbohydrates and proteins, including all essential amino acids. Biochemical composition varies greatly among *B. rapa* members. We are the first to carry out a multifactor evaluation of valuable morphological and biochemical traits of *B. rapa* accessions from the core collection of Vavilov Institute of Plant Genetic Resources (VIR, 96 samples) in eco-geographical study (South China and Leningrad region of Russia) under field trials and under a greenhouse conditions. By means of the developed SSR (simple sequence repeats) markers which are in linked disequilibrium with QTL of morphological and biochemical traits, the molecular genetic analysis was firstly carried out in leafy, rooted, and oilseed *B. rapa* genotypes from the VIR core collection. As a result, new genetic sources for quality parameters have been found among the samples investigated. In average, the samples did not differ noticeably on plant morphology (leaf length, leaf width, petiole size, hairiness, colour) in field tests when growing in South China and in Leningrad region, whereas in a greenhouse at higher plant density the leaf size decreased, e.g. in Chinese cabbage, pak-choi, wutacai, and turnip the leaves were 5-12 cm longer and wider in the field tests. Under field trials, we revealed the significant and high significant correlations between dry matter (DM) and ascorbic acid (AA) level ($r = 0.51$, $p < 0.05$), DM and chlorophyll a (Chla) content ($r = 0.59$, $p < 0.05$), DM and carotenoids (Cd) ($r = 0.55$, $p < 0.05$), DM and carotins (Cn) ($r = 0.67$, $p < 0.05$), DM and β-carotene (β-C) ($r = 0.59$, $p < 0.05$), DM and protein (P) level ($r = –0.49$, $p < 0.05$); Chla and Chlb ($r = 0.93$, $p < 0.001$), Chla and Cd ($r = 0.59$, $p < 0.05$), Chla and Cn ($r = 0.49$, $p < 0.05$), Chla and β-C ($r = 0.99$, $p < 0.001$); Chlb and β-C ($r = 0.92$, $p < 0.001$); Cd and Cn ($r = 0.49$, $p < 0.05$), Cd and β-C ($r = 0.63$, $p < 0.05$); Cn and β-C ($r = 0.49$, $p < 0.05$). In the greenhouse, the significant and high significant r values were as follows: $r = –0.59$ ($p < 0.05$) for DM and AA, $r = 0.58$ ($p < 0.05$) for DM and Chlb, $r = –0.53$ ($p < 0.05$) for DM and Cd, $r = 0.71$ ($p < 0.001$) for DM and Cn; $r = –0.59$ ($p < 0.05$) for AA and Chlb, $r = 0.83$ ($p < 0.001$) for AA and Cd, $r = 0.58$ ($p < 0.05$) for AA and P; $r = 0.74$ ($p < 0.001$) for Chla and Chlb, $r = 0.67$ ($p < 0.05$) for Chla and Cn, $r = 0.95$ ($p < 0.001$) Chla and β-C; $r = –0.48$ ($p < 0.05$) for Chlb and Cd, $r = 0.87$ ($p < 0.001$) for Chlb and Cn, $r = 0.64$ ($p < 0.05$) for Chlb and β-C; $r = 0.63$ ($p < 0.05$) for Cn and β-C. The semi-headed Chinese cabbage Syaobaikou and Dunganskaya, pak-choi Mayskaya, and especially Ching Pang Yu Tsain with a distinctly high level of chlorophylls and carotene, are indicated as new promising genetic sources for valuable biochemical parameters under both field and greenhouse conditions. Their indices in the field trials and greenhouse tests were 5.44-7.03 and 4.20-5.40 %, respectively, for DM (that is higher as compared to mean value for the crop), 12.61-24.66 and 24.12-33.23 % for P, 32.56-46.46 and 30.00-61.47 mg/100 g for AA, 33.35-110.64 and 53.82-95.99 mg/100 g for Chla, 12.20-53.80 and 17.89-44.78 mg/100 g for Chlb, and 1.80-6.75 and 3.00-6.04 mg/100 g for β-C. It is confirmed that the SSR

markers BRMS051, KS51082, BRMS043 and KS50200 may effectively screen collection accessions and breeding material for desired morphological and biochemical traits. Our original data allow to practically implement an association mapping strategy and identify genetic determinants of morphological and biochemical quality characteristics using unique *B. rapa* collection preserved in VIR.

Keywords: *Brassica rapa*, morphological and biochemical quality characteristics, molecular markers, screening of plant collection

Turnip (*Brassica rapa* L.) is the wide-spread species on the globe, which includes economically important premature ripening and productive oil, vegetable, fodder crops with valuable chemical content. Low level of fats and high content of water makes them low-calorie, and a significant amount of biologically active substances (vitamins, enzymes, etc.) positively contribute to human health, stimulates the immune system and prevents the development of cardiovascular diseases. In N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) search for sources of improved morphological signs and biochemical quality among *B. rapa* collection is being carried out. Genetic analysis of these valuable quantitative trait loci (QTL) is possible due to significant genetic variability of the crop. The biochemical composition of plants within the *B. rapa* species varies greatly [1-5]. In the past decade, the character of basic metabolite accumulation, including biologically active substances, in previously poorly studied East Asian *B. rapa* wild cabbage plants and varieties have been reported [6, 7]. The explanation for the widest variability of morphological features in *B. rapa* is suggested by K. Lin et al. [8], who found unique genes in three morphotypes of *B. rapa*, the root turnip and short-stage form, whose genomes were sequenced and compared with the genome of the headed napa cabbage.

Earlier [9-12], we mapped the loci, responsible for seven morphological and five biochemical quality traits in population lines of doubled haploid leaf, root and oil crops of *B. rapa* L. In total, 140 QTL were mapped (taking into account the control by one locus of the same characteristics under the different cultivation conditions or several characteristics simultaneously), determining the formation of these agriculturally important properties in the lines of *B. rapa* doubled haploids under field conditions and in a greenhouse. Molecular markers, genetically linked to the studied QTL, are identified, and a block genomic structure of the genetic component construction (chromosome loci and linkage groups), involved in the expression of morphological and biochemical quality attributes, is discussed. However, the achieved results not fully withhold the nature of inheritance and molecular genetic control of these traits, being of interest for studying the genetic diversity in *B. rapa*.

A specific of this paper is that a multifactorial complex assessment of morphological and biochemical quality traits that make *B. rapa* plants valuable was carried out for the first time. Analysis of the genomes of *B. rapa* various forms using molecular markers, which are in linkage disequilibrium with QTL of morphological and biochemical features, made it possible to identify promising genetic sources for quality selection in *B. rapa*. In this case, the detected molecular markers can serve as an effective tool in the mass screening of the collection samples and selection material.

The objective of the work was the morphological, biochemical and molecular genetic evaluation of the *Brassica rapa* L. collection of VIR in various ecological and geographical zones under field conditions and in the greenhouse.

Techniques. A total of 96 samples of different origin from the main VIR collection, covering botanical diversity of *B. rapa* species, were examined using taxon description by C.E. Specht and A. Diederichsen [13], with the exception of ssp. *sylvestris* (Lam.) Janchen separation as a subspecies different from ssp. *oleifera*. All samples were evaluated under the long-term field trials in the Pushkin Branch of VIR (St. Petersburg) and at the South China Experimental Station of Enza Zaden

company (Guangzhou, Guangdong, China) in 2010, and also in 2013-2016 in the winter greenhouse (Pushkin Branch of VIR). The planting scheme was 70×30 cm in the field tests and 25×20 cm in the greenhouse. Morphological description and biochemical evaluation were carried out according to the previously presented methods [3, 4, 14, 15] on 20 plants of each sample.

DNA was isolated from young green leaves of plants by D.B. Dorokhov and E. Kloke protocol [16]. PCR screening was carried out as previously described [17]. Reaction mixture (25 μl) contained 10× incubation buffer (2.5 μl), 0.5 μl of each dNTP (10 mM), 1 μl of primer (10 pmol/μl), 0.5 μl of Taq DNA-polymerase (5 unit/μl) (Sibenzyme, Russia) and 20 ng of gDNA. PCR amplification was performed in a DNA thermocycler (Bio-Rad, USA) with an individual program for each type of markers in accordance to genetic marker database of vegetable crops (http://vegmarks.nivot.affrc.go.jp/). The results were visualized by DNA electrophoresis in a 1.8 % agarose gel with ethidium bromide. A documentation system (Bio-Rad, USA) was used for registration of the patterns.

The data were processed using Statistica 6.0 software (StatSoft Inc., USA). The significance level $p \leq 0.05$ was used.

Results. Samples of *B. rapa* collection (Table 1) were evaluated under conditions of a greenhouse (seeding on February 1) and in field trials (seeding in Guangzhou on May 25, in Pushkin on June 25) for a number of morphological signs, which determine the productivity and consumer appeal of plants. These were leaf blade length, width, color, pubescence, the nature of the surface, as well as the petiole length and width in the best developed leaf of the middle canopy.

1. The sample list of the main collection of the *Brassica rapa* L. species from the world collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR)

Subspecies	Variety	VIR accession number	Name	Origin
ssp. *pekinensis* (Lour.) Hanelt (napa)	Dungan	139	Dungan	Kazakhstan
	Xiao	53	Local	Kazakhstan
		74	Xiao Bai Kou	China
		89	Dou-shaped early ripening	China
	Chirimen	100	Hikoshima spring	Japan
	Nagasaki	238	Nagoya Market	Japan
	Shantung	58	Bi-Ze	Kirghizia
		210	Kiriba Santo	Japan
		108	Local	China
	Kasin	132	Kasin	Japan
		247	Hasinbechu	Korea
	Chosen	122	Len Sin Dzon	China
		207	Chosen	Japan
	Aichi	63	Local	China
		131	Aichi	Japan
	Nozaki	111	Nozaki early	Japan
		327	Nozaki Harumaki	Japan
	Kaga	103	Kaga	Japan
		88	Ju Sin Bao Tou Bai Zai	China
	Hotoren	127	Hotoren	Japan
	Chi Fu	48	Wong-Bok	The Netherlands
		110	Matsushima	Japan
	Kensin	222	Kensin	Japan
	Granat	164	Michihli	Canada
		71	He Tou Ven	China
	Da Zin Kou	56	Da Zin Kou	China
		128	Zushita	Japan
		198	Local	China
ssp. *chinensis* (L.) Hanelt (Chinese)	Piorbuy	75	Piorbuy	China
	Suesman	77	Suesman	China
		Vr.930	Mayskaya 8	China
	Taisai	46	Tai Na	Russia
		106	Yanzai	China
		214	Nicanme Jukijiro Taisai	Japan
	Yu Tsai (var. utilis)	195	Local	China
		203	Ching Pang Ju Tsai	China

Table 2 (continued)

var. *rosularis* (Tsen & Lee)	Ta Gu Zai	84	Hee Yu Ta Zai	China
Hanelt (rosette)		129	Ta Gu Zai	China
var. *narinosa* (Bailey) Hanelt	Chrysanthemum	154	Chrysanthemum heart	China
(broad-beaked mustard)		213	Bitamin na	Japan
var. *purpuraria* (Bailey) Bailey				
(purple)		391	Xing Yang	China
ssp. *nipposinica* (Bailey) Hanelt	Mibuna	115	Mibuna	Japan
(Japan)	Mizuna	159	Mizuna	Japan
		241	Shiroguki Kyona	Japan
ssp. *rapa* L. f. *Komatsuna*	Komatsuna	215	Uzuki Komatsuna	Japan
(leaf turnip Komatsuna)		242	Goseki Late	Japan
	Kurona	264	Kurona	Japan
Japan leaf vegetables	Mana	372	Bansei Mana	Japan
	Sirona	98	Osaka Market	Japan
		217	Okute Osaka Shirona	Japan
	Hiroshimana	335	Hiroshimana	Japan
Stable hybrids between sub-		96	Shantai	China
species		302	Gurin Debu	Japan
		331	White Long Petiole	Japan
		436	Benrina	Japan
ssp. *rapa* L.	Chinese	163	Local	China
(root turnip)	Ostersundomskiy	307	Ostersundomskiy	Russia
	Bortfeldskiy	385	Bortfeldskiy	Ukraine
	Karelian	738	Karelian	Russia
	Grobovskaya	821	Grobovskaya	Russia
	Milanskaya white	826	Milanskaya white	Russia
	Petrovskaya	830	Petrovskaya	Russia
	Teltower	894	Teltower	Germany
	Norfolk			
	violet-headed	984	Norfolk	France
	Volynskiy	1050	Volynskiy	Ukraine
	Golden globe	1283	Golden globe	The Netherlands
ssp. *oleifera* (DC.) Metzger		68	Local	China
f. *annua* (spring cress)		1	Kun Min ai u-zai	China
		2	Hue Zin u-zai	China
		11	Gute	Finland
		13	Local	Argentina
		25	Zsjan Su U uan-uzai 5082	China
		63	Pahsi	India
		106	Lotni mustard	India
		108	Arlo	Sweden
		114	Local (tetraploid)	Pakistan
		163	LGL	Pakistan
		192	Mustard	Nepal
		248	Local	Spain
		251	Vat-cawte	Tanzania
		301	BHLS	Nepal
		339	Jui-cai-tai	China
		374	Local 88/47	Bhutan
ssp. *oleifera* (DC.) Metzger		166	Root mustard	Tunisia
f. *biennis* (winter cress)		337	U-zai-zsi	China
ssp. *dichotoma* (Roxb.) Hanelt		53	Local toria	India
(brown sarson)		100	Local	Nepal
		135	Ds 17	India
		161	Toria selection	Pakistan
		205	Sarson	Pakistan
ssp. *trilocularis* (Roxb.) Hanelt		131	Type 1	India
(yellow sarson)		188	Palton sarson 66	India
		299	Sangam	India
		338	Chen-du-ai-u-zai	China
ssp. *sylvestris* (Lam.) Janchen		176		Italy
(wild sylvan cress)		218	Nabo silvestre	Peru

The collection average length and width of the leaf blade under field conditions in China and cultivation in the Pushkin Branch of the VIR were not significantly different, whereas the dimensions of the leaf blade were much smaller in the greenhouse (Table 2). At the same time, in China, the size of the leaf blade was significantly larger in Chinese cabbage, broad-beaked mustard and purple cabbage (all cultures of South China origin), as well as in root turnips, being slightly more in South China rosette cabbage and cress. The dimensions of

the leaf blade of the napa cabbage and turnip, as well as the length of the blade were greater during the cultivation in Leningrad region. Obviously, this is due to the very high summer temperatures in South China (35 °C and above), which limit the rapid growth of vegetative organs in some cultures. In the napa cabbage, the size of the leaf blade authentically exceeded the population average. In field tests in China, Pushkin and in the greenhouse, the length was 38.22±1.61, 41.82±1.03 and 31.87±0.71 cm, respectively, and width was 19.33±0.80, 25.02±0.78 and 16.02±0.45 cm. The leaf size of the turnip under field conditions also exceeded the average for the collection, while in the greenhouse it did not differ from the average for the species. The leaf blade of the cultivated cress was significantly less (by 30-40 %, regardless of the cultivation conditions) than the population average, and the wild cress and rosette cauliflower were the smallest. The leaf blades of napa, Chinese, broad-beaked mustard cabbages, turnip, cultivated cress under field conditions were 5-12 cm longer and wider than in the greenhouse, the leaf blades in rosette and Japanese cabbage, as well as in wild cress under the same conditions practically did not differ in size.

2. Average size of leaf in *Brassica rapa* L. grown under contrast conditions

Crop	Length, cm			Width, cm		
	field trial		P, greenhouse	field trial		P, greenhouse
	SCh	P		SCh	P	
Leaf blade						
Napa cabbage	38.22±1.61	41.82±1.03	31.87±0.71	19.33±0.80	25.02±0.78	16.02±0.45
Chinese cabbage	26.10±2.58	21.36±1.08	16.13±1.16	19.20±1.30	16.33±1.06	11.18±0.58
Rosette cabbage	14.50±0.50	12.30±2.00	13.93±1.11	11.50±0.50	10.45±1.05	10.05±1.03
Broad-beaked mustard and purple cabbage	24.70±1.86	21.50±1.98	16.73±0.42	22.30±2.61	18.60±0.38	11.17±0.42
Japanese cabbage	18.00±3.06	24.63±2.20	24.10±7.03	9.67±2.40	9.50±3.10	8.37±1.19
Turnip	24.78±3.14	19.12±1.39	19.10±0.83	19.00±1.78	12.26±0.91	15.95±0.95
Leaf turnip	27.58±2.09	33.84±2.63	21.79±1.01	18.75±1.22	23.51±1.32	12.45±0.67
Cress	19.04±1.13	16.67±0.52	12.75±0.48	13.72±0.64	12.96±0.47	10.38±0.40
Wild cress	13.50±0.50	11.50±1.00	14.03±2.01	11.50±0.50	9.50±0.50	11.85±1.86
The collection average	25.75±1.09	26.34±1.20	20.63±0.85	16.92±0.53	17.61±0.67	12.85±0.35
LSD_{05}	3.31	3.62	2.55	1.60	2.04	1.05
Petiole						
Chinese cabbage	16.00±1.79	15.41±1.74	12.35±1.35	3.33±1.39	3.39±0.23	2.06±0.09
Rosette cabbage	9.00±2.01	10.50±0.10	12.28±1.65	2.20±0.25	1.95±0.35	1.51±0.09
Broad-beaked mustard and purple cabbage	21.00±1.53	13.87±1.25	15.73±1.73	3.67±0.34	3.40±0.15	1.64±0.08
Japanese cabbage	16.30±2.17	14.43±0.66	15.76±3.06	0.97±0.03	1.10±0.15	1.03±0.09
Turnip	21.25±1.36	23.39±1.04	30.90±2.54	2.00±0.29	1.63±0.15	1.44±0.09
Leaf turnip	14.80±1.84	12.68±1.66	11.11±0.95	2.90±0.28	3.81±0.32	1.97±0.14
Cress	16.20±0.85	15.63±0.58	14.71±0.80	1.10±0.07	1.16±0.10	0.71±0.05
Wild cress	14.00±3.01	10.50±0.50	15.15±3.16	0.90±0.10	0.75±0.25	0.64±0.13
The collection average	16.53±0.64	16.07±0.65	16.33±1.37	1.98±0.14	2.04±0.13	1.29±0.07
LSD_{05}	1.93	1.95	4.11	0.43	0.41	0.22

Note. SCh — Southern China, Guangzhou (Guangdong Province), P — Pushkin Branch of N.I. Vavilov All-Russian Institute of Plant Genetic Resources — VIR, St. Petersburg). Field trials were carried out in China in 2010, in Pushkin in 1997-2016, greenhouse tests were conducted in 2013-2016. For Latin descriptions of cultures, see Table 1. The results were obtained by analyzing 96 samples from the main VIR collection. Average (X) and average errors (±Sx) are given.

The population average length of the petiole under all cultivation conditions was stable, the width of the petiole in Southern China and Leningrad region differed slightly, but in the greenhouse was significantly smaller (see Table 2). In broad-beaked mustard and purple cabbage, Japanese cabbage, leaf turnip and wild cress the length of the petiole in Southern China was much larger than in Leningrad region, in wild cress — slightly more, and in turnip and rosette cabbage — less than in Leningrad region. In Chinese cabbage, leaf turnip and cress the petiole was shorter in the greenhouse, than in the field; in the remaining cultures it was longer (in turnip — 2 times as long as the population average). All samples of napa cabbage had the whole sessile leaves without petioles. A long, relatively wide petiole was typical of root turnips, broad-beaked mustard

and Japanese cabbage (15-30 % longer than the collection average), a short petiole was characteristic of cauliflower. In the samples, the length of the petiole under field conditions and in the greenhouse basically differed insignificantly (by 1-4 cm), except for Japanese cabbage and root turnip, in which the petiole in the greenhouse was 10-15 cm longer than in field. The widest petiole was found in specimens of Chinese and broad-beaked mustard cabbage and turnip, the thinnest ones were typical of Japanese cabbage and cress. In field tests, the petiole was significantly wider in all crops, except for Japanese cabbage and wild cress.

The leaf surface in the specimen differed depending on the botanical affiliation from smooth to folded or slightly, medium and strongly wrinkled, the nature of the pubescence varied (from absence to a strong manifestation), the color of the blade varied from light to dark green.

3. The samples of *Brassica rapa* L. distinguished by the size of the food organs when grown under contrast conditions

Sample, VIR accession number	Leaf length, cm			Leaf width, cm			Petiole length, cm		
	field trial		P, greenhouse	field trial		P, greenhouse	field trial		P, greenhouse
	SCh	P		SCh	P		SCh	P	
Napa cabbage (no petiole)									
Kiriba Santo, k-210	55.0±2.6	45.9±3.3	36.3±4.2	22.0±1.7	22.6±1.5	14.8±1.2			
Chosen, k-207	50.6±2.8	42.1±2.1	37.9±2.7	25.6±2.4	20.2±1.9	18.1±2.6			
Crop average	38.2±1.6	41.8±1.0	31.8±0.7	19.3±0.8	25.0±0.8	16.0±0.4			
Chinese cabbage									
Tai-na, k-46	24.2±2.3	22.8±2.7	16±3.1	19.0±2.6	18.2±2.2	13.8±2.8	13±1.8	22.8±3.0	16.7±2.5
Nicanme Jukijiro Taisai, k-214	26.5±2.8	23.3±2.5	12.9±2.8	21.5±1.4	18.1±1.8	10.3±1.6	25.4±2.4	19.5±2.7	17.1±2.0
Crop average	26.1±2.5	21.3±1.1	16.1±1.2	19.2±1.3	16.3±1.1	11.2±0.6	16.0±1.8	15.4±1.7	12.3±1.3
Leaf turnip									
Goseki Late, k-242	40.3±3.7	40.5±2.9	25.9±3.2	21.5±2.8	23.1±2.0	9.5±1.9	16.6±2.3	11.5±2.2	9.6±1.6
Bansei Mana, k-372	50.4±3.2	45.2±3.6	22.6±2.6	36.8±3.4	26.9±2.6	15.4±1.4	9.1±2.1	9.8±2.1	8.4±1.2
Crop average	27.6±2.1	33.8±2.6	21.8±1.0	18.7±1.2	23.5±1.3	12.4±0.7	14.8±1.8	12.7±1.7	11.1±0.9

Note. SCh — Southern China, Guangzhou (Guangdong Province), P — Pushkin Branch of N.I. Vavilov All-Russian Institute of Plant Genetic Resources — VIR, St. Petersburg). Field trials were carried out in China in 2010, in Pushkin in 1997-2016, greenhouse tests were conducted in 2013-2016. For Latin descriptions of cultures, see Table 1. The results were obtained by analyzing 96 samples from the main VIR collection. Average (X) and average errors (±Sx) are given.

As genetic sources, we selected samples in which the size of the food organs was significantly higher than the average population index (Table 3). In a number of samples, the length and width of the leaf and petioles under contrasting conditions stably exceeded the mean values for the crop, which indicated their high adaptive ability. A slight variability in these features was noted in samples of napa cabbage Kiriba Santo, Chosen, Chinese cabbage Tai-na, Nicanme Jukijiro Taisai, local Chinese turnips (k-163), which formed a commercial root yield and a high-quality salad leaf rosette. The present study confirmed, that leaf and semi-headed varieties of napa cabbage are more suitable for growing in a greenhouse, unlike the typical headed varieties, as reported earlier [6]. Adaptive properties of the collection samples of other species during comparative tests in the greenhouse and under field conditions were evaluated for the first time.

The absence of pubescence and bright color increase the consumer qualities of *B. rapa* leaf crops. In the best specimens, pubescence was absent or very poor, and the color of the leaf varied from bright light green to bright dark green.

Biochemical assay has shown (Table 4) that the dry matter content in the studied samples averaged 7.77±0.25 % under field conditions and significantly exceeded the same value in the greenhouse (5.36±0.15 %), moreover, in all crops, except for napa cabbage, the excess (by 30-140 %) was significant (see Table 4). The dry matter content authentically differ in various species and varied from 5.65 % in napa cabbage to 11.24 % in leaf turnips in field studies, and from 4.44 % in Chinese cabbage to 6.62 % in turnips in the greenhouse. Thus,

the variability in the dry matter content under field conditions was significantly higher than in the greenhouse.

4. The content ($X \pm Sx$) of nutrients and biologically active substances in *Brassica rapa* L. crops under different growth conditions

Crop	Dry matter, %	Proteins, %	Ascorbic acid, mg/100 g	Chlorophyll, mg/100 g		Carotinoids, mg/100 g	β-Carotene, mg/100 g
				a	b		
Field conditions (2006-2016)							
Cabbage:							
napa	5.65±1.47	23.73±3.86	50.45±14.82	26.21±12.04	11.72±5.94	10.30±4.28	1.82±0.85
Chinese	9.99±1.91	24.85±2.73	65.21±13.36	64.51±11.96	29.03±9.87	17.60±3.62	4.12±0.67
rosette	9.41±1.98	27.80±3.24	58.03±13.69	98.87±9.06	55.46±6.79	18.74±2.05	6.34±0.63
broad-beaked mustard and purple	8.36±3.29	29.09±2.00	46.24±5.93	74.71±4.91	37.69±6.83	16.18±2.58	4.56±0.19
Japanese	9.17±0.10	23.49±3.01	46.24±1.36	86.47±6.86	40.73±5.54	21.43±3.50	5.39±0.26
Turnip:							
root, leaves	8.81±1.62	24.66±3.82	71.91±10.29	78.26±25.09	43.16±20.32	15.56±4.75	4.89±1.53
leaf	11.24±3.67	21.35±1.28	77.52±10.26	98.61±4.12	47.15±3.83	20.71±10.1	6.24±0.83
Cress	7.45±1.34	27.95±3.13	69.74±21.15	81.83±11.69	47.29±14.08	12.99±4.30	5.05±0.89
Wild cress	10.64±0.73	17.99±1.04	68.68±4.80	75.25±0.64	41.09±1.09	10.77±1.02	4.55±0.03
The collection average	7.77±0.25	24.87±0.39	58.70±1.65	59.06±3.27	30.63±2.09	14.06±0.55	3.77±0.19
LSD_{05}	0.77	1.17	4.95	9.83	6.29	1.64	0.56
Greenhouse (2014)							
Cabbage:							
napa	4.84±0.08	31.06±0.48	35.00±1.44	48.15±2.54	18.23±1.32	23.07±0.76	2.97±0.16
Chinese	4.44±0.13	24.12±0.98	30.00±4.21	73.30±4.49	36.03±2.88	27.85±3.78	4.46±0.30
rosette	4.72±0.28	23.65±0.90	19.00±3.01	63.22±0.78	33.34±0.33	20.46±0.56	4.09±0.01
broad-beaked mustard and purple	5.40±0.18	20.46±0.58	19.00±1.15	64.11±5.09	36.77±3.63	16.93±3.38	3.78±0.31
Japanese	5.44±0.12	27.23±1.51	38.00±5.20	87.60±6.67	37.41±3.47	36.39±2.42	5.45±0.38
Turnip:							
root, leaves	6.62±0.17	25.32±0.67	15.00±0.63	77.26±4.03	50.29±3.46	14.65±1.08	4.70±0.26
leaf	4.46±0.23	27.36±0.98	37.00±4.54	57.62±5.23	27.85±6.78	25.19±2.81	3.56±0.30
Cress	6.08±0.14	27.69±0.55	20.00±0.53	65.70±1.45	46.11±2.37	11.79±0.56	4.02±0.07
Wild cress	5.80±0.28	25.32±2.98	21.00±0.71	78.24±9.88	56.08±12.01	15.28±4.28	4.73±0.49
The collection average	5.36±0.10	27.50±0.40	27.03±1.16	63.24±1.71	35.15±1.72	19.77±0.94	3.88±0.10
LSD_{05}	0.29	1.2	3.47	5.12	5.16	2.81	0.31

Note. The protein content is indicated per dry weight, biologically active substances — per wet weight. The tests were carried out in Pushkin Branch of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg). For Latin descriptions of cultures, see Table 1. The results were obtained by analyzing 96 samples from the main VIR collection. Average (X) and average errors ($\pm Sx$) are given

We did not find the same patterns for protein level. In field studies, its content was significantly lower than the collection average in wild cress and much higher in broad-beaked mustard and purple cabbage (see Table 4), while in the greenhouse the broad-beaked mustard cabbage, on the contrary, had the lowest index with the highest in the napa cabbage. At the collection average, the protein content increased in the greenhouse, but the range of variability (11 %) persisted regardless of the growth conditions. In Chinese cabbage, turnip and cress, the protein content in comparative tests was practically stable, in napa and Japanese cabbages, leaf turnip and cress the parameter was higher in the greenhouse, in rosette, broad-beaked mustard and purple cabbage — under field conditions. As for the last group of plants, this may be due to their relatively slow growth or to a characteristic manifestation of the analyzed sign, which is common to these botanically closely related crops.

B. rapa plants are important sources of ascorbic acid, carotenoids, chlorophylls. In our studies, the content of ascorbic acid in *B. rapa* samples varied quite widely. The minimum values corresponded to the average for the white cabbage, and the maximum values exceeded them several times. At the collection average, the indexes were 2 times higher under field conditions (58.7±1.65 vs. 27.03±1.16 mg/100 g), and this was noted for all crops, except for Japanese

cabbage which had not so significant difference. In fact, the ascorbic acid content in rosette and broad-beaked mustard cabbage, root turnip and cress during the cultivation in the field was 3 times higher and more than in the greenhouse. The largest accumulation of ascorbic acid in the field was observed in Chinese cabbage, turnip and cress, in the greenhouse — in napa and Japanese cabbages and leaf turnip.

At the collection average, carotenoid accumulation was higher in the greenhouse (19.77±0.94 vs. 14.06±0.55 mg/100 g, LSD_{05} 2.81 and 1.64, respectively). This is especially important in the winter and spring periods, when food is poor in vitamins. The richest sources of carotenoids were greenhouse plants of Japanese cabbage, followed by Chinese cabbage, wild cress and leaf turnip. Under field conditions, the samples of Japanese, Chinese, rosette cabbage and leaf turnip were distinguished by this feature. In napa cabbage and wild cress, the excess of carotenoids in the greenhouse was more than 2-fold relative to that recorded in the field. In rosette, broad-beaked mustard and purple cabbage, cresses and turnips, the amount of carotenoids was relatively stable. Approximately 20 % of the carotenoid fraction are carotenes. Actually in carotene 80-90 % fraction is represented by the β-form. On the average, the content of β-carotene differed little (3.88±0.1 and 3.77±0.19 mg/100 g in the greenhouse and in the field, respectively). Carotene accumulation was stable in Chinese and Japanese cabbage, turnip, wild cress, whereas in napa cabbage it was higher in the greenhouse (2.97±0.16 vs. 1.82±0.85 mg/100 g, LSD_{05} 0.56 and 0.31, respectively), in rosette, broad-beaked mustard and purple cabbage, leaf turnip it was higher in the field, and the maximum accumulation was characteristic of Japanese, Chinese and rosette cabbage.

Chlorophyll plays a significant role in dietary nutrition: eating green leaves increases blood hemoglobin and erythrocytes. At the collection average, the content of chlorophylls *a* and *b* was insignificantly higher in the greenhouse (the sum of 63.24±1.71 and 35.15±1.72 vs. 59.06±3.27 and 30.63±2.09 mg/100 g). The practically stable content of chlorophylls (especially of chlorophyll *a*) was noted in Japanese cabbage, turnip and wild cress, its increment in the greenhouse was found both in napa and Chinese cabbages, and in the filed — in rosette and broad-beaked mustard cabbage, turnip and cress. With a deficiency of fresh salad vegetables in the winter to spring time, mainly fast growing crops (Peking and Chinese cabbage) are cultivated in greenhouses, which are high valuable due to the ability to accumulate green pigments under these conditions. As greenhouse crops, it also reasonable to recommend rosette, broad-beaked mustard, Japanese cabbage and leaf turnip, which are significantly superior to the napa cabbage in the chlorophylls content (their maximum level was noted in rosette and Japanese cabbage and leaf turnip).

The correlation analysis of biochemical characteristics showed that the amount of dry matter is significantly and unidirectionally associated with the chlorophylls, carotenes and β-carotene levels, regardless of cultivation conditions (Table 5). The interdependence between the content of dry matter and ascorbic acid, as well as carotenoids, was significant, but multidirectional (direct in field and reverse in the greenhouse). Significant invers correlations were found between the dry matter and protein content in the field. In the greenhouse, there was a significant inverse correlation between the content of ascorbic acid and chlorophyll *b*, and direct correlation for carotenoids, protein. A relationship between the amount of chlorophylls *a* and *b*, as well as their relation to the accumulation of carotenoids and carotenes (especially β-carotene) was highly reliable. An essential correlation was noted between the content of carotenes and β-carotene. Thus, samples that combine a high content of chlorophylls and carotenes can be obtained quite easily, while selection for a simultaneously high content of pro-

tein and ascorbic acid will cause difficulties.

5. Correlation coefficients between biochemical characteristics in *Brassica rapa* L. under different growth conditions

Parameter / Parameter	DM	AA	Chl a	Chl b	Cd	Ca	β-Ca	P
Field conditions (2006-2016)								
Dry matter (DM)		0.51*	0.59*	0.36	0.55*	0.67*	0.59*	–0.49*
Ascorbic acid (AA)			0.29	0.26	–0.02	0.33	0.30	–0.39
Chlorophyll a (Chl a)				0.93**	0.59*	0.49*	0.99**	0.16
Chlorophyll b (Chl b)					0.32	0.37	0.92**	0.28
Carotinoids (Cd)						0.49*	0.63*	0.09
Carotene (Ca)							0.49*	0.01
β-Carotene (β-Ca)								0.16
Proteins (P)								
Greenhouse (2014)								
Dry matter (DM)		–0.59*	0.31	0.58*	–0.53*	0.71**	0.36	–0.02
Ascorbic acid (AA)			–0.11	–0.59*	0.83**	–0.42	–0.09	0.58*
Chlorophyll a (Chl a)				0.74**	0.21	0.67*	0.95**	–0.31
Chlorophyll b (Chl b)					–0.48*	0.87**	0.64*	–0.37
Carotinoids (Cd)						–0.36	0.29	0.22
Carotene (Ca)							0.63*	–0.18
β-Carotene (β-Ca)								–0.25
Proteins (P)								

Примечание. The tests were carried out in Pushkin Branch of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg).

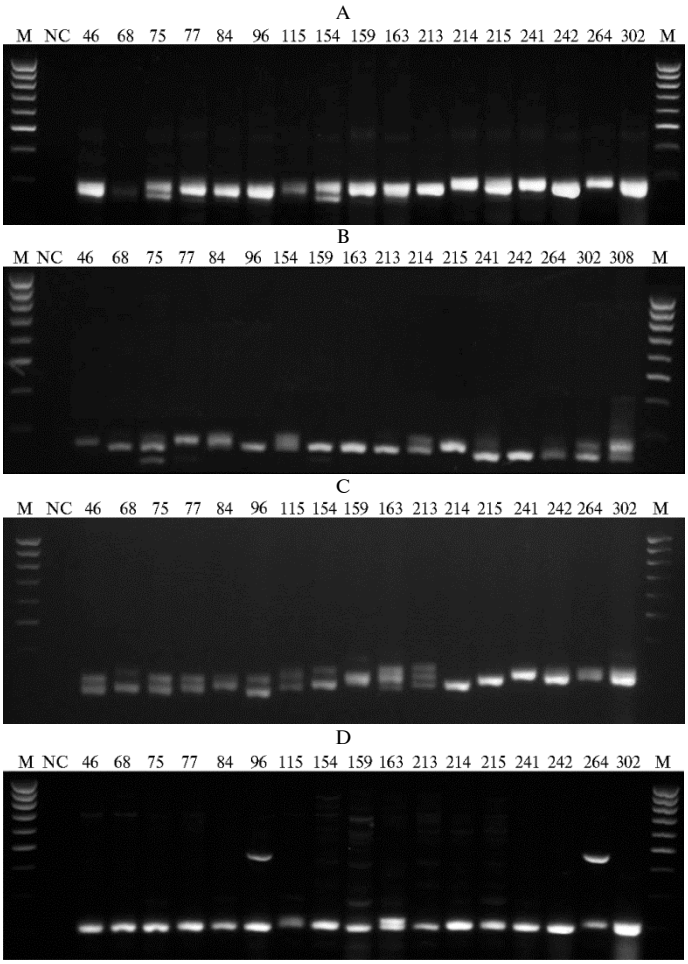
*, ** Significant ($p < 0.05$) and highly significant ($p < 0.001$) correlation coefficients.

Based on the data obtained, we revealed new genetic sources of the studied biochemical features with 5.44-7.03 and 4.20-5.40 % for dry matter (which exceeds crop averages), 12.61-24.66 and 24.12-33.23 % for protein, 32.56-46.46 and 30.00-61.47 mg/100 g for ascorbic acid, 33.35-110.64 and 53.82-95.99 mg/100 g for chlorophyll *a*, 12.20-53.80 and 17.89-44.78 mg/100 g for chlorophyll *b*, and 1.80-6.75 and 3.00-6.04 mg/100 g β-carotene in field conditions and a greenhouse, respectively. These are samples of semi-headed napa cabbage Xiao Bai Kou and Dungan, Chinese cabbage Mayskaya and Ching Pang Yu Tsai, which was particularly high in chlorophylls and carotene.

For molecular studies we used 8 SSR markers, suitable for subsequent screening of samples of the world collection. The markers were located on A03 (3 markers), A05 (1 marker), A06 (2 markers) and A09 (2 markers) chromosomes, encompassing linkage groups in which, according to our QTL analysis [10-12, 18] the loci are located, which control the morphological and biochemical quality features of the species. The unevenness of the markers assignment on chromosomes indirectly indicates the presence of the genome blocks of co-adapted gene suggested by us, and the existence of co-adapted blocks of genes in the genome as a whole. The presence of such blocks in the genome of *B. rapa* is confirmed by our earlier studies, which established the genetic determinants of *B. rapa* resistance to different races of *Xanthomonas campestris* pv. *campestris* causing black rot [18-20]. The effectiveness of the molecular markers selected by us should be noted, as some markers (for example, BRMS043 and BRMS034) were proved to be effective molecular genetic descriptors of the species for resistance to black rot. We used these markers to identify the collection specimens of *B. rapa* resistant to each race of *X. campestris* separately and to the pathogen as a whole [18, 21, 22].

Using 8 molecular markers to study 18 *B. rapa* samples, we detected 26 polymorphic SSR fragments from 122 to 410 bp in size. Marker BRMS051 (Fig., A) with the expected size of the 262 bp amplicon in 77 % of the samples was linked to the length and width of the leaf blade, at that in 58 % of these samples its width was 20 cm and more. In 60 % of cases, alleles which produced fragments of 262 bp and 280 bp, were associated. Fragment (allele) 280 bp was manifested in all lines with an average length of leaf blade (up to 20 cm), in all

rosette cabbage samples, in all samples and varieties of Chinese cabbage and in 60 % of stable hybrids, the parent form of which was Chinese cabbage. Note that these variants of Chinese cabbage had a green (but not light or dark green) color of the leaf. Fragment (allele) 262 bp was found in 75 % of samples of turnip.



The results of PCR analysis of *Brassica rapa* L. samples using SSR markers BRMS051 (A)**, KS51082** (B)**, BRMS043** (C) **and KS50200** (D)**:** 46, 68, 75, 77, 84, 96, 115, 154, 159, 163, 213, 214, 215, 241, 242, 264, 302 and 308 are samples (VIR catalog numbers), NC — negative control, M — M16-DNA marker 100 bp (10 fragments from 100 to 1000 bp, Sibenzym, Russia). The collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR, St. Petersburg).

The KS51082 (see Fig. B) was linked to the QTL of the length of the petiole. Samples k-115, k-163 and k-214 with the expected fragment 282 bp had long and very long petiole (22-40 cm). A 282 bp amplicon was revealed in all samples of Japanese cabbage and in 50 % of samples of turnip.

Using the marker BRMS043 (see Fig. B), we found the samples with the expected size of 318 bp amplicon. In total of 70 % of these samples the leaf surface was smooth and slightly wrinkled. All samples with a pair of fragments (alleles) 285 and 305 bp had smooth or slightly wrinkled leaves. Samples k-115, k-154, k-163 and k-213, combining fragments 285 and 318 bp, were outstanding on the β-carotene content of 5.1-6.2 mg/100 g. In the presence of 318 bp amplicon, 71 % of the variants had a fairly long or long leaf blade (26-44 cm) of medium width (16-23 cm), and in 75 % of samples with short leaf blades (11-23 cm) a fragment (allele) 305 bp was manifested. Allele 285 bp was found in all samples of Chinese cabbage and a hybrid of Chinese and napa cabbages, and a fragment of 318 bp was revealed in 60 % of Japanese cabbage samples. The same allele (318 bp) was found in half of the samples of leaf turnip, and in 75 % of leaf turnip we found allele corresponding to the 305 bp fragment.

The use of the molecular marker Ol12-F02 linked to the QTL for pubescence and color of the leaf blade [7] revealed four fragments, 200, 185, 175, and 140 bp. Molecular analysis of the same samples with the BRMS014 marker showed the presence of two amplicons (263 and 280 bp) and its linkage to QTL of leaf color (from light green to green) in the most part of the

samples (80 %) of those in which these amplicons appeared. The same pair of amplicons was found in 70 % of Chinese cabbage samples and its hybrids.

Screening with the KS50200 marker (see Fig. D) revealed fragments 292, 280 and 260 bp. Samples that showed 260 bp amplicon in 75 % of cases had a dark green leaf blade 15-20 cm wide with a smooth, sometimes slightly wavy edge. A 280 bp amplicon was found in hybrids of Chinese and napa cabbages, and also in 75 % of leaf turnips (in the latter, the width of the blade is, at average, 20 cm, the color is dark green, the edge is from smooth to slightly wavy). In Chinese cabbage (75 % of samples with a light green and green color and a smooth edge of the leaf blade with a width of 15-21 cm), a fragment corresponding to the 292 bp allele appeared. A fragment of this size was found in 66 % of samples of Japanese turnips with a sharp-toothed cut of the leaf edge.

Screening with the BRMS034 marker linked to the color of leaf blade, as we previously reported when mapping populations of the doubled haploids lines [12], showed the presence of amplicons 122 and 144 bp (in the forms with an 144 bp amplicon the leaves were dark green), however, the studied samples did not show a reliable relationship between the detected amplicons and the sign.

In the screening with BRMS042, the amplicon of expected size (380 bp) was found for all samples. In two of the three samples of broad-beaked mustard cabbage, a 410 bp amplicon was revealed, which also appeared in one sample of Chinese cabbage (k-46). Japanese cabbage and leaf turnip produced 620-170 bp amplicons, which were not typical for the remaining samples of the studied collection.

Thus, we carried out a complex (morphological, biochemical, molecular genetic) assessment of *Brassica rapa* quality traits in environmental tests with different methods of cultivation. Note that the analysis of the *B. rapa* genomes by molecular genetic markers that are in disequilibrium linkage with corresponding QTL of the leaf color, the content of total protein, β-carotene, carotenoids, ascorbic acid, dry matter, has been carried out by anyone never before. These studies confirmed the possibility of using the SSR markers found by us to screen collection and breeding material for morphological and biochemical features. In addition, the obtained original results allow us to proceed to the practical implementation of associative mapping and identification of specific genetic determinants defining the manifestation of a number of agriculturally important and economically significant qualitative traits, using the unique material of *B. rapa* from the collection of N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR).

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NEW GENERATION HYBRIDS OF WHITE CABBAGE (*Brassica oleracea* L. convar. *capitata* var. *alba* DC) BASED ON DOUBLED HAPLOIDS

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Abstract

Presently, cabbage breeding is mainly focused on F_1 hybrids necessitating constant parental lines to be obtained. Doubled haploid (DH) technology based on isolated microspore in vitro culture is widely used to produce pure lines of brassica crops. This method allows us to rapidly develop homozygous lines, in contrast to time-consuming traditional breeding for heterosis in cross-pollinating crops which takes 7 to 10 years for annuals and 14 to 20 years for biennial plants. One of the objectives of DH technology is to provide the all possible number of doubled haploid plants that allows more fully encompass the spectrum of genetic recombination, including the recessive locus. The aim of our study was to evaluate economically important traits in white cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba* DC) constant doubled haploid lines of late ripening and to improve the technology for producing DH based F_1 hybrids. Eleven breeding lines of late ripening cabbage were used to obtain doubled haploid lines from microspore in vitro culture. Of the obtained lines, twelve doubled haploid genotypes were selected for further use based on evaluation of ploidy and combining ability. Seed progeny was reproduced by hybridization of regenerated plants in a climatic chamber (2014-2015). We used the schemes of creating self-incompatible lines and two-line-based hybrids. In the field trials (Moscow region, 2014-2015), the doubled haploids and their hybrid combinations were compared to the standard (Severyanka F_1) for the main valuable characteristics (i.e. the content of dry matter, nitrates, and vitamin C). The field resistance to Fusarium wilt, alternariosis, and pest damage were determined at cabbagehead technical maturity. The resistance to clubroot was assessed under artificial infection. There was a direct relationship of the average number of chromosomes to the number of chloroplasts in the stomata guard cells and the length of guard cells. The frequency of spontaneous doubling of the chromosomes numbers varied from 50.0 % to 87.5 % in different geotypes. A total of 11 to 73 % produced lines were high self-incompatible. Their geitonogamic pollination in the topcrosses resulted in 42 hybrid combinations. The model of F_1 hybrid most fully responding to consumer market demands was developed. Ten promising hybrid combinations which matched the model parameters in two-year field testing were recommended for variety testing. Hybrids were characterized by uniformity, high biochemical quality, the resistance to major diseases and pests and the yield of 104.60±8.27 t/ha. The dry matter content reached to 10.5 %, the sugar content was about 4.21-5.10 %, and ascorbic acid level ranged from 21.12 to 38.70 mg%. Both the highest level of ascorbic acid (92.0 mg%) and the smallest nitrate accumulation (33 mg/kg) were characteristic of one hybrid combination.

Keywords: white cabbage, *Brassica oleracea* L., doubled haploid lines, heterosis F_1 hybrids, in vitro isolated microspore culture, self-incompatibility, DH-technology, spontaneous doubling, ploidy

The most common culture among plants of the *Brassica oleracea* L. species is white cabbage due to a complex of biological and economically useful properties. F_1 hybrids with high market quality and resistance to harmful pathogens are most in demand [1]. At present, the creation of heterosis hybrids of white cabbage is based on cytoplasmic male sterility (CMS) and the crossing of self-incompatible inbred lines. Seeds from the intercross inside the same line are not formed, but crosses with plants of another line give 100 % hybrid seeds. Incompatibility is manifested on the style: incompatible pollen does not germinate at all or the pollen tubes, appearing and reaching the surface of the papilla of stigma,

curl and thicken [2]. Such lines are propagated and maintained by manual geitenogamic pollination of buds [3, 4]. The main modern requirements for culture are the storability of cabbage-head, the suitability for mechanized harvesting, resistance to diseases (club root disease, vascular bacteriosis, fusarial head blight, etc.) and pests [5]. Intensive cultivar changing requires the acceleration of the selection process [6].

White cabbage is a cross-pollinated plant with a 2-year growth cycle. In traditional selection, inbred lines of white cabbage are obtained through forced self-pollination for 6-12 generations, which significantly extends the selection process. To speed up the selection, doubled haploids (DH-technologies) are used [7-12], in particular a microspores culture in vitro, which not only ensures the homozygosis of the DH-line, but also enhances morphogenesis by using the entire spectrum of genetic recombinations including recessive traits. The technology is based on the ability of microspores to switch from gametophytic development to sporophyte under the influence of stress factors (high temperature, high osmotic pressure, etc.). Due to sporophyte type division, microspores form embryoids that develop into haploid plants (Hs) or doubled haploids (DH) [13, 14], with the involvement of which in the selection of pure lines reduces the time spent on crossing [15]. For vegetable crops, including cabbage, domestic DH-technology [16] has been developed, but it needs to be optimized for specific genotypes.

In plants developed after regeneration from isolated microspore cultures ploidy differs: alongside with doubled haploids, haploids, tetraploids, and mysoploids are presented [17]. In this case only homozygotic diploid plants arising as a result of spontaneous doubling of the number of chromosomes can be directly used in breeding programs [18]. There are several methods for analyzing ploidy. The classical method of chromosome counting in cells of meristematic root tissue or in dividing cells of young buds is most accurate [19, 20]. However, it is very complex and time-consuming and is not suitable for large scale screening [21]. Quantitation of nuclear DNA content and ploidy by flow cytometry [22] is carried out easily and quickly with a minimal amount of leaf tissue, but it requires considerable expenditures for equipment and reagents [23]. The count of the number of chloroplasts in the guard cells of the stomas is considered as a fairly simple and cheap method that can be used for most crops, including cabbage. It is shown that there is a high correlation between the number of chloroplasts in the stomata guard cells and the ploidy of plants [24]. Thus, the optimization of DH-approach for breeding and seed production remains relevant [25-28].

For the first time in Russia, using the culture method of isolated microspores in vitro, we obtained DH-populations of white cabbage, in which forms with predetermined properties were detected, which enabled us to select promising hybrid combinations in a short time.

The purpose of this work was to evaluate the constant lines of white cabbage doubled haploids on a set of agriculturally important traits and to improve the technology for creating heterotic F_1 hybrids on their basis.

Technique. The lines of doubled haploids were obtained via the isolated microspore in vitro culture using 11 selection lines of late-ripening white cabbage (*Brassica oleracea* L. *convar. capitata* var. *alba* DC). Donor plants were grown in a climatic chamber at 20-22 °C and 16 h/8 h (day/night) with illumination of 9000 lux. Buds harvested at the initial stage of flowering were sterilized for 30 sec in 96 % ethanol, then 5 min in a 50 % aqueous solution of the commercial preparation Belizna (KhimAlians, Russia) with the addition of 1 drop Tween 20 (PanReac Quimica S.L.U, Spain) per 100 ml, followed by 3-fold washing in sterile distilled water. Sterile buds 4-5 mm in size were transferred to Lichter's medium with half concentration of components ($^1/_2$NLN) and 13 % su-

crose, pH 5.8 [29], 30 buds per 6 ml medium, and homogenized. The microspore suspension was passed through a nylon filter with a cell size of 40 μm and precipitated for 5 min at 125 g in an Eppendorf 5804R centrifuge (Eppendorf AG, Germany). Microspores were washed twice in $^1/_2$NLN. The washed microspores were placed in Petri dishes of 6 cm in a diameter (10 buds per dish) with 5 ml of the nutrient medium of the above composition and incubated for 2 days in the dark at 32 °C. The incubation was then carried out at 25 °C in the dark to form embryoids. When the embryoids reached the stage of large globules, the heart-shaped or torpedo phase, they were placed in Petri dishes on Gamborg's medium (B5) [30] supplemented with 0.5 % glucose, 0.5 % sucrose and phytogel (3 g/l).

The shoots that embryoids formed were separated, placed on $^1/_2$ Murashige-Skoog medium (MS) with 2 % sucrose and 3 g/l phytogel, and cultured at 25 °C and a photoperiod of 14 h (2500 lux, fluorescent lamps). Plants with normally developed leaves and root system were transferred to vegetation vessels with a mixture of peat and perlite (7:3) and covered with perforated plastic cups for adaptation in vivo. Regenerated plants grown under the same conditions as donor plants [32] were evaluated for ploidy and combination ability.

The number of chromosomes in the cells of the meristematic root tissue was calculated by spreading method. Root tips of 0.5-1.0 cm in length were placed in a solution of α-bromide-naphthalene (1 μl per 10-15 ml) and kept for 12 hours in the cold. The roots were then transferred to a Clarke's fixative (alcohol and glacial acetic acid at a 3:1 ratio) and left for 1 hour in the cold, then thoroughly washed in water and transferred to 0.3 ml of a mixture of enzymes (10 ml citrate buffer, pH 4.5, cellulase 0.2 g, 5S pectinase 0.2 g, macerozyme 0.25 g, dreizelase 0.1 g) with the addition of cellulase 0.006 g and 2.7 ml of citrate buffer. The specimen was covered and put on a water bath (37 °C) for 1 hour. After softening the roots, the enzyme mixture was replaced with water. The white tip of the root was separated and pipetted onto the glass. A drop of acetic acid and a freshly prepared Clarke's fixative were added and the preparation was dried in air. The preparations were examined using an Axio Imager A2 fluorescence microscope (Carl Zeiss, Germany) with filter kits for DAPI (Zeiss Filter set 1, Carl Zeiss, Germany) [33].

For indirect determination of ploidy, chloroplasts were counted in the guard stomatal cells and their length was measured using an Axio Imager A2 microscope (Carl Zeiss, Germany) with fluorescence (filter set BR 490 and 515) [34]. The epidermal layer of cells was removed from the underside of the leaves, washed in distilled water, placed on a slide in a drop of water, covered with a cover slip and viewed under a microscope. Photos of at least 20 pairs of stomatal cells in each plant were used for documentation and chloroplast counting was performed. Visualization, measurements and calculations were carried out using an AxioVision software (Carl Zeiss, Germany).

Seed progenies were obtained by crossing regenerants in an artificial climate chamber during the winter-spring 2014-2015 (under the same conditions as in cultivating donor plants). The scheme for creating self-incompatible lines and obtaining two-line hybrids of white cabbage was used [35]. Field trials with seeds were laid out in 2014-2015 on experimental plots prepared according to standard for vegetable crops [36]. Seeds were sown in the third decade of April in cassettes with a cell diameter of 5×5 cm. In the third decade of May, the seedlings were planted in an open soil per 10 plants in 2-fold replication. The plantation scheme is 70×50 cm.

At technical ripeness of the head, lines of doubled haploids and hybrid combinations were characterized for a number of agriculturally important traits

(the weight and size of the head, the diameter of the rosette of leaves, the height of the outer and inner cabbage stump, the size of the leaf), comparing with the standard (Severyanka F_1). The vegetation period was estimated as time for maturing 90 % of the heads, after which the yield was harvested. The location of the lower leaves of the rosette, the color of the leaves, the intensity of the wax coating, the state of the surface of the leaf blade, the venation pattern were assessed visually. Biochemical parameters were determined in the integrated sample of the most typical heads, i.e. dry matter — after drying to constant weight, nitrates —by potentiometry, vitamin C — iodometrically [37].

Field resistance to fusarium wilt, alternaria, and pest damage were studied under natural field conditions at technical ripeness of the head. Resistance to the club root disease (damage, %) was evaluated under artificial infection with a randomly distributed placement of plants in 2-fold replication per 10 pcs in each variant [38].

Statistical processing included the determination of the arithmetic mean (X) and the mean error (m).

Results. Among the 10 white cabbage selection lines only 5 lines were responsive to introduction into culture in vitro. As a result, from 20 to 1000 regenerant plants per parent line were obtained from isolated microspores in vitro.

It was found that haploid cells in the in vitro culture were unstable, and tended to endomitosis (doubling of chromosomes without nuclear fission) with formation of cells of different ploidy. This made it possible to divide all the studied samples into groups according to the ploidy in accordance with the proposed indicators (Table 1). It was shown that the frequency of spontaneous doubling of the number of chromosomes in regenerative plants varied from 50.0 to 87.5 % depending on the genotype. In the works of other authors, the doubled haploid frequency in white cabbage was 10-40 % [39], 21-67 % [40], and 50-70 % [41]. In our studies, the proportion of haploid and tetraploid plants ranged from 0 to 25.0 %.

1. Ploidy of regenerated plants of late-ripening white cabbage (*Brassica oleracea* L. *convar. capitata* var. *alba* DC) based on the results of cytological analysis ($X \pm m$)

Ploidy	NCGC, psc.	GCL, nm
Haploids ($n = 9$)	9.00±0.63	20.45±0.80
Diploids ($2n = 18$)	13.73±0.80	24.15±0.55
Tetraploids ($4n = 36$)	24.53±1.46	34.12±0.70

Note. n — the number of chromosomes; NCGC — the number of chloroplasts in stomata guard cells, GCL — stomata guard cells length.

In all variants, we observed a direct relationship between the average number of chromosomes, the number of chloroplasts in the stomata guard cells, and the length of these cells. It is reported that in plants the number of chloroplasts correlates with the number of chromosomes, that is, the number of chloroplasts in diploid plants is about half as many that in haploids [34]. According to the literature, *B. oleracea* ssp. plants have the following number of chloroplasts in the stomata guard cells: 6-9 for haploids, 10-15 for diploids, and 20-25 for tetraploids [42]. The results of our studies confirmed that the calculation of the number of chloroplasts could be the simplest and fastest method to estimate plant ploidy.

The scheme we used for obtaining heterosis hybrids F_1 was based on sporophytic physiological self-incompatibility of the lines of white cabbage doubled haploids. Artificial geitenogamic pollination of flowers, with bud preemasculation, resulted in varying manifestation of self-incompatibility in lines of regenerated plants which was determined by the number of seeds (high for 0-1 pcs/seedpod, medium for 2-5 pcs/seedpod, low or absent for > 5 pcs/seedpod). Depending on the genotype, from 11 to 73 % of the lines of doubled haploids

2. Complex evaluation of the main agriculturally important traits in prospective hybrid combinations based on doubled haploid lines of late-ripening white cabbage (*Brassica oleracea* L. *convar. Capitata* var. *alba* DC) ($X \pm m$, Moscow region)

Hybrid combination	Vegetation, days	Head weight, kg	Yield, t/ha	Stump size, cm			Biochemical parameters				Plant infection, %			Pest damage, %
				inner		outer	dry matter, %	sugars, %	vitamin C, mg%	nitrates, mg/kg	club root disease	fusarium wilt	Alternaria blight	
				length	width	height								
3-3-3×1-19-1	160	2.76±0.26	78.66±7.41	4.57±0.39	5.07±0.29	4.72±0.80	10.50	5.10	38.70	81	6.3±0.4	1.5±0.1	0.6±0.2	6.5±1.0
2-45-1×1-18-2	165	2.72±0.19	77.52±5.42	6.80±0.36	4.60±0.18	5.93±0.61	9.00	5.00	92.00	33	12.5±2.7	2.7±0.6	2.0±0.5	7.2±0.7
2-45-1×5-13	165	3.18±0.02	90.63±0.57	9.00±1.00	4.00±0.50	7.00±0.40	9.90	4.40	35.20	59	15.0±1.8	0.5±0.1	1.5±0.1	11.8±2.5
3-3-3×2-331	160	2.26±0.18	64.41±5.13	8.12±0.55	6.43±1.02	9.33±3.79	10.06	4.42	26.40	100	25.0±5.2	5.0±0.8	3.0±0.2	7.0±0.5
11-124-1×2-307	158	2.41±0.35	68.69±9.98	6.26±1.28	4.57±0.43	5.36±0.69	10.13	4.85	37.60	80	17.5±2.2	3.8±0.1	3.3±0.2	6.0±0.2
5-13-2×2-307	165	2.49±0.48	70.97±13.68	6.19±0.96	4.00±0.51	6.00±1.33	9.85	4.36	21.12	124	20.0±4.9	6.6±0.3	1.6±0.4	6.6±0.1
3-3-3-2×1-19-2	160	3.18±0.07	90.63±2.00	7.00±0.60	5.00±0.40	10.00±0.10	9.52	4.21	24.64	69	31.0±2.5	7.5±0.1	2.5±0.5	10.0±1.3
2-331×11-1-1	165	3.09±0.34	88.07±9.69	8.25±0.53	5.17±0.34	12.85±0.99	9.60	4.40	33.40	105	15.0±1.0	3.1±0.7	2.2±0.2	3.5±0.8
11-68×5-13	158	2.99±0.28	85.22±7.98	6.43±0.29	3.07±0.29	7.57±0.96	9.80	4.90	33.40	100	38.0±3.6	5.0±0.4	3.0±0.1	10.6±0.5
2-331×5-13	165	3.67±0.29	104.60±8.27	8.94±0.40	4.34±0.29	9.86±1.99	9.80	4.70	37.00	89	7.5±0.8	1.0±0.1	0.5±0.1	6.0±0.1
Severyanka F_1 (standard)	160	2.98±0.54	84.93±15.39	9.00±0.90	5.00±0.40	5.00±0.20	9.00	4.10	31.70	109	45.0±2.7	0.5±0.1	2.0±0.5	11.0±1.5

showed high self-incompatibility, and from 0 to 29 % — medium self-incompatibility. It was these lines (12 genotypes) that were further used in the creation of heterotic hybrids F_1 (lines with no self-incompatibility were involved in other breeding programs). As a result of the topcross in the artificial climate chamber, 42 hybrid combinations were obtained on the basis of self-incompatibility.

For further planning experiments, the target model F_1 of the hybrid was compiled for the main agrotechnical and consumer characteristics. For the model, the vegetation period is 150 days or more (from planting the seedlings to the technical ripeness of the head); heads are equal in maturation terms, transportable, suitable for mechanized harvesting, medium sized, rounded, dense, weighing 2.5-3.5 kg, with excellent internal structure, small internal stump, with dry matter content of 9-10 %, high vitamin C and sugars level, and low nitrates, resistant to major diseases and pests.

In the complex evaluation of all samples within 2 years, 10 promising hybrid combinations were identified under field conditions by the agriculturally important traits (Table 2). The vegetation of hybrids lasted for 158-165 days, which corresponded to the late maturation period. Yields ranged from 64.41±5.13 to 104.60±8.27 t/ha, which made the cultivation of these hybrids cost-effective. The inner stump was small, that corresponded to the standard pattern, and in most cases inner stump turned out to be almost 2 times smaller than the standard, due to which the eatable part of the head increased. Among the hybrids the samples with high outer stump (from 9.33±3.79 to 12.85±0.99 cm) were detected which made them suitable for mechanized harvesting. All hybrids were characterized by a high content of dry matter (from 9.0 to 10.5%). The amount of ascorbic acid ranged from 21.12 to 38.70 mg%. An exception was a hybrid combination 2-45-1×-18-2 with ascorbic acid content of 92.0 mg%, which was almost 3 times higher than in the remaining samples. In the same hybrid combination, the accumulation of nitrates was the lowest (33 mg/kg). In the remaining samples, their amount was from 33 to 124 mg/kg, which did not exceed the MPC 500 mg/kg for wet weight (GOST R 51809-2001) of late-ripening white cabbage. Sugar content (4.21-5.10 %) exceeded the standard in all hybrid combinations.

Consequently, all hybrid combinations had high biochemical indices. Therefore, in the analysis it is necessary to proceed from their comprehensive assessment, which also includes productivity and resistance to major diseases and pests. Note that the hybrids remained mostly low-injured by major diseases and pests, which indicates their relative field resistance.

Thus, from isolated microspore culture in vitro, we obtained regenerated plants of white cabbage with different ploidy. There was a direct dependence between the average number of chromosomes and the number of chloroplasts in the stomata guard cells, and also the length of these cells. The frequency of spontaneous doubling in culture varied from 50.0 to 87.5 % and was the largest for doubled haploids. The involvement of homozygotic lines of such haploids in the breeding allowed us to create hybrid combinations of late-ripening white cabbage for a short time (2-3 years), which correspond to the declared requirements of competitive F_1 hybrids.

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APPLICATION OF MULTIDIMENSIONAL METHODS TO SEPARATE VARIETIES ON THEIR RESPONSE TO ENVIRONMENT FACTORS

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Abstract

Till now, the areas under rice crops are mostly occupied with the limited number of varieties. For enriching genetic biodiversity, it is necessary to improve selection of unique rice genotypes, and provide ecologically-based location of each variety. Now the efficiency of breeding is decreasing because of incomplete characterization of potentially donor genotypes. Presently, the domestic standards for competitive state trail do not cover a detailed study of the samples, since the developed varieties are tested at a single level of mineral nutrients with no estimation of a response to stressful influences and yield production sustainability. That leads to rejection of those highly productive samples for which such conditions are not optimal. In the present work we firstly summarized methods to comprehensively characterize adaptive plasticity of rice plants under contrast conditions (i.e. different dates for planting, various levels of mineral nutrition and stressors). In a multifactorial experiment with 19 combinations of the factors tested, we investigated yield variability in 24 Russian rice (*Oryza sativa* L.) varieties. The samples were planted on April 15, May 15, or June 15 and grown at optimum ($N_{120}P_{60}K_{60}$) and excess ($N_{240}P_{120}K_{120}$) fertilizer rates, in thin and dense crops (200 or 300 plants per square meter, respectively), under artificial salinization (0.35 % NaCl added to the soil at tillering). The data were processed using cluster and discriminant analysis. The multidimensional statistical methods allow us to clasterize the varieties into four groups with the closest characteristics as influenced by the full set of studied factors, and then to allocate distinct factors for the most precise discrimination between the samples. A standard cultivation was found to be less effective for developing plant plasticity. It is more correct to compare samples when the conditions are favorable for plant performance and productivity potential. Stresses, in combination with favorable factors, contribute to an increase in trait variability and dispersion, resulting in more accurate dividing varieties into groups. In our case study, with the use of «step-by-step analysis back» module we reduced the number of discriminating factors to two ones adequate for 100 % reliable allocation of typical representatives of the groups. High mineral levels and water deficit were enough to truly classify 88 % of the samples. This is sufficient in genetic research where it is necessary to select the most typical representatives. Samples of the groups 1 and 3 have been classified correctly, and only three varieties of the group 2 have got to another cluster. The discriminant analysis also shows distance of each variety from the center of the group. Samples with the minimum distance are the most typical representatives which can be used as genetic sources of desired traits, as contrast parental forms in hybridization, or involved in marker-assisted selection and GTL mapping. Early planting, dense crops, high fertilizer rates, and lack of water were the factors which mostly influenced on the clear separation of the samples into clusters according to how the varieties responded to external environment. The virtual «ideal variety» (a model) and Kurchanka variety were grouped in the same cluster, and the varieties from the group 1 were close to the «ideal variety» on the response to environment. Despite high yield production, the dispersion in the group 3 which includes Kurchanka and the model variety was 3 times as much as in other groups. Therefore, stability of the varieties was lower in this cluster (group 3) as compared to the first and the second clusters (groups 1 and 2).

Keywords: rice, *Oryza sativa*, multidimensional methods, claster analysis, estimation of breeding material, discriminant analysis

Currently the areas under rice crops are mostly occupied with the limited

number of varieties. For enriching their genetic diversity, it is necessary to improve effectiveness of evaluation and selection of unique genotypes, and provide ecologically based location of each variety [1-3]. Apart from that, the efficiency of breeding is decreasing because of incomplete characterization of parental material. Existing system does not provide for a detailed study of samples provided for competitive variety testing [4-5]. Productivity of the developed varieties is tested at a single level of mineral nutrition, which leads to rejection of those highly productive samples for which such nutrient status is not optimal. This approach prevents identification of potential yield production of a sample, norm of reaction of a variety to stressful influences and donors of high functional activity of genetic systems, determining productivity and adaptability [6-7], and does not provide for evaluation of stability of the developed forms [8-10]. As a result, the most valuable forms, which has taken years of selectionist efforts, does not find a use.

Potential productivity of rice, similar to many other crops, has been increasing very slowly since the latter half of the 20th century. Further increase in rice production may be achieved by intensification or promotion to regions with lower or higher temperatures, and areas with salinized or flooded soils [11-13]. Climatic changes will cause increase in average temperatures by the mid-21st century. Apart from that, short-term temperature variations, not common to the regions will be observed more often (climatic changes in tropical zone have already resulted in decreased rice yields). Temperature increase by 1 °C leads to decrease in yields by more than 10 % [14, 15]. Due to sea level rise expansion of territories with salinized soils is forecasted [16-18]. Thus, not only potential productivity shall be improved, but stability of yields and comprehensive stress resistance shall also be ensured in order to increase the crop production [19, 20]. Varieties with high potential productivity are more vulnerable to abiotic stress factors. That's why plant growing in many countries is focused on optimal and stable yield production, rather than on maximum yields [21-23].

Evaluation of plant stability is normally performed during growing in various environmental conditions or using contrast agrotechnical approaches [24-26]. However, stress levels (salinization, high or low temperatures) are hardly ever used in experiments [27-28] and contrast planting dates are rarely studied [29-31]. This significantly depletes the information obtained.

Mathematical processing is normally performed using analysis of variance and regression analysis [32-34]. Multivariate statistics methods for statistical evaluation of results have not been used till recent time [35-37]. Their introduction in breeding material and promising variety studies will ensure more effective identification of samples suitable for a broad cultivation area [38-40]. Multivariate statistics methods allow grouping of samples with the most similar responses to impact of various factors, determination of environmental conditions ensuring the most accurate differentiation of samples and requiring minimum expenditures for experiment conduct [41-43], exclusion of less informative variants and identification of minimum number of variants ensuring 100 % reliable assignment of a sample to the respective adaptability group [44-46].

In this work we have developed a breeding material evaluation system, including contrast planting dates, various mineral nutrition and stress levels, based on the fusion of previously proposed methods. Application of multivariate statistics methods for processing of results has significantly increased the informative value of the data.

The study was aimed at grouping of Russian rice varieties by the response to a complex of environmental factors and determination of conditions ensuring the most effective performance of such grouping.

Techniques. A total of 24 Russian rice (*Oryza sativa* L.) varieties were investigated in a multifactorial experiment (established at the growing site of

All-Russian Research Institute of Rice in 2004-2006). The plants were grown in lysimeter experiments at optimum ($N_{120}P_{60}K_{60}$) and increased ($N_{240}P_{120}K_{120}$) mineral nutrition levels; with spaced (200 plants per 1 m^2) and close (300 plants per 1 m^2) planting; with various planting dates (April 15, May 15, June 15); at salinization created artificially during the tillering phase by introduction of NaCl into the soil up to the concentration of 0.35 %; under lack of moisture (watering was ceased during the paniculation phase). The sample included 30 plants of a variety per the experiment variant. Planting was performed in single-row plots, 10 plants per row; the distance between the rows was 10 cm. The experiments were performed in triplicate, with randomized location of plots.

Productivity (grain weight per plant) was evaluated in all plants in the experiment. Growing of plants at optimum mineral nutrition level ($N_{120}P_{60}K_{60}$) with spaced (200 plants per 1 m^2) planting performed on May 15 served as a control in all variants. The seeding rate was 200 plants per 1 m^2 in all cases, except for close planting.

The data were processed using cluster and discriminant analysis and Statistica 6.0 software (StatSoft, Inc., USA) [36, 37, 39].

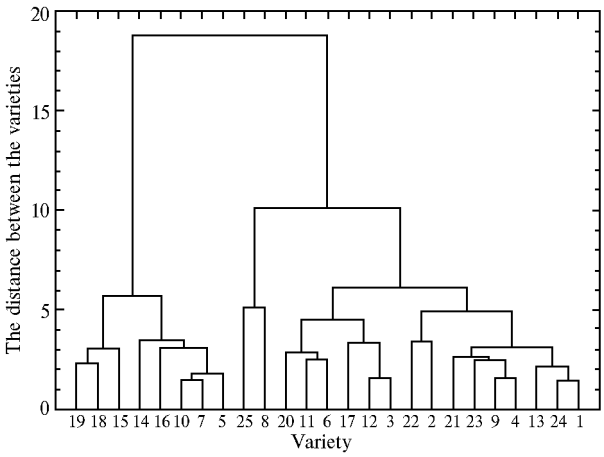

Fig. 1. Clusterization of Russian rice (*Oryza sativa* L.) varieties by the response to environmental factors based on productivity evaluation (Warda method)**:** 1 — Ametist, 2 — Boyarin, 3 — Dalnevostochnii, 4 — Druzhnii, 5 — Zhemchug, 6 — Izumrud, 7 — Kasun, 8 — Kurchanka, 9 — Lider, 10 — Liman, 11 — Pavlovskii, 12 — Primorskii, 13 — Rapan, 14 — Sadko, 15 — Serpantin, 16 — Snezhinka, 17 — Sprint, 18 — Strelets, 19 — Fakel, 20 — Fontan, 21 — Khazar, 22 — Jupiter, 23 — Yantar, 24 — group average, 25 — model (ideal) variety.

Results. Our experiment ensured analysis of organization of genetic systems, determining attraction of photosynthesis products from stem and leaves to panicle, their microdistribution between grain and chaff, effectiveness of soil nutrition, resistance to close planting, salinization and lack of moisture, variability of ontogenesis durations [1, 2]. Using cluster analysis of the data on productivity of varieties in all variants of the experience they were divided into 4 groups with different response to the studied environmental factors (Fig. 1).

During the next stage evaluation of significance of inter-group differences by the response to a complex of growing conditions was required, using discriminant analysis. Discriminant analysis is based on plotting of trait-function linear combinations, where each trait or experiment variant has its coefficient (contribution). We have plotted three discriminant functions using Statistica 6.0 software for separation of groups. The first two functions considered more than 97 % of initial dispersion of the experiment variants. Thus, the values of the remaining function could be omitted in solving of further problems, as it considered less than 3 % of dispersion.

Evaluation of significance of discrimination of variety groups, performed using χ-square test, has demonstrated that only the first discriminant function is effective for their separation. The possibility of absence of inter-group differences for this function was below the significance level permissible in biological studies ($p < 0.05$). However, even with the use of the first discrimination function, uniform grouping of varieties by stability of productivity in various environmental

conditions was not achieved in all experiment variants.

Suitability of the experiment variants for identification of intervarietal differences was also evaluated using discriminant analysis (Table 1).

1. Standardized coefficients of discriminant functions during evaluation of differences between the studied rice (*Oryza sativa* L.) varieties by the response to environmental factors

Environmental conditions (experiment variant)	First discriminant function	Second discriminant function
Early planting, 2004	0.12	–0.48
Close planting, 2004	0.57	–1.21
Late planting, 2004	0.55	0.81
Optimum planting date, 2004	0.54	–1.56
Salinization, 2004	–0.27	3.82
High mineral nutrition level, 2004	–0.38	–1.12
Early planting, 2005	0.06	0.09
Salinization, 2005	0.88	–0.07
Late planting, 2005	0.54	–0.18
Close planting, 2005[a]	–2.63	–1.24
Lack of moisture, 2005[a]	–2.16	–1.09
High mineral nutrition level, 2005[a]	–3.03	0.41
Optimum planting date, 2005	0.26	0.11
Early planting, 2006[a]	1.98	2.93
Optimum planting date, 2006	0.35	1.45
Close planting, 2006	–0.40	–2.20
High mineral nutrition level, 2006[a]	–1.36	–0.48
Late planting, 2006	–0.89	–0.85
Salinization, 2006	0.18	1.98
Total percentage of considered dispersion	91	97

N o t e. [a] — the experiment variants with minimum contribution to inter-group differences.

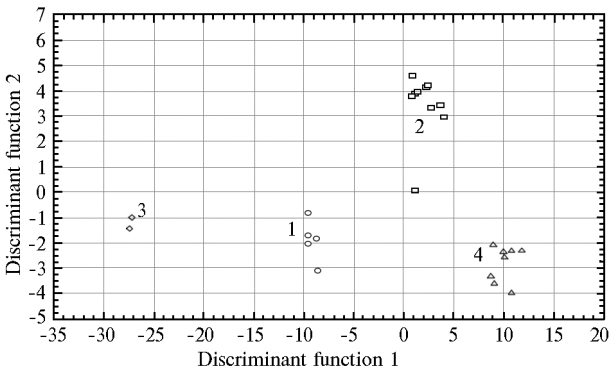

Fig. 2. Cluster distribution of Russian rice (*Oryza sativa* L.) varieties in two discriminant functions by the response to all studied environmental factors: 1 — first cluster (G_1:1), 2 — second cluster (G_2:2), 3 — third cluster (G_3:3), 4 — fourth cluster (G_4:4). Similarity measure is Mahalanobis distance (D^2).

Absolute values of standardized coefficients of discriminant functions allow determination of contribution of a certain variable or the experiment variant to the final function and their role in inter-group differences. In our case maximum absolute values were observed for five experiment variants with maximum contribution to separation of groups by the response to environmental conditions: early planting in 2006, high mineral nutrition level in 2005 and 2006, close planting and lack of moisture in 2005. It should be noted that standard growing conditions during all years of the study allowed differentiation of samples to a lesser extent, which was confirmed by relatively low absolute values of coefficients of discriminant functions.

Evaluation of similarity of the response to environmental conditions of the selected groups by means of calculation of distance between their centroids was one of the discriminant analysis results. Mahalanobis distance (D^2) acted as a similarity measure; it was the largest between the first, the second and the fourth groups, which indicates the most significant genetic differences between them (Fig. 2).

A variety model with maximum grain weight per plant in all studied experiment variants was also included in the analysis for identification of a group

of varieties with maximum productivity. The model (ideal) variety and Kurchanka variety were grouped in the same cluster. Varieties of the first group were the most similar to the model variety in terms of productivity in all experiment variants, i.e. demonstrated the most stable high productivity in the studied conditions. The first group included varieties Dalnevostochnii, Izumrud, Pavlovskii, Primorskii, Fontan; the second — Ametist, Boyarin, Druzhnii, Lider, Rapan, Sprint, Khazar, Jupiter, Yantar; the third — Kurchanka variety and ideal variety model; the fourth — varieties Zhemchug, Kasun, Liman, Sadko, Serpantin, Snezhinka, Strelets, Fakel. Evaluation using F-test has demonstrated significance of differences between the third and the fourth groups (clusters) (null hypothesis probability 0.035), as well as between the first and the fourth groups (null hypothesis probability 0.048). The differences between the first and the second group of varieties were insignificant.

Absolute values of standardized coefficients of discriminant functions not only allow determination of contribution of a certain variable to this function, but also ensure reduction of the number of studies variants. For example, if an absolute value of standardized coefficients of discriminant functions of a variable (the experiment variant) is small, it can be excluded from the analysis. This procedure is referred to as determination of informative list of traits. We used it for determination of environmental conditions ensuring the most comprehensive identification of inter-group differences in case of reduction of the experiment variants.

The most significant reduction of the experiment variants ensures backward step-by-step analysis which we used to solve the problem. It has been established that even two variants of the experiment allow reliable grouping of varieties by the response to environmental conditions. Maximum high productivity of samples was obtained in 2005 in variants with high mineral nutrition level and lack of moisture, as compared with the value in 2004 and 2006. Thus, environmental conditions favorable for implementation of variety productivity potential allow correct comparison of samples. Stress development against the background of other favorable factors expands the range of trait variability and dispersion in the experiment, which results in more reliable grouping of varieties.

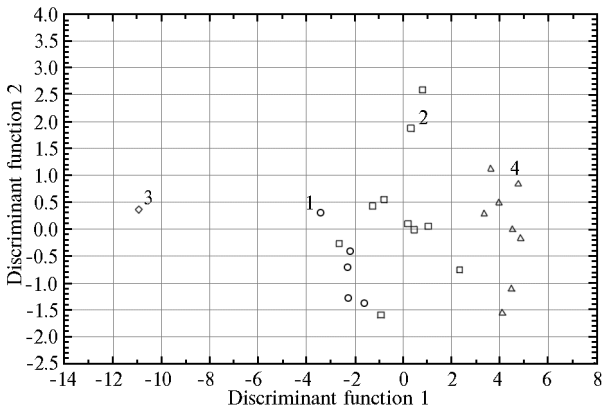

Fig. 3. Cluster distribution of Russian rice (*Oryza sativa* L.) varieties in two discriminant functions by the response to high mineral nutrition level and lack of moisture: 1 — first cluster (G_1:1), 2 — second cluster (G_2:2), 3 — third cluster (G_3:3), 4 — fourth cluster (G_4:4).

Genetic studies normally require identification of the most typical representatives of each cluster (group). In this case assignment of borderline samples to a wrong group does not impair the work effectiveness. Thus, our task is maximum reduction of the number of experiment variants and to preserve accuracy of identification of typical cluster representatives at the same time.

According to the results of clustering of the studied rice varieties using two selected experiment variants (high mineral nutrition level and lack of moisture), 88% of samples were classified correctly, which is acceptable for genetic studies. At that, complete match with the evaluation results for all experiment variants was observed in the first

and the third groups; only three samples from the second group were assigned to another cluster (one to the fourth group, and two to the first group) (Fig. 3, Table 2).

Discriminant analysis also demonstrates the distance between each variety and a group centroid. Samples with minimum distance from centroid are the most typical group representatives, which can be used as the studied trait gene sources or contrast forms during selection of pairs for hybridization or molecular marking and quantitative trait gene localization.

2. Correctness of assignment of rice (*Oryza sativa* L.) varieties to clusters (groups) during discriminant analysis with reduced number of the experiment variants against the initial value

Group	Percentage of correct assignments	G_1:1	G_2:2	G_3:3	G_4:4
G_1:1	100.0000	5	0	0	0
G_2:2	70.0000	2	7	0	1
G_3:3	100.0000	0	0	2	0
G_4:4	100.0000	0	0	0	8
Total	88.0000	7	7	2	9

Note. G_ — group of samples, G_1:1 — the number of samples in the first group (during analysis of all experiment variants), assigned to the group in case of reduction of the number of analyzed variants during discriminant analysis.

3. The experiment variants identified during forward step-by-step analysis, joint application of which ensures reliable assignment of rice (*Oryza sativa* L.) variety to groups with different adaptability and stability of productivity

Variant	F	p
High mineral nutrition level, 2005	53.856	0.000
Lack of moisture, 2005	4.736	0.021
Early planting, 2004	3.855	0.038
Optimum planting date, 2005	3.089	0.068[a]
Late planting, 2006	2.069	0.158[a]
Close planting, 2005	4.271	0.029
High mineral nutrition level, 2006	2.198	0.141[a]
Optimum planting date, 2004	1.638	0.233[a]
Close planting, 2006	1.179	0.359[a]
Salinization, 2004	1.011	0.422[a]

Note. [a] — variants with unreliable discrimination of samples; F — *F*-test, p — possibility of absence of intergroup differences when using the experiment variant.

In order to obtain more accurate results, for example, for development of data bases on the contribution of genetic systems to productivity or adaptability, the number of the experiment variants shall be increased up to the values ensuring reliable separation and classification of samples. This task can be performed using forward step-by-step analysis, which reduces the number of experiment variants to a lesser extent. Application of this analysis allowed us to identify ten variants, ensuring 100 % effective assignment of samples to groups with different adaptability and stability of productivity (Table 3).

Thus, we have established that standard growing conditions allow differentiation of rice variants by the response to environmental factors to a lesser extent. Experiments ensuring more significant range of trait variability in case of external effects shall be established for determination of sample stability and plasticity. High mineral nutrition level, close planting, lack of moisture during the maturation phase and early planting acted as such conditions in our study. Varieties Dalnevostochnii, Izumrud, Pavlovskii, Primorskii, Fontan, Ametist, Boyarin, Druzhnii, Lider, Rapan, Sprint, Khazar, Jupiter, and Yantar (the first and the second clusters) have demonstrated the greatest stability of productivity during discriminant analysis, and we recommend to use them as sources for the trait “stable high productivity in various environmental conditions”. Varieties Kurchanka, Zhemchug, Kasun, Liman, Sadko, Serpantin, Snezhinka, Strelets,

and Fakel (the third and the fourth clusters) were less stable, notwithstanding that a model ideal variety was assigned to one of the groups during clustering.

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CARBOHYDRATE COMPOSITION OF FLAX MUCILAGE AND ITS RELATION TO MORPHOLOGICAL CHARACTERS

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Abstract

Mucilage of flax is a valuable product for food, medicine and biocomposites production. Each direction of use needs special characteristics of seeds, so it is necessary to describe flax polymorphism of mucilage carbohydrate composition to determine the effect of seed color and pleiotropic effects of genes controlling it on the mucilage chemical composition. The originality of the work consists in the use of lines genetic collection with identified seed color genes and methods of multivariate and nonparametric statistics to identify patterns of influence of seed color on the mucilage composition. Seed mucilage polysaccharide composition was evaluated in 29 lines and three cultivars of flax (15 lines had red-brown seeds of the wild type, 9 lines had yellow seeds and 7 lines had modified brown seeds). For some lines the genetic control of seed color was known (from 8 lines with yellow seeds 4 lines had gene *s1*, 4 ones had gene *YSED1*; 5 lines with yellow hue of seeds had gene *pf1*).Water extraction of mucilage performed for 2 hours at 20 °C. After freeze drying monosaccharide composition was examined by gas chromatography. Generally, mucilage contained more pectin (pect = rhamnose (Rha) + galacturonic acid (GalA), 38-64 %) than arabinoxylans (AX = arabinose (Ara) + xylose (Xyl), 10-38 %). In the most of lines maximal and minimal percent of pectin was caused by rhamnogalacturonan (RG1b = 2 × Rha), except of the variety Orshanskii 2 which had an exceptionally high content of GalA and accordingly, homogalacturonan (HGA = GalA-Rha). Increase of AX was caused by extending of the core (Xyl), but there were lines with increased branching (Ara) or proportionally increased the whole molecule. Ratio Ara:Xyl was about 0.23 (0.05-0.30). Its extreme values did not always correspond to the AX content. Percent of RG1b was approximately twice higher than that of AX. But there were lines with more AX than RG1b. Galactose (Gal) was about 15 % of mucilage sugars, fucose (Fuc) was about 3.5 %. In average glucose (Glc) was 3.6 % of mucilage but it varied greatly (from 1.3 to 11.2 %, *Cv* = 79 %). Factor analysis revealed two main factors. The factor 1 showed antagonism of AX, Ara, Xyl with pectins, Gal, and GalA. The factor 2 showed antagonism of HGA with Fuc and Ara:Xyl. Mann-Whitney U rank test showed the significant decrease of AX, Ara, Xyl and conversely the increase of GalA, Gal, HGA, RG1b and RG1b:AX in brown seeds. Yellow seeds had significantly higher percent of AX, Xyl, Fuc and conversely lower percent of RG1b, HGA, GalA and Gal. Lines homozygous for the gene *s1* contained significantly more Glc, AX, Ara, Xyl and less Gal, RG1b, Rha, GalA at lower RGb:AX. No significant differences in the composition of mucilage for lines carrying genes *YSED1* and *pf1* were identified. For the first time, by nonparametric and multivariate statistics we revealed a complete difference between lines groups with brown seeds having the greatest load on the factor 1 (much pect, GalA and Gal) and yellow seeds, and also homozygotes for the gene *s1* having the lowest load on the factor 1 (much AX, Ara, Xyl).

Keywords: *Linum usitatissimum*, genetic collection, genes of seeds colours, flax mucilage, arabinoxylan, rhamnogalacturonan 1

Flax is an ancient industrial crop. Russia occupies the second place after Canada on the area of oil flax cultivation [1]. In the last decade, its unconventional use in the manufacture of bakery and pastry products, including specialized products [2-4], has sharply increased. The most important substance determining the quality of baked goods is mucilage, constituting about 5 % of

the weight of flax seeds [5]. It is also used separately as an egg white substitute [6]. Biscuits made from flax flour with a high content of arabinoxylans have a greater specific volume, openness, are more pliable and springy than similar products with a low content of arabinoxylans, and exceed the wheat flour standard according to these parameters [7]. In medicine, flaxseed mucilage is used at gastrointestinal disorders, hypercholesterolaemia, atherosclerosis, diabetes, nephritis and hormone-dependent cancer [8, 9]. It has been shown that long chains of homogalacturonan or linear rhamnogalactouronan are required for immunomodulating effect of pectins [10].

In the European Union, Canada and the USA, biocomposite materials are being intensively developed, completely consisting of plant raw materials, where fibre acts as a reinforcing component, and flax mucilage as a binder [11, 12].

Mucilage is formed in the secondary cell wall of the epidermal cells of the flax seed cover [13], and promotes their spread by the animals, adhesion to the soil, attracting soil microorganisms in the rhizosphere [14, 15], and is also used in seedling feeding [16]. In the flax mucilage, the content of polysaccharides is 83.3 %, with 4.6 % of proteins and 11.8 % of total ash [17]. The chemical composition of polysaccharides depends on the method of the mucilage extraction. It is obtained mainly from whole seeds, less often from flour [18] and seed cake [19], and then in most cases it is precipitated by a nonpolar solvent, such as ethanol or acetone, but this technique has been shown to disrupt the functional properties of mucilage [20]. No standard method has been developed to investigate the composition of carbohydrates (in contrast to proteins). The mucilage polysaccharides are analyzed using the method of size exclusion chromatography (SEC), in which the substances are separated according to their molecular weight (M_w), and the homogeneity of the obtained fractions is evaluated, followed by the assessment of the monosaccharide composition of each fraction using gas chromatography [21]. More often, the monosaccharide composition of mucilage is determined without separation into fractions, as a pooled sample based on these fractions differs from the original sample in the rheological properties [21]. The composition of mucilage includes seven basic monosaccharides, xylose (Xyl), arabinose (Ara), rhamnose (Rha), galacturonic acid (GalA), galactose (Gal), fucose (Fuc) and glucose (Glc) [21].

The polysaccharides of yellow flax seeds have been most thoroughly studied [22, 23]. It has been shown that their mucilage consists of a neutral (M_w 1.16×10^6 g/mol, 75.00 % of the total amount) and two acidic (M_w 6.52×10^5 g/mol, 3.75 %, M_w 1.35×10^4 g/mol, 21.25 %) fractions, but they are also not homogeneous [19]. Neutral polysaccharides of the seed mucilage are pentosan and (galacto)arabinoxylan (AX) in a ratio of Ara:Xyl 1:5. The main polymer chain is formed by Xyl residues to which Ara and Gal are attached; also, Fuc and Rha with GalA are a part of the polysaccharide (Rha and GalA may form a part of the next fraction). Acid polysaccharides are represented by the rhamnogalacturonan 1 (RG1) pectin with a different molecular weight. Its main chain (RG1 backbone — RG1b) is built from alternating hexoses of Rha and GalA; the Gal residues forming the lateral branches are linked with Rha; in addition, Fuc and Xyl are found in RG1. The fractions of flax mucilage differ in their molecular weight and the ratio of sugars.

The branching of mucilage polysaccharides is evaluated by the degree of methylation. For example, it was shown for AX that 72.5 % of Xyl residues had two additional branches, 2.5 % — one branch, and 25.0 % are included only in the skeleton chain of AX. No one residue of Xyl was found, which would be a lateral branch. Gal, Fuc and Xyl may be terminal sugars, whereas Ara participates in the lengthening of the lateral chain [23]. Some of the Rha

residues are found only in the skeleton of RG1, the rest have a lateral branch, whereas GalA serves exclusively as a link in the main chain of this pectin [W. Cui et al., 1994, cited from 24].

The genotype of flax significantly affects the content [25] and the composition of mucilage. Five of six samples with yellow seeds had fewer acid and more neutral polysaccharides than five of the six brown-seed varieties, but one sample from each group had the opposite mucilage composition [26]. This may indicate both a different genetic control of the yellow-seedness, and the influence of other genes on the composition of polysaccharides. Genetic control of the biosynthesis and secretion of mucilage was studied using *Arabidopsis thaliana* L. as a model object. Forty four genes have been sequenced that affect these processes. The detected genes were divided into four following groups: the outer integument differentiation regulators; genes of mucilage synthesis and secretion; genes involved in maintaining the structure and modification of mucilage; genes involved in the differentiation of cells secreting the mucilage [14].

To determine the patterns of the seed coloration effects on the mucilage composition, for the first time we used lines from the genetic collection with known seed color genes, as well as multi-dimensional and nonparametric statistics. For the first time it has been established that in lines carrying the *s1* gene, responsible for the absence of anthocyanins in the whole plant with a pleiotropic effect on the yellow coloration of the seeds, there are much more arabinoxylans and glucose in mucilage than in other lines.

The aim of this study was to establish the polymorphism of flax (*Linum usitatissimum* L.) for the carbohydrate composition of mucilage, as well as to evaluate the correlation between this parameter with the color of the seeds and the pleiotropic effect of the genes controlling it.

Techniques. The study used seeds of 28 lines created in the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) and 3 recognized varieties grown in the fields of the VIR's laboratories in Pushkin (Leningrad region) in 2001-2007. Three varieties and 12 lines had red-brown seeds (wild type), 9 lines — yellow seeds, and 7 lines had a changed brown color of the seeds. Ten seeds from each line were soaked for 2 hours in 2 ml of double-distilled water (20 °C, with stirring for the first hour). The monosaccharide composition of the obtained mucilage solution (the amount of arabinose, xylose, rhamnose, galacturonic acid, fucose, galactose and glucose) was studied using a GCMS-QP5050A gas chromatograph/mass spectrometer (Shimadzu Corp., Japan) after freeze drying, methanolysis and silylation by a standard procedure [28].

The proportion of polysaccharides was calculated based on the composition of each of them: AX = Ara + Xyl (arabinoxylan), RG1b = 2 × Rha (the main chain of rhamnogalacturonan 1 taking into account approximately equal molecular weight of the RG1b monomers, i.e. Rha and GalA); HGA = GalA – Rha (a linear polymer homogalactorunan was determined by the difference in total amount of GalA and the amount included in the composition of RG1b expressed by Rha), pect = Rha + GalA (pectin). The ratios of Ara:Xyl and RG1b:AX were also calculated.

The data was processed using the Microsoft Excel package (primary statistics), the Statistica 7.0 programs (StatSoft, Inc., USA; factor analysis, variance analysis, Student's *t*-test), SPSS 13.0 (nonparametric statistics) [29-33].

Results. The flax varieties and lines used in the study are described in Table 1. In the samples studied, the yellow color of the seeds was controlled by different genes. The gk-103, gk-136, gk-351 and k-391 samples were homozygous for *s1*, which inhibits the anthocyanin color of the whole plant with a pleiotropic effect (white stellate corolla, yellow anthers). The gk-351 (gk-136 × gk-

121) line also carried the *rs1* gene (light yellow-brown seeds), which was hypostasized by the *s1* gene. In the gk-159, gk-390, gk-391 and gk-395 lines, the seed color was determined by the dominant *YSED1* gene, in gk-173 — by the recessive *ysed2* gene, and not by the allelic *YSED1*. The yellow seedness of the gk-129 line was the result of the interaction of two genes: the main *pf-a*^{d} gene (seeds with a yellow tinge, pink corolla, orange anthers), and *yspf1* modifier (in the *pf-a*^{d} *yspf1* genotype the seeds are yellow). It should be noted that the gk-159 and gk-160 lines, as well as gk-390, 393 and 394 originated from common ancestors.

1. Varieties and lines of flax (*Linum usitatissimum* L.) from the VIR collection, used to assess the effect of seed colour on the composition of the mucilage obtained from them

Cat. No	Parentage, seed color	Phenotype	Genotype
	Reb-brown seeds		
gk-2	l-1 from k-48 (Altgauzen breeding station, Russia)	d	
gk-22	l-3-2 from k-562 (Pskov ridge, Russia)	d	
gk-79	l-1-2 from k-5408 (Pechora ridge, Russia)	d	
gk-91	l-1 from k-5522 (Palkinsky ridge, Russia)	d	
gk-130	l-1 from k-6577 (Medra, Czech Republic)	d	
gk-109	l-3-2 from k-6099 (Makovi M.A.G., Argentina)	d	
k-6807	Orshansky 2 (Republic of Belarus)	d	
gk-160	L-2-1 from k-7659 (a biological admixture in the Bionda variety, Germany)	m	
gk-125	l-5-1 from k-6296 (Koto, USA)	m	
k-8409	Kinelsky 2000 (Russia)	m	
k-7822	Cyan (Russia)	m	
gk-132	l-1 from k-6608 (Currong, Australia)	m	
gk-396	l-1-1 from i-605311 (Agt1393/02, Czech Republic)	m	
gk-393	l-2 from i-595808 (a biological admixture in the Linola variety, Canada)	m	
gk-394	l-3 from i-595808 (a biological admixture in the Linola variety, Canada)	m	
	Yellow seeds		
gk-103	l-4 from k-5896 (Lin 255, the Netherlands)	m	*s1*
gk-136	l-1 from k-6634 (Mermilloid, Czech Republic)	m	*s1*
gk-351	l-1 from (gk-136 × gk-121, Russia)	m	*s1, rs1*
gk-159	l-1-1 from k-7659 (Bionda, Germany)	m	*YSED1*
gk-390	l-1 from i-595808 (a biological admixture in the Linola variety, Canada)	m	*YSED1*
gk-391	l-1-2 from i-601679 (Eyre, Australia)	m	*YSED1*
gk-395	l-1 from i-601680 (Walaga, Australia)	m	*YSED1*
gk-173	l-1 from i-548145 (Ottawa 2152, Germany)	m	*ysed2*
gk-129	l-2 from k-6392 (Bolley Golden, USA)	m	*pf-a*^{d}, *yspf1*
	Yellow-brown or spotted seeds		
gk-141	l-1 from k-6815 (K-6, Russia), dark yellow-brown seeds	d	*pf1*
gk-143	l-1 from k-6917 (Versailles, France), yellow-brown seeds	d	*pf-a*^{d}
gk-176	l-1 from (gk-141 × gk-103), Russia, yellow-brown seeds	d	*pf1*, *s1*
gk-255	l-3 from (gk-121 × gk-141), Russia, yellow-brown seeds	m	*pf1*, *rs1*
gk-121	l-1-1 from k-6272 (L. Dominion, Northern Ireland), light yellow-brown seeds	m	*rs1*
gk-65	l-3 from к-3178 (local, Tver oblast) red-brown seeds with yellow specks	m	*ora1*
gk-124	l-1 from к-6284 (Stormont Mothley, Northern Ireland), red-brown seeds with a yellow spot	d	*f*^{e}

Note. k — the VIR catalogue numbers, gk — numbers in the VIR's catalogue of the genetic collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg); d — long-fibred flax (tough flax), m — intermediate (linseed flax). Only genes that affect the color of seeds are indicated.

The gk-141 and gk-143 lines with a modified brown color of the seeds were homozygous for the two different alleles of the *pf1* gene, i.e. *pf1-pf1* and *pf-a*^{d} (*pf1,* the seeds are dark yellow-brown; *pf-a*^{d}, seeds with a color ranging from yellow to dark yellow-brown). The gk-121 line carried the *rs1* gene, controlling the light yellow-brown color of the seeds. The gk-65 sample was homozygous for the *ora1* gene, which is responsible for the appearance of yellow flecks on red-brown seeds, as well as the orange color of anthers. The gk-124 line contained the *f*^{e} gene, which determines the presence of a yellow spot in red-brown seeds, a light blue corolla, and grey anthers. Two lines with yellow-brown seeds had a hybrid origin, such as gk-176 (gk-141 × gk-103, the *s1* and *pf1* genes) and gk-255 (gk-121 × gk-141, the *pf1* and *rs1* genes) [27].

For all the samples studied, arabinoxilan averaged 26.4 % of the total amount of mucilage sugars (with variations from 7.9 to 38.4 %). The highest

content of AX was found in all lines carrying the *s1* gene (gk-103, ggk-136, gk-351, gk-176), in two of the four lines with the *YSED1* gene (gk-159, gk-391), in gk-173 carrying the *ysed2* gene and the gk-160 line with red-brown seeds. The sample from gk-351 was characterized by the maximum Ara content. In two related lines, gk-159 and gk-160, as well as in gk-132, a high content of Ara and Xyl was found. In the remaining lines, xylose was predominated in the mucilage composition. Consequently, branching was increased in the gk-351 line, a proportional increase in the size of the polysaccharide molecule occurred in gk-159, gk-160 and gk-132, and in the remaining cases the chain elongated without branching.

The minimum content of AX was seen in 5 brown-seed samples (gk-393, gk-394, k-396, k-6807 and k-8409). In the k-8409 line, this was the result of a proportional decrease in the Ara and Xyl content; whereas in the gk-394 line, the length of the main chain reduced to a greater extent, while in gk-393, gk-396 and k-6807, the amount of AX decreased at the expense of lateral branches. The ratio of Ara:Xyl reflected the degree of AX branching and averaged 0.23, varying from 0.05 to 0.30. In the yellow-seeded variant of gk-129, the degree of branching of AX was 5 times lower than in the remaining samples. Weak branching of AX was also observed in the arabinoxylan-deficient samples of gk-393, gk-396 and k-6807. The lines with the extremal content of AX, such as gk-394, gk-351 and gk-143, had the largest degree of branching (Table 2).

2. Carbohydrate composition of mucilage (%) from seeds of different lines and varieties of flax (*Linum usitatissimum* L.) from the VIR collection

Cat. No	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	HGA	pect	AX	Ara:Xyl	RGb:AX
Reb-brown seeds												
gk-2	6.2	24.4	3.9	22.6	25.3	16.2	1.4[a]	1.0[a]	49.7	28.8	0.27	1.70
gk-22	4.6	20.6	3.0	21.9	30.9	17.3	1.7[a]	10.3	51.4	26.5	0.21	1.55[a]
gk-79	4.8	21.3	2.9[a]	21.9	30.6	16.9	1.7[a]	9.4	51.9	26.6	0.22	1.60[a]
gk-91	5.4	22.4	2.7[a]	23.6	28.1	15.8	2.0[a]	5.7	50.5	29.1	0.23	1.54[a]
gk-130	4.0	20.8	2.9[a]	16.2	33.4	15.2	7.5	12.5[b]	54.2	20.2	0.24	2.07
gk-109	4.7	19.5	3.1	19.5	33.1	18.0	2.0[a]	13.6[b]	52.7	24.2	0.24	1.62[a]
к-6807	2.1	25.7	2.9[a]	13.1	39.2[b]	14.1	2.9	13.5[b]	64.9[b]	15.2	0.16	3.38
gk-160	7.7[b]	20.1	3.3	28.2[b]	26.4	11.3	3.1	6.3	46.5	35.8[b]	0.27	1.12[a]
gk-125	4.8	23.6	3.1	20.0	28.6	18.7	1.3[a]	5.0	52.2	24.8	0.24	1.90
k-8409	2.9	27.3	4.1	12.1	32.9	17.5	3.1	5.7	60.2	15.0	0.24	3.63
k-7822	3.6	26.5	4.3	14.3	32.1	15.9	3.3	5.6	58.7	17.9	0.26	2.96
gk-132	7.3[b]	20.9	2.5[a]	30.9[b]	22.9	12.5	2.9	2.0[a]	43.8	38.3[b]	0.24	1.09[a]
gk-396	1.5[a]	26.7	3.4	8.8[a]	34.1	21.8[b]	3.8	7.4	60.8	10.2[a]	0.17	5.23
gk-393	0.9[a]	29.3[b]	3.9	6.9[a]	35.1	22.1[b]	1.7[a]	5.9	64.4[b]	7.9[a]	0.14	7.44[b]
gk-394	2.3	29.4[b]	4.6	7.9[a]	34.6	19.8	1.4[a]	5.1	64.0[b]	10.2[a]	0.29[b]	5.76
Yellow seeds												
gk-103	6.2	17.1[a]	3.4	30.9[b]	21.3[a]	11.6	9.5	4.2	38.3[a]	37.1[b]	0.20	0.92[a]
gk-136	6.7	18.4[a]	3.6	29.5[b]	20.2[a]	10.4[a]	11.2[b]	1.8[a]	38.6[a]	36.2[b]	0.23	1.01[a]
gk-351	7.8[b]	19.8	6.1[b]	26.2	25.7	11.7	2.7	5.9	45.5	34.1[b]	0.30[b]	1.16[a]
gk-159	7.7[b]	18.9	3.6	30.7[b]	22.5[a]	11.4	5.2	3.6	41.3	38.4[b]	0.25	0.98[a]
gk-390	4.0	26.7	3.4	15.8	32.0	16.8	1.3[a]	5.4	58.7	19.8	0.26	2.69
gk-391	7.0[b]	21.3	3.9	29.5[b]	21.7[a]	13.6	2.9	0.4[a]	43.1	36.5[b]	0.24	1.17[a]
gk-395	4.8	22.7	4.0	21.6	27.1	13.3	6.5	4.3	49.8	26.4	0.22	1.72
gk-173	6.0	21.2	4.6	29.6[b]	25.9	10.4[a]	2.3[a]	4.7	47.1	35.6[b]	0.20	1.19[a]
gk-129	1.0[a]	24.8	3.5	21.4	29.6	18.0	1.8[a]	4.8	54.4	22.4	0.05[a]	2.22
Yellow-brown or spotted seeds												
gk-141	5.8	22.7	3.5	22.2	26.3	16.0	3.5	3.6	48.9	28.1	0.26	1.62[a]
gk-143	6.6	23.9	3.8	22.0	25.0	16.1	2.7	1.1[a]	48.9	28.6	0.30[b]	1.67
gk-255	5.1	21.6	2.9	22.6	27.7	16.5	3.6	6.1	49.3	27.7	0.22	1.56[a]
gk-176	7.0[b]	17.1[a]	3.4	28.5[b]	22.8	9.6[a]	11.7[b]	5.7	39.9[a]	35.5[b]	0.25	0.96[a]
gk-121	3.5	22.5	3.5	17.5	33.6	17.1	2.3[a]	11.0	56.1	21.0	0.20	2.15
gk-65	5.8	19.4	2.8[a]	27.1	29.9	12.7	2.4[a]	10.5	49.3	32.8	0.21	1.18[a]
gk-124	5.7	25.3	3.1	21.4	26.3	16.8	1.4[a]	0.9[a]	51.6	27.1	0.27	1.87
$X_{av.}$	5.0	22.6	3.5	21.4	28.5	15.3	3.6	5.9	51.2	26.4	0.23	2.15
LSD	0.9	1.6	0.3	3.3	2.3	1.6	1.3	1.7	3.5	4.2	0.02	0.72
Cv, %	40	15	20	33	17	21	79	62	14	33	22	71

Note. k — the VIR catalogue numbers, gk — numbers in the VIR's catalogue of the genetic collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg). Ara — arabinose, Xyl — xylose, Rha — rhamnose, GalA — galacturonic acid, Fuc — fucose, Gal — galactose, Glc — glucose, HGA — homogalacturonan (HGA = GalA – Rha), pect — pectin (pect = Rha + GalA), AX — arabinoxylan (AX = Ara + Xyl); Ara:Xyl — the ratio of arabinose and xylose, RG1b:AX — the ratio of rhamnogalacturonan I and arabinoxylan. $X_{av.}$ — the mean for the whole sample, *Cv* — coefficient of variation, a — minimum values, b - maximum values.

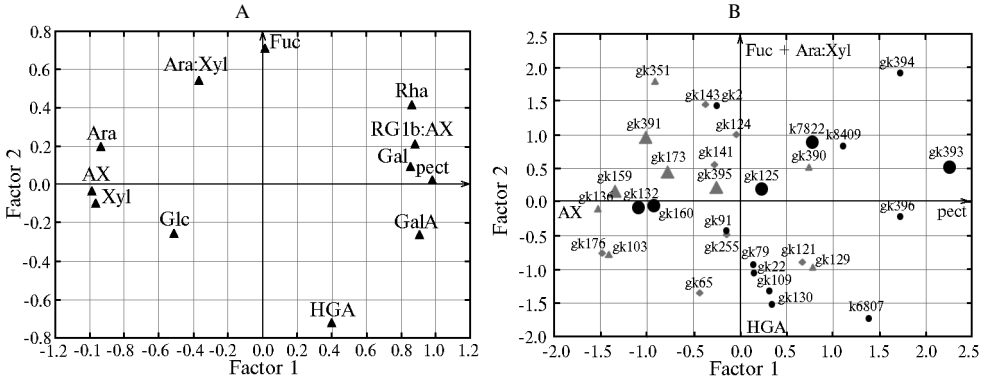
On average in the sample, pectins accounted for 51.2 % of the total amount of mucilage sugars (from 38.3 to 64.9 %, *Cv* = 14 %) (see Table 2). Brown-seeded samples of the gk-393, gk-394, gk-396, k-6807, k-8409, k-7820 lines and yellow-seeded sample of gk-390 produced the greatest amount of pectins. Of interest, the gk-393, gk-394 and gk-390 lines originated from the same variety, Linola. Yellow-seeded lines with the *s1* gene (gk-136, gk-103, gk-176) and the *YSED1* gene (gk-159) were characterized by the lowest content of pectins. Both the maximum and the minimum amounts of pectins were caused by the content of RG1b (with the exception of the sample of k-6807, in which a high proportion of GalA and, accordingly, HGA was observed). An increased amount of HGA was also found in the gk-109 and gk-130 lines with red-brown seeds and in the gk-121 line with light yellow-brown seeds. The minimum proportion of HGA in mucilage was found in the wild-type gk-2 line, the yellow-seeded sample of the gk-391 line, and in the lines with a modified color of seeds, such as gk-124 and gk-143.

In most lines, the content of RG1b in mucilage was higher than that of AX: the RG1b:AX ratio averaged 2.2, with a median of Md = 1.6. However, this parameter varied greatly (*Cv* = 71 %), because there were samples (gk-393, gk-394, gk-396), in which the amount of RG1b was 7.4-5.5 times higher than that of AX, while in the yellow-seeded lines carrying the *s1* gene (gk-103, k-176, k-136) and the *YSED1* gene (gk-159), RG1b and AX were present in equal portions (see Table 2). Such a high variability in the ratio of acid and basic polysaccharides in the mucilage from flax seeds is consistent with the data presented by W. Cui et al. [26].

The portion of galactose in mucilage on average in the sample was 15.3 % of the total amount of mucilage carbohydrates with a variation from 9.6 to 22.1 %. The maximum content of Gal was found in the gk-393, gk-394, gk-125 lines with brown seeds, while the minimum in the yellow-seeded samples of the gk-173 (the *ysed2* gene), gk-136, gk-103, gk-176, gk-351 (the *s1* gene), gk-159 (the *YSED1* gene) lines and in the gk-160 line with brown seeds. Fucose accounted for an average of 3.5 % (ranging from 2.5 to 6.1 %). The highest values of this parameter were observed for yellow seeds from the gk-351 (the *s1*, *rs1* genes) and gk-173 (the *ysed2* gene) lines, and brown seeds from the gk-394, k-7822 lines, while the lowest values were seen for the gk-132, gk-91, gk-130, gk-79, gk-22, k-255, gk-65 and k-6807 lines. The glucose content varied greatly within the sample studied (*Cv* = 79 %), averaging to 3.6 % (Md = 2.7 %), whereas for the gk-130 line and the yellow-seeded gk-159 and gk-395 lines (the *YSED1* gene), this parameter was almost twice as high, i.e. > 5 %, and in the lines carrying the *s1* gene (gk-103, gk-136 and gk-176), it reached 9.5-11.7 % (see Table 2). In the observed disproportion, the origin of Glc can be associated with the xyloglucans of the primary cell wall, which is more fragile in the mutant lines, and also with starch extracted from the deeper layers of the seed (endosperm).

Using the principal component analysis, we have revealed two factors influencing the composition of mucilage in the lines studied (Fig.). The first factor (F1) determined the ratio of the two main polysaccharides in the mucilage, i.e. of pectins and pentosans (AX). The analysis identified the antagonism between AX, Ara, Xyl, on the one hand, and between pect, GalA, Gal — on the other. This factor determined about 60 % of the total variability. Based on this, two groups of lines were identified: with the highest percentage of AX, such as yellow-seeded samples of the gk-136, gk-103, gk-159, gk-391, k-351, gk-173 lines, their relatives (gk-160 and gk-176), as well as the gk-132 line; with the highest proportion of pectins, such as wild-type samples of the gk-393, gk-394, gk-396, k-6807, k-8409, k-7822 lines and yellow-seeded samples of the gk-129, gk-390 lines. The second factor (F2) determined the ratio of HGA with Fuc and

Ara:Xyl. There was an antagonism revealed between HGA, on the one hand, and Fuc, Ara:Xyl, on the other hand. F2 was responsible for about 15 % of the total variability, with the samples divided into the following groups: the first one with the highest content of HGA (k-6807, gk-130, rk-65, gk-22, gk-79, gk-121, gk-109) and the smallest ratio of Ara:Xyl (gk-129, gk-103); the second group with the largest percentage of Fuc (gk-351), the maximum ratio of Ara:Xyl (gk-2, gk-143, gk-394) and with the lowest content of HGA (gk-124, gk-391).



The distribution of sugars and polysaccharides in the mucilage of seeds in the studied lines of flax (*Linum usitatissimum* L.) (A**) and these very lines according to these parameters (**B**) using the principal component analysis:** Factor 1 — a ratio of pectins and pentosans, Factor 2 — a ratio of HGA with Fuc and Ara:Xyl; Ara — arabinose, Xyl — xylose, Rha — rhamnose, GalA — galacturonic acid, Fuc — fucose, Gal — galactose, Glc — glucose, HGA — homogalacturonan (HGA = GalA – Rha), pect — pectin (pect = Rha + GalA), AX — arabinoxylan (AX = Ara + Xyl), RG1b — ramnogalacturonan 1. Circles, triangles and diamonds (B) mark the lines with red-brown, yellow, yellow-brown or spotted seeds, respectively. Large icons correspond to the lines with large seeds.

Within the system of two factors, two groups of lines were distinguished, where the first group consisted of the yellow-seeded lines (gk-136, gk-103, gk-159, gk-391, gk-351, gk-173) and their relatives (gk-160, rgk-176) with the highest percentage of AX; the second group included the brown-seeded lines (gk-22, gk-79, gk-109, gk-130) and a sample of the gk-121 line with the highest proportion of pectins and an increased content of HGA in them, as well as the gk-129 line with a low Ara:Xyl ratio.

The multi-dimensional scaling method made it possible to divide the characteristics and lines between groups equal to those obtained by the factor analysis (data not shown).

To determine the effect of morphological features of flax on the carbohydrate composition of mucilage, we used the rank Mann–Whitney U test (Table 3). A significant decrease in the proportion of arabinoxylan (percentages of AX, Ara, Xyl) and increase in pectins (GalA, Gal, HGA, pect, RG1b:AX), as well as an increased factor loadings of F1 were found in mucilage extracted from brown seeds compared to that from the rest ones. In yellow seeds, the content of AX, Xyl, and Fuc was significantly higher, while that of pect, HGA, GalA and Gal lower, also, low loadings of F1 were revealed. In oil flax, a small yet significant excess of the Fuc portion was noted (Table 3).

Lines homozygous for the *s1* gene had significantly higher values for Glc and arabinoxylan (AX, Ara, Xyl) and lower values for Gal and pectins (pect, Rha, GalA, RGb:AX), as well as low values of factor loadings by F1. We revealed no significant differences in the mucilage composition for the lines carrying the *YSED1* and *pf1* genes (see Table 3).

The analysis of variance and Student's *t*-test confirmed the conclusions drawn based on the rank Mann-Whitney U test (data not shown).

3. A comparison of the carbohydrate composition of mucilage (%) in the lines of flax (*Linum usitatissimum* L.) with various seed coloring and type of use (using the Mann-Whitney U test)

Characteristic	Presence	*n*	Ara	Xyl	Rha	GalA	Fuc	Gal	Glc	HGA	pect	AX	RGb:AX	F1
Oil use	Yes	20	4.8±0.5	21.6±1.9	22.9±0.8	28.2±1.1	3.7±0.2	15.2±0.8	3.6±0.6	5.3±0.6	51.1±1.8	26.4±2.3	2.35±0.41	1.15±0.53
	No	11	5.2±0.4	21.2±1.2	22.2±0.8	29.2±1.4	3.2±0.1	15.6±0.7	3.5±1.0	7.0±1.5	51.3±1.8	26.3±1.6	1.78±0.18	0.68±0.48
	p		0.97	0.71	0.68	0.80	0.04*	0.71	0.46	0.48	0.80	0.84	0.93	0.90
Seeds:														
brown	Yes	15	4.2±0.5	17.9±1.9	23.9±0.9	31.2±1.1	3.4±0.2	16.9±1.1	2.7±0.4	7.3±1.0	55.1±1.7	22.0±2.4	2.84±0.50	0.52±0.25
No	Нет	16	5.7±0.4	24.8±1.2	21.5±0.7	26.1±1.0	3.7±0.2	13.9±1.0	4.4±0.9	4.6±0.8	47.5±1.5	30.5±1.5	1.50±0.13	–0.48±0.19
	p		0.02*	0.01*	0.08	0.002*	0.18	0.02*	0.15	0.03*	0.00*	0.01*	0.03*	0.01*
yellow	Yes	9	5.7±0.7	26.1±1.8	21.2±1.0	25.1±1.3	4.0±0.3	13.0±0.9	4.8±1.2	3.9±0.6	46.3±2.3	31.8±2.3	1.45±0.21	–0.63±0.29
No	Нет	22	4.6±0.4	19.5±1.4	23.2±0.7	29.9±0.9	3.4±0.1	16.3±0.6	3.1±0.5	6.7±0.8	53.2±1.4	24.2±1.8	2.44±0.36	0.26±0.20
	p		0.10	0.03*	0.14	0.01*	0.03*	0.02*	0.28	0.02*	0.02*	0.03*	0.06	0.03*
Homozygous for the gene:														
s1	Yes	4	6.9±0.3	28.8±1.0	18.1±0.7	22.5±1.2	4.1±0.7	10.8±0.5	8.8±2.1	4.4±0.9	40.6±0.6	35.7±0.6	1.01±0.05	–1.33±0.14
No	Нет	27	4.7±0.4	20.3±1.3	23.3±0.6	29.4±0.8	3.5±0.1	16.0±0.6	2.8±0.3	6.1±0.7	52.8±1.2	25.0±1.6	2.32±0.30	0.20±0.18
	p		0.01*	0.02*	0.003*	0.01*	0.32	0.01*	0.01*	0.56	0.003*	0.02*	0.003*	0.003*
YSED1	Yes	4	5.9±0.9	24.4±3.5	22.4±1.6	25.8±2.4	3.7±0.1	13.8±1.1	4.0±1.2	3.4±1.1	48.2±3.9	30.3±4.4	1.64±0.38	–0.46±0.46
No	Нет	27	4.8±0.4	21.0±1.3	22.7±0.7	28.9±0.9	3.5±0.1	15.6±0.6	3.5±0.6	6.3±0.7	51.6±1.4	25.8±1.7	2.23±0.31	0.07±0.19
	p		0.35	0.44	1.00	0.22	0.26	0.29	0.60	0.08	0.41	0.38	0.60	0.32
pf1	Yes	5	5.1±1.1	23.3±1.3	22.0±1.3	26.3±1.2	3.4±0.1	15.2±1.5	4.6±1.8	4.3±0.9	48.3±2.3	28.4±2.1	1.61±0.2	–0.30±0.36
No	Нет	26	4.9±0.4	21.1±1.5	22.8±0.7	29.0±1.0	3.6±0.1	15.3±0.7	3.4±0.5	6.2±0.7	51.7±1.5	26.0±1.8	2.26±0.32	0.06±0.20
	p		0.63	0.52	0.96	0.22	1.00	0.96	0.24	0.39	0.26	0.67	0.67	0.49

N o t e . *n* — the numer of the studied samples; Ara — arabinose, Xyl — xylose, Rha — rhamnose, GalA — galacturonic acid, Fuc — fucose, Gal — galactose, Glc — glucose, HGA — homogalacturonan (HGA = GalA – Rha), pect — pectin (pect = Rha + GalA), AX — arabinoxylan (AX = Ara + Xyl); Ara:Xyl — the ratio of arabinose and xylose, RG1b:AX — the ratio of rhamnogalacturonan 1 and arabinoxylan, F1 — loadings by Factor 1. The mean ($X_{av.}$) and standard errors of mean (±SE) are given; p — the likelihood of similarity between alternative groups (the availability or lack of a characteristic).

* Differences between alternative groups by the analyzed parameter are significant at $p < 0.05$.

Therefore, the studied lines of flax have the wide polymorphism in terms of the composition of mucilage. Yellow seeds (the *s1* gene) compared to the brown ones contain on average a higher level of neutral polysaccharides (arabinoxylans), while brown ones were higher in acid polysaccharides (pectins). However, there are lines that have the opposite ratio of acid and basic fractions of the mucilage. The impact of other genes (*YSED1* and *pf1*), controlling the changed color of seeds in flax, on the composition of extracted mucilage was not revealed. For the first time, we demonstrated the way to use the results of determination of the factor loadings on the lines as an independent complex characteristic, allowing sampling by a set of characteristics.

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BIOLOGICAL EFFECTS IN WHEAT (*Triticum vulgare* L.) UNDER THE INFLUENCE OF METAL NANOPARTICLES (Fe, Cu, Ni) AND THEIR OXIDES (Fe_3O_4, CuO, NiO)

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Abstract

In recent decades, the development of nanotechnology has led to the need for a thorough study of ultrafine metal security. It is known that many of ultrafine metals have pro-oxidant and toxic properties. However, no studies have been performed to comprehensively compare of how the metal and metal oxide nanoparticles (NP) affect plants. We first examined complex morphophysiological parameters in wheat (*Triticum vulgare* L.) seedlings exposed for 2 days to spherical nanoparticles (NPs) of Fe^0 or Fe_3O_4, Cu^0 or CuO, and Ni^0 or NiO at 0.0125 to 1.0 M concentrations. Analysis of metric characteristics showed that the sensitivity to Cu^0 NP and Ni^0 NP was much higher than that to their oxides (CuO, NiO). NiO NP and CuO NP had no lethal effects at all tested concentrations though caused a significant (more than 2-fold) reduction in most of the growth parameters. At low (less than 0.05 M) levels of Fe NP and Fe_3O_4 NP the seedlings showed a significantly stimulated growth as compared to control. In contrast, the Cu^0 NP, CuO NP, Ni^0 NP and NiO NP caused toxic effect on growth which increased as the metal level elevated. The analysis showed a high sensitivity of roots, as the first target for the toxic agents, to low metal concentrations. At low Cu^0 NP, CuO NP, NiO NP and Ni^0 NP levels in the medium, the root growth was 19 times, 7.4 times, 4.8 times and 2.2 times lower as compared to control. Basing on morphological parameters, the nanoparticles were arranged in the following ascending order of their toxicity for growth of the main root and the first leaf in *T. vulgare*: $Fe_3O_4 \rightarrow Fe^0 \rightarrow NiO \rightarrow CuO \rightarrow Ni^0 \rightarrow Cu^0$. Analysis of photosynthetic pigments showed that a 2-day exposure to Fe NP and Fe_3O_4 NP led to generally more positive and stable effects on pigments as compared to copper and nickel. In the presence of less than 0.05 M metal the seedlings were green with a marked stimulation of pigmentation. At the same time, there was the strongest negative effect of the Cu NP on chlorophyll a (22.0-33.0 %), and Ni NP on chlorophyll b (16.0-68.0 %). The influence of CuO toward lower chlorophyll content was dose-dependent: a statistically significant decrease in chlorophyll a was observed at 0.05, 0.1 and 0.5 M (9.0-21.5 %), and in chlorophyll b at 0.0125 and 0025 M (4.0-15.0 %). NiO NP had insignificant inhibitory effect on chlorophylls at 8.7 % decrease. Carotenoids were less sensitive to tested nanoparticles as compared to chlorophylls. Analysis of MDA content in the seedlings showed that nanoparticles influenced lipid peroxidation in the roots rather than in leaves. The effect of MDA accumulation in roots was the most apparent after exposure to some NPs, especially Ni^0 NP, Su^0 NP and CuO NP which caused MDA increase exceeding control by 17.0 %, 25.0 % and 18.7 %, respectively. The Fe^0, Fe_3O_4 and NiO NPs did not affect the MDA content, whereas Fe_3O_4 NP reduced the MDA level by 30.0 %. Thus Fe^0, Fe_3O_4, Cu^0, CuO, Ni^0 and NiO nanoparticles selectively affect cell metabolism and exhibit different biological activity depending on chemical composition and concentrations.

Keywords: *Triticum vulgare* L., metal nanoparticles, growth rates, photosynthetic pigments, malondialdehyde, lipid peroxidation

Despite the multi-level intracellular protection of cells from stress, when affected with adverse factors, the concentration of reactive oxygen species (ROS)

in plants increases and a cascade mechanism of oxidative stress is triggered, leading to the destruction of vital cellular components and to cell death. Plants are constantly exposed to ROS, especially in the presence of nanoparticles (NPs) of metals [1]. Using various mechanisms, most species have the ability to adapt to NPs of metals [2]. However, these are not sufficient to prevent oxidative stress and the manifestation of cyto- and genotoxic effects.

It has been shown that the Cu^0 nanoparticles at a high concentration (1,000 mg/l) adversely affect the growth of the bean seedlings, penetrate through the cell membrane and form aggregates with cellular components [3]. Nanoparticles containing Cu^0 and Cu_2O can block water channels by adsorption and increase the inflow of ROS into the roots of onions, which in turn disrupts the cell division and metabolism [4]. In an experiment with *Triticum aestivum*, the inhibition of shoot growth after exposure to the Cu^0 NPs at various concentrations [5] was described. It was also reported that as the concentration of the Cu^0 NPs and their agglomerates increased, the rates of bioaccumulation of this element in plants was elevated.

Previously, we have compared the effects of the Fe^0 NPs (diameter 80±5 nm), Fe_3O_4 (width 50-80 nm, height 4-10 nm) and the $FeSO_4$ (II) solution on common wheat (*Triticum vulgare* L.) plants [6]. At the same time, the greatest susceptibility (decrease in seed germination, suppression of leaf growth, and decrease in the amount of pigments in seedlings) was recorded with increasing ferric sulfate (II) concentration in the medium, and there were differences observed between the effects of nanoparticles, such as the germination activity was more strongly affected by Fe^0 NPs, and the length of the leaves and the amount of pigments by Fe_3O_4 NPs.

Toxicity of water-soluble metal nanoparticles is associated primarily with a high redox potential of ions and oxidation of biological molecules. The most important factor is the ability of nanoparticles to induce oxidative stress, i.e. to generate ROS [7]. When studying the oxidative damage of plant cells after treatment with the CuO NPs, it was established that the effect occurred not in ions of copper, but in its oxide, because of its limited solubility in the growth medium [8]. In the roots of tomato plants, after incubation with NiO NPs, a high pool of ROS and increased lipid peroxidation (by 39.3 to 49.5 % vs. control) were found in the protoplast [9]. However, an attempt to distinguish between oxidative induction either by NiO oxide or nickel ions failed, since both of these compounds were available in the root cells. It has been suggested that Ni^{2+} ions generate in plants the $HO^{\bullet-}$ radical through the Haber-Weiss reaction.

In recent years, it has been established that oxidative stress can be caused by nanoparticles of many metals (silver, gold, iron), or ferrites, as well as oxides of zinc, nickel, copper, iron, titanium and silicon. The main attention is focused on studying the absorption of nanometals and plant growth rates after exposure to nanoparticles of metals and their oxides. Comprehensive studies revealing the mechanisms of phytotoxic properties of nanoparticles of transition metals (iron, copper and nickel) in comparison with their oxide forms have not been previously conducted.

In this paper, we present for the first time an approach to a comparative assessment of the toxicity of nanoforms based on Fe, Cu, and Ni, taking into account not only the morphological characteristics of plants, but also important physiological stress markers such as the activity of the photosynthetic apparatus and the degree of peroxide oxidation of membrane lipids.

The aim of the study was to assess morphophysiological indices in wheat seedlings in response to the effects of the nanoparticles of iron, copper, nickel

and their oxides at various concentrations.

Techniques. The research used commercially available spherical nanoparticles of iron Fe^0, copper Cu^0, nickel Ni^0 and their Fe_3O_4, CuO and NiO, produced by electric explosion of a conductor in an argon atmosphere. Material research testing of the preparations (particle size, polydispersity, volume, quantitative content of fractions, surface area) included electronic scanning, translucent and atomic force microscopy using LEX T OLS4100, JSM 7401F, JEM-2000FX (JEOL, Japan). Particle size distribution was investigated using a Brookhaven 90Plus/BIMAS ZetaPALS and a Photocor Compact nanoparticles analyzer (Photocor, Russia) in lyosoles obtained after treatment with the ultrasonic disperser UZDN-2T (NPP Akadempribor, Russia) for 30 min (35 kHz , 300 W, amplitude of oscillations of 10 μA).

Wheat (*Triticum vulgare* L.) seeds were disinfected with 0.01 % $KMnO_4$ solution for 5 min and, after triple washings with distilled water, placed on a substrate of filter paper in plastic Petri dishes (9 cm in diameter), 20 pieces each, at a distance of at least 0.5-1.5 cm from each other. For the relative synchronization of growth, equally sprouted seeds were chosen and transferred to wet filter paper in separate plastic dishes. Within 1 day, plants were in new conditions without additional influences. On day 3, 5 ml of a suspension of the nanoparticles of Fe^0, Cu^0, Ni^0 and Fe_3O_4, CuO и NiO oxides in dilutions to a metal concentration of 0.0125 to 1.0 M were added to the dishes with sprouts. The suspensions were pre-sonicated at 35 KHz in a Sapphire TTC bath-type source (ZAO PKF Sapphire, Russia) for 30 min. A group of control plants was kept in distilled water. In this setting, shoots were not contacted with nanoparticles, which could enter the plant only through the roots. After the addition of the nanoparticle suspension, to uniformly distribute them in the media, the Petri dishes were shaken in the ST-3M thermal shaker (Elmi, Latvia) for 5 min. The control and study samples prepared this way were left in a climatic chamber (Agilent, USA) at 12-hour illumination, 22±1 °C and humidity 80±5% for 3 days, and not allowing to dry [6].

On day 5, after the breakthrough of the coleoptile, physiological and biochemical indices were evaluated. In 10 sprouts, the ruler was used to measure the length of the 1st leaf (from the base to the apex of the main leaf) and the main root (from the root neck to the tip of the main root) within the accuracy of 1 mm. Three plants from each variant were selected to determine the content of photosynthetic pigments (PPs) and oxidative stress indicators.

The amount of PPs was measured in ethanol extracts using the spectrophotometric method according to the standard procedure [10]. The leaves of the sprouts (one leaf from each of 10 sprouts from each sample) weighing 30 g were quickly cut with scissors and homogenized in a chilled mortar with $CaCO_3$, calcined quartz sand and a small amount (2-3 ml) of 96 % ethyl alcohol. After precipitation (the K-23 centrifuge, GDR), about 20 ml of 96 % ethyl alcohol was re-added to the resulting suspension and homogenized once again. For maximum extraction of pigments, this procedure was repeated 2-4 times (to almost complete transparency of the extractant) and the extracts obtained for each sample were combined. Insoluble particles were separated by centrifugation, and 96 % ethanol was added to the extract until the predetermined final volume. The absorbance of the extracts was determined on a single-beam automated photometer KFK-3 (Russia) at various wavelengths (665 nm, 649 nm and 450.5 nm) corresponding to maximum absorbance of chlorophyll *a*, chlorophyll *b* and carotenoids.

The lipid peroxidation (LPO) was assessed by the accumulation of the product of the malonic dialdehyde (MDA) reaction with thiobarbituric acid (TBA)

(Lenreactiv, Russia). The evaluation was carried out using a method based on the measurement of a colored trimethine complex using trichloroacetic acid (TCA; Reachim, Russia) at λ = 532 nm [11]. To accomplish this, 100 mg of wheat tissue was homogenized with 200 μl of 20 % TCA. The resulting homogenate was centrifuged for 5 min at 12,000 g. The supernatant was used as a sample for analysis. 100 μl of the supernatant were added to each of the two tightly closed test tubes. 100 μl of 20 % TCA was added to one of the samples, it served as a control in spectrophotometric measurements. To another sample, 100 μl of 0.5 % TBA solution was added. The samples were incubated in a boiling water bath (100 °C) for 30 min, and then cooled at room temperature.

Lab experiments were performed in a 3-fold biological replication, analytical determination for each sample in triplicate. The reliability of the experimental data was assessed by mathematical statistics using Statistica 10.0 application software package (StatSoft, Inc., USA). The arithmetic mean (*M*) with the standard error of the mean (*m*) are presented.

Results. The results of nanoparticles qualification are shown in Table 1.

1. The characteristics of metal-containing nanopowders used in the study (*M*±*m*)

Preparation	Average size, nm	Specific surface (S_{spec}), m^2/g	ζ- potential, mV
Fe^0 [1]	70±4.12	7.7	13±0.5
Fe_3O_4[1]	76±3.11	10.1	19±0.5
Cu^0 [2]	54±2.06	12.5	31±0.1
CuO[2]	65±2.45	14	47±0.1
Ni^0 [1]	57±1.15	4.5-6.0	25±0.5
NiO[2]	68±2.21	12	29±0.5

N o t e. Manufacturers: 1 — OOO Plasmotherm (Russia), 2 — Powder Advanced Technologies LLC (Russia).

The linear growth of plants is an important indirect characteristic of the intensity of cell division or stretching. We analyzed the metric indices in sprouts after 2 days of exposure to different concentrations of NPs of metals and their oxides (Fig. 1).

The high resistance of plants was observed when affected with iron-containing nanoparticles, which was expressed in a significant

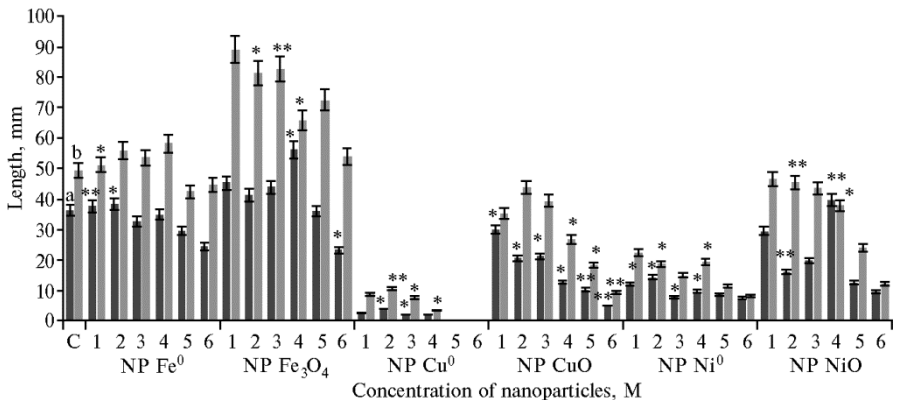

Fig. 1. Length of the main root (a) **and 1st leaf** (b) **in wheat (*Triticum vulgare* L.) sprouts after a 2-day incubation with nanoparticles of metals and their oxides in different concentrations:** 1 — 0.0125 M, 2 — 0.025 M, 3 — 0.05 M, 4 — 0.1 M, 5 — 0.5 M, 6 — 1.0 M; C — control.
*, ** Differences vs. control are significant at $p \le 0.05$ and $p \le 0.01$, respectively..

promotion of growth of sprouts in comparison to the control. Therefore, in the presence of 0.0125 M Fe^0 NPs, the length of the 1st leaf and the main root exceeded the values in the controls by 3.5 ($p < 0.01$) and 3.6 % ($p < 0.05$), respectively. In addition, nanoparticles promoted root growth (by 5.7 %, $p < 0.05$). At the same time, a significant increase in the length of the 1st leaf compared to the control was observed in the presence of Fe_3O_4 NPs at concentrations of 0.025 and 0.05 M, by 39.0 ($p < 0.05$) and 40.0 % ($p < 0.01$), respectively. A 2-

fold increase in the metal concentration (0.1 M) promoted the growth of the root and leaf (by 35.0 and 25.0 %, respectively, $p < 0.05$). At the maximum concentration of the Fe_3O_4 NPs (1.0 M), the root length decreased by 56.0 % ($p = 0.05$).

On the contrary, when using nanoparticles of Cu^0 and CuO, Ni^0 and NiO, a toxic effect developed which was enhanced with an increase in the amount of metal in the solution.

The presence of the Cu^0 NPs in the aqueous phase in concentrations of 0.5 and 1.0 M completely inhibited the growth of the sprout and root and even in minimal dilutions it markedly decreased this value. A significant inhibition of the growth of the 1st leaf was noted in options with 0.025; 0.05 and 0.1 M Cu^0 NPs with 4.7 ($p < 0.01$), 6.5 and 14.5 times ($p < 0.05$) decrease vs. control, respectively, and the growth of the root at 0.025 and 0.05 M Cu^0 NPs decreased 9.8 and 19.0 times ($p \leq 0.05$). The addition of 0.05, 0.1 and 1.0 M CuO NPs significantly affected the leaf length resulting in 1.8-fold, 2.7-fold ($p < 0.05$) and 5.3-fold ($p < 0.01$) decrease, respectively, when compared to the control samples. The main root was 1.2-7.4 times ($p < 0.05$) shorter than in the control for all dilutions of nanoparticles.

The nanoparticles of Ni^0 at doses of 0.025 and 0.1 M caused a significant 2.6-fold and 2.5-fold ($p < 0.05$) decrease in the leaf length. The root of the plants developed poorly at all the concentrations of Ni^0 NPs and was 2.5-4.8-fold shorter than in the control ($p < 0.05$). The effect of nanoparticles of nickel oxide turned out to be weaker and was debatable: NiO NPs at concentrations of 0.025 and 0.1 M inhibited the growth of the aerial part by 8.0 ($p = 0.01$) and 3.4 % ($p < 0.05$), at concentrations of 0.025 and 0.05 M slowed the growth of the root part 2.2 and 1.8 times, whereas the concentration of 0.1 M, on the contrary, led to an increase in the root length by 7.0 %.

The content of PPs in plants can be a more informative indicator of the toxicity of NPs compared with growth rates. In our experiments, the amount of PPs varied depending on the type and concentration of nanoparticles and was not always correlated with the dynamics of the length of the 1st leaf.

After 2 days of exposure of sprouts to the Fe^0 and Fe_3O_4 nanoforms, the effect on the pigment content was generally more favorable and stable than when using copper- and nickel-based nanomaterials. Thus, after the incubation in Fe^0 NPs and Fe_3O_4 NPs at a concentration of < 0.05 M, the green color of the sprouts was preserved and even the synthesis of pigments increased. The content of chlorophylls *a* and *b* was significantly increased compared to the control: by 1.5-7.6 and 2.6-16.5 % ($p \leq 0.05$), as influenced by 0.0125-0.05 M Fe^0 NPs, and by 2.0-11.7 ($p < 0.05$) and 1.0 % ($p = 0.001$) at 0.0125 and 0.05 M Fe_3O_4 (Table 2). The sum of chlorophylls increased by 1.9-7.0 % under the influence of both forms of nanoparticles. At 0.5 and 1 M, the amount of chlorophyll *a* decreased for Fe^0 NPs and Fe_3O_4 on average by 13.0-17.0 and 2.0 % ($p \leq 0.05$), while the content of chlorophyll *b* did not change significantly.

With a similar effect of Cu^0 NPs, along with a significant suppression of the growth and the development of a dark brown coloration of the leaves, a decrease in the content of green pigments was recorded on average by 19.0 % ($p < 0.05$). Cu^0 NPs caused a decrease in the chlorophyll *a* content by 22.0-33.0 %, even at a concentration that was 4 times lower than the maximum sublethal dose (0.0125 M) (see Table 2). At the same time, the amount of chlorophyll *b* remained close to the control or changed insignificantly.

In the option with the CuO NPs, an overall decrease in the total amount of pigments even after exposure to low concentrations (< 0.05 M) was 11.8 % vs.

control. It should be noted that the CuO NPs influenced the amount of chlorophyll *b* (4.0-30.0 %) to a greater extent than the Cu^0 NPs. A statistically significant decrease in the chlorophyll *a* content vs. control, i.e. by 9.0 % ($p < 0.05$), 10.5 % ($p = 0.01$) and 21.5 % ($p = 0.05$), was noted at 0.05, 0.1 and 0.5 M, respectively, and in the chlorophyll *b* content by 4.0 % ($p < 0.05$) and 15.0 % ($p = 0.05$) at 0.0125 and 0.025 M, respectively.

2. The content of photosynthetic pigments (mg/g wet weight) **in wheat (*Triticum vulgare* L.) leaves after 2 days of incubation of sprouts with nanoparticles** (NP) **of metals and their oxides in different concentrations** ($M \pm m$)

Option	NP concentration, M	Chlorophyll *a*	Chlorophyll b	Carotenoids
Control		1.585±0.14	0.591±0.16	0.278±0.04
Fe^0	0.0125	1.611±0.04*	0.607±0.03*	0.281±0.01*
	0.025	1.609±0.01***	0.708±0.01*	0.397±0.05*
	0.05	1.716±0.05*	0.622±0.02**	0.304±0.11
	0.1	1.244±0.22	0.473±0.21	0.296±0.04*
	0.5	1.402±0.21*	0.441±0.18	0.273±0.05*
	1.0	1.352±0.18*	0.463±0.12	0.268±0.12
Cu^0	0.0125	1.295±0.10*	0.569±0.22	0.369±0.08
	0.025	1.209±0.03*	0.595±0.09	0.372±0.12
	0.05	1.192±0.02*	0.586±0.04*	0.281±0.05*
	0.1	1.284±0.06*	0.579±0.09	0.299±0.02*
Ni^0	0.0125	1.581±0.20	0.588±0.02**	0.275±0.13
	0.025	1.448±0.03*	0.508±0.07	0.269±0.01*
	0.05	1.452±0.14*	0.406±0.04*	0.276±0.04*
	0.1	1.434±0.05**	0.383±0.03***	0.273±0.05*
	0.5	1.394±0.94	0.397±0.15	0.254±0.03**
	1.0	1.398±0.18	0.352±0.01*	0.272±0.04*
Fe_3O_4	0.0125	1.621±0.04*	0.597±0.07	0.288±0.19
	0.025	1.796±0.03**	0.548±0.08	0.281±0.13
	0.05	1.583±0.23	0.552±0.12	0.289±0.16
	0.1	1.534±0.12	0.581±0.14	0.273±0.14
	0.5	1.564±0.01**	0.563±0.06	0.286±0.05**
	1.0	1.552±0.02*	0.534±0.08	0.292±0.03*
CuO	0.0125	1.524±0.09	0.567±0.04**	0.285±0.15
	0.025	1.548±0.31	0.512±0.03*	0.251±0.12
	0.05	1.452±0.05*	0.476±0.05**	0.259±0.02**
	0.1	1.434±0.02**	0.453±0.07	0.243±0.01*
	0.5	1.304±0.05*	0.491±0.18	0.254±0.03*
	1.0	1.352±0.14	0.483±0.12	0.255±0.04*
NiO	0.0125	1.581±0.10	0.590±0.03*	0.273±0.12
	0.025	1.579±0.38	0.568±0.02*	0.251±0.01*
	0.05	1.452±0.30	0.646±0.15	0.269±0.03*
	0.1	1.374±0.16	0.583±0.05**	0.263±0.05*
	0.5	1.396±0.09	0.521±0.18	0.274±0.16
	1.0	1.162±0.19	0.613±0.15	0.252±0.03*

*, **, *** Differences between mean values in the control and in the test option are statistically significant at $p \le 0.05$, $p < 0.01$, $p < 0.001$, respectively.

The Ni^0 nanoparticles had an effect on the content of both forms of chlorophyll (*a* + *b*) in plants of *T. vulgare* comparable to that of CuO NPs. On average, the value decreased by 18.5 %, and with an increase in the metal concentration in the media, the effect was enhanced. It was found that chlorophyll *b* is more susceptible to the Ni^0 NPs. In the test option, a dramatic decrease in its amount (by 16.0-68.0 %) was observed: at a minimum concentration (0.0125 M) of the Ni^0 NPs, the parameters were similar to the control ($p = 0.01$) followed by a dose-dependent decrease by 45.5 % ($p < 0.05$), 54.0 % ($p = 0.001$), and 68.0 % ($p < 0.05$) in the presence of 0.05, 0.1 and 1.0 M Ni^0 NPs. The chlorophyll *a* content was no more than 10.0 % reduced compared to control (up to 1.434-1.452 mg/g wet weight vs. 1.585 ± 0.04 mg/g in the control) (see Table 2). Nanoparticles of NiO exerted an insignificant inhibitory effect, which was manifested in a decrease in the content of chlorophylls *a* + *b* by 8.7 % as compared to the control. The amount of chlorophyll *b* was similar to that in the control, not only at initial concentrations of nickel oxide (0.0125 and 0.025 M,

$p \leq 0.05$), but also at 0.1 M ($p < 0.01$). At the same time, a decrease in the amount of chlorophyll *a* lowered down to 36.0 % in comparison to the control. One of the reasons for this might be the limited transport of the NiO NPs to the shoot from the roots.

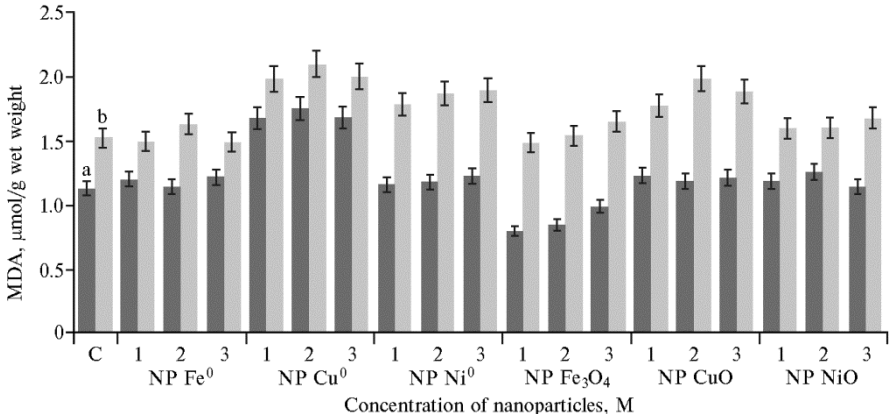

Fig. 2. The content of malonic dialdehyde (MDA) in leaves (a) **and roots** (b) **after a 2-day incubation of wheat (*Triticum vulgare* L.) sprouts with nanoparticles of metals and their oxides in different concentrations:** 1 — 0.025 M, 2 — 0.05 M, 3 — 0.1M; C — control ($p \leq 0.05$).

However, carotenoids in the leaves of the *T. vulgare* sprouts appeared to be less susceptible to the NPs of metals compared to chlorophylls (see Table 2). Therefore, the accumulation of yellow pigments within the normal range (0.1-0.5 mg/g wet weight) was observed, and only in few cases there was a slight change in this value. In the option with the Cu^0 NPs, the plants differed from the control ones based on the increased content of carotenoids (by 14.5 %). In contrast, a relatively small (less than 10.0 %), but a stable decrease of this value was revealed in the sprouts at concentrations of 0.05 M CuO NPs and Ni^0 NPs, as well as at 0.5 M Fe^0 NPs (by 10.0, 3.5 and 3.0 %, respectively).

The obtained data indicate that a very probable cause of lesser resistance to the majority of the investigated nanoparticles (Ni^0, Cu^0 и CuO) is the increasing intensity of the oxidative stress in plants influenced by metals. The assessment results demonstrated that the MDA content in the leaves and roots of the control plants was in the range from 0.79±1.01 to 1.75±0.14 and from 1.48±0.22 to 2.11±1.5 μmol/g wet weight. In the presence of nanoparticles, this value changed in the roots more markedly than in the leaves. The effects of the Fe^0, Fe_3O_4 and NiO nanoparticles led to virtually no increase in the MDA content in wheat roots (Fig. 2). In contrast, the effects of some nanoparticles, especially Ni^0, Cu^0 and CuO, contributed to a significant accumulation of MDA in the root part of plants with an increase in the analyzed parameter by 17.0, 25.0 and 18.7 % ($p < 0.05$), respectively.

A significant increase in LPO (by 33.0 % vs. control) in the leaves was observed for the effects of NPs of Cu^0 (see Fig. 2), correlating to an increase in the content of carotenoids (see Table 2), which under the conditions of our experiment did not probably participate in preventing the oxidation of lipid fatty acids in cell membranes. Under the same conditions, the formation of MDA at treatment with the Fe_3O_4 NPs had only a minor tendency to decrease, i.e. by 30.0 % compared to control. The absence of significant changes in LPO in the leaves after exposure to the Ni^0, CuO and NiO nanoparticles can also be due to a decrease in the carotenoid pool (see Table 2).

Therefore, the obtained results allow to conclude that in wheat sprouts held for 2 days in the media containing nanoparticles, there were changes oc-

curred in several parameters simultaneously.

The sensitivity of sprouts to nanoparticles of pure metals Cu^0 and Ni^0 turned out to be much higher than to their oxides CuO and NiO. Of interest, none of the concentrations of the CuO and NiO NPs produced a lethal effect. All the wheat plants when exposed to the highest concentrations (0.5 and 1.0 M) of these nanoforms remained viable, despite a significant (more than 2-fold) decrease in most growth parameters. In addition, the analysis revealed a higher sensitivity of the root system to nanoparticles as the first target for the toxic effect of metals. The NPs of metals and oxides can be arranged by increasing their toxic effect on the growth of the main root and 1st leaf of the *T. vulgare* sprouts in the following order: $Fe_3O_4 \rightarrow Fe^0 \rightarrow NiO \rightarrow CuO \rightarrow Ni^0 \rightarrow Cu^0$.

The analysis of PPs showed that the strongest negative effect on the chlorophyll *a* content was observed for the Cu^0 NPs (22.0-33.0 %), on chlorophyll *b* — for the Ni^0 NPs (16.0-68.0 %), on carotenoids — for the CuO NPs (10.5 %). These same nanoparticles caused a decrease in the total amount of chlorophylls in the sprouts, which could have a common cause associated with the development of oxidative stress, since chloroplasts are the main source of ROS generation under stress conditions. Apparently, the copper-containing nanoparticles could directly participate in the generation of hydroxyl radicals (in the Fenton and Haber-Weiss reactions) and singlet oxygen in the cells [12], which led to the oxidative destruction of pigments, or could release copper ions and thereby induce metabolic effects, such as to disrupt the stroma and grana of chloroplast membranes [13]. The data obtained are consistent with the conclusions of the studies, in which there was a general decrease in the total amount of PPs after treatment with copper oxide nanoparticles [14-16]. The decrease in the chlorophyll *a* content without affecting the chlorophyll *b* level has also been previously shown [16]. The observed decrease in the chlorophyll *b* content after exposure to the Ni^0 NPs is probably caused by nickel ions involved in indirect damage to the light accumulating complex [8, 17].

It appears that the changes in the carotenoids content in our experiments was due to the lower sensitivity of the enzymes involved in their synthesis to nanoparticles, and also to the active role of these pigments in the neutralization of the ROS.

When analyzing the quantitative changes in LPO products, it can be concluded that the processes of lipid peroxidation when exposed to the iron-containing nanoparticles are insignificant and hardly differ from those in intact plants, which indicates a non-significant development of oxidative stress [9, 18]. This can be explained by the primary non-specific response to stress as one of the components of passive adaptation of the plant. The accumulation of oxidized fatty acid products was detected in the roots, as influenced by the Cu^0, Ni^0 and CuO nanoparticles. Other researchers have obtained similar data for the Cu^0 and CuO NPs and considered LPO as one of the most important toxicity mechanisms associated with exposure to nanometals [15, 16].

It is known that in plants the pore size of the cell wall varies from 2 to 30 nm [20], while the size of ions and water molecules is about 0.28 nm. Therefore, the studied NPs of metals remain probably on the surface and act indirectly. M. Whitby et al. [21] reported that damage to cells in plants is associated with the aggregation of NPs on their surface, which creates a barrier for apoplastic transport. However, many studies reported the possibility of the penetration of even larger NPs inside the cells, as well as the appearance of new pores for the transport of metals. For example, T.D. Deryabina [22] has proved that spherical Cu^0 NPs 80 ± 15 nm in size may push through the plasmalemma and accumulate in vacuoles.

Previously it has been reported that nanoparticles can penetrate through the cell membrane and form aggregates with cellular components [3], and their oxides can block water channels through adsorption and increase the penetration of ROS [4]. A high pool of ROS and a rise in LPO in the protoplast under the effects of NiO were found and it was suggested that the Ni^{2+} ions could generate the $HO^{\bullet-}$ radical in plant cells through the Haber-Weiss reaction [12]. The toxicity of Cu^{O} and CuO nanoforms, as well as the Cu^{2+}/Cu^{+} ions, which were released from them, has been revealed [5]. However despite all these facts, it appeared to be impossible to accurately differentiate the effects of oxidative induction by metals, their oxides and ions. The oxidative properties of the CuO NPs are probably due to the good solubility of this oxide down to Cu^{2+} and Cu^{+} ions [23, 24]. The higher toxicity of the Cu^{0} NPs as compared to CuO NPs evolves probably from their lower mobility in the media [13, 24]. In addition, it is necessary to take into account the probable interaction of NPs with biomolecules, including DNA [25].

Therefore, the investigation of the growth processes, the content of pigments and products of lipid peroxidation showed that metabolic changes in the *Triticum vulgare* L. wheat in the presence of nanometals and their oxides depended both on the chemical nature of the metal and on the concentration of the nanopreparation used. The results provide additional evidence on selectivity during the activation of one or another metabolic reaction determined by the properties of the nanomaterial. In addition, the redox upset in the presence of Ni^{0}, Cu^{0} and CuO can be attributed to a non-specific reaction of plants, since such changes are typical for a variety of stress types.

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CYTOGENETIC EFFECTS IN *Allium cepa* L. RESULTED FROM SEPARATE AND COMBINED EXPOSURE TO Cu, Zn AND Ni

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Abstract

Heavy metal contaminations of agricultural lands necessitate the study of phyto- and genotoxic effects in plants of different types. The impact of elevated concentrations of biologically essential metals, e.g. Cu and Zn, and the metals with a pronounced toxic effect even at low concentrations is of special importance. Generally, the model objects (e.g. *Allium cepa* L.) are used to simulate the impact of one of the metals. In this case the concentration chosen is greater (100 times or more) than the levels of actual contamination and maximum permissible concentrations in different environments. Data on the combined action of metals at concentrations actually existing in the environment are practically not reported, which prevents the development of standards to limit their impact on ecosystems and agrobiocenoses. Our objective was to compare the cytogenetic changes in the root meristem of *Allium cepa* exposed to different concentrations of Cu, Zn and Ni separately and combinedly. Experiments were carried out in 4-fold replication. The roots of onion plants (10 per replication) were germinated in distilled water (control) or $Cu(NO_3)_2 \cdot 3H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ and $Ni(NO_3)_2 \cdot 6H_2O$ solutions. Salt concentrations corresponded to the maximum permissible concentrations in water for fish-farming (Cu — 0.001 mg/l, Ni and Zn — 0.01 мg/l) and for household purposes (Cu and Zn — 1.0 mg/l, Ni — 0.02 mg/l). Chromosome aberrations were viewed in 180-790 cells of onion root tips in fresh crush preparations after acetic-orcein staining. We estimated the mitotic index calculated as the fraction of mitotic cells to the total number of cells in the root meristem, the frequency of aberrant cells, and the types of chromosomal aberrations. The influence of each element and their various combinations on cytogenetic parameters was compared that allowed us to determine the coefficient of antagonism. It has been shown that Cu, Zn and Ni ions, as depending on their properties, inhibited cell division in onion root meristem to varying degrees. At relatively low concentrations of metals, equal to the MPC in water for fish-farming, the frequency aberrant cells was about 3 to 7 times higher as compared to the control. An increased metal concentrations (1000-fold, 100-fold, and 2-fold for Cu, Zn and Ni, respectively) did not lead to a proportional increase in the frequency of aberrant cells which was only two times as much as that in control. Changes in the mitotic index were also disproportionate to the metal concentration in the solution. When combined, the metal ions had lower genotoxic effect as compared to their individual activity. The differences between separate and combined effects are indicative of ion competition. The calculated coefficients of antagonism in the experiment ranged from 0.20 to 0.40.

Keywords: *Allium cepa* L., heavy metals, genotoxic effect, coefficients of antagonism

Intensification of economic activity and degradation processes may lead to increase in pollutant content, including heavy metals (HM) in natural environments. For example, heavy metal soil contamination values in several regions of the Russian federation are rather high. The greatest soil contamination (as a percentage of examined area) is observed with regard to Cu (3.8 %) and Ni (2.8 %), while this parameter for Zn is lower (0.2 %) [1].

HM toxicity for living organisms is determined by their concentration and physical and chemical properties, including migration capacity and bioavailability, which finally determine the value of chemical elements accumulation in

organs and tissues [2]. Accumulation of HM in soil affects growth and development of plants [3, 4], as well as soil microbiota [5], which leads directly or indirectly to farm crops yield loss. To date, the main mechanisms of HM fixation in solid phase of soil have been determined [6]. It ensures development of measures aimed at decrease of HM content in the soil solution and reduction of toxicity risk for agricultural plants. Phytotoxic effect has been studied sufficiently for only a few metals [7-9]. The lack of information on the patterns of biological systems response to effect of a wide range of HM complicates evaluation of their actual hazard for plants and animals.

As a rule, experimental studies are conducted using model objects, with impact of one metal in various concentrations, including high ones, which is aimed at quantitation of the effect. However, in natural conditions elevated concentrations of metals with negative impact on cenosis components are generally lower than those selected for an experiment. Polyelemental impact is considered even to a lesser extent, as in this case forecasting of risks is much more difficult, because of possible mutual interactions of metals and synergism or antagonism processes [10]. The studies of barley and wheat sprouts [11] have demonstrated decrease in phytotoxic effect of Al^{3+} ions in the presence of Fe^{3+} ions, which may be explained by induced synthesis of proteins similar to transferrin or lactoferrin in animals, organic acids or phytochelatins, inactivating Al^{3+} ions. Sensitivity of barley varieties to the effect of Al ions of genetic pleiotropic nature has been detected [12].

The impact of elevated concentrations of biologically essential metals (Cu, Zn) or the metals with a pronounced toxic effect even at low concentrations (Ni) is of special importance for agricultural practice. It has been established that these elements have pronounced mutagenic properties [13-15]. Their compounds may cause both clastogenic and aneugenic effects (including disturbances of mitosis and cytokinesis) [16]. Inhibition of mitosis induced by Ni and Zn is explained by redistribution of cell (tissue, organism) energy resources for metallothionein synthesis, while Cu ions increase mitotic activity [17]. Significant correlation between DNA damage and Cu content was detected [18].

Special attention is paid to search of phytoremediation agents [19, 20], examination of heavy metal consumption and distribution patterns in various parts of plants [21, 22], including depending on the varietal characteristics [23], examination of selective transport of elements from rhizosphere to leaves in ontogenesis [24], mechanisms of heavy metal detoxication in various plant species [25, 26].

Examination of genotoxicity of various chemical substances for cytogenetic and morphometric parameters of *Allium cepa* L. test object is of importance [27-29]. The data on toxic effects of Cu, Zn and Ni compounds, both independently (at $CuCO_3$ concentration of 5 mg/l, $ZnCl_2$ concentration of 10 mg/l, $NiCO_3$ concentration of 0.7 mg/l) [17] and in various combinations, were obtained using this test system. It has been noted that in case of joint effect of metal ions on root meristem of *Allium cepa* the mitogenicity in tissue changes and protein synthesis in cells increases.

As a rule, rather high concentrations of metals are selected for work. For example, according to R.F. Garipova [17], these significantly exceeded the maximum permissible values in water for fish-farming (100-1000-fold) and household (more than 2-fold) purposes. Examination of effects of small metal concentrations and determination of their genotoxicity value is more relevant, as it is important from both scientific and practical point of view, especially in case of polyelemental contamination. Apart from that, the lack of data on antagonism coefficients for various combinations of elements prevents comprehensive determination

of interdependent effect; at that, synergism and antagonism shall be taken into account in determination of risk of heavy metal impact on living organisms.

We first evaluated the impact of Cu, Zn and Ni in concentrations, corresponding to their maximum permissible content in water of various economic purposes, on cytogenetic parameters of *Allium cepa* L. This approach ensures comparison of HM effects at concentrations, differing 1000-fold, 100-fold, and 2-fold, and identification of mutual inhibition of toxic effect of various metals in case of their combined action.

Our work focused on examination of the impact of Cu, Zn and Ni on development of cytogenetic effects in root meristem of *Allium cepa* L. at various concentrations of metals, in conditions of mono- and polyelemental contamination, as well as determination of antagonism coefficients.

Techniques. Plants of *Allium cepa*, variety Stuttgarter Riesen, were the study object. The experiments were conducted in quadruplicate in laboratory conditions. A total of 10 pcs of planting onion of similar size (1.5-1.7 cm) were selected for each replication of the experiment variant. The roots of onion plants were grown in distilled water (control) or $Cu(NO_3)_2 \cdot 3H_2O$; $Zn(NO_3)_2 \cdot 6H_2O$ and $Ni(NO_3)_2 \cdot 6H_2O$ solutions. During examination of joint effect the solution contained $Zn^{2+} + Ni^{2+}$, $Zn^{2+} + Cu^{2+}$, $Cu^{2+} + Ni^{2+}$ and $Zn^{2+} + Ni^{2+} + Cu^{2+}$ ions. Salt concentrations corresponded to the maximum permissible concentrations in water for fish-farming purpose (MPC_{ff}) (0.001 mg/l for Cu ions, 0.01 mg/l for Ni and Zn ions) and in water for household purpose (MPC_h) (1 mg/l for Cu and Zn, 0.02 mg/l for Ni).

Chromosome aberration analysis was conducted in root meristem of onion plants. To do so, the roots of onions about 1 cm long were cut off in 72 hours, fixed in acetic alcohol (3:1) and washed with 80 % alcohol. Fresh crush preparations with acetic-orcein staining were examined. Mitotic index (MI), i.e. the proportion of cells at mitosis stage in the total number of root meristem cells, as well as aberrant cells frequency (ACF) and types of chromosome aberrations were considered. A total of 180-790 ana-telophase cells were reviewed in each variant.

The effect of heavy metals was evaluated by a deviation of variant average values of the respective parameters from their control values. Antagonism coefficient (AC) was calculated as per the formula [31]:

$$AK = S_{Me1+Me2}/(S_{Me1} + S_{Me2}),$$

where $S_{Me1+Me2}$ is the proportion of aberrant cells in case of joint effect of two metals minus the spontaneous aberration background value (the proportion of aberrant cells in control); S_{Me1} and S_{Me2} are the proportion of aberrant cells in case of effect of one metal minus the spontaneous aberration background value (the proportion of aberrant cells in control). In case of effect of three metals antagonism coefficient was calculated as per the formula:

$$AK = S_{Me1+Me2+Me3}/(S_{Me1} + S_{Me2} + S_{Me3}).$$

Statistical processing of the data was performed using Microsoft Excel. Significance of differences between variant was determined based on paired two-sample *t*-test for mean values. Mean values (X) and standard error of mean ($\pm x$) are presented in the tables.

Results. It has been shown that Cu, Zn and Ni ions present in the germination solution inhibited cell division in onion root meristem to varying degrees (Table 1).

Genotoxic effect of metals essential for plants (Cu and Zn) was nearly similar, while genotoxic effect of Ni was significantly higher: ACF (%) in onion root meristem at Cu concentration of 0.001 mg/l has increased 5.3-fold as compared to the control, at Zn concentration of 0.01 mg/l 4.7-fold (see Table 1). In

case of increase of Cu and Zn concentration up to 1 mg/l (MPC_h) ACF increased 11.3-fold and 8.9-fold, respectively, as compared to the control (see Table 1). A 7.3-fold and 13.4-fold ACF increase was observed for Ni at MPC_{ff} and MPC_h, respectively.

1. Cytogenetic parameters in case of effect of Cu, Zn and Ni in various combinations on root meristem of *Allium cepa* L. ($X \pm x$)

Variant	The number of anatelophase cells reviewed	Aberrant cells frequency, %	Mitotic index, %	Chromosome aberration type
Water for fish-farming purpose				
Cu	725±7	3.10±0.39[a, b]	14.53±0.58[b, d, e]	m, f
Zn	500±5	2.72±0.46[a, b, e]	10.91±0.41[a, e]	m, f
Ni	424±4	4.23±0.60[a, c, d, e]	10.43±0.38[a, d, e]	m, f
Cu + Zn	585±6	1.78±0.21	9.91±0.48	m, f
Zn + Ni	550±5	2.58±0.51	12.41±0.66	m, f, 3p
Ni + Cu	279±3	2.92±0.49	7.82±0.65	m, f
Cu + Zn + Ni	571±6	2.82±0.34	9.09±0.47	m, f
Control	781±8	0.58±0.23	15.12±0.73	m
Water for household purpose				
Cu	314±4	6.56±0.82[a, b, d, e]	7.24±0.46[a, d]	m, f
Zn	333±3	5.16±0.90[a, b, d, e]	8.77±0.41[a, e]	m, f, 3p
Ni	438±4	7.76±1.37[a, d, e]	9.96±0.40[a, c, d, ед]	m, f, g
Cu + Zn	391±4	3.03±0.20	8.97±0.45	m, f
Zn + Ni	324±3	3.04±0.39	7.35±0.34	m, f
Ni + Cu	177±2	3.83±0.80	6.32±0.42	m, f
Cu + Zn + Ni	347±4	3.85±0.54	7.13±0.40	m, f
Control	781±8	0.58±0.23	15.12±0.73	m

Note. Concentrations equal to maximum permissible concentrations in water for fish-farming (MPC_{ff}) and household (MPC_h) purpose were used. MPC_{ff}: Cu — 0.001 mg/l, Zn — 0.01 mg/l, Ni — 0.01 mg/l; MPC_h: Cu — 1 mg/l, Zn — 1 mg/l, Ni — 0.02 mg/l; control — distilled water; f — fragment, m — bridge, g — lagging, 3p — tripolarity.

[a, b, c, d, e] The differences are significant at $p < 0.05$ with regard to control , Cu + Zn, Zn + Ni, Ni + Cu and Cu + Zn + Ni, respectively.

MI decrease was observed with increase of metal salt concentrations in the solution from MPC_{ff} up to MPC_h. MI decreased 2.0-fold in case of Cu and 1.3-fold in case of Zn; the differences were insignificant for Ni (10.43±0.38 and 9.96±0.40 for MPC_{ff} and MPC_h, respectively). Thus, MI changes were not proportional to increase in metal content in the solution. Similar phenomena were observes for other metals, for example, Cd [32, 33].

HM salt effect on plants is characterized with complexity and a wide range of targets, which explains various disturbances observed at all organization levels of a biological object [17, 34]. Zn and Cu are bioactive (essential) chemical elements of major importance, involved in various metabolic processes. The role of Ni is known to a lesser extent. As a rule, Ni is considered as a toxic element for most plants [8]; its participation in some enzymatic reactions in legumes has been confirmed. The majority of plant species is characterized by high resistance to excess of metal salts in soils due to metal consumption limitation mechanisms and their detoxication in cells. According to laboratory studies, HM in 10^{-6}-10^{-3} M concentrations cause cytogenetic disturbances in apical meristem cells of *Allium cepa* L. [16]. The rating of HM salts with regard to their ability to induce chromosome aberrations is as follows:

$$ZnSO_4 > Pb(CH_3COO)_2 > Al(NO_3)_3 = NiSO_4 > CdCl_2 > CuSO_4.$$

At that, the strongest antimitotic effect was observed for Ni (the percentage of chromosome lagging, multipolar anaphases and K-mitoses increased nearly 70-fold as compared to the control). Ni ions also were the strongest inducers of cytokinesis disturbance [16].

In our study chromosome lagging was only detected for Ni in concentrations equal to MPCh (see Table 1). Two types of aberrations, i.e. acentric fragments and bridges, were observed by us during anaphase and telophase.

Simultaneous presence of two metals in growth solution decreased the

toxic effect of each of them. In variants with the solution containing Cu ions chromosome aberration frequency in onion root cells in the presence of Zn decreased significantly, i.e. 1.7-fold and 2.2-fold for MPC_{ff} and MPC_h, respectively, as compared to ACF for Cu. In combination of Cu and Ni ions ACF decreased less significantly, i.e. 1.06-fold and 1.70-fold. In case of simultaneous effect of Cu, Zn and Ni in concentration equal to MPC_{ff} ACF did not reach the values of individual effect of metals (for Cu and Ni) or was comparable with them (for Zn) (see Table 1). In case of MPC_h and joint effect of metals ACF was always below the values of their individual effect (see Table 1). ACF_{Ni} and $ACF_{Cu+Zn+Ni}$ differed 2-fold.

2. Antagonism coefficient in case of effect of Cu, Zn and Ni in various combinations on root meristem of *Allium cepa* L.

Variant	MPC_{ff}	MPC_h
Cu + Zn	0.31	0.26
Zn + Ni	0.37	0.24
Ni + Cu	0.40	0.27
Cu + Zn + Ni	0.28	0.20

N o t e. Concentrations equal to maximum permissible concentrations in water for fish-farming (MPC_{ff}) and household (MPC_h) purpose were used. MPC_{ff}: Cu — 0.001 mg/l, Zn — 0.01 mg/l, Ni — 0.01 mg/l; MPC_h: Cu — 1 mg/l, Zn — 1 mg/l, Ni — 0.02 mg/l. Antagonism coefficient was calculated by aberrant cells frequency.

Mitotic index for MPC_{ff} in case of joint effect of metals was below MI in case of individual effect, except for MI of Zn + Ni, where increase of the parameter was observed as compared with both MI of Zn and MI of Ni. On the contrary, the changes of MI for MPC_h in case of joint effect of two metals varied, and in case of joint effect of three metals (Cu, Zn and Ni) MI was lower than that of Zn or Ni. Such differences in response to separate and combined presence of metals in the solution suggest competition of metal ions [34]. At that, a new type of chromosome disturbances (tripolarity) was observed in Zn + Ni (MPC_{ff}) pair. In case of metal concentration increase up to MCP_h tripolarity was only observed in Zn.

Antagonism coefficient values calculated by ACF (Table 2) varied from 0.20 (for Cu + Zn + Ni) to 0.27 (for Ni + Cu) at MCP_h. At MPC_{ff} this parameter was from 0.28 (for Cu + Zn + Ni) to 0.40 (for Ni + Cu). It should be noted that maximum and minimum antagonism coefficient values, irrespective of concentrations, were observed in the same components of metal mixture.

Thus, genotoxic effect of heavy metal ions on root meristem cells of *Allium cepa* has been established at the concentrations of elements, corresponding to the respective MPC values in water for fish-farming and household purposes. Even at relatively low concentrations of metals (0.001 mg/l for Cu, 0,01 mg/l for Ni and Zn) the aberrant cells frequency was about 3 to 7 times higher as compared to the control. Mutual inhibition of effects, which was the most prominent in Cu + Zn variant, was observed in case of joint effect of metal ions. Increase in metal concentration up to MPC_h led to 2-fold increase in genotoxicity values, but was disproportionate to the increase in concentration of elements. At that, different spectra of chromosome aberration for essential (Cu, Zn) and toxic (Ni) elements were observed (chromosome lagging was detected for Ni at MPC_h). Antagonism coefficient values (by aberrant cells frequency) at MPC_h varied depending on the combination of metals. The revealed patterns of individual and joint effect of metals on root meristem shall be considered during monitoring of both water ecosystems and soil biocenosis subject to joint effect of heavy metals, as well as during evaluation of environmental risk for various ecosystems under multiple-factor effect of chemical elements.

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EFFECT OF Al_2O_3 NANOPARTICLES ON SOIL MICROBIOCENOSIS, ANTIOXIDANT STATUS AND INTESTINAL MICROFLORA OF RED CALIFORNIAN WORM (*Eisenia foetida*)

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Abstract

With the accumulation of experimental data it is evident that nanomaterials, which are widely used in human activity, look promising for agronomy. However, available publications on a comprehensive assessment of biological risks arising from nanoherbicides, nanofertilizers, etc., in particular on how the metal nanoparticles affect geobionts, are very limited. In the model experiments with California red worms *Eisenia foetida* as test organisms we studied the influence of aluminum oxide nanoparticles on soil biocoenosis and their biodegradation. We found an increase in mortality of worms up to 20 % at the maximum dosage of aluminum oxide nanoparticles introduced into the soil. Assay of antioxidant defense enzyme activity in the *E. foetida* revealed an increased superoxide dismutase and catalase level as influenced by the studied nanoparticles. The positive effect of their vermicomposting was shown. At increasing content of aluminum oxide nanoparticles (the nanoparticle dosage of 50, 100, 300, and 3000 mg/kg in the groups 1, 2, 3, and 4, respectively), the 61.7-67.6 % reduction in soil microorganism counts was found without vermicomposting vs. 55.6-61.3 % under vermicomposting. The number of microorganisms in the soil decreased in the groups 1, 2, 3 and 4 by 42.8, 52.4, 61.9 and 76.2 % for fungi, by 64.3, 77.9, 78.6 and 85.7 % for nitrogen-fixing bacteria, and by 22.7, 38.6, 84.1 and 86.4 % for bacteria cultured on starch-and-ammonia agar. The number of cellulolytic bacteria increased by 6.9 % in the group 1 and decreased by 16.7, 12.5 and 25.0 % in the groups 2, 3 and 4, respectively. A similar trend was observed under the influence of aluminum oxide nanoparticles on the *E. foetida* intestinal microflora. As the soil content of aluminum oxide nanoparticles increased from 50 to 3000 g/kg, the total number of microorganisms in the *E. foetida* intestine decreased by 9.7 to 43.2 %. In this, fungi decreased in the groups 1, 2, 3 and 4 by 18.0, 20.0, 39.0 and 40.0 % as compared to control. The number of nitrogen-fixing bacteria was insignificant in the control samples and decreased in the *E. foetida* intestine in the groups 1, 2, 3 and 4 by 16.0, 60.0, 78.8 and 80.0 %. The cellulolytic bacteria counts increased in the intestine of *E. foetida* (by 16.0 %) at minimum nanoparticle dosages, whereas in the groups 2, 3 and 4 this index was lower by 8.0, 32.0, 25.0 and 40.0 %. The number of bacteria cultured on starch-and-ammonia agar decreased in the *E. foetida* intestine in groups 1, 2, 3 and 4 by 13.3, 46.7, 60.0 and 73.3 %. Therefore, our data indicate dose-dependent effects of aluminum oxide nanoparticles and gradual development of their toxicity toward soil and intestinal microflora at increasing levels in the soil. The negative impact of the aluminum oxide nanoparticles on soil biocenosis was shown that was manifested in its depletion, leading to soil degradation and decreased fertility. We confirmed the necessity for complex assessment of nanoparticle biotoxicity in a variety of habitats. The antioxidant system activity in the presence of Al_2O_3 nanoparticles is indicative of *E. foetida* adaptability to stress caused by these agents.

Keywords: nanoparticles, *Eisenia foetida*, catalase, superoxide dismutase, microorganisms

In recent years, a sufficient number of studies have been accumulated to

substantiate the prospects for the use of nano-materials in microelectronics [1], energy physics [2], chemical [3], food [4], pharmaceutical [5], medical industry [6-8]. Nanoparticles (NPs) of metals are also of great practical importance in agriculture (in particular, in plant growing and for increasing soil fertility), where they can be used as nano-herbicides [9], nano-pesticides [10], as plant growth stimulants [11] and nano-fertilizers [12].

Among the various nano-materials, nanoparticles of aluminum and its oxides attract attention. About 250 minerals containing aluminum are known and used in various fields, including in the agricultural sector, the possibility of using them in the nanoform is being actively studied at the present time [13, 14]. However, there is only a limited number of works on the integrated assessment of biological risks associated with aluminum nanoparticles [15-19].

One of the bioindicator species in determining the potential toxicity of chemicals in soils [20, 21] is a red Californian worm (*Eisenia fetida*). Worms play an important role in the circulation of substances and the formation of soil structure, improving the degradation of organic substrates, mixing soil layers and enhancing aeration [22, 23].

In the presented study, using the red Californian worm as a test object, we first demonstrated the dose-dependent action of aluminum oxide nanoparticles, the development of a bactericidal effect on the soil microflora and intestinal microbiocenosis of *E. foetida* with an increase in Al_2O_3 nanoparticle content in the soil, as well as the manifestation of adaptation reactions in *E. fetida*.

The aim of our work was to study the influence of Al_2O_3 nanoparticles on soil microbiocenosis, vermicomposting and the state of the antioxidant system and intestinal microflora in a red Californian worm.

Techniques. The study used a preparation of aluminum oxide nanoparticles (NPs of Al_2O_3, Advanced Powder Technologies, Ltd, Russia), obtained by electric explosion of a conductor in an argon atmosphere. The size of nanoparticles is 54 nm, the Z-potential is 30±0.1 mV. The nanoparticle powder composition included: Al_2O_3 (95 %), sorbed nitrogen and hydrocarbons (3 %), water (2 %). Material certification of the preparations included electronic scanning and transmission microscopy on instruments JSM 7401F and JEM-2000FX (JEOL, Japan). X-ray phase analysis was performed on a DRON-7 diffractometer (NPO Burevestnik, Russia).The zeta-potential of the particles was determined on a Photocor Compact-Z analyzer (Photocor, Russia).

A preliminary assessment of the biological activity of NPs Al_2O_3 in vitro was carried out in the inhibition test of bacterial bioluminescence. Suspension samples of nanoparticles were prepared in the concentration range of 0.00625-4.0 M and sonicated for 30 min. The genetically engineered luminescent strain *Echerichia coli* K12 TG1, constitutively expressing the genes of *luxCDABE* of the sea microorganism *Photobacterium leiongnathi* 54D10 (lyophilized preparation Ecolum, NVO Immunotech, Russia) was used. Immediately prior to the beginning of the *E. coli* K12 TG1 experiments, the bacteria were revived with chilled distilled water, kept at 2-4 °C for 30 min, after which the temperature of the bacterial suspension was adjusted to 15-25 °C. The bacterial luminescence inhibition test was carried out using an Infinite PROF200 microplate analyzer (Tecan Group, Ltd, Switzerland), dynamically recording the luminescence intensity of the resulting mixtures for 180 min with an interval of 5 min. The results of the influence of nano-materials on the intensity of bacterial bioluminescence (I) were estimated by the formula:

$$I = \frac{Ik_{0\ min} \times Io_{n\ min}}{Ik_{n\ min} \times Io_{0\ min}},$$

where Ik and Io are the intensity of the luminescence of the control and test sam-

ples at the 0 and *n*-th minutes of the measurement.

Toxicity was studied according to the OECD Guidelines for the Testing of Chemicals (OECD, 1984, 2004) on laboratory earthworm cultures (red Californian hybrid) *Eisenia foetida* Andrei Bouche. All the selected individuals were reproductive. As the substrate, artificial soil was used (70 % quartz sand, 20 % clay, 10 % peat). Groups (n = 10 each) were formed from the individuals of the same weight. In groups I, II, III and IV, NPs of Al_2O_3 were added to the soil at doses of 50, 100, 300, 3000 mg/kg, respectively; V group served as a control (without NPs). NPs were introduced into the substrate before the worms were placed into, mixed with a household mixer and adjust to a humidity of about 85 %. At the same time, the ground soil was incubated with NPs Al_2O_3 in the same dosages without vermicomposting. The experiment was carried out in 3 replicates.

The antioxidant activity of catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) in worms were determined on day 14 of the experiment on an automatic biochemical analyzer CS-T240 (Dirui Industrial Co., Ltd, China) with commercial Randox biochemical sets (Randox, USA).

Influence of NPs on the worm intestinal microflora and soil microflora was assessed on day 14. For purification of intestines, worms were kept in a plastic container on wet filter paper for 1 day, then, using a sterile scalpel, the intestine taken and placed in a sterile tube. The soil samples were also taken into sterile test tubes. Microbiological studies were carried out within one day after sampling. Meat-peptone agar (MPA) was used to determine the total count of microorganisms. Microorganisms metabolizing mineral nitrogen were isolated on starch-ammonia agar (SAA), microscopic fungi on Chapek medium, nitrogen fixators on Ashby medium, cellulosolytic microorganisms on Getchinson medium [24, 25]. Nystatin (40 μg/ml) was added to prevent fungal growth on SAA, and penicillin (50 μg/ml) was added to prevent bacterial growth on Chapek medium. Inoculations were conducted in triplicate.

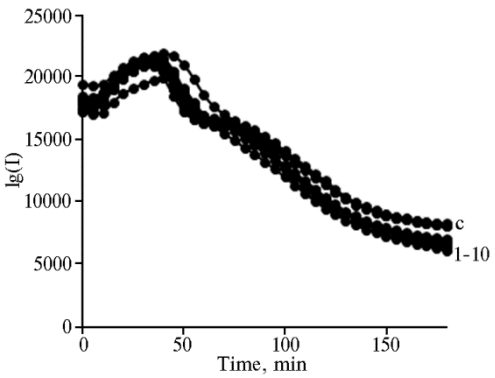

Fig. 1. Dynamics of luminescence (I) *of Echerichia coli* K12 TG1 with cloned genes of *luxCDABE Photobacterium leiongnathi* 54D10 upon contact with Al_2O_3 nanoparticles in different concentrations: 1 — 4.0 M, 2 — 2.0 M, 3 — 1.0 M, 4 — 0.5 M, 5 — 0.25 M, 6 — 0.1 M, 7 — 0.05 M, 8 — 0.025 M, 9 — 0.0125 M, 10 — 0.00625 M, c — control.

Statistical analysis of the data was carried out using the Statistica 10.0 software package (StatSoft Inc., USA). The arithmetic mean (M) with the standard error of the mean (m) are presented.

Results. The contact of *E. coli* K12 TG1 with increasing concentrations (0.00625-4.0 M) NPs Al_2O_3 did not cause suppression of the luminescence of bacteria for 60 min, which made it possible to characterize $NPsAl_2O_3$ as non-toxic (Fig. 1). After 180 min, the bioluminescence was reduced by 20-30 % compared to the control values, which characterized the studied NPs as being slightly toxic for living cells. The absence of cytotoxic and genotoxic affection of nanoparticles of aluminum compounds on a living cell in in vitro experiments has been earlier reported [26, 27]. Reducing the luminescence of bacteria with the prolongation of contact up to 180 min suggests the presence of a chronic toxic effect of NPs which requires time for development. Nanoparticles of other metals demonstrate similar chronic biotoxicity [28-30].

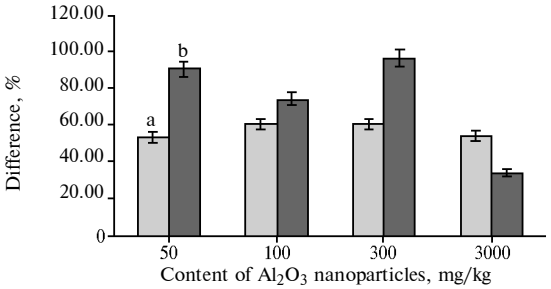

Fig. 2. The difference between the activity of superoxide dismutase (a) **and catalase** (b) **in *Eisenia fetida* in control and experiment after 14 days of cultivation in artificial soil with different content of Al_2O_3 nanoparticles.**

To assess the biotoxicity of NPs of Al_2O_3 to *E. foetida*, the activity of antioxidant defense enzymes was studied. Low and medium doses (50-300 mg/kg) of nanoparticles caused a manifest induction of catalase activity with maximum at 300 mg/kg. Further increase in the load of nanoparticles on soil (up to 3000 mg/kg) led to CAT inhibition. A similar result was obtained by H.Y. Liang et al. [31]. At the same time, only at 3000 mg/kg of Al_2O_3 NPs, an increase in CAT activity as compared to the control, was significantly different from that in the other groups. For SOD, the increment of activity was approximately the same for different concentrations of NPs of Al_2O_3, that is, we did not observe a dose-dependent effect (Fig. 2).

It is known that aluminum compounds are capable of activating the expression of SOD and CAT genes and, as a consequence, the enzymes themselves [32]. The uncontrolled increase in the concentration of the oxidant active forms under the action of a toxic agent in the cells promoted the enhancement of the functions of the defense mechanisms, which serves as an important element of the body reaction to the toxic dosages of the substance in the environment [33].

In studies of W. Sun et al. [34], the SOD activity in earthworms increased with moderate ecological stress and decreased with severe ecological stress. That is, NPs of aluminum has a prolonged effect, and the process of relaxation of the system (return to normal) can take a certain time after the elimination of the toxicant. Acting as a primary antioxidant defense system, CAT and SOD catalyze the conversion of reactive oxygen species to less active or inert forms [35, 36]. In our experiments, the effect of Al_2O_3 NPs on SOD in *E. foetida*, as in the case of CAT, was accompanied by an increase in activity with an increment in the Al_2O_3 content from 0 to 3000 mg/kg.

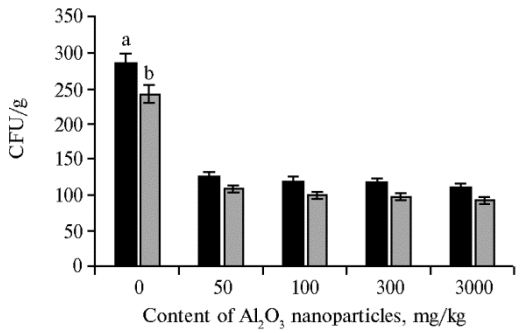

Fig. 3. Total counts of microorganisms in the soil after 14 days of incubation with Al_2O_3 nanoparticles in different dosages without vermicomposting (a) **and under vermicomposting** (b)**.**

A decrease in enzyme activity at dosage of 3000 mg/kg was accompanied with the increase in mortality of the test object reaching 20 % compared to 100 % of the survival in other groups.

The ability of soil components to interact with NPs metals, bind them and reduce bioavailability levels the toxic effect of NPs on *E. foetida*. Dissolved or solid particles of some organic substances can agglomerate and sorb NPs in the soil matrix and thereby reduce their bioactivity [37, 38].

Counts of both soil microorganisms and intestinal microorganisms of worms varied after 14 days of incubation with increasing amounts of Al_2O_3 NPs. As the Al_2O_3 NPs dose increased from 50 to 3000 mg/kg, the total number of microorganisms consistently decreased in the soil by 61.7-67.6 % without vermicomposting and by 55.6-61.3 under vermicomposting, and in the *E. foetida* intestine by 9.7-43.2 % (Fig. 3, 4). At the same time, the minimum dosage

(50 mg/kg) of Al_2O_3 NPs reduced the total number of soil microorganisms, but did not affect the intestinal microbiocenosis of *E. betida*, which was due to the action of barrier and adaptive mechanisms in this soil habitant. A dose dependent decrease in the amount of soil microflora, due to changes in metabolic processes in the cell, is specifically of other types of nanoparticles [39].

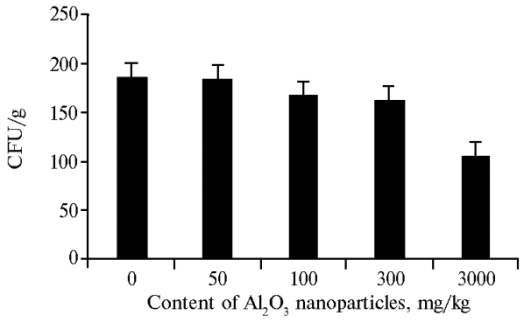

Fig. 4. The total number of microorganisms in the intestine of *Eisenia fetida* after 14 days of cultivation with Al_2O_3 nanoparticles in different dosages.

There were differences in the toxic effect of Al_2O_3 NPs on microorganisms in vitro and in vivo: at comparable doses bactericidal properties in vivo were shown, while in vitro absent. The development of the toxic effect was determined by the time factor and characteristics of growth medium.

In control samples of soil with vermicomposting and in the intestine quantitative analysis revealed the dominance of bacteria, while the number of microscopic fungi was several orders lower.

When incubating with Al_2O_3 NPs, the number of all groups of microorganisms was changing. The number of fungi in the soil decreased by 42.8, 52.4, 61.9 and 76.2 % in I, II, III and IV groups, respectively, compared to control, and in the intestines of *E. foetida* — by 18.0 and 20.0 % in groups I and II, and by 39.0 and 40.0 % in groups III and IV (Table 2). That is, with the increase in the content of Al_2O_3 NPs, the number of microorganisms decreased steadily which indicates a bactericidal effect on the soil and intestinal microflora. There was a decrease in the resistance of fungi to the action of NPs, especially when studying the microbiocenosis of the intestine of *E. foetida*. Similar results on the effect on microscopic fungi are known for iron nanoparticles [40]. Soil fungi, as a rule, are more resistant to heavy metals than bacteria [41].

The number of nitrogen fixing bacteria was insignificant in control samples and under the influence of Al_2O_3 NPs decreased in the soil in I, II, III and IV groups, respectively, by 64.3, 77.9, 78,6 and 85,7 %, in the intestine of *E. foetida* — by 16.0, 60.0, 78.8 and 80.0 %. The amount of cellulosolytic bacteria increased in the soil and intestine of *E. foetida* with minimal dosages of nanoparticles by 6.9 and 16.0 %, respectively. At the same time, the number of these bacteria in group II decreased by 16.7 and 8.0 %, in III — by 12.5 and 32.0 %, in IV — by 25.0 and 40.0 %.

2. The number of different microorganisms in the soil and intestine of *Eisenia fetida* during vermicomposting after 14 days after the application of Al_2O_3 nanoparticles

Group	Microscopic fungi, thousand CFU/g	Bacteria		
		cellulose-destroying, million CFU/g	utilizing the mineral nitrogen, million CFU/g	nitrogen fixing, million CFU/g
		Soil		
Control	21.0±3.20	72.0±9.70	44.0±6.50	14.0±4.20
I	12.0±2.60	77.0±4.30	34.0±2.10	5.0±0.70
II	10.0±2.10	60.0±6.40	27.0±3.10	3.1±0.20
III	8.0±0.65	63.0±8.20	7.0±0.80	3.0±0.13
IV	5.0±0.40	54.0±2.50	6.0±0.60	2.0±0.10
		Intestine		
Control	5.0±1.60	25.0±1.60	15.0±0.30	4.1±0.80
I	4.1±1.20	29.0±2.40	13.0±1.20	2.4±0.40
II	4.0±0.45	23.0±1.02	8.0±0.50	2.0±0.20
III	3.1±0.10	17.0±2.20	6.0±0.20	1.1±0.30
IV	3.0±0.20	15.0±1.50	4.0±0.90	0.8±0.04

According to the literature data, metal NPs in low doses are capable of stimulating the growth of individual bacteria groups, whereas in high doses they have a bactericidal and bacteriostatic effect, causing damage to the integrity of the cell membrane and changes in the functioning of intracellular systems [42]. It is also known that an influence of some NPs on the viability of nitrogen-fixing bacteria and the colonization of substrates by them is determined by the charge of NPs [43]. The number of microorganisms using mineral forms of nitrogen, especially actinomyces, is an important indicator, since some actinomyces are typical symbionts of invertebrates, including earthworms involved in the transformation of nutrient components of the soil [44].

Analysis of the soil microflora and intestinal microflora of *E. foetida* after 14-day incubation showed a decrease in the number of bacteria on SAA, as influenced by Al_2O_3 NPs: by 22.7, 38.6, 84.1 and 86.4 % for soil, and by 13.3, 46.7, 60.0 and 73.3 % for the intestine of worms in I, II, III and IV groups, respectively. As a result, there was a general tendency to the decrease of microorganism number in soil and intestines under the influence of Al_2O_3 NPs as the dosage of nanoparticles increased. The exception was the minimum dose of NPs: in this case there was an increase in the number of cellulose-destroying bacteria.

Thus, the model experiment confirmed the adverse effect of Al_2O_3 nanopreparations on soil biocenosis, manifested in its impoverishment which is accompanied by degradation and a decrease in fertility. The adaptive abilities of the red Californian worm (*Eisenia fetida*) under introduction of Al_2O_3 nanoparticles into the soil have been established based on antioxidant system parameters. The positive effect of vermicomposting is shown to reduce the toxicity of nanoparticles in the soil. In general, the obtained results confirm the need for a comprehensive assessment of the biotoxicity of nanoparticles in different habitats.

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FEATURES OF *Lonicera caerulea* L. REPRODUCTIVE BIOLOGY

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Abstract

Blue-berried honeysuckle (*Lonicera caerulea* L. s. l.) of the Honeysuckle family (*Caprifoliaceae*) is a high-quality berry plant which has been actively cultivated in recent years in various countries with temperate climate. Blue-berried honeysuckle is valued for ultraearly fruit ripening, as well as high content of antiscorbutic vitamin and biologically active phenolic compounds with antioxdidant, immunomodulating, antibacterial, antiviral, antifungal, antiallergic and other activities widely used in medicine, cosmetic surgery, food industry and agriculture. Variation of reproductive ability of *L. caerulea* cultivars and selections in different conditions of introduction in Russia and abroad prevents realization of potential fruitfulness of industrial plantations. For the purpose of finding out possible causes of decrease in fruit set and weight, morphological full-value of pollen, self-fertilization and interpollination were assessed, self-incompatibility of this species cultivars was studied. Blue-berried honeysuckle cultivars of various ecological-geographical and genetic origin widespread in West Siberia and north-east China were studied, which belong to the subspecies *L. caerulea* subsp. *altaica* (Pall.) Gladkova (syn. *L. altaica* Pall.), *L. caerulea* subsp. *venulosa* (Maxim.) Worosh. (syn. *L. edulis* Turcz. ex Freyn), *L. caerulea* subsp. *kamtschatica* (Sevast.) Gladkova [syn. *L. kamtschatica* (Sevast.) Pojark., *L. caerulea* var. *kamtschatica* (Sevast.) Pojark.]. Self-pollination and interpollination of 8 honeysuckle cultivars (Salyut, Berel', Goluboe vereteno, Zolushka, Tomichka, Parabel'skaya, Pamyati Gidzyuka and Kamchadalka) were studied by data of field research in 1999-2002 on the experimental plot in Novosibirsk. Cytoembryologic research, including that of microsporogenesis products and possible meiosis disturbance of 19 honeysuckle cultivars, was conducted from 1999 to 2014. The results obtained showed a decrease in pollen fertility to 0.8 % connected with anomalies in meiosis. Significant variations in fruit set (0.9-64.0 %), weight and seed quantity were determined by the genetic control of self-incompatibility under self-pollination and in different variants of cross-pollination of blue-berried honeysuckle cultivars. As morphological manifestations of self-incompatibility, anomalies in growth of pollen tubes in style tissues after transpollination of closely related cultivars and autogamy were determined. The main causes of loss cultural plantations productivity of blue honeysuckle may be low pollen fertility in some cultivars *L. caerulea* subsp. *altaica* and partial self-incompatibility with the cross-pollination varieties due to their closely related origin. The cultivars for effective pollination of blue-berried honeysuckle plantations were selected (selection from different families). For varieties that are derived from the parent form Start — Goluboe vereteno, Sinyaya ptica, Lazurnaya, Zolushka, Gerda, Berel' best pollinators may be cultivars derived from the initial form of Del'fin — Tomichka, Parabel'skaya, Pamyati Gidzyuka, Vasyuganskaya, Narymskaya. It is necessary to include additional cultivars of pollinators when planting high-yielding varieties Barhat, Salyut, Berel' or new cultivars produced with their involvement which are characterized by low pollen fertility.

Keywords: *Lonicera caerulea*, pollen sterility, self-fertilization and interpollination cultivars, self-incompatibility, fruit set, seed production, pollen tubes

In recent decades, in temperate climate countries, the culture of blue-berried honeysuckle has been intensively developing (*Lonicera caerulea* L. s. l., *Caprifoliaceae*). The value of this species is due to early ripening, high content of vitamin C [1, 2] and biologically active phenolic compounds [3-5], which thanks to their antioxidant [6-8], immunomodulating [1], antibacterial [8], antiviral [1], antifungal [2], antiallergic and other activities [1] are widely used in medicine

[9], cosmetology, food industry and agriculture. In Russia, significant advances have been achieved in selection of blue honeysuckle. More than 100 varieties of *L. caerulea* have been entered in the State Register of Breeding Achievements Approved for Use.

A unique collection of blue-berried honeysuckle has been formed at the University of Saskatchewan (Canada), including 32 varieties of Russian selection, 50 species from nurseries and scientific institutions in North America and Russia, and original wild-type material collected during expedition scientific tours in Japan (Hokaido Island), Canada, the USA. Based on this collection, a group of outstanding varieties was created in Canada, including those suitable for mechanized harvest [10]. Marketable gardens of blue-berried honeysuckle (*L. caerulea* subsp. *emphyllocalyx*) have been established in Japan on Hokkaido since 1970 [11]. At present, internal production of honeysuckle in Japan is about 200 t/year (85 ha), the biology of this subspecies is being actively investigated [12]. Successful introduction of blue-berried honeysuckle is being implemented in the USA [13]. Numerous studies are underway in European countries, such as Poland [14-17], Czech Republic, Slovakia [9, 18], Romania [19], Estonia [20], Lithuania [21]. Significant progress has been made in the industrial cultivation of berries and their processing in China [22, 23]. For establishing industrial plantations of blue-berried honeysuckle in Heilongjiang Province, mainly Russian varieties are used, as well as seedlings of wild plant forms, such as *L. boczkarnikowae* Plekh. (syn. *L. regelyana* Boczkarn.), growing in the northeast of China [22], and *L. caerulea* subsp. *venulosa* (Maxim.) Worosh. (syn. *L. edulis* Turcz.ex Freyn.) from the natural populations of the Lesser Khingan mountain range.

Despite the interest of researchers to *L. caerulea*, there are almost no investigations on reproductive biology of this species. Lack of knowledge about the peculiarities of the biology of pollination and fertilization leads to grave mistakes in the implementation of programs for the wide introduction of a new culture into production. Our survey of nurseries in Heilongjiang Province, conducted in 2014, showed that only one variety of Russian selection breeds on an industrial scale, i.e. Berel', which was isolated based on a set of economically valuable signs in the collection plantations of several farms in the northeast of China. The analysis of data on the productivity of *L. caerulea* in Heilongjiang Province, as well as at various points of introduction in Russia [23, 24] revealed a wide range of variability in the reproductive capacity of varieties and selected forms of blue-berried honeysuckle.

The study of biological features of the productivity development in the *L. caerulea* varieties was conducted in 1994-2004 in the Central Siberian Botanical Garden of SB RAS (CSB, Novosibirsk, Russia). The findings have been partially published [25, 26], but the number of professionals dealing with blue-berried honeysuckle in Russia and abroad continues to increase. Therefore, consideration of the evidence on the biology of pollination of *L. caerulea*, obtained earlier and supplemented in recent years, remains relevant.

We were the first to establish that the reasons for the decline in productivity of the blue-berried honeysuckle cultivated crops are partial incompatibility in the cross pollination of varieties caused by their closely related origin and the low quality of pollen in some hybrids of *L. caerulea* subsp. *altaica*.

The aim of the present work was to identify possible reasons for the decrease in the potential productivity of blue-berried honeysuckle and choose the varieties for joint cultivation, and to achieve it, we evaluated male gametophyte fertility, self-sterility, interpollination, fruit weight and seed growing potential, and also investigated the incompatibility in the *Lonicera caerulea* L. varieties of various geographical and genetic origin.

Techniques. Studies were conducted in the Central Siberian Botanical Garden of SB RAS, in the forest-steppe zone of the south of Western Siberia, in a continental climate with a moderate supply of heat and moisture. We studied varieties and selected forms of blue-berried honeysuckle of different ecogeographical and genetic origin.

Field trials were conducted in 1999-2002. Pollen fertility, fruit set and weight and seed growing potential were assessed in self- and interpollination in 8 varieties (Salyut, Berel', Goluboe vereteno, Zolushka, Tomichka, Parabel'skaya, Pamyati Gidzyuka and Kamchadalka) of three subspecies with a tetraploid set of chromosomes ($2n = 36$): *L. caerulea* subsp. *altaica* (Pall.) Gladkova (syn. *L. altaica* Pall.), *L. caerulea* subsp. *venulosa* (Maxim.) Worosh. (syn. *L. edulis* Turcz. ex Freyn), *L. caerulea* subsp. *kamtschatica* (Sevast.) Gladkova [syn. *L. kamtschatica* (Sevast.) Pojark., *L. caerulea* var. *kamtschatica* (Sevast.) Pojark.] [27]. Controls were variants obtained from natural open pollination. All variants of pollination were investigated in three replications on different plants of the same variety, choosing branches of the same age.

Honeysuckle forms compact inflorescences, consisting of two closely arranged flowers (double-flowered), the pollination of which gives one infructescence, that is why gauze coverings were used to isolate not individual flowers, but at least 100 double-flowered plants in each replication (under each gauze cage). The flowers were isolated 3-5 days before the beginning of flowering. Pollination was performed in the mass flowering phase 2-3 times under each gauze cage with an interval of 1-2 days. In the period of full maturation, the number of set fruits, the average weight of one fruit in each pollination option, the number of completed seeds and undeveloped ovules were recorded. The results were reported separately for each gauze cage. Pollen was harvested from colored yellow buds, dried under diffused light, tested for fertility and stored in a desiccator in glass vials until pollination [28].

In 1999-2014, in 19 varieties of blue-berried honeysuckle, pollen fertility was assessed by staining with acetocarmine [29, 30]. The results were recorded using the Primo Star light microscope (Carl Zeiss, Germany). The proportion of normal pollen grains from the total number of studied ones was determined.

The best pollinators were considered the varieties that provided the percentage of set fruit higher than in control, equal or close to it. Varieties that ensured setting of 50-70 % of the fruit as compared to control were allocated to a group of admissible pollinators. Varieties for which the setting of fruit was below 50 % vs. control were attributed to poor pollinators [28].

Observations of the growth of pollen tubes in pestle tissues in each pollination option were carried out by standard methods [32]. Pistil styles were recorded 6, 12, 24, 36 hours after pollination. The preparations of germinating honeysuckle pollen tubes were stained with fluorochrome (aniline blue). The preparations were analyzed using a ML-2B fluorescent microscope with the MFN-10 camera adapter (LOMO, Russia).

Mean (X) and standard error of the mean (S_x) were calculated using mathematical statistics [31] and the Microsoft Excel software package.

Results. Data on variety studied in the paper is presented in Table 1.

The success of fertilization largely depends on the viability of the pollen grains. The analysis of pollen fertility (1999-2014) showed its high morphological adequacy in varieties of the Kamchatka and Primorsky Krai origin. When stained with acetocarmine, pollen fertility in the *L. caerulea* subsp. *kamtschatica* samples averaged 96.2±1.5 % with a coefficient of variation (Cv) of 4.1 %. For the *L. caerulea* subsp. *venulosa* samples, fertility of pollen grains was 91.2±3.4 %, although there were more pronounced fluctuations by years

(Cv = 9.2 %).

1. The geographical and genetic origin of the *Lonicera caerulea* L. varieties used in the study

Name	Origin	Taxon
Goluboe vereteno Sinyaya ptitsa Zolushka Lazurnaya	Seedlings from open pollination of the selected form Start from the Kamchatskaya population	*L. caerulea* subsp. *kamtschatica* 2*n* = 36
Gerda	A seedling from pollination of the Sinyaya ptitsa variety with a mix of pollen from a seedling of the wild blue-berried honeysuckle from the Kamchatskaya population	
Kamchadalka	A seedling from open pollination of the selected form from the Kamchatskaya population	
Salyut Galochka Sirius Barkhat	Seedlings of wild honeysuckle from the Rudnyi Altai (Eastern Kazakhstan, Leninogorsk district)	*L. caerulea* subsp. *Altaica* 2*n* = 36
Ognenny opal Selena	Seedlings from open pollination of the selected form of honeysuckle from the Rudnyi Altai	
Berel'	A seedling from pollination of the selected form № 12-19 (Sirius) from the Rudnyi Altai with a mixture of pollen taken from the Goluboe vereteno, Sinyaya ptitsa and Lazurnaya varieties	*L. caerulea* subsp. *altaica* × *L. caerulea* subsp. *Kamtschatica* 2*n* = 36
Bakcharskaya	A seedling from open pollination of the selected form № 15-63 from Primorsky Krai	*L. caerulea subsp. Venulosa* 2*n* = 36
Tomichka Pamyati Gidzyuka Narymskaya Vasyuganskaya Parabel'skaya	Seedlings from open pollination of the selected form № 68-2 (Del'fin) of a seedling of wild honeysuckle from the Dalnegorsky district of Primorsky Krai	

Significant variability in pollen fertility (0.8 to 98.6 %, Cv = 67.2 %) was observed in the varieties of the Altaic origin and a hybrid obtained with their participation (Berel'). A large number of defective pollen grains were found during the investigation of pollen in the Galochka, Sirius, Barkhat, Salyut and Berel' varieties. A high quality of pollen, which did not depend on growing conditions, was typical for the Ognenny Opal (91.6 to 97.5 %) and Selena (87.3-96.6 %) varieties. The Berel', Salyut, Sirius, Galochka and Barkhat varieties also had very poor pollen productivity, i.e. a low number of microspores was formed. In the samples of the Sirius and Barkhat variety, most of the anthers did not contain microspores. Both on ready-to-open and fully opened flowers, most of the anthers were green and sluggish.

In our additional studies [33], these samples showed numerous abnormalities at different stages of meiosis, which could lead to low fertility of the pollen grains. Reduced fertility of pollen grains was also observed in hybrids obtained with the participation of these varieties [34]. Among the hybrids in which the Berel' and Salyut varieties were used as female parents, samples were found with a high-fertile (up to 97 %) and low-fertile (down to 2-37 %) pollen. At the same time in hybrid families, where the low-fertile variety acted as a paternal form, all seedlings showed high fertility of pollen. This indicates a probable inheritance of the sterility of the pollen grains along the maternal line and the need for mandatory quality control of pollen in selection work, as well as when choosing pollinators for joint cultivation in the blue-berried honeysuckle farm-scale plantations.

Many researchers devoted their investigations to self-incompatibility in blue-berried honeysuckle [35-37]. It has been established that the samples of this species belong to self-sterile plants, and when they are forced to self-pollination within the clone, the fruits are not set at all, and instead fruits with uncompleted seeds with low germination or small seedless fruits are set. Self-incompatibility in the representatives of *L. caerulea* is manifested by stopping the growth of pollen

tubes in the lower half of the pistil style [38]. Taking into account the results given in other papers [39, 40], we may suggest a gametophytic type of incompatibility reaction in *L. caerulea*. Self-incompatibility directly affects the productivity, because it serves as the basis for genetic control of sexual reproduction of plants [41, 42]. Single-variety plantings, as well as unfavorable weather conditions during flowering and fruit ripening, which hamper interpollination by insects, are responsible for almost complete infertility. In this regard, it is necessary to study specifically the degree of self-sterility and, for introduction, select forms with the greatest manifestation of self-fertility in the genotype for further consolidation of this trait in the offspring by methods of selection.

The samples of *L. saerulea* we studied appeared to be virtually self-fertile. After forced self-pollination of flowers, the formation of 0.9 to 10.5 % of fruits was observed (Table 2). The Kamchadalka, Goluboe vereteno, Parabel'skaya and Pamyati Gidzyuka varieties were characterized by the highest degree of self-fertility; in certain years, during self-pollination, the set of fruit in these cultivars was 20.0, 15.9, 15.6 and 15.2 %, respectively [43]. The Salyut and Berel' varieties, obtained based on the samples from Altai, as well as the Zolushka variety of the Kamchatka origin, had a low degree of self-fertility. In most varieties, the formation of a small number of fruits after self-pollination was observed. During the period from flowering to ripening, they slightly increased in size, reaching $^1/_3$-$^1/_2$ of the weight of fruits obtained from interpollination. With the onset of the mass ripening, the fruits acquired a typical moderately blue color. The analysis of seed productivity showed that they were seedless to a greater degree (Table 3). The set seeds failed to germinate and produce plants.

2. The set of fruit in the blue-berried honeysuckle (*Lonicera caerulea* L.) plants of different varieties depending on the pollinating variety ($X \pm S_x$, Novosibirsk, 1999-2002)

Pollinated variety	Pollinating variety								Control
	1	2	3	4	5	6	7	8	
Berel'	0.9±0.76	39.6±2.89	24.5±5.34	29.6±4.70	36.2±3.21	29.6±5.25	26.7±5.09	44.6±1.82[a]	59.0±1.55
	1.5	67.1	41.5	50.2	61.7	50.5	45.6	75.7[a]	
Goluboe vereteno	23.3±4.05	9.3±2.01	28.9±8.26	39.0±3.86	41.0±7.66	39.5±8.46	11.4±3.64	41.6±3.46[a]	52.9±6.47
	43.9	17.5	49.2	65.7	69	66.5	19.2	70.0[a]	
Zolushka	27.3±14.45	14.0±6.81	3.2±0.71	42.6±1.67[a]	40.2±7.62[a]	32.2±6.02	24.4±5.85	42.6±5.64[a]	54.7±6.80
	49.9	25.6	5.8	77.9[a]	73.5[a]	58.8	44.6	77.8[a]	
Kamchadalka	13.4±6.10	23.6±4.65	20.6±3.02	8.1±4.93	29.9±3.49[a]	28.0±5.73[a]	18.6±6.45	16.8±4.33	37.7±7.01
	35.6	62.7	54.6	21.5	79.4[a]	74.4[a]	49.5	53.5	
Pamyati Gidzyuka	24.5±6.29	38.0±4.16[a]	27.8±4.41	26.4±7.20	10.5±2.99	20.0±3.73	12.0±3.91	15.8±2.52	51.8±2.49
	47.3	73.5[a]	53.7	51.1	20.4	38.6	23.2	30.5	
Parabel'skaya	25.4±3.48	42.1±7.48[a]	45.2±2.62[a]	40.4±4.03[a]	20.6±4.36	8.2±3.26	13.3±3.77	31.3±3.85	54.9±5.46
	46.3	76.7[a]	82.4[a]	73.6[a]	37.6	14.9	24.2	57.1	
Salyut	16.0±6.40	27.2±3.02	30.2±4.28	31.7±3.90	38.4±6.28[a]	27.1±3.83	3.4±1.17	31.1±7.39	53.9±5.84
	29.7	50.4	56	58.7	71.1[a]	50.2	6.3	57.6	
Tomichka	35.1±3.47	53.4±3.23[a]	38.0±8.44	41.1±8.96	38.1±6.53	25.5±6.52	18.3±5.11	6.0±1.33	64.0±6.86
	54.9	83.4[a]	59.3	64.2	59.5	39.9	28.5	9.4	

Note. 1 — Berel', 2 — Goluboe vereteno, 3 — Zolushka, 4 — Kamchadalka, 5 — Pamyati Gidzyuka, 6 — Parabel'skaya, 7 — Salyut, 8 — Tomichka; control — open pollination. Above the line — the average rate of fruit set, %; under the line — vs. control, %; a — the best options of pollination based on the compatibility.

The set of fruits in autogamy and open interpollination varied widely over the years of studies. This is due to the negative impact of unfavorable weather conditions (a prolonged drop in temperature down to 3 C, rain with snow) on fertilization, which was observed during the flowering period in 2001 and 2002. The most stable percentage of useful ovary was seen in the Berel' and Pamyati Gidzyuka varieties.

The investigation of the mutual variability of forms and varieties of *L. cepinella*, carried out earlier in various research institutes in Russia and abroad, showed in most cases the successful repollination of forms belonging to the *Caeruleae* subsection and those having the same set of chromosomes [27, 36,

37, 44]. Crossability between di- and tetraploid honeysuckle species from the *Caeruleae* subsection is feasible, however, the fruit set and yield of full seeds were very low, and the viability of triploid ($2n = 27$) hybrids was reduced [12, 27]. Consequently, the use of samples with different numbers of chromosomes as pollinators is unacceptable.

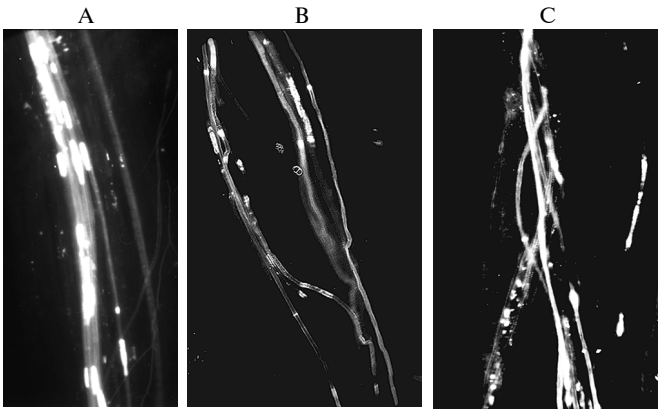
3. The weight of fruit and number of seeds in blue-berried honeysuckle (*Lonicera caerulea* L.) plants of different varieties depending on the pollinating variety ($X \pm S_x$, Novosibirsk, 1999-2002)

Pollinated variety	Pollinating variety								Control
	1	2	3	4	5	6	7	8	
Berel'	0.35±0.01	0.61±0.01	0.56±0.06	0.60±0.10	0.64±0.03	0.57±0.04	0.45±0.06	0.62±0.03	0.63±0.02
	0	12.0±1.1	7.1±2.4	6.7±3.04	8.0±0.2	7.7±3.3	4.8±0.9	8.9±1.2	10.2±1.6
Goluboe	0.44±0.07	0.37±0.02	0.60±0.10	0.67±0.06	0.73±0.09	0.67±0.03	0.40±0.03	0.73±0.10	0.92±0.03
vereteno	4.1±0.9	1.1±0.2	4.1±3.2	8.7±6.2	14.8±0.1	6.4±2.2	1.4±0.07	17.4±4.3	15.8±1.5
Zolushka	0.50±0.06	0.50±0.01	0.47±0.08	0.70±0.10	0.76±0.19	0.60±0.10	0.43±0.30	0.80±0.03	0.84±0.05
	1.9±0.5	1.8±0.5	0.9±0.05	5.8±2.1	5.9±1.5	5.9±1.6	1.8±0.5	9.9±3.5	9.8±3.1
Kamchadalka	0.44±0.01	0.70±0.10	0.60±0.10	0.24±0.06	0.68±0.14	0.52±0.08	0.43±0.01	0.60±0.03	0.74±0.06
	5.2±3.7	9.5±1.8	4.6±1.2	0.3±0.3	11.9±0.3	7.6±1.5	4.8±2.4	3.3±1.8	9.7±1.6
Pamyati	0.50±0.10	0.72±0.09	0.58±0.09	0.46±0.03	0.25±0.07	0.37±0.06	0.32±0.07	0.43±0.10	0.81±0.05
Gidzyuka	4.7±2.4	13.2±3.7	8.2±3.0	7.3±2.7	1.2±1.1	4.6±2.7	2.4±0.9	3.7±1.2	15.1±0.8
Parabel'skaya	0.54±0.06	0.64±0.08	0.78±0.08	0.63±0.05	0.41±0.04	0.31±0.02	0.30±0.02	0.65±0.08	0.67±0.03
	6.9±2.3	18.3±1.6	15.2±3.7	10.4±2.7	5.4±2.3	1.5±0.6	3.2±1.6	9.4±2.1	13.3±1.6
Salyut	0.36±0.08	0.51±0.07	0.49±0.05	0.55±0.09	0.51±0.09	0.42±0.04	0.30±0.04	0.56±0.10	0.57±0.03
	4.0±2.8	6.0±1.0	5.2±0.3	6.9±2.0	5.1±1.4	3.2±1.4	0.8±0.5	7.6±0.6	6.6±1.1
Tomichka	0.42±0.01	0.53±0.04	0.57±0.09	0.54±0.09	0.46±0.09	0.36±0.09	0.31±0.07	0.21±0.04	0.59±0.01
	8.5±0.6	13.0±2.1	12.3±0.1	7.5±3.6	10.2±4.6	3.7±1.7	6.6±5.0	0.8±0.8	12.9±2.8

Note. 1 — Berel', 2 — Goluboe vereteno, 3 — Zolushka, 4 — Kamchadalka, 5 — Pamyati Gidzyuka, 6 — Parabel'skaya, 7 — Salyut, 8 — Tomichka; control — open pollination. Above the line — the average rate of fruit set, %; under the line — vs. control, %; a — the best options of pollination based on the compatibility.

In our studies, with the artificial interpollination of the *L. caerulae* varieties under investigation, the proportion of the set fruit, their weight and the number of seeds varied greatly, depending on the pollinating variety (see Table 3). Low fruit setting was typical for variants, where Berel' and Salyut were used as pollinating varieties. The poor quality of pollen negatively affected the fertilization. Combinations of pollination, where closely related varieties were used as parental forms, such as Goluboe vereteno × Zolushka, Zolushka × Goluboe vereteno, Pamyati Gidzyuka × Parabel'skaya, Pamyati Gidzyuka × Tomichka, Parabel'skaya × Pamyati Gidzyuka, Tomichka × Parabel'skaya and Berel' × Zolushka, had a relatively low grade of setting (25.6 to 48.6 %) along with a small fruit weight and number of seeds (see Table 2).

This partial compatibility or semi-compatibility in honeysuckle can presumably be due to the gametophytic control of the reaction of incompatibility of pollen and pestle tissues. According to the hypothesis by E.M. East, A.J. Mangelsdorf [45] and molecular studies of recent years [42], the plants of the *S1S2* genotype contain both alleles of incompatibility in the diploid cells of the style, and either the *S1* or *S2* allele in self-pollination of the haploid pollen grains. Both the pollen of *S1* and pollen of *S2* are suppressed in the tissues of the style carrying the same factors. When the plants with gametophyte control of incompatibility within the family are repollinated, which is possible in the above-mentioned cases, the crossing of the *S1S2* × *S2S3* heterozygote may occur, resulting in a partial weakening of the incompatibility barrier due to the presence among the pollen grains both those of *S2* that is incompatible with the pistil tissues and those of *S3* that do not cause such a reaction. Depending on how the *S*-alleles of the *S*-locus (locus of incompatibility) were distributed in the offspring, we could observe the intervariety sterility, partial or complete compatibility of the male gametophyte and pistil tissues during the repollination of closely related varieties.



The growth of pollen tubes in the pistil style in blue-berried honeysuckle (*Lonicera caerulea* L.) plants: A — open pollination (the Zolushka variety, ×365), B — pollination of closely related varieties (Zolushka × Goloboe vereteno, ×277), C - self-pollination (the Tomichka variety, ×295) (the ML-2B microscope, LOMO, Russia, Novosibirsk, 2000).

Observations of the growth of pollen tubes in the pistil tissues showed that in repollination of the varieties having no common genetic origin the growth of pollen tubes (as in open interpollination) was characterized by the mass character and a clear orientation towards the ovules. Straight and brightly fluorescent against the background of the pistil tissues, the pollen tubes grew as a dense bundle closer to the center of the column (Fig., A). Callose plugs were of regular rectangular shape and of the same size and arranged evenly along the entire length of the tube. In variants of crossing closely related varieties, as well as in self-pollination, there was a lack of a clear direction of growth of the pollen tubes to the base of the pistil (see Fig., B). Their bending, twisting and weak luminescence just after passing through the $^{1}/_{2}$-$^{2}/_{3}$ column were noted. Worm-like or clavate-shaped callose plugs were arranged irregularly and brightly fluoresced on the subtle background (in some cases) of the pollen tubes. The pollen tubes in all the observed variants of autogamy had a fuzzy outline, the callose plugs were numerous and irregularly shaped (see Fig., C), which confirms the findings from the investigations by M.N. Plekhanova [38].

4. Potential mutual interpollination of the blue-berried honeysuckle (*Lonicera caerulea* L.) varieties of different ecological and geographical origin based on the experiments conducted in the forest-steppe zone of the south of Western Siberia (Novosibirsk, 1999-2002)

Pollinated varieties	Best pollinators	Acceptable pollinators	Poor pollinators
Berel'	Tomichka	Goluboe vereteno	Salyut
		Kamchadalka	Zolushka
		Parabel'skaya	
		Pamyati Gidzyuka	
Goluboe vereteno	Tomichka	Kamchadalka	Berel'
		Pamyati Gidzyuka	Salyut
		Parabel'skaya	Zolushka
Zolushka	Pamyati Gidzyuka	Parabel'skaya	Berel'
	Tomichka		Salyut
	Kamchadalka		Goluboe vereteno
Kamchadalka	Pamyati Gidzyuka	Goluboe vereteno	Berel'
	Parabel'skaya	Zolushka	Salyut
		Tomichka	
Pamyati Gidzyuka	Goluboe vereteno	Zolushka	Berel'
		Kamchadalka	Salyut
			Parabel'skaya
			Tomichka
Parabel'skaya	Goluboe vereteno	Tomichka	Berel'
	Zolushka		Salyut
	Kamchadalka		Pamyati Gidzyuka
Salyut	Pamyati Gidzyuka	Goluboe vereteno	Berel'
		Zolushka	
		Tomichka	
		Kamchadalka	
Tomichka	Goluboe vereteno	Zolushka	Berel'
	Kamchadalka	Pamyati Gidzyuka	Salyut
			Parabel'skaya

We divided the varieties into groups (Table 4). The best pollinators were varieties and selected forms that had a remote genetic origin and were characterized by the high pollen fertility. The group of poor pollinators included primarily the varieties with the low-fertile pollen and closely related forms.

Based on the estimations of genetic origin, the Goluboe vereteno, Sinyaya ptitsa, Lazurnaya, Zolushka, Gerda and Berel' varieties were obtained from the Start parent form (*L. caerulea* subsp. *kamtschatica*) [43], while the Tomichka, Parabel'skaya, Pamyati Gidzyuka, Vasyuganskaya and Narymskaya varieties were derivatives of the Del'fin parent form (*L. caerulea* subsp. *venulosa*) [46]. The results of the studies allow suggesting a more high-grade interpollination and realization of potential productivity in the honeysuckle plantings when used in joint plantings of varieties from different selection families.

This rule can also be applicable to newly created varieties. For example, in recent years, the Bakcharsky velikan, Chulymskaya, Gordost' Bakchara, Sil'ginka, Bakcharskaya Jubileinaya and other varieties were created by plant breeders and have become widely spread [46]. As they are of the closely related origin (derived from subsequent generations of the Del'fin form), varieties from another family must be used as pollinators for them.

When establishing the plantations with high-yielding varieties, such as Salyut, Barkhat, Berel' and hybrids with their participation, characterized by poor pollen quality, it is necessary to include additional pollinating varieties.

Thus, pollen fertility in the blue-berried honeysuckle (*Lonicera caerulea* L.) varieties of different ecological and geographical origin varies considerably. Low fertility and very low pollen productivity are characteristic for a part of the representatives of *L. caerulea* subsp. *altaica* from the Rudny Altai. The use of varieties with a high content of sterile pollen as pollinators leads to reduced fruit setting and a decrease in their weight. All studied varieties of *L. saerulea* were self-sterile. Over the years of research, open pollination in the conditions of the forest-steppe zone in the south of Western Siberia ensured on average the setting of 38-64 % of the fruit. When crossing the closely related varieties of blue-berried honeysuckle, > 50 % decrease in the set of fruit, a decrease in fruit weight and seed productivity were found vs. open pollination. In the variants obtained by crossing closely related blue-berried honeysuckle varieties and in autogamy, similar growth anomalies in the pollen tubes in the pistil tissues were observed, which were apparently related to the gametophytic control of self-incompatibility. The varieties were selected to provide a more productive pollination during joint cultivation.

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METHOD OF DETERMINATION OF PEAR LEAF AREA ON LINEAR MEASUREMENTS BY CALCULATION OF CORRECTION FACTORS AND VARIATION STATISTICS APPROACH

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Abstract

Morphological study of foliage in fruit crops are mainly conducted to study photosynthetic activity as related to leaf area. Currently, there are different methods to determine this parameter with varying accuracy. In the paper, we first compared the accuracy of assessing leaf area in pear by two methods based on a relationship between the leaf size (S) and its linear dimensions, the length (L) and the width (W). These ways were the use of a conversion factor (correction coefficient) and the regression analysis. The following 10 pear genotypes of different ripening were involved to measure the leaf linear parameters: *Pirus communis* L. — varieties Beurre Giffard, Vega (early ripening); varieties Williams, Chernomorskaya Yantarnaya (summer ripening); Beurre Bosk, Rassvet, hybrid № 8520, Nart; *Pirus serotina* Rehd. — varieties Kilchu and Choo-chen-sok (autumn ripening). The correction by means of conversion factor was based on a similarity of the investigated leaf shape to relevant geometrical figure. Under this model, the leaf area calculation as $S = 0.69 \times (LW)$ was the most exact. This formula allows us to fast and exactly estimate the intact leaf size in pear trees and in other fruit crops with the same leaf shape to determine its changes throughout long time without destruction. Under the regression analysis procedure, the independent variables were L, W, L^2, W^2, and LW. Of these, the latter (LW) was optimum, resulting in a linear regression equation $Y = 0{,}922581 + 0{,}660898 \times (LW)$ based on which the MS Excel 7.0 program has been developed. This program allows us to find the sum of leaf areas or to determine an individual leaf area. Additionally, we found the indicators of leaf size and shape, and the averages for the sample, and also suggested graphics displaying leaf area. The scale for estimation was developed as a nomogram. Radial diagram with the special scale marked as area units against L and W units was also offered to simplify extensive research when more than 50-100 estimations required. Thus, due to close positive correlation between leaf linear dimensions and area, it is possible to practically apply conversion factor and regression equations, including developed nomogram and radial diagram, for calculation of leaf areas with the minimum error under natural conditions. The developed models may be helpful to measure area of oval, ovoid and unlobed leaves in southern fruit crops (e.g. apple, pear, cherry, plum), subtropical crops (citrus, feijoa, persimmon, tea), and ornamental wood bushes and grassy plants used for landscape gardening. Computerized technology promotes acceleration and simplification of the calculations.

Keywords: pear, *Pirus communis* L., *Pirus serotina* Rehd., genotype, leaf plate, leaf length, leaf width, leaf area, conversion factor, regression analysis

The investigation of morphological features of the plant, their variability and varietal stability allows obtaining detailed information about the genotype as a whole and its interaction with the environment. The correlation between productivity and the varietal resistance to the main stress factors is unquestionable, but it is influenced by other factors, primarily the activity and the nature of the assimilation apparatus.

A leaf is an organ of a higher plant, functionally designed for photosynthesis, transpiration and gas exchange. Providing the plant with plastic substances and participating in the continuous transport of substances along conductive structures, it plays a multi-functional role in supporting the vital activity, devel-

opment and adaptation of the plant organism. Among other vegetative organs of the plant, the leaf is the structure most active in the metabolic relation and with diverse morphology [1-3]. Morphological examination of the leaf apparatus of fruit crops is carried out mainly in connection with the study of photosynthetic activity, with one of the metric indices being used, i.e. the area of the leaf blade [4]. Modern computer technology and statistical methods allow to increase the accuracy of the analysis and reveal patterns that could not have been detected earlier due to serious inaccuracy.

Currently, experience has been accumulated in the application of methods which, with varying degrees of accuracy, make it possible to determine the leaf area, such as weight method, planimetric method, method of standards, determination of the area by the specific weight of leaf cutouts, electrographic powder method. Such developments have several significant drawbacks. They are either labour-intensive and inefficient, or their use is associated with the separation of leaves from the plant and the inability to follow-up them. Therefore, more attention is paid to the mathematical method of calculating leaf area by its linear dimensions. Several methods for measuring the area of leaves have been described. Their common advantage consists in deducing on the basis of regression mathematically grounded leaf area formulas for different species and varieties of crops during mass determinations [5-8]. N.Ph. Konyaev applied the linear regression method which allows deriving leaf area formulas for various vegetable crops [9]. The disadvantage of this method is that, if the formulas drawn based on the length and width or just the length of the leaf are equal, the individual deviations of the area are larger in the cases where the formula is drawn based on just the length of the leaf and lesser when it is drawn based on the length and width. To improve accuracy, it is advisable to derive formulas for each variety separately.

V.Ya. Volkov and N.Ph. Selevtsev proposed a series of three mathematical models for determining the area of cucumber leaves based on their length and width at different stages of growth [10]. The length of the leaf in this case is the length of the line of the segment (part) of the leaf blade pattern from the tip to the point perpendicular to the intersection with the line of the pattern along the width of the blade, closer to the leaf petiole. The method is applicable only to the specific shape of the leaf blade, taking into account its growth characteristics. N.S. Robbins и D.M. Pharr applied the multiple regression method, taking into account the characteristics of growth and growing conditions [11]. The length of the cucumber leaf was measured from the tip to the end point of the central vein. However, in this case it is required to derive an individual formula for each variety not only based on the genotype characteristics, but also considering the growth conditions.

A method for determining the area of a strawberry leaf was developed using a special scale based on the methods of variational statistics [12]. Its advantage consists in revealing the correlation between the leaf area defined by the planimeter and its parameters. This correlation is expressed by an equation and is used to calculate the area of the strawberry leaf along the central vein, and also to obtain a scale for practical use. The disadvantage of the method is that for calculating the error of estimate not more than 5 %, it is required to calculate the leaf area in the range from 15 to 220 cm^2.

In this paper, for the first time we used two methods for estimating the area of the leaf surface for the pear crop, such as based on the scaling ratio and by means of regression equations relating the leaf area with its linear dimensions (length and width).

The objective of the study is to determine the correction factor and obtain a regression model for calculating the leaf area without separating it from

the plant in different pear varieties with a minimum possible error.

Techniques. Investigations were carried out at the collection site (planted in 1998) of the All-Russian Research Institute of Floriculture and Subtropical Crops in 2001-2004. The leaf biometric parameters were studied on 10 varieties of pear of different maturation periods: *Pirus communis* L. — Beurré Giffard, Vega (early summer ripening); Williams, Chernomorskaya Yantarnaya (summer ripening); Beurré Bosk, Rassvet, hybrid № 8520, Nart; *Pirus serotina* Rehd. — Kilchu and Choo-chen-sok (autumn ripening). Leaves were taken from the middle part of the crown for each variety, 10 pcs. each in 3 replicates [14]. Each leaf was measured using a ruler along the central vein and width at the widest position, the leaf area was determined by planimetry using palettes (15×10 cm film plates with 0.5 cm^2 squares drawn).

Data on the length and width of the leaf blade was used to determine the conversion factor and calculate the area based on the methods of variation statistics [15-22]. The relationship between the actual area of the leaf and its parameters was expressed with an equation used for the theoretical calculation of the area of the pear leaf. When constructing the regression model, the following parameters were used as regressors: length (L), square length (L^2), width (W), square width (W^2) and LW.

The data processing was performed by the correlation analysis and regression analysis, descriptive statistics, using the Statistica for Windows 5.5 [23] software package and Microsoft Excel 7.0.

Results. The method for determining the area of the leaf using the estimated coefficient is based on the correlation between the shape of the leaf (as an indicator of the genotype feature) and a geometrical figure describing it (elliptical, narrow or back-ovoid, oval, round, lancet-shaped) [24, 25]. The variety of shapes of leaf blades suggests a wide variation in the choice of linear dimensions. In most cases, two indicators are used, such as the length and the width, which have a high correlation (0.98) with the area of the leaf surface [26]. Having determined the shape of the figure which fits the leaf, one can calculate the coefficient between its actual area, measured by the direct (planimetrical) method, and the area of the figure [27-29].

The coefficient is defined as the ratio of the actual area to the area of the rectangle with x and y sides:

$$K = S/L \times W, \quad (1)$$

where K is the coefficient (dimensionless quantity), L is leaf length (cm), W is the leaf width (cm), S is the leaf area (cm^2) determined by a direct method.

To simplify the coefficient calculation, one can calculate it by the length or width:

$$K = S/L^2, \quad (2)$$

$$K = S/W^2. \quad (3)$$

The method of the calculated coefficient does not require complicated computer equipment, it can be easily used in the field. In addition, long-term observations are feasible in this case, for example, to determine changes in the leaf area during plant development from the initial stages to its dieback.

Using the formulas (1), (2), (3), we determined three variants of the estimated coefficient, i.e. K_1, K_2, K_3. For each of them, three samples of 100 values were obtained, which were subjected to statistical processing with the calculation of the mean (X) and the coefficient of variation (Cv, %), which varied from 1.18 (for K_1) to 2.57 (for K_2). The smaller was the coefficient of variation, the smaller were the deviations of each value of the sample from the calculated mean. The analysis showed a slight difference (0.11-0.65 %) when applying for-

mulas with the estimated coefficient for different varieties. With calculations using one formula in 10 pear varieties, the minimum error was 0.1 %. The most accurate way to determine the area of pear leaves using the estimated coefficients is to calculate it based on LW using the following formula: S = 0,69 × (LW). This formula can be used to quickly calculate the area of leaves of pear and other fruit crops with a similar shape of the leaf blade. The proposed method allows to observe the changes in the leaf area over a long period and determine the photosynthesis rate (the amount of organic matter accumulated by leaves over a certain period of time).

The search for an optimal mathematical model by variational statistics methods for the theoretical determination of the leaf area was carried out by linear and multiple regression analysis. The tests showed that for different pear varieties the inclusion of the width in the regression model provided a more accurate calculation of the leaf area ($R^2 = 0.98$). A similar method of estimating the area of a leaf is required to study the relationship between the area of the leaf blade and the growth of the plant. The area of one leaf or multiple leaves of one plant, calculated using the formulas, can exactly match or slightly or substantially deviate from the values obtained with a planimeter. This is explained by individual plant differences and age-related changes in the leaf shape. The closer the points are relative to the line of the regression curve, the lesser are the deviations from the actual area.

The formulas can be drawn both from the length and width measurements and the measurement of just the length of the leaf. Based on their equivalence and accuracy, the individual deviations of the area in our tests turned out to be greater when the formula was derived from the length of the leaf and lesser - from the length and width. It was reasonable to derive formulas for each variety separately, because cross replacement of formulas for different varieties decreased the accuracy of determining the area. If a minimum error was observed for calculating the area of a leaf using one formula for several varieties, then this formula could be considered a common one for the pear crop. The analysis of the obtained data established a close positive correlation between the parameters of the length, width and area of the leaf blade, which allowed to calculate the equations of the linear and curvelinear (parabolic) regression of the leaf area of the pear.

The selection model needed a balance between the accuracy of calculation and a variability factor (the smallest number of variables needed for the calculation). Having estimated the determination coefficient (R^2), Fisher's *F*-test, the mean square error (mS) for each regression calculation, we found several equations suitable for calculating the area of the pear leaf (see Table). After step-by-step selection, the most accurate was the following equation: $Y = -6.613161 - 0.272064 \times (LW) + 4.9738 \times W$ ($R^2 = 97.91$) at $p < 0.01$.

The equation $Y = 0.922581 + 0.660898 \times (LW)$ was less accurate ($R^2 = 97.13$ %), but since the correlation coefficient between the area and the product of the length of the leaf and its width turned out to be high ($r = 98.5$), we applied this mathematical model of linear regression.

Regression models describing the dependence of the leaf area on its length and width in pear varieties

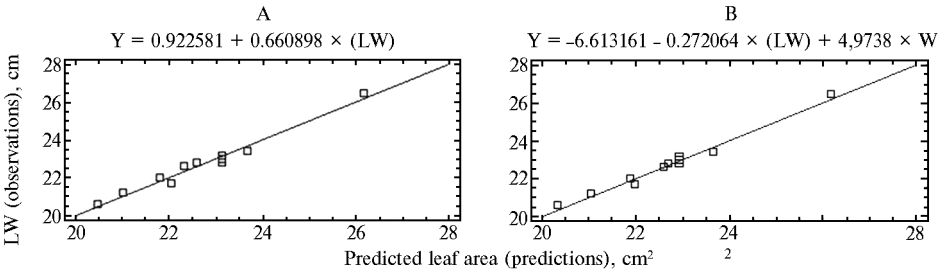
Model	R^2	mS	*F*
Length-based model:			
$Y = -32.7338 + 6.89464 \times L$	87.21	59.4	191.01
Width-based model:			
$Y = -11.565 + 8.37592 \times W$	97.54	66.44	1111.15
Model based on the product of length and width:			
$Y = 0.922581 + 0.660898 \times (LW)$	97.13	66.16	950.95

Table (continued)

Model based on the square length and width:			
$Y = -4.87168 + 0.426423 \times L^2$	87.67	59.72	199.15
$Y = 5.65063 + 1.01831 \times W^2$	98.13	66.84	1473.21
Model based only on the length and its square:			
$Y = 434.525 - 108.44 \times L + 7.1152 \times (L^2)$	91.16	41.05	139.22
Model based on the length, width, their squares:			
$Y = -6.613161 - 0.272064 \times (LW) + 4.9738 \times W$	97.91	33.34	632.81
$Y = 267.743 + 4.32894 \times L^2 - 68.7042 \times L + 6.70968 \times W$	99.09	22.15	940.88
$Y = 271.486 + 0.773545 \times (LW) + 4.01169 \times L^2 - 66.374 \times L$	98.89	22.45	775.85
$Y = 55{,}3646 \times W^2 + 0.286783 \times (LW) - 39.9336 \times W + 21.5484 \times L - 1.36161 \times L^2$	99.99	3148.99	140398.98
$Y = 66.3173 - 0.557339 \times L + 0.286783 \times (LW) - 28.8807 \times W + 4.15485 \times W^2$	99.17	16.89	752.95

N o t e. Y — the area, L — length, W — width; R^2 — the determination coefficient; mS — mean sum of squares of the regression; *F* — the value of the Fisher test.

The analysis of the diagrams (Fig.) and summing of the obtained data showed that regression models with a single LW measurement can serve to accurately calculate the leaf area in different pear varieties.



Diagrams representing the equations of linear (A) **and multiple** (B) **regression for models of calculating the area of a pear leaf based on its length** (L) **and width** (W)**.**

The values of Fisher's *F*-test derived from the analysis of variance, indicate that the deviation from linearity was due to random selective variation and the zero hypothesis about the absence of a linear relationship was rejected. As $F_f > F_{st}$, there was a significant difference between varieties at 1.0 %. All models were statistically significant ($p < 0.01$), the significance level for the interaction of traits was 99.0 %. Based on the resulting regression equation, we developed a program for calculating the leaf area in MS Excel 7.0. The method we proposed for calculating the area of pear leaves makes it possible to construct a scale by calibrating it in units of area. To accomplish this, the equation obtained must be solved with respect to L and W. Applying over the scale a pear leaf along its length with adjustment for its width, we derive the leaf area. To speed up and simplify the procedure with 50-100 measurements taken, a radial diagram can be built. The arcs are drawn over the diagram through each 1 cm of the leaf length. To determine the area, one should attach a leaf with the central vein to the start of the diagram (0 point) and, having determined the length, adjust for the width (or, using the biometric data previously obtained, calculate the area by the diagram).

The developed methods can be used to determine the area of oval, ovoid and non-dissected leaves in southern fruit crops (apple, pear, cherry, plum) and subtropical crops (citrus, feijoa, persimmon, tea), woody, shrubby and herbaceous plants.

Therefore, the most accurate way to determine the area of the pear leaves is a calculation based on a single measurement of the LW. A close positive relation between the length and width of the leaf blade makes it possible to accurately calculate in the field the leaf areas for different pear varieties using the methods of the estimated coefficient, regression equation, nomogram and radial diagram with a minimum error. Computer technology allows to speed up and simplify these calculations.

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